SEAGRASSES UNDER TIMES OF CHANGE

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SEAGRASSES UNDER TIMES OF CHANGE

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EDITORIAL
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Editorial: Seagrasses Under Times of Change

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Keywords: seagrasses, climate change, eutrophication, responses of seagrasses to single and combined stressors, spatial-temporal modeling

The Editorial on the Research Topic

Seagrasses Under Times of Change

Awareness of the ecological importance of seagrasses is growing due to recent attention to their role in carbon sequestration as a potential blue carbon sink (Fourqurean et al., 2012; Bedulli et al.), as well as their role in nutrient cycling (Romero et al., 2006), sediment stabilization (James et al., 2019), pathogen filtration (Lamb et al., 2017), and the formation of essential habitats for economically important marine species (Jackson et al., 2001; Jones et al.). Despite their importance and the increasing public and scientific awareness of seagrasses, simultaneous global (e.g., ocean warming, increase in frequency and severity of extreme events, introduction and spread of invasive species) and local (e.g., physical disturbances, eutrophication, and sedimentation) anthropogenic stressors continue to be the main causes behind the ongoing global decline of seagrass meadows (Orth et al., 2006; Waycott et al., 2009).

Degradation of seagrass ecosystems entails the loss of the associated biota, primary productivity, and local fisheries, and increased sediment re-suspension and beach erosion, processes that result in severe ecological and socio-economic consequences not only for seagrass meadows but also for neighboring ecosystems and human inhabitants (Erftemeijer and Lewis, 2006; Joseph et al., 2019; Moksnes et al., 2021).

Will climate change exert diverging effects on different seagrass species? Will ocean warming eventually exceed the adaptive potential of local seagrass species resulting in a shift of their biogeographic ranges? Does eutrophication cause similar stress as exposure to thermal stress? Do seagrass populations with different "histories" respond differently to stress? Can we suggest new improvements for conservation and management of local meadows that will enhance resilience to the predicted and unpredicted scenarios of change?

In this issue dedicated to seagrasses under times of change, we have collected 17 studies authored by 104 seagrass researchers from around the world that are trying to answer many of these and other questions.

In this Research Topic, readers will find studies that compare the responses of seagrasses to single and combined stressors in their environment and their interactions through multi-stressor laboratory experiments, field studies, and spatial-temporal modeling, ranging from the cellular level (Nguyen, Yadav et al.) to ecosystem processes (e.g., Helber, Procaccini et al.; Helber, Winters et al.).

We are particularly happy to see the diversity of the seagrass studies presented here. This includes:

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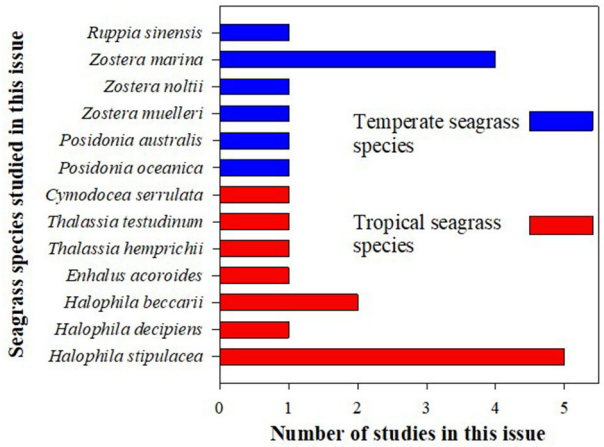

FIGURE 1 | The number of species that were studied in this issue. Blue and red bars represent temperate and tropical species, respectively.

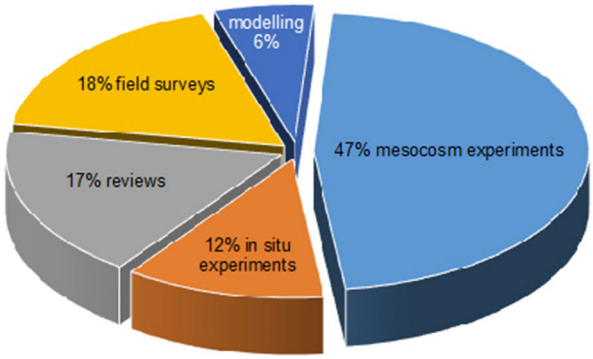

FIGURE 2 | The types of studies that were published in this issue.

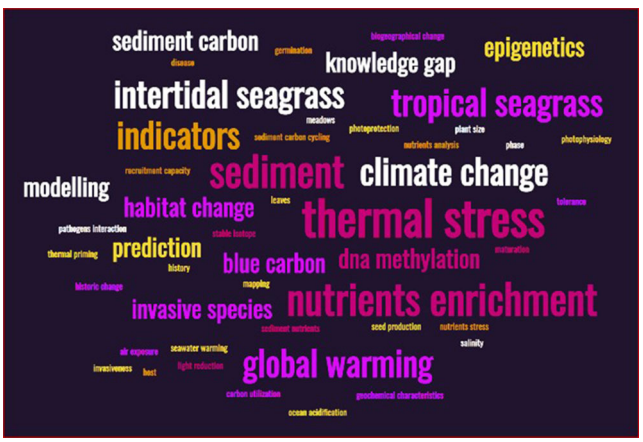

FIGURE 3 | A word cloud representing the 4 prominent keywords used by each one of the 17 studies. The size of font/word is in relation to the number of times that term/word was repeated. We realize that some words are related/similar, but this is because of the word choice is made by a paper's authors, and thus thought all original words should be included as is.

1. Species diversity (**Figure 1**): While research efforts have traditionally focused on native (usually temperate; blue bars in **Figure 1**) "flagship" seagrass species (e.g., *Posidonia oceanica*, *P. australis, Zostera marina, Z. muelleri*), this collection includes studies on a total of 13 different seagrass species, and a large proportion of the publications here are on tropical seagrass species (*Thalassia, Halophila, Cymodocea*, *Enhalus*; red bars in **Figure 1**).
2. Study type (**Figure 2**): Although this is a collection of only 17 publications focused on studying seagrass under changing times, this collection represents a diverse group of types of studies. The number of experimental studies (∼60% of studies in this issue) goes to show that we have gone way beyond just documenting the loss of seagrasses, and much effort is going into understanding the mechanisms and improving our ability to predict changes.

Mesocosm simulations (i.e., single or multi-stressor experiments under controlled laboratory conditions) account for half the studies (Artika et al.; Fang et al.; Lowell et al.; Nguyen, kim et al.; Nguyen, Yadav et al.; Premarathne et al.; Viana et al.). These mesocosms allowed authors to study seagrass responses under the combination of thermal and nutrient stress much more realistic scenarios than previous efforts that focused usually only on one stressor. In addition to the use of mesocosms, we present here papers using modeling (Beca-Carretero et al.; Rock and Daru), *in situ* nutrient manipulations (Helber, Procaccini et al.; Helber, Winters et al.), field surveys (Bertelli et al.; Duffin et al.; Gu et al.), and reviews of historical data (Green et al.; Winters et al.) that have identified gaps of knowledge that can direct future efforts (Rock and Daru; Winters et al.). While molecular studies have emerged as "standard use" in recent years, at least in the field of seagrasses, epigenetics is rather a new field (Jueterbock et al.; Nguyen, kim et al.), and the early exposure of seeds to ocean acidification (Lowell et al.) offers some practical potential in the field of restoration. Studies also include some promising new methods such as thermal priming (Nguyen, kim et al.) that offer some future potential in active restoration efforts (e.g., "super seagrasses").

As the word cloud (**Figure 3**) shows, we still have many topics which at least in this issue, did not get enough attention (i.e., the small-sized words).

In conclusion, extreme thermal events (e.g., heat waves or increasing seawater average temperature) and eutrophication-related stressors, such as light attenuation or nutrient increase, were, among others, the two main factors studied in this Research Topic. They both caused responses in photo-physiology, morphology, and tissue nutrient content in the diverse tropical and temperate species studied. Studies in this Research Topic have highlighted that coexisting species (even in the same meadow) assert different responses to stress or resilience capacity due to their different life-history traits. Moreover, it was shown that the same species growing in different environments or coming from different geographic areas can have contrasting responses to the same stress. Therefore, the conservation and management of seagrass meadows will depend on local studies that focus on the responses and resilience of the specific

species and populations in the area. This Research Topic has also demonstrated high levels of plasticity exhibited by certain species to adverse environmental conditions, and that regular and consistent long-term monitoring of seagrass sites is needed to detect significant declines and plan conservation policies. Modeling species distribution under future temperature and salinity conditions project an increase in invasive species and a dramatic change of species composition in an exemplary study for the Mediterranean.

We hope that this Research Topic has not only answered some of the initial questions but has opened new research lines that generate a better understanding of seagrass loss in these changing times. This knowledge is needed to make effective decisions for the conservation of seagrass meadows worldwide.

AUTHOR CONTRIBUTIONS

GW initiated and led the writing. DW, MT, HR, and IV edited and improved earlier versions. All authors contributed to the article and approved the submitted version.

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Responses of Invasive and Native Populations of the Seagrass *Halophila stipulacea* to Simulated Climate Change

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Climate change fuels invasions of plant species and displacement of local plants. Little is known about the ecophysiological adaptation of the invasive species, and their ability to cope with the changing conditions in their new habitat. *Halophila stipulacea*, a tropical seagrass native to the Gulf of Aqaba (GoA; northern Red Sea), became a Lessepsian migrant spreading within the eastern Mediterranean where it could potentially outcompete local species. We analyzed temperature records in the last 35 years and show that water temperature has increased faster in the eastern Mediterranean Sea compared to GoA, suggesting that *H. stipulacea*'s invasive success is associated with adaptation to thermal warming. Furthermore, we compared the responses of native (Eilat, Israel) and invasive (Limassol, Cyprus) *H. stipulacea* plants to current (26°C) and predicted thermal maxima (29 and 32°C) in a controlled experimental microcosm. Morphological and photo-physiological results showed negative effects of heat stress on the native plants while un-affected/or even enhanced performance in their invasive counterparts. Gene expression, studied for the 1st time in *H. stipulacea,* pointed to differences in the molecular responses of two populations to thermal stress. Results predict that sea warming will cause vast reductions in *H. stipulacea* meadows growing in the GoA while it will facilitate *H. stipulacea's* spread within the Mediterranean Sea.

Keywords: thermal stress, global warming, *Halophila stipulacea*, invasive species, Lessepsian migrant, tropical seagrass

INTRODUCTION

Seagrass meadows thrive worldwide in shallow sedimentary shorelines, where they fulfill important ecological services (including high primary productivity, production, and burial of organic carbon, nutrient cycling, and sediment stabilization) estimated at US$ 2.8×10^6 km^{-2} yr^{-1} (Costanza et al., 2014). Yet, seagrass ecosystems are facing a global crisis due to both direct (reduced water quality, coastal development, and poor land use) and indirect (climate change) anthropogenic perturbations (Orth et al., 2006; Waycott et al., 2009; Short et al., 2011). Loss of seagrass ecosystems

entails devastation of the associated biodiversity and primary productivity, reduction of local fishing grounds, enhancement of coastal erosion, and loss of major carbon sinks culminating in ecological and socio-economic dislocations (Wyllie-Echeverria and Cox, 1999; Orth et al., 2006; Fourqurean et al., 2012; Costanza et al., 2014; Arias-Ortiz et al., 2018). Increasing seawater temperatures associated with global warming directly affect seagrasses in several ways, including changes in growth rates, physiological functions, patterns of sexual reproduction, phenology, and seed germination (Marbà et al., 1996; Orth et al., 2006; Marbà and Duarte, 2010; Jordà et al., 2012). In addition, ocean warming and the associated increase in the frequency, duration and severity of heat waves (Easterling et al., 2000; Schär et al., 2004; Oliver et al., 2018) act indirectly by facilitating the establishment and spreading of invasive species (Stachowicz et al., 2002; Bradley et al., 2010) alongside reducing the biotic resistance of native communities to invader establishment (Diez et al., 2012).

The seagrass *Halophila stipulacea* (Forsskål) Ascherson (order Alismatales, family Hydrocharitaceae) is a dioecious, small tropical species native to the Red Sea, Persian Gulf, and Indian Ocean (den Hartog, 1970; Green and Short, 2003). Within the northern Gulf of Aqaba (GoA), one of the focal regions of this study, *H. stipulacea* is the most dominant, and sometimes only seagrass species (El Shaffai, 2011; Mejia et al., 2016), forming extensive mono-specific meadows found in both shallow and deep environments (1–50 m depth; Sharon et al., 2011; Winters et al., 2017), in soft sediments ranging from fine sand to gravel (Mejia et al., 2016; Rotini et al., 2017).

Following the opening of the Suez Canal in 1869, *H. stipulacea* soon became a Lessepsian migrant (den Hartog, 1972; Lipkin, 1975a) and has since become established in many parts of the eastern Mediterranean Sea (Lipkin, 1975a,b; van der Velde and den Hartog, 1992; Gambi et al., 2009; Katsanevakis and Tsiamis, 2009; Sghaier et al., 2011). Surprisingly, in 2002 *H. stipulacea* was also reported from the Caribbean Sea (Hector and David, 2004), and merely a decade later it was found in most Eastern Caribbean island nations as well as along the coast of the continental South American (Willette and Ambrose, 2009, 2012; Maréchal et al., 2013; Vera et al., 2014; Willette et al., 2014; Steiner and Willette, 2015; van Tussenbroek et al., 2016; Scheibling et al., 2018). In the Caribbean, *H. stipulacea* has been shown to displace native Caribbean seagrass species (e.g., *Syringodium filiforme, Halodule wrightii,* and *Halophila decipiens*) by physically displacing local Caribbean seagrass species by monopolizing their spaces (Willette and Ambrose, 2012), leading to a major change in the Caribbean's seagrass landscape (Steiner and Willette, 2015).

Although *H. stipulacea* was included in the "100 Worst Invasive Alien Species in the Mediterranean" (Streftaris and Zenetos, 2006), in this basin, evidence for its "invasive" characteristics are scarce. Observations point toward a relatively limited "invasion success" in this region, as can also be inferred from the limited number of studies reporting competitive displacement of native Mediterranean seagrasses by the invasive *H. stipulacea* (Georgiou et al., 2016). However, some evidence of the potential competitive displacement by the invasive or "alien" *H. stipulacea* does exist. Sghaier et al. (2014) showed that a small patch (0.2 ha) of *H. stipulacea* in Cap Monastir Marina (eastern Tunisian coast), grew to cover more than 2.2 ha in only 4 years, and, in the process, displaced 50% of the native *Cymodocea nodosa*. More evidence of the future potential of *H. stipulacea* in the Mediterranean, comes from its slow (12 km yr^{-1}; Chiquillo et al., 2018) but continuous spread and expansion in this basin, moving westwards and northwards (Lipkin, 1975a; Gambi et al., 2009, 2018; Sghaier et al., 2011).

As *H. stipulacea* seems to be highly adapted to a wide range of environmental conditions (Gambi et al., 2009; Sharon et al., 2011) it has been predicted that the ongoing tropicalization of the Mediterranean Sea (i.e., becoming warmer and saltier; Bianchi and Morri, 2003; Borghini et al., 2014; Ozer et al., 2017) accompanied by the recent doubling of the Suez Canal (Galil et al., 2015) can facilitate *H. stipulacea* to outcompete local seagrass species (Sghaier et al., 2014) or to occupy newly available habitat following extirpation of local Mediterranean temperate seagrass species (Marbà and Duarte, 2010; Jordà et al., 2012; Chefaoui et al., 2018; Marín-Guirao et al., 2018; Savva et al., 2018).

In addition to the changing Mediterranean, *H. stipulacea* is also facing climate change within its native range, e.g., the northern GoA (Fine et al., 2013). Surrounded by deserts, the GoA receives very little rain and practically no runoffs from neighboring arid lands, creating an oligotrophic and warm tropical sea (Winters et al., 2006; Reiss and Hottinger, 2012). While studies have highlighted the potential effects of ocean warming on local coral reefs (Fine et al., 2013), little is known about the thermal tolerance of local *H. stipulacea*. In the GoA, where the tropical *H. stipulacea* is often found in meadows neighboring coral reefs (Mejia et al., 2016; Winters et al., 2017), loss of *H. stipulacea* could lead to increased sediment re-suspension, nutrient, and particulate loading into the water column (Orth et al., 2006) with adverse consequences to neighboring coral reefs due to algal overgrowth (Genin et al., 1995). Since tropical seagrass meadows also modify seawater carbonate chemistry by taking up CO_2, potentially mediating the effects of ocean acidification (Unsworth et al., 2012), loss of *H. stipulacea* meadows in the GoA would also enhance the decalcification process of neighboring corals (Hoegh-Guldberg et al., 2007). The fact that many reef fish species tend to hide in the structurally complex coral reefs to avoid predators, but forage in nearby structurally simple tropical seagrass meadows (Beck et al., 2006), entails that loss of seagrass meadows in the GoA will have cascading effects on local biodiversity.

Increased seawater temperatures can alter growth rates and other physiological and biochemical functions of seagrass (Short and Neckles, 1999; Beca-Carretero et al., 2018). With photosynthesis being among the first processes to be affected by elevated temperatures (Sharkey, 2005), several studies on the effects of thermal stress on seagrasses have focused on changes in photophysiological parameters (Ralph, 1998; Campbell et al., 2006; Winters et al., 2011). While studying gene expression is emerging as an important tool in seagrass thermal stress studies (Procaccini et al., 2012; Davey et al., 2016), so far, this was only applied in temperate seagrass species (Bergmann et al., 2010; Winters et al., 2011; Davey et al., 2016;

Marín-Guirao et al., 2016), with no molecular studies ever performed in tropical seagrass species.

In both the eastern Mediterranean and the northern GoA, understanding the thermal tolerance of local *H. stipulacea* populations is crucial for seagrass conservation and management. The objective of the present study was to test whether *H. stipulacea* plants from native and invasive populations differ in performance and ecophysiology under thermal conditions that simulate current and future climate changes. We first analyzed temperature records and warming trends in the northern GoA (Eilat, Israel) and the eastern Mediterranean Sea (Limassol, Cyprus). We then collected plants from both native (GoA) and invasive (Limassol) *H. stipulacea* populations and compared their physiological, biochemical and molecular responses to current (26°C) and predicted thermal maxima (29 and 32°C) in a microcosm setup. Results suggest a rapid adaptation of the invasive population to the ongoing warming of the Mediterranean Sea. We predict that sea warming will cause vast reductions in *H. stipulacea* meadows growing in the GoA while facilitating their spread within the Mediterranean Sea. These results have important implications for biodiversity management and conservation in both of these regions.

MATERIALS AND METHODS

Plant Materials and Habitat Descriptions

Halophila stipulacea plants (with shoots, attached roots, and rhizomes) were collected at 4 m depth from both an invasive (Limassol, eastern Mediterranean Sea, Cyprus; 34°42′20″ N, 33°07′24″ E; **Figures 1A,B,D**) and from a native population (Eilat, northern GoA, Israel; 29°32′47″ N, 34°57′51″ E; **Figures 1A,C,E**) on September 29th 2017 and October 3rd, respectively (n = 200 plants collected at each site; **Figure 1A**). The population growing in Limassol ("dream café" site; **Figures 1B,D**), came from a shallow area 100 m from shore that was protected by detached breakwaters. In this area we found three seagrass species: *Cymodocea nodosa*, residual patches of *Posidonia oceanica* and the invasive of *H. stipulacea* mixed in-between them (**Figure 1D**). Sediment at this site was sandy and the slope was very gentle. Similarly, the population growing in Eilat, came from a shallow meadow, 100 m from shore that is the northern-most tip of the GoA. In this area ("North Beach" site), *H. stipulacea* grows in extensive mono specific meadows that start at 2 m and extend to deeper waters (30 m, Winters et al., 2017). Sediment at this site was sandy and the slope was very gentle (Mejia et al., 2016). In both sites, the *H. stipulacea* plants that were collected, were at least 2 m away from each other in order to avoid collecting samples from the same clone. During collection, water temperatures at both sites were around 25–26°C.

Environmental Conditions at the Collection Sites

While environmental conditions at the collection site in Eilat (e.g., seawater temperatures, salinity, sediment type, Kd and nutrient levels) have been described before (Mejia et al., 2016; Winters et al., 2017) and are well-monitored in nearby sites for nearly 20 years by the National Monitoring Program at the Gulf of Eilat (NMP-Israel[1]), no long-term dataset exists for anywhere near the Cyprus site. Thus, comparisons between the long-term conditions at the two sites were based on two data sets. To compare seawater salinities between the two sites, the Bio-ORACLE global dataset (Tyberghein et al., 2012; Assis et al., 2018) was used to compare minimum, maximum, and average values of water salinities over a relatively long-term data set (2000–2014, based on monthly averages; Supplementary materials S1). To compare environmental temperatures and rates of warming between study locations in Eilat and Limassol, we obtained daily sea surface temperatures (SSTs) for the period between 1982 and 2017 from the NOAA dOISST.v2 dataset at www.ngdc.noaa.gov (Smith, 2016). We used Advanced Very High-Resolution Radiometer (AVHRR) only data, due to its longer temporal span and because it has been shown to outperform other datasets in coastal areas (Lima and Wethey, 2012). For each of the two locations, daily climatology parameters were produced by averaging corresponding daily values over the entire time span. Average warming rates were computed as the slope of the linear regression of seasonally detrended SST versus time (Lima and Wethey, 2012). Seasonal variability was removed by subtracting from each day its corresponding climatology, i.e., the average temperature for that same day computed over the entire period under analysis (Chatfield, 2016). To compensate for temporal autocorrelation that could cause an underestimation of the standard errors of the warming rates, we followed Foster and Rahmstorf (2011) and reduced the data per effective degrees of freedom, using a conservative approach where autocorrelation structure was treated as an autoregressive moving average process by applying the ARMA (1,1) model (Foster and Rahmstorf, 2011). Warming rates for February and August were computed based on averaged daily values for those months. All calculations were done in R Core Team (2018).

Mesocosm Facility

Collected plants were transferred to our seagrass dedicated microcosm facility in zip lock bags filled with seawater (**Figure 2A**; recently described in Oscar et al., 2018). Here plants were planted in 15 aquaria (40 cm width, 33 cm height, 45 L of seawater in each aquarium) layered with 20 L (∼7 cm high) of natural sediment (sediment collected from the shores of the GoA, Eilat; **Figure 2A**) and filled with artificial seawater (Red Sea Salt, Israel). Each aquarium contained 20 individuals (i.e., 10 invasive and 10 native plants) with invasive and native populations separated by plastic dividers (insert in **Figure 2A**). A total of 5 aquaria were assigned for each treatment and considered as experimental replicates (n = 5 in each treatment). Salinity was maintained at 40 PSU (partial salinity units, equivalent to the average salinity of the GoA's seawater; The National Monitoring Program at the Gulf of Eilat, 2014[1]) by adding purified water to compensate evaporation. In order to retain the quality of experimental seawater, 20% of the total volume of water in each aquarium was replaced by newly prepared seawater every week. This microcosm facility is fully controlled

[1]http://www.meteo-tech.co.il/EilatYam_data/ey_data.asp (accessed July 3, 2017)

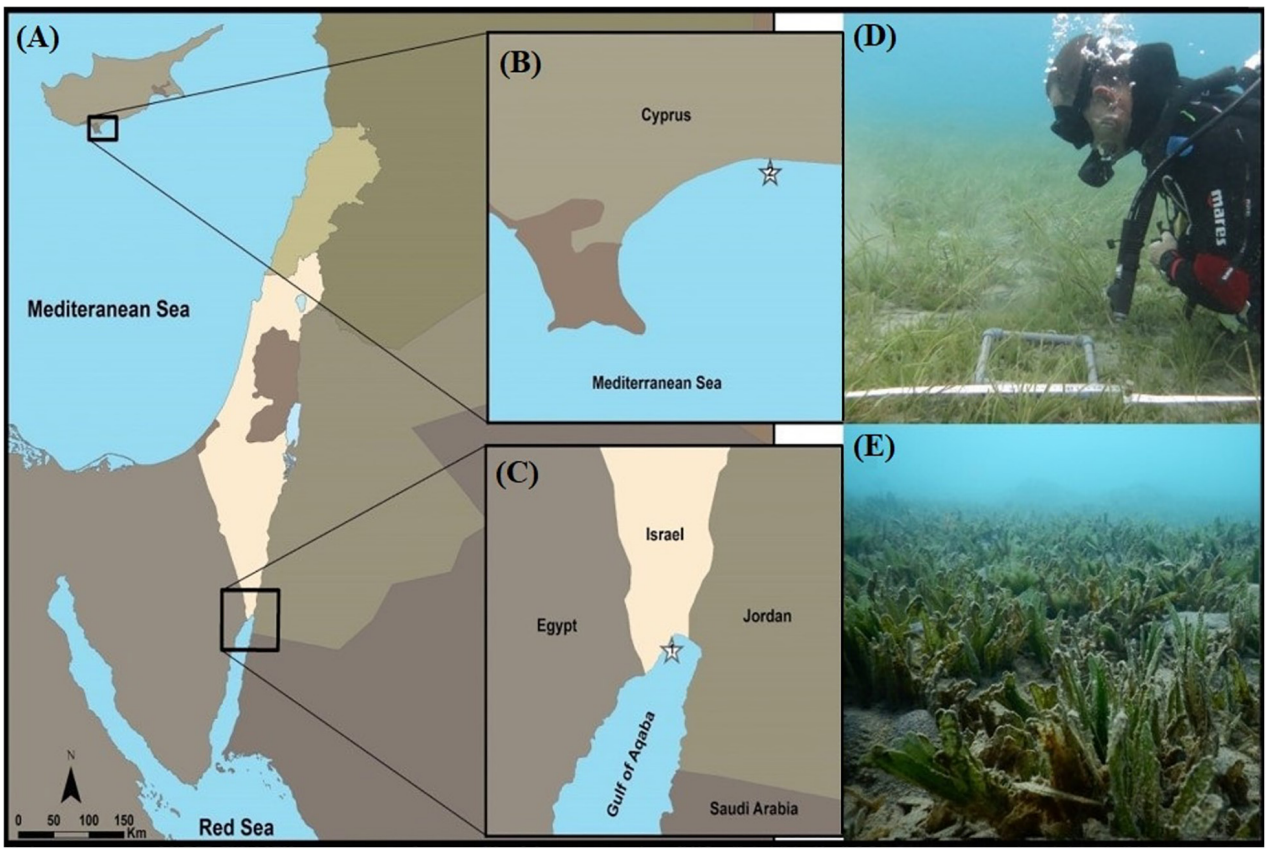

FIGURE 1 | Sampling sites of *Halophila stipulacea* **(A–C)**: (1) The native population, North Beach site, Eilat, northern Gulf of Aqaba (GoA, northern Red Sea, Israel). (2) The invasive population, growing at the Dream Café site, Limassol (eastern Mediterranean Sea, Cyprus). **(D)** Invasive *H. stipulacea* plants growing intermixed with plants of the native Mediterranean seagrass *Cymodocea nodosa* at the Dream café site (Photo: Yuval Sapir). **(E)** *H. stipulacea* growing in its native habitat in the GoA (Photo: Hung Manh Nguyen). Depths of both plant collection sites 3–4 m.

for temperature and light (ProfiLux 3.1T eX Controller, GHL; GHL, Germany). Irradiance was provided by T5 fluorescent lamps (Osram Lumilux HO 865/54W cool daylight with the color temperature of 6500 degrees Kelvin) that were set to a 10:14 h light: dark cycle, with intensities of 120 μmol photons s^{-1} m^{-2} measured at the bottom of the aquaria.

Water temperatures were measured automatically every 1 h with PT1000 thermal sensors (GHL, Germany) connected to two ProfiLux 3.1T eX GHL controller systems (GHL, Germany). In each one of the three treatments (detailed below), water temperature was followed over time using two sensors, situated in two different aquaria belonging to the same treatment (**Figure 2A**). Salinity and temperature were also checked manually every day using the WTW Multi 340i Portable Meter (Xylem Analytics, Weilheim in Oberbayern, Germany). Each one of the 15 aquaria, was fit with a submersible pump to provide gentle water movement within the aquarium.

Experimental Treatments

Following 3 weeks of the acclimation period, heating treatments were applied. These included maintaining plants at 26°C (just under the current maximal summer water temperatures in Eilat; Winters et al., 2006), and two predicted climate change scenarios, including 29 and 32°C. The experiment was started by 1 week of gradual increases of the temperatures in the control tanks (from 25 to 26°C) and in the two thermal stress treatments (from 25 to 29°C, and from 25 to 32°C, at heating rates of 1 and 0.5°CC day^{-1}, respectively, **Figure 2B**). Plants were kept for 2 weeks at these high temperature treatments (29 and 32°C), after which, water temperatures were gradually ramped down (at cooling rates similar to those mentioned above) to 26°C at the end of the experiment, with measurements at T4 taken 24 h after reaching control temperatures (**Figure 2B**). Plants exposed to the control treatment were kept at 26°C throughout the entire experiment (**Figure 2B**).

Plant Performance

Plant performance was estimated by measuring a total number of shoots (shoots $plant^{-1}$), horizontal growth rates (mm $week^{-1}$), and leaf surface area (cm^2) before, during, and after the thermal stress treatment (**Figure 2B**). For this, three plants per population in each aquarium were randomly selected for measuring the total number of shoots every week, which were averaged into a single biological replicate (i.e., within each treatment, each

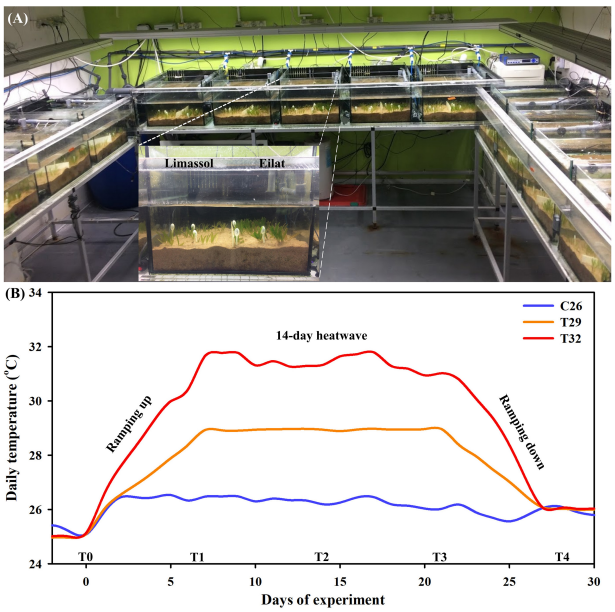

FIGURE 2 | The seagrass dedicated mesocosm facility, containing 15 aquaria fully controlled for water temperature, light, and salinity **(A)**. Aquaria were layered with natural sediment and filled with artificial seawater. Each aquarium contained 10 invasive and 10 native *H. stipulacea* plants, separated by plastic dividers (**A**, inset). Average water temperature profiles (n = 2 sensors in two tanks from each treatment) during the microcosm experiment (day 0–28) **(B)**. Shown are the three thermal treatments (26°C [control], 29°C and 32°C), the time points (T0–T4) on which measurements and samples were taken, and the different stages of the experiment – 6-days of ramping up the temperature, the 14-day heat wave, followed by 6-days of ramping temperatures down again to control levels (26°C).

aquarium = one biological replication). In addition, three plants were randomly selected per aquarium and used to assess horizontal growth rates once per week. For this, the distance from the basal leaf sheath to the tip of the three youngest leaves was measured and these three measurements were averaged into a single biological replicate. Finally, mature intact leaves (i.e., fully grown, often the third youngest shoot with leaf tip; broken leaf tips were not used) were detached from one plant per population in each aquarium once a week, scanned on a digital scanner (CanoScan LiDE 120, Canon U.S.A., Inc.) and leaf surface area was estimated by using ImageJ (Abràmoff et al., 2004).

Biochemical Measurements

Scanned leaves that were used for leaf surface area measurements (mentioned above) were subsequently used for measuring changes in chlorophyll *a* and *b* contents. Chlorophylls were extracted in 100% methanol according to Wellburn (1994) and adapted to a 96 well microplate reader (Epoch 2 Microplate Spectrophotometer, Biotek) as described in Warren (2008). After scanning, leaves were immediately weighed and 50 mg (fresh weight) of tissue was homogenized into small pieces before transferring into 1.5 mL tubes. 1 mL of 100% methanol was added into the tube, covered with aluminum foil, and kept at 4°C overnight. 200 μL of chlorophyll extract was then transferred into a 96-well plate (CELLSTAR®, Greiner Bio-One) and the absorbance read at 470, 652, 665, and 750 nm. Chlorophyll *a* and *b* contents were calculated according to Tran et al. (2018).

Effects of thermal stress on biochemical parameters were studied over time (**Figure 2B**) by measuring changes in the total protein content (Elavarthi and Martin, 2010). For these measurements, 50 mg of the fourth youngest leaves were ground thoroughly to a fine powder using stainless steel beads (SSB32, Next Advance) in a 1.5 mL Eppendorf Safe-Lock Tube in a Bullet Blender tissue homogenizer (Next Advance). A volume of 0.5 mL of 0.2M potassium phosphate buffer (pH 7.8; Carlo Erba reagents) containing 0.1 mM Ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich), 0.5% (w/v) Polyvinylpyrrolidone (PVPP, Sigma-Aldrich), and 1% Protease inhibitor cocktail (Sigma-Aldrich) to neutralize phenol effect (Zhang et al., 2007) were added to the sample. The tube then was centrifuged at 13,500 *g* at 4°C for 20 min, and an approximately 800 μL of the supernatant was transferred to a new 1.5 mL reaction tube (Greiner Bio-One) and stored at 4°C until being used for measuring protein content. Total protein contents were determined using the Bradford assay (Bradford, 1976) adapted for microplate readers using bovine serum albumin (BSA, Sigma-Aldrich) as the protein standard according to Tran et al. (2018).

Photosynthetic Functionality

The photophysiological effects of the heat wave treatment were assessed by measuring the effective quantum yield of photosystem II (PSII) measured as $\Delta F/F'_m = (F'_m - F/F_m)$ using pulse amplitude–modulated (PAM) chlorophyll fluorometer (Diving-PAM, Walz, Germany) as commonly applied in many previous studies (Ralph and Gademann, 2005; Saroussi and Beer, 2007; Winters et al., 2011). All PAM measurements were made midday, and taken underwater using the leaf distance clip on the base of the 2nd youngest leaf (n = 5 randomly chosen shoots at each time point from each population and treatment) and after removal of epiphytes. Irradiance levels at this position ranged between 130 and 150 μmol photons m^{-2} s^{-1} (measured with the micro quantum sensor, Apogee, United States).

Sampling for RNA, RNA Preparation, Primer Design, and Quantitative Real-Time PCR (qRT-PCR) Assays

Fully mature leaves (2nd youngest shoot; approximately 50–100 mg fresh weight) from each biological replicate were randomly collected on day 21, after 2 weeks of exposure to the heat-stress (T3), and day 28, at the end of the experiment (T4), 24 h after temperatures had returned to the control temperature (26°C) (**Figure 2B**). Samples were quickly cleaned from epiphytes and blotted dry using tissue paper, inserted into 1.5 mL Eppendorf Safe-Lock Tubes, and snap frozen immediately in liquid nitrogen before being stored in −80°C until extraction of RNA.

Total RNA was isolated from young fully mature leaves of *H. stipulacea* control (26°C) and heat treated (29 and 32°C) plant samples using the TRIzol method (2M guanidine thiocyanate, 4M ammonium thiocyanate, 3M sodium acetate,

38% phenol, 5% glycerol) (Kant et al., 2006). The total RNA quantity and quality were assessed with a NanoDrop spectrophotometer (ND-1000; NanoDrop Technologies) and its integrity was checked by running on a 1% agarose gel electrophoresis. Extracted total RNA was then treated with RNase-free DNase I (EN0525, Thermo Fisher Scientific) to eliminate genomic DNA. cDNA was synthesized from 1 μg of DNase I treated total RNA using the High-Capacity cDNA Reverse Transcription Kit (Catalog no. 4368813, Applied Biosystems) according to the manufacturer's instructions. The resulting cDNA was diluted 1:10 prior to quantitative real-time PCR (qRT-PCR) assays.

The expression of heat shock protein 70 (Hsp70), Superoxidase dismutase [Mn] (SOD), Photosystem II protein D1 (PsbA), and Photosystem II protein D2 (PsbD) genes were analyzed by qRT-PCR. The gene-specific primer pairs for Hsp70 and UBQ (Ubiquitin – Reference gene) were designed with Primer Express v2.0 (Applied Biosystems, United States) using the recently sequenced and annotated *H. stipulacea* transcriptome (Oscar et al., unpublished data). Ubiquitin has commonly been used in seagrass studies as a reference gene and has been shown to be stable in a several seagrass species exposed to different salinity, pH, light, and thermal conditions (Serra et al., 2012; Marín-Guirao et al., 2016). Primer sequences for SOD were taken from Winters et al. (2011), while primer sequences for PsbA and PsbD were taken from Marín-Guirao et al. (2016) (see **Table 1** for primer sequences).

Real-time PCR was performed with the ABI PRISM 7500 Sequence Detection System (SDS, Applied Biosystems) using SYBR Green to monitor double-stranded DNA (dsDNA) synthesis. Each reaction contained 5 μl PerfeCTa SYBR Green FastMix (Quanta Biosciences), 4 μl (20 ng) cDNA and 300 nM of gene-specific primer in a final volume of 10 μl. PCR amplifications were performed using the following conditions: 95°C for 30 s, 40 cycles of 95°C for 5 s (denaturation) and 60°C for 35 s (annealing/extension). Data were analyzed using the SDS 1.3.1 software (Applied Biosystems). To check the specificity of annealing of the primers, dissociation kinetics was performed at the end of each PCR run. The experiment was performed with three biological replicates and each reaction was performed in triplicates. The relative quantification values for each target gene were calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) using the Ubiquitin gene as an internal reference (Serra et al., 2012; Marín-Guirao et al., 2016). To ensure the validity of the $2^{-\Delta\Delta CT}$ method, twofold serial dilutions of cDNA were used to create standard curves. The amplification efficiency (E) values of the target and reference genes were shown to be approximately equal, with all $E > 0.91$, and all linear fit $R^2 > 0.95$ (Livak and Schmittgen, 2001).

Statistical Analysis

For plant performance, biochemical and photosynthetic functionality measurements, results from within each tank (biological replicates) were averaged and considered as one experimental replicate. In total, 5 experimental replicates (n = 5 aquaria) from each of these measurements were used for statistical analyses. For gene expression results, three experimental replicates (n = 3) were used to statistically determine the effects of treatments within each gene at each time-point.

GLM (general linear model)-repeated measures analyses were conducted on plant performance, biochemical and photosynthetic functionality results from each population separately using the statistical package IBM SPSS Statistics (v. 20) to test the overall effects of different temperatures (control 26°C, treatment 29°C, and treatment 32°C). Normality of data was tested using the Shapiro–Wilk test and homogeneity of variances were verified using Levene's (1960). When parametric assumptions were not met, data were transformed using the Johnson transformation (Johnson, 1949) by using the statistical analysis package SPC for Excel (BPI Consulting, LLC, v.5). Whenever a significant difference was detected, Tukey HSD's multiple comparison *post hoc* test was applied.

Effects of different experimental temperatures on each gene of interest (GOI) were analyzed separately for each population at each time-point using Pair-wise comparison test with Permutational Multivariate Analyses of Variance (PERMANOVAs) with Primer 6 v.6.1.16 and

TABLE 1 | Primer pairs used in *Halophila stipulacea* quantitative real-time PCR assays.

Gene name	Symbol	Function	Primer sequence	References
Ubiquitin	Ubi	Protein degradation (Housekeeping gene)	F 5′-CCTTGGTTCCAGAAATAGC-3′	Present study
			R 5′-GTTCAACCCATGGCATAC-3′	
Heat shock protein 70	Hsp70	Molecular chaperone	F 5′-ACCCTTATGACCGATGAG-3′	
			R 5′-CAGCCTGGTTCTTTGTATC-3′	
Superoxidase dismutase [Mn]	SOD	Antioxidant	F 5′-ATGGGTGTGGCTTGCTTA-3′	Winters et al., 2011
			R 5′-ATGCATGCTCCCATACATCT-3′	
Photosystem II protein D1	Psb A	The reaction center protein in PSII	F 5′-GACTGCAATTTTAGAGAGACGC-3′	Marín-Guirao et al., 2016
			R 5′-CAGAAGTTGCAGTCAATAAGGTAG-3′	
Photosystem II protein D2	Psb D	The reaction center protein in PS II	F 5′-CCGCTTTTGGTCACAAATCT-3′	
			R 5′-CGGATTTCCTGCGAAACGAA-3′	

Shown are the full and abbreviated (symbol) gene names, the function associated with this gene, the forward (F) and reverse (R) sequences of the primers used and the reference from which this primer sequence was taken from.

PERMANOVA + v.1.0.6 software package (PRIMER-E, Ltd.) (Anderson et al., 2008).

RESULTS

Environmental Conditions at the Two Sites

Comparisons between the long-term environmental conditions at the two sites that were based the Bio-ORACLE global dataset (Supplementary materials S1) demonstrated that salinity was very similar between the sites, with an annual average of 39.2 and 38.8 [Practical Salinity Scale (PSS)] for Cyprus and GoA sites, respectively (results not shown). Differences in minimal and maximal water salinity between the sites were even smaller (data not shown).

Long-term (1982–2017) analysis of daily SSTs from both sampled locations showed similar patterns in summer maxima. In both sites, highest temperatures were observed during August, with average water temperatures in August reaching 26.92 ± 0.02°C SE in Cyprus and 27.41 ± 0.02°C SE in the northern GoA (**Figures 3A,B**). During February, the coldest month in both locations, differences were much larger, with average water temperatures in Cyprus reaching 16.61 ± 0.01°C SE, while in the subtropical GoA, February temperatures did not go below 21.27 ± 0.01°C SE (**Figures 3A,B**).

In both sites, average warming rates over the multi-decade were significantly positive (regression analysis, $p < 0.05$), indicating that sea surface warming is taking place, but with some clear differences between the sites (**Figures 3C,D**). While in Cyprus both winter (February) and summer (August) warming trends were very similar (0.31 ± 0.17°C SE/decade and 0.32 ± 0.11°C SE/decade, $p < 0.05$, respectively; **Figure 3D**), in GoA August warming was more than double of that observed in February (0.46 ± 0.18°C SE/decade and 0.21 ± 0.15°C SE/decade, $p < 0.05$, respectively; **Figure 3C**). Seasonally detrended SST regression analysis continued to show that both sites have been warming significantly, although this data shows that warming has been faster in Cyprus (0.36 ± 0.06°C SE /decade, $p < 0.05$) compared with the northern GoA)0.26 ± 0.06°C SE /decade, $p < 0.05$) (**Figures 3E,F**).

Plant Performance

Results from the number of shoots throughout the experiment (**Figures 4A,B**) demonstrate the negative effects of increased water temperature on both native and invasive plants. Native plants suffered massive reductions in shoot number (**Figure 4A**) resulting in a significant difference between control plants and

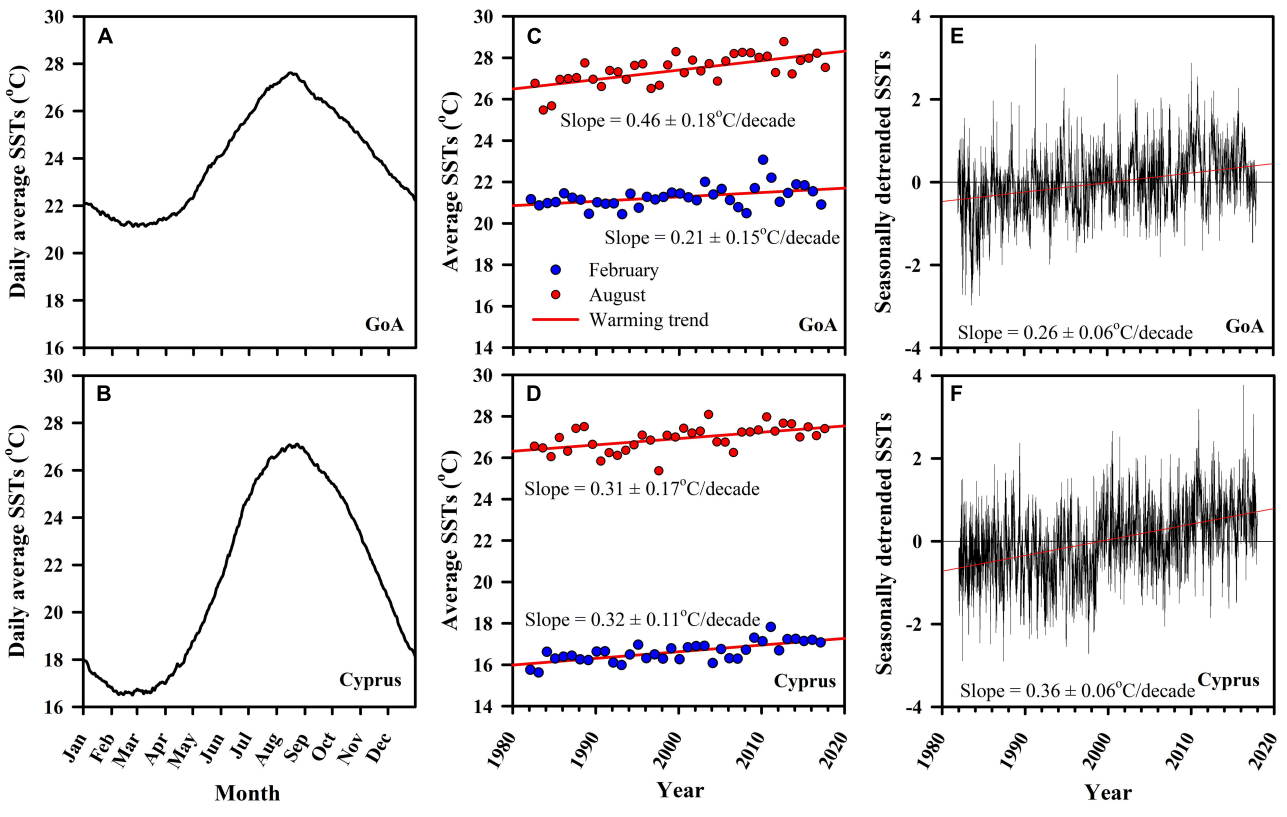

FIGURE 3 | Long-term monitoring of sea surface temperatures (SSTs) for both the native (GoA: **A,C,E**) and invasive (Cyprus: **B,D,F**) *H. stipulacea* collection sites for the period between 1982 and 2017 from NOAA dOISST.v2 dataset (www.ngdc.noaa.gov; Smith, 2016). Shown are **(A,B)** daily averages (Jan–Dec) of SSTs, **(C,D)** computed warming rates (°/decade) for February (winter; blue circles) and August (summer; red circles) with associated slopes of the linear regression, alongside the **(E,F)** seasonally detrended SSTs versus time with their associated slopes of the linear regression for the warming rates (°/decade).

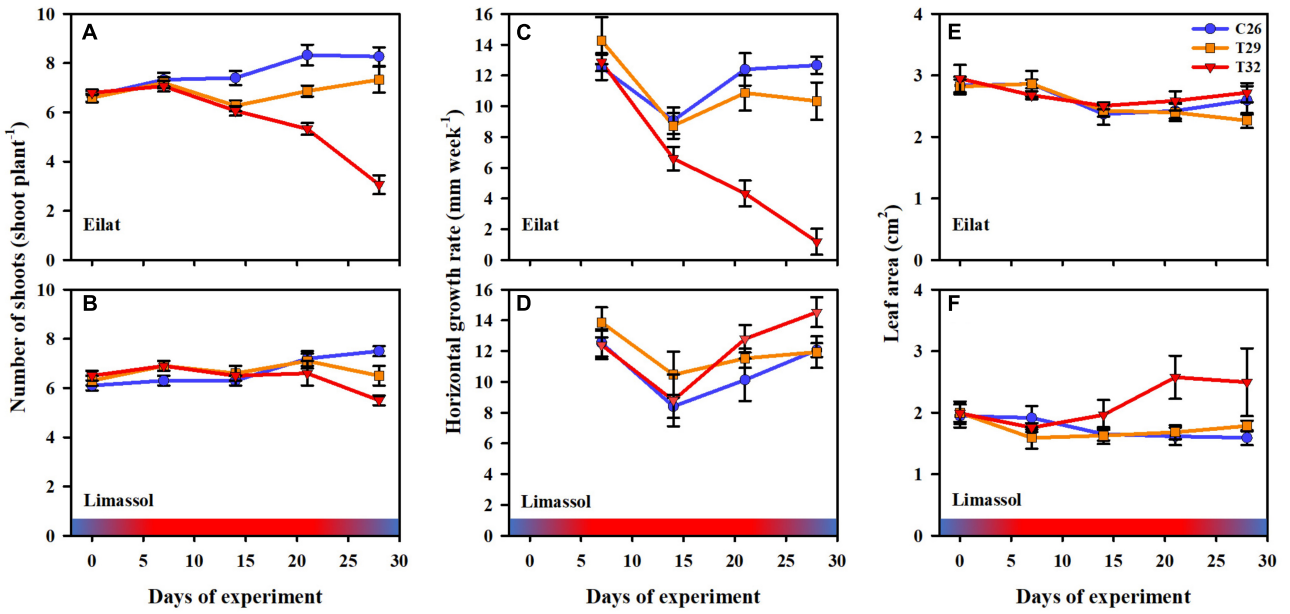

FIGURE 4 | Effects of a simulated heat wave on plant performance of native **(A,C,E)** vs. invasive **(B,D,F)** populations of *H. stipulacea*. Shown are changes over time (days of experiment) in the **(A,B)** number of shoots (shoot plant^{-1}), **(C,D)** horizontal growth rates (mm week^{-1}) and **(E,F)** leaf area (cm^2). In all parameters, results represent $n = 5$, mean ± SE. Gradient color bars indicate the experimental heat wave.

TABLE 2 | GLM-repeated measures analyses and Multiple comparisons with Tukey HSD *post hoc* results from different populations (Eilat vs. Limassol).

Population	Measurements	df	*F*	*p*	*Post hoc*	Multiple comparisons
Eilat	Chl *a* and *b*	2	10.992	**0.002**	Tukey HSD	C26 = T29 ≠ T32
Limassol	Chl *a* and *b*	2	1.406	0.283	Tukey HSD	C26 = T29 = T32
Eilat	Protein	2	1.478	0.267	Tukey HSD	C26 = T29 = T32
Limassol	Protein	2	15.337	**0.000**	Tukey HSD	C26 = T29 ≠ T32
Eilat	Number of shoots	2	24.567	**0.000**	Tukey HSD	C26 ≠ T29 ≠ T32
Limassol	Number of shoots	2	0.828	0.460	Tukey HSD	C26 = T29 = T32
Eilat	Horizontal growth	2	31.386	**0.000**	Tukey HSD	C26 = T29 ≠ T32
Limassol	Horizontal growth	2	1.107	0.362	Tukey HSD	C26 = T29 = T32
Eilat	Leaf area	2	1.402	0.284	Tukey HSD	C26 = T29 = T32
Limassol	Leaf area	2	5.362	**0.022**	Tukey HSD	C26 = T29, C26 = T32, T29 ≠ T32
Eilat	$\Delta F/F'_m$	2	20.806	**0.000**	Tukey HSD	C26 = T29 ≠ T32
Limassol	$\Delta F/F'_m$	2	0.341	0.717	Tukey HSD	C26 = T29 = T32

Significant difference ($p < 0.05$) are in bold and underlined. n = 5 biological replicates.

heated plants (GLM-repeated measures, $p < 0.001$, **Table 2**). Results from Tukey HSD *post hoc* multiple comparisons detected significant differences among the three experimental conditions, indicating that exposure to 29°C was already warm enough to stress the native plants significantly (C26 ≠ T29 ≠ T32). In contrast, while the number of shoots of the invasive plants decreased slightly over time when exposed to thermal stress (**Figure 4B**), no significant difference was detected with GLM-repeated measures or the Tukey HSD *post hoc* multiple comparisons, suggesting a higher thermal tolerance level of the invasive plants.

Similarly, increased water temperature seemed to have little or no effect on the growth rates of invasive plants as heated plants were able to maintain their growth rates similar or even slightly higher than growth rates of control plants (**Figure 4D**), compared with the exposure of the native plants to 32°C which dramatically slowed their growth rates (GLM-repeated measures, $p < 0.001$, **Figure 4C** and **Table 2**). Indeed, even during the recovery phase, native plants that were previously exposed to 32°C almost stopped growing (**Figure 4C**). Native plants that were exposed to 29°C heated plants, significantly reduced their number of shoots, but this reduction was much less than in the 32°C treatment (**Figure 4C**).

Results from the measurements of leaf surface area show big differences in the response to the increased water temperature. The fact that native leaves start bigger than their invasive counterparts corresponds to them being larger year-round also in the field (Nguyen et al., in review). In native

plants, no significant difference among treatments were found (**Table 2**). However, in the invasive plants, extreme increased water temperature of 32°C, surprisingly caused increases to their leaf sizes (**Figure 4F**). Indeed, significantly larger leaves were observed in 32°C heated invasive plants (GLM-repeated measures, $p = 0.022$ and Tukey HSD *post hoc* Multiple comparisons: C26 = T29, C26 = T32, T29 ≠ T32, **Table 2**). This phenomenon is actually in correspondence with the growth rate results (**Figure 4D**) strengthening the hypothesis about a higher thermal tolerance of the invasive plants in comparison to their native counterparts.

Biochemistry

Chlorophyll *a* and *b* contents were similar in the two populations at the beginning of the experiment (T0, approximately 0.28 mg g FW^{-1}; **Figures 5A,B**). Results (**Figure 5**) demonstrate that the 29°C heated plants (native and invasive) maintained their biochemical contents (i.e., chlorophyll *a*, chlorophyll *b* and proteins) similar to their control plants. Indeed, in both populations, the GLM-repeated measures and Tukey HSD *post hoc* multiple comparisons (**Table 2**) showed no differences between the chlorophyll *a*, chlorophyll *b* and proteins contents of control vs. 29°C treatment (C26 = T29, **Table 2**).

On the other hand, the responses of native and invasive plants to the 32°C warming scenario showed big differences from T2 onwards (**Figures 5A,B**). While under stress of 32°C chlorophyll *a* and *b* contents degraded significantly in the native plants (from 0.28 to 0.20 mg g FW^{-1}, **Figure 5A**; GLM-repeated measures $p = 0.002$, Tukey HSD *post hoc* multiple comparisons C26 = T29 ≠ T32, **Table 2**), in the invasive plants, chlorophyll *a* and *b* content increased (from 0.23 to 0.50 mg g FW^{-1}, **Figure 5B**), but this increase was found to be non-significant (GLM-repeated measures $p = 0.283$, Tukey HSD *post hoc* multiple comparisons C26 = T29 = T32, **Table 2**).

A similar result was evident from the protein contents (**Figures 5C,D**). In the native plants, there was a strong trend of reduction in protein content of plants exposed to the 32°C warming scenario (GLM-repeated measures, $p = 0.267$, Tukey HSD *post hoc* multiple comparisons, C26 = T29 = T32, **Table 2**), indicating a possible thermal threshold response.

In contrast, compared to 26 and 29°C treatments, plants from the Mediterranean actually increased significantly their protein contents over time when exposed to the 32°C warming scenario (GLM-repeated measures, $p < 0.001$, Tukey HSD *post hoc* multiple comparisons, T26 = T29 ≠ T32, **Figure 5D** and **Table 2**).

Photosynthetic Functionality

In terms of photosynthetic functionality, results demonstrate differences between the response of native and invasive populations of *H. stipulacea* to the extreme thermal stress. At first, as observed in both populations, increased water temperature

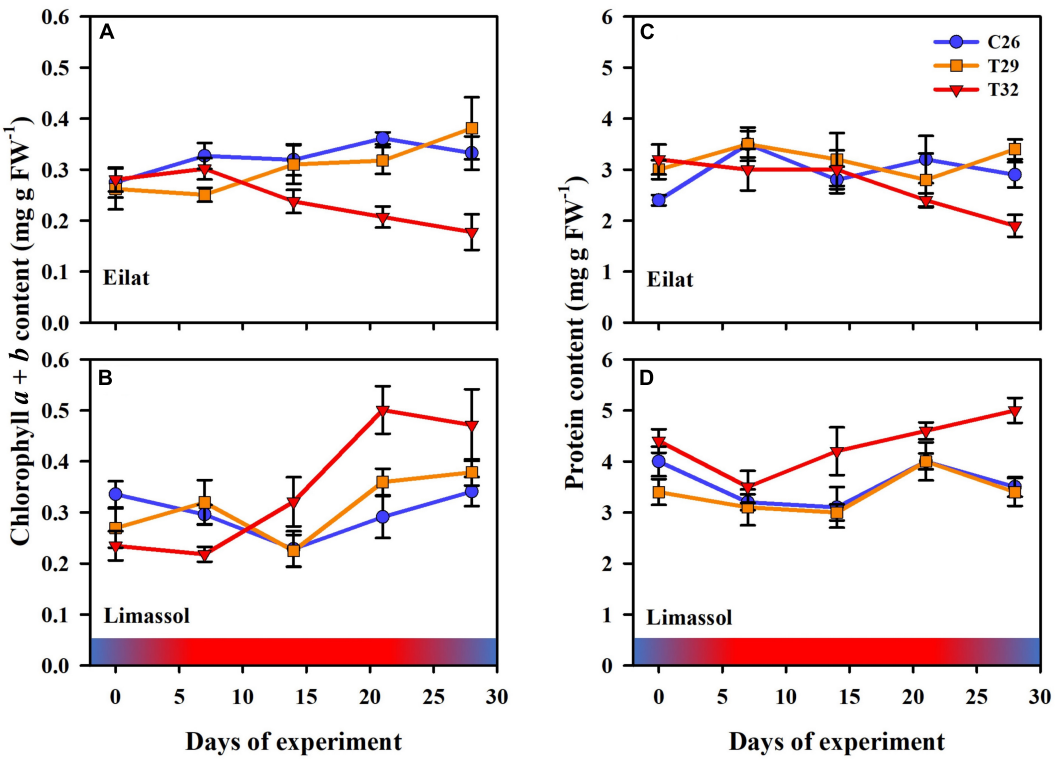

FIGURE 5 | Effects of a simulated heat wave on plant biochemical indicators of native **(A,C)** vs. invasive **(B,D)** populations of *H. stipulacea*. Shown are changes over time (days of the experiment) in the **(A,B)** chlorophyll *a* and *b*, and **(C,D)** protein contents. In all parameters, results represent $n = 5$, mean ± SE. Gradient color bars indicate the experimental heat wave.

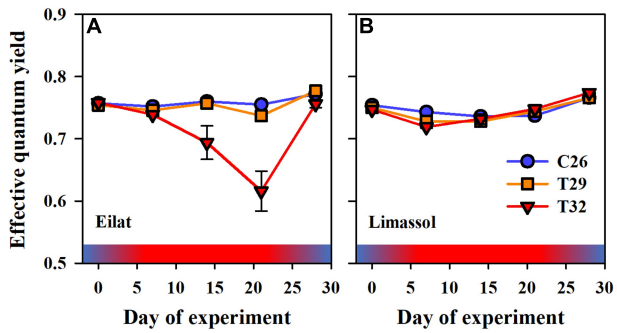

FIGURE 6 | Effects of a simulated heat wave on plant photosynthetic functionality of native **(A)** vs. invasive **(B)** populations of *H. stipulacea*. Shown are changes over time (days of experiment) in the effective quantum yield $\Delta F/F'_m$. In all parameters, results represent $n = 5$, mean ± SE. Gradient color bars indicate the experimental heat wave.

to 29°C did not result in any significant difference between heated plants in comparison to control ones (**Figures 6A,B**). Interestingly, while the extreme thermal condition (i.e., 32°C) lowered significantly the effective quantum yield in native plants (**Figure 6A**, GLM-repeated measures, $p < 0.001$, Tukey HSD *post hoc* multiple comparisons, T26 = T29 ≠ T32, **Table 2**), oppositely it had no negative effect on the invasive plants (**Figure 6B**). This contrasting phenomenon suggests a clear difference in thermal tolerance between the two populations.

Gene Expression

Gene expression results from this study provide the very first insight into some of the molecular responses of *H. stipulacea* to thermal stress. Results include the expression of three different gene categories including a molecular chaperone that is expressed in response to stress (HSP70), photosynthesis related-genes (psbD and psbA) and an antioxidant related-gene (SOD; **Figure 7**).

All studied genes were significantly influenced by population, treatment or their interactions (**Table 3**). Overall, expression patterns were lower under moderate stress (29°C) as compared to the more severe thermal stress of 32°C (**Figure 7**). Native plants expressed significantly more HSP70 during the stressful condition (T3) both at 29°C and significantly more at 32°C (**Figure 7A**), compared with the invasive plants where exposure to both temperatures did not cause a significant upregulation of HSP70 (**Figure 7C**). Returning plants from both populations to control temperatures at T4 entailed a shift to down-regulation of HSP70, with plants previously exposed to 32°C having significantly stronger down-regulation compared with control plants (**Figures 7A,C**; Pair-wise comparison, $p < 0.001$, **Table 3**). Down-regulation of plants previously exposed to 32°C was stronger in native compared with invasive plants (**Figures 7B,D**).

During the heat stress condition (T3), both native and invasive plants showed up-regulation of psbD and down-regulation of psbA (**Figures 7A,C**). The up-regulation of psbD was significant only for the invasive plants (**Figures 7A,B**, Pair-wise comparison, $p < 0.001$, **Table 3**). When water temperatures returned to the control level (**Figures 7B,D**), the 32°C invasive heated-plants shifted their expression from a very weak down-regulation during the heat stress itself, to a significant upregulation of psbA (**Figure 7D**, Pair-wise comparison, $p < 0.001$, **Table 3**). No change was found in the expression pattern of the native plants (**Figure 7B**).

Lastly, thermal-induced expression of the antioxidant-related gene (SOD) concurs with other genes showing the invasive plants were more responsive to increased water temperature. Both native and invasive plants significantly up-regulated SOD to compensate with the increased damage caused by thermal stress (**Figures 7A,C**), with a much stronger up-regulation found for the invasive population. Returning plants to control water temperatures (T4) resulted in reversing the upregulation of SOD found during the heat wave (T3, **Figures 7A,C**) to a significant down-regulation during of SOD in T4 (**Figures 7B,D**). In both populations, there was a stronger down-regulation of SOD during T4 in plants that were previously exposed to the higher temperatures (32°C). The fact that this downregulation of SOD was only significant in the invasive plants (**Figure 7D**) indicates that the invasive plants might be more thermally plastic than the native ones.

DISCUSSION

Invasiveness of plants may be accompanied by rapid evolution due to selection in the colonized habitat (examples from land plants – Maron et al., 2004; Novy et al., 2013; Sultan et al., 2013). Here we demonstrate that plants of invasive seagrass population may have undergone an additional adaptation to the changes in the colonized habitat. Plants of *H. stipulacea* from the invasive population performed overall better than plants from the native population in a common garden simulated thermal stress experiment. We show that while native plants were negatively affected in photo-physiological and growth responses by thermal stress, invasive plants did not suffer and might have even benefited from it. We also show that the putative mechanism of adaptation to thermal stress could be the differential gene expression. Taken together, these results hint toward rapid evolution of *H. stipulacea* plants following their Lessepsian migration through the Suez Canal. This process could possibly work through strong selection on the colonizing genotypes during (e.g., the warm the ballast water of ships) or after their transport (post-introduction evolution – through exposure to many heat waves). Such a process would help to maximize the performance and establishment of the invasive population in their new environment (Molina-Montenegro et al., 2018).

Our results support the hypothesis that climate change amplifies the spread of invasive plants (Bradley et al., 2010; Zelikova et al., 2013). The long-term analysis of daily SST concurs with previous studies in the GoA (Fine et al., 2013) and the eastern Mediterranean Sea (Rilov, 2016; Ozer et al., 2017). In both of our studied sites, average SST warming during the last 35 years was higher than the global mean coastal SST trend of 0.17 ± 0.11°C/decade (Liao et al., 2015). In the GoA, the temperature has been warming at a rate 1.63 times faster than

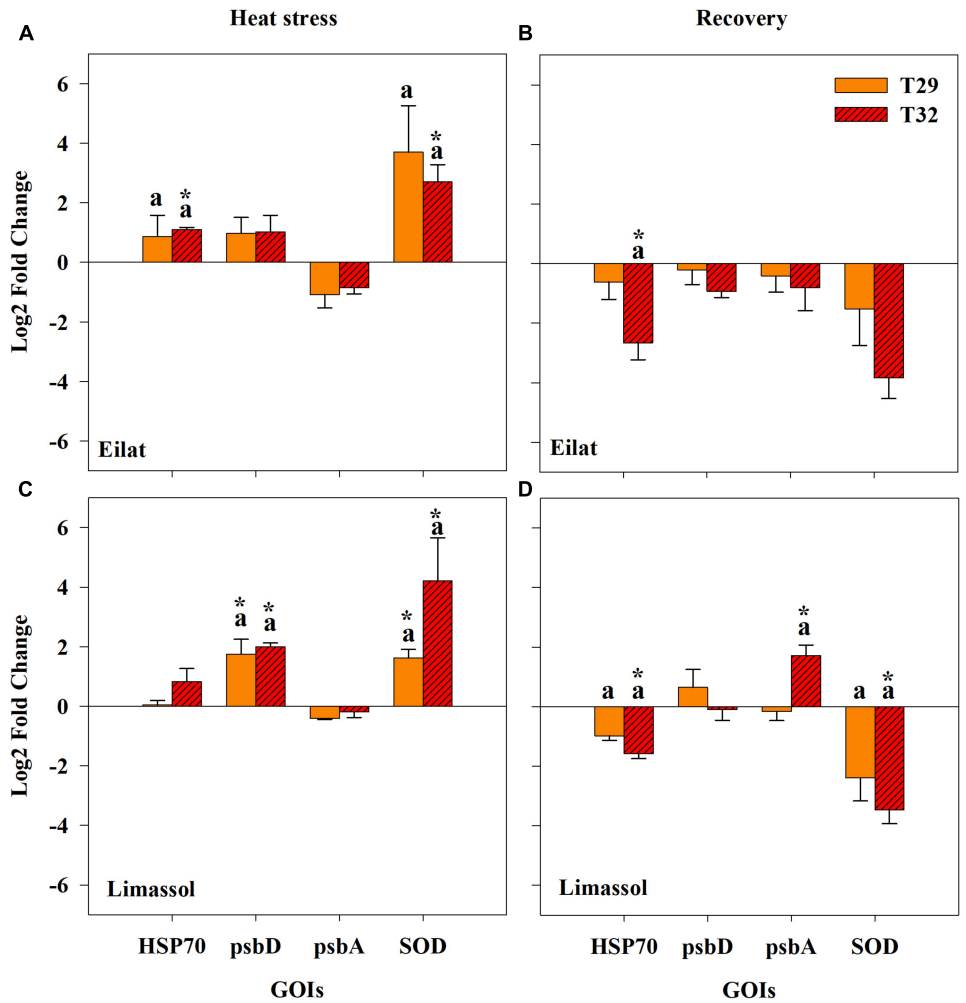

FIGURE 7 | Effects of a simulated heat wave on plant gene expression (log2 scale) in native **(A,B)** and invasive **(C,D)** populations of *H. stipulacea* plants ($n = 3$; mean ± SE). Results of pair-wise comparison tests are presented on top of each column: an asterisk (*) indicates significant differences between control vs. heated plants ($p < 0.001$) and letters represent differences between 29 and 32°C-heated plants. Shown are expression patterns during the middle of the heat stress (T3; **A,C**) and following 24 h after the return to the control temperature (T4; **B,D**). Gene abbreviations are found in **Table 1**.

this global coastal average, but in Cyprus, warming has been occurring at even faster rates, 2.25 times faster than this global coastal average SST trend.

Under assault for at least a century, seagrasses may be nearing a crisis with respect to global sustainability (Waycott et al., 2009; Hyndes et al., 2016). In addition to increasing mean temperatures (Jordà et al., 2012), seagrass ecosystems will also face increasing climate variability that will affect the frequency, duration and intensity of summer heat waves (Schär et al., 2004; Diez et al., 2012; Oliver et al., 2018), with a projected longer duration of warm periods (Jordà et al., 2012; Oliver et al., 2018). Furthermore, these global climatic changes have been predicted to exacerbate invasions (Buckley and Csergő, 2017).

The results from our microcosm experiment suggest that native and invasive populations of *H. stipulacea* might differ in their evolutionary trajectories under future projections of climate changes. Plants from the native population of GoA showed signs of stress during and after the exposure to high temperatures (i.e., 32°C) in most of the measured parameters: they lost high numbers of shoots per plants (**Figure 4A**), reduced their growth rates (**Figure 4C**) and leaf sizes (**Figure 4E**). The plants from the invasive population of Cyprus, on the other hand, kept the number of shoots constantly (**Figure 4B**), their growth rates were similar to control plants (**Figure 4D**), and surprisingly doubled their leaf sizes under the same high temperature of 32°C; these results concur with those of Georgiou et al. (2016) that also showed increased growth rates of *H. stipulacea* from Cyprus exposed to increased water temperatures. Biochemical assays further suggested that GoA plants suffered at simulated high temperature and could not be able to maintain their chlorophylls as well as their protein contents (**Figures 5A,C**). In contrast, exposure to thermal stress caused Cyprus plants to increase significantly their photosynthetic pigments and protein (**Figures 5B,D** and **Table 2**). Application of PAM fluorometry

TABLE 3 | Pair-wise comparison results on ΔCt data of genes of interests (GOIs).

Population	GOI	Groups	T3			T4			Pair-wise comparison	
			t	$p_{(perm)}$	Unique perms	*t*	$p_{(perm)}$	Unique perms	T3	T4
Eilat	HSP70	C26, T29	0.96857	0.3955	10	0.35353	0.5008	10	C26 = T29, T29 = T32, C26≠T32	C26 = T29≠T32
		C26, T32	2.786	**0.0001**	10	1.8645	**0.0001**	10		
		T29, T32	0.8154	0.4036	10	1.9625	**0.0001**	10		
	psb D	C26, T29	1.0957	0.4996	10	0.22217	0.8006	10	C26 = T29 = T32	C26 = T29 = T32
		C26, T32	1.1073	0.4119	10	1.1669	0.3063	10		
		T29, T32	0.10778	0.8018	10	1.4035	0.2912	10		
	Psb A	C26, T29	1.574	0.1028	10	0.70632	0.7049	10	C26 = T29 = T32	C26 = T29 = T32
		C26, T32	1.7734	0.0964	10	0.85223	0.507	10		
		T29, T32	0.56742	0.596	10	0.3809	0.9	10		
	SOD	C26, T29	1.9009	0.1016	10	0.46151	0.3964	10	C26 = T29, T29 = T32, C26≠T32	C26 = T29 = T32
		C26, T32	3.2119	**0.0001**	10	1.7064	0.0963	10		
		T29, T32	0.73228	0.61	10	1.6567	0.1013	10		
Limassol	HSP70	C26, T29	0.3616	0.8061	10	1.1231	0.2895	10	C26 = T29 = T32	C26 = T29, T29 = T32, C26≠T32
		C26, T32	1.5214	0.2037	10	1.8723	**0.0001**	10		
		T29, T32	1.6395	0.0971	10	2.6359	0.1018	10		
	psbD	C26, T29	2.0624	**0.0001**	10	1.0817	0.3031	10	C26≠T29 = T32	C26 = T29 = T32
		C26, T32	3.1588	**0.0001**	10	0.2165	0.7953	10		
		T29, T32	0.65765	0.6019	10	1.0192	0.2948	10		
	psbA	C26, T29	1.4273	0.2965	10	0.58878	0.4973	10	C26 = T29 = T32	C26 = T29≠T32
		C26, T32	0.47392	0.6903	10	4.2527	**0.0001**	10		
		T29, T32	1.1579	0.3033	10	3.4207	**0.0001**	10		
	SOD	C26, T29	3.0314	**0.0001**	10	1.0241	0.496	10	C26≠T29 = T32	C26 = T29, T29 = T32, C26≠T32
		C26, T32	2.0587	**0.0001**	10	1.9148	**0.0001**	10		
		T29, T32	1.7459	0.0984	10	1.0689	0.4032	10		

Significant differences ($p < 0.05$) are in bold and underlined.

strengthens our conclusions as significant reduction in ΔF/Fm′ was only found for native plants (**Figure 6**).

The results from this study suggest that native and invasive *H. stipulacea* plants might underwent different evolutionary pathways, and potentially will further diverge in their evolutionary trajectory given that the current trends of increased sea temperatures continue. While the plants from the northern GoA suffered greatly from simulated increases in seawater temperatures, the plants from the invasive population from the eastern Mediterranean did not suffer physiologically and seemed to take advantage of simulated seawater warming.

We speculate that if these plants are representative of larger populations, future increase in seawater temperature will threaten the native populations and benefit the invasive ones. Further exploration of this prediction should use multiple populations from both seas, in order to assess the width of the phenomenon found here in representing plants from one site in each sea.

Although It has been suggested that the range of expansion of *H. stipulacea* in the Mediterranean will be limited by the 15°C sea surface isotherm experienced during winter seasons (reviewed by Gambi et al., 2009), our results are in agreement with the those of Georgiou et al. (2016) that predicted that the rising winter and summer temperatures in the Mediterranean Sea (Ozer et al., 2017) will not be a limiting factor of the expansion of *H. stipulacea* in the Mediterranean Sea.

The work presented here, alongside that of Georgiou et al. (2016) represent the only two studies that experimentally investigated the effects of temperature on *H. stipulacea*, shedding light on the gap of knowledge in this ecologically important species compared with the numerous published studies on the effects of thermal stress on both temperate and tropical species (Bergmann et al., 2010; Winters et al., 2011; Tutar et al., 2017; George et al., 2018).

Our gene expression results provide some very first molecular knowledge regarding the effects of temperature on *H. stipulacea.* Changes in the expression level of heat shock protein (HSP70) in our study shed light on some of the molecular mechanisms behind the physiological differences in our experimental plants. Increases in expression of HSP70 as a result of thermal stress were also shown in other seagrass species including *Zostera marina*, *Posidonia oceanica,* and *Cymodocea nodosa* (Bergmann et al., 2010; Marín-Guirao et al., 2016). The increase in expression of HSP70 in *H. stipulacea* plants from the invasive population under

simulated increased temperature, suggests that invasive plants might have mechanisms in place to cope with sea warming. The significantly higher expression levels of SOD in the invasive population could give these plants the protection from reactive oxygen species (ROS) needed to continue normal photosynthesis under thermal stress (Lesser, 1997; Sharkey, 2005). The fact that psbA (the chloroplast gene that codes for the photosystem II protein D1) tends in both populations to be only weakly down-regulated during the thermal stress, might indicate the possibility that photosystem II was not the main site of damage of the heated plants.

While we did not include genotyping data in this current study, a recent study on reconstructing the invasion history of the *H. stipulacea* applied 2bRAD sequencing to several populations, including the two populations used here (Chiquillo et al., unpublished data). Results from that study clearly demonstrate that genetic diversity in the Mediterranean Sea is actually lower than that in the GoA. The fact that the invasive Cyprus *H. stipulacea* population, with lower genetic diversity, did better under thermal stress than its native GoA higher diversity counterpart (Chiquillo et al., unpublished data), does not concur with those of Ehlers et al. (2008) that showed that *Zostera marina* populations with higher genotypic diversity survived thermal stress better than populations with reduced genotypic diversity. One possible explanation for this could be a high pre-existing genetic diversity in the native GoA range (as shown by Chiquillo et al., unpublished data) and the later filtering of genotypes that could be locally pre-adapted in the new invasive range to climate change (Molina-Montenegro et al., 2018).

With the recent doubling of Suez Canal (Galil et al., 2015, 2017) and the ongoing tropicalization and warming of the Mediterranean (Bianchi and Morri, 2003; Borghini et al., 2014) a process that is happening even faster in the eastern Mediterranean (Ozer et al., 2017), *H. stipulacea* could potentially become more prevalent in these waters in the coming years. This prediction is quite probable considering that conditions in the Mediterranean Sea are becoming less favorable for its temperate seagrass species (Jordà et al., 2012; Chefaoui et al., 2018; Marín-Guirao et al., 2018; Savva et al., 2018) and more welcoming to tropical species (Sghaier et al., 2014; Georgiou et al., 2016; Gerakaris et al., 2019).

In the Caribbean Sea, *H. stipulacea* has been demonstrating its negative characteristic by invading the entire Caribbean (Willette and Ambrose, 2009, 2012; Maréchal et al., 2013; Vera et al., 2014; Willette et al., 2014; Steiner and Willette, 2015; van Tussenbroek et al., 2016; Scheibling et al., 2018). Studies in the Caribbean have shown that *H. stipulacea* is physically displacing local Caribbean seagrass species (e.g., *Syringodium filiforme*, *Halophila decipiens,* and *Halodule wrightii*; Willette and Ambrose, 2012), thus changing the Caribbean's seagrass landscape (Steiner and Willette, 2015). Taking into account what is already happening in the Caribbean Sea, combined with our knowledge on the changing Mediterranean Sea (Jordà et al., 2012; Rilov, 2016; Ozer et al., 2017; discussed above), our results presented here demonstrate clearly that the spreading of *H. stipulacea* in the Mediterranean Sea has a high potential threat to biodiversity of the Mediterranean region.

While the spread of *H. stipulacea* in the Mediterranean Sea is worrying, there is also a threat to the native sub-tropical populations in the GoA. The National Monitoring Program at the Gulf of Eilat (NMP-Israel) has been collecting high-resolution data of local marine ecosystems since 2004[2], but most of this program's efforts have been focused on the coral reefs in the region, while local *H. stipulacea* meadows have only very recently been included in this program.

Results from our study highlight the importance of long-term, coordinated monitoring of this important engineering species, in both native and invasive populations of *H. stipulacea*. It should be noted that we used only one population from each basin. While running simulated thermal stress experiments with multiple temperatures and populations is complicated enough (3 temperatures × 2 populations in the present study), including more populations in such experiments would be necessary in the future to account for the genetic variability (Procaccini et al., 1999) and different thermal tolerances that might exist in *H. stipulacea* populations in both the Mediterranean and the Red Sea. Thus, we call for further studies to include multiple populations from each basin to reconfirm the results presented here.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

HN, YS, and GW conceived and designed the experiments. HN, NY, and SB performed the experiments. HN and GW analyzed the results. HN, FL, YS, and GW wrote the manuscript. All authors reviewed the manuscript.

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[2] http://www.iui-eilat.ac.il/Research/NMPAbout.aspx

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Stress Memory in Seagrasses: First Insight Into the Effects of Thermal Priming and the Role of Epigenetic Modifications

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While thermal priming and the relative role of epigenetic modifications have been widely studied in terrestrial plants, their roles remain unexplored in seagrasses so far. Here, we experimentally compared the ability of two different functional types of seagrass species, dominant in the Southern hemisphere, climax species *Posidonia australis* and pioneer species *Zostera muelleri*, to acquire thermal-stress memory to better survive successive stressful thermal events. To this end, a two-heatwave experimental design was conducted in a mesocosm setup. Findings across levels of biological organization including the molecular (gene expression), physiological (photosynthetic performances and pigments content) and organismal (growth) levels provided the first evidence of thermal priming in seagrasses. Non-preheated plants suffered a significant reduction in photosynthetic capacity, leaf growth and chlorophyll *a* content, while preheated plants were able to cope better with the recurrent stressful event. Gene expression results demonstrated significant regulation of methylation-related genes in response to thermal stress, suggesting that epigenetic modifications could play a central role in seagrass thermal stress memory. In addition, we revealed some interspecific differences in thermal responses between the two different functional types of seagrass species. These results provide the first insights into thermal priming and relative epigenetic modifications in seagrasses paving the way for more comprehensive forecasting and management of thermal stress in these marine foundation species in an era of rapid environmental change.

Keywords: seagrasses, thermal priming, gene expression, *Posidonia australis*, *Zostera muelleri*, epigenetic

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INTRODUCTION

Plants, as sessile organisms, have developed sophisticated mechanisms to efficiently respond to environmental changes as they cannot quickly escape from potentially stressful conditions. Some of these mechanisms are included within the concept of stress memory, which is defined as the capacity of plants experiencing recurrent stress to "remember" past stressful events and prepare to respond in a better way when stressful conditions occur again (Bruce et al., 2007). Many terrestrial plants exposed to cyclic or episodic perturbations have shown increased tolerance when stress recur,

a response referred to as hardening, priming, conditioning or acclimation (Zwiazek, 1991; Goh et al., 2003; Biber et al., 2009). This phenomenon includes by stress-induced structural, genetic and biochemical modifications that may lead to phenotypes with increased resistance (Baldwin and Schmelz, 1996; Bruce et al., 2007; Jaskiewicz et al., 2011; Yakovlev et al., 2011). It has been suggested that stress memory can last from several days to months and even years, and in some cases, it can be transmitted to the next generation (Baldwin and Schmelz, 1996; Iqbal and Ashraf, 2007; Rendina González et al., 2018).

Understanding of plant priming, including the length of plant stress memory as well as the mechanisms involved, remains largely unknown (Crisp et al., 2016). Molecular modifications are recognized as major mechanisms underlying stress memory in plants via activating, enhancing or speeding up responses to coping with environmental stressors (Crisp et al., 2016). Studies dealing with multiple stressors have also discovered an increasing number of epigenetic mechanisms responsible for the formation of stress memory in plants (Kinoshita and Seki, 2014; Dodd and Douhovnikoff, 2016; He and Li, 2018). Epigenetic modifications can alter gene expression without changing the underlying DNA sequence and occur in the form of DNA methylation, histone modifications and non-coding micro RNAs (Bossdorf et al., 2008; Bonasio et al., 2010). Epigenetic variations have the potential to increase phenotypic plasticity and accelerate adaptation to recurring stressful conditions (Verhoeven et al., 2016; Richards et al., 2017). DNA methylation is the most frequently studied and best understood epigenetic mechanism in plants. Several studies have revealed that environmental stress can result in an increase or decrease in cytosine-methylation throughout the genome and at specific loci to mediate environmentally-responsive and stress-responsive gene expression (Wada et al., 2004; Yaish et al., 2011; Dowen et al., 2012; Greco et al., 2013; Secco et al., 2015).

Seagrasses are a unique group of marine plants that have colonized the marine environment for thousands of kilometers of the sedimentary shorelines from sub-Artic to tropical regions over the past 60–90 million years ago (Les et al., 1997; Short et al., 2007). As foundation species of coastal ecosystems, seagrasses fulfill important ecosystem services including sediment stabilization, coastal protection, nutrient cycling, water quality improvement, fishery maintenance, and carbon sequestration, among others (Orth et al., 2006; Fourqurean et al., 2012; Nordlund et al., 2016). Despite their crucial functional role in the Earth ecosystem, seagrass meadows are declining due to rapid environmental changes driven by human activities (Orth et al., 2006; Waycott et al., 2009). Data from numerous studies across the globe have shown that seagrasses were disappearing worldwide at a rapid rate of 110 km^2 per annum between the period of 1980 and 2006, which resulted in a loss of 29% of the total world seagrass population (Waycott et al., 2009). Indeed, ten seagrass species (∼14%) have already been listed at risk of extinction, while three species have been listed as endangered (Short et al., 2011). Some seagrass species are even predicted to go extinct by the end of this century, as is the case of the Mediterranean endemic *Posidonia oceanica*, as a consequence of warming trends and extreme oceanic events (Marbà and Duarte, 2010; Chefaoui et al., 2018). The situation is expected to worsen as a consequence of ongoing climate change (Waycott et al., 2009; Arias-Ortiz et al., 2018). One of the consequences of climate change in the marine environment is the ocean warming, a gradual increase in the mean of seawater temperature. However, climate change also gives way to extreme oceanic events (i.e., marine heatwaves), which have become conspicuous in the last few decades (Oliver et al., 2017, 2018). Marine heatwaves (MHW) are generally defined as extreme warm periods that last for at least 5 days with a level of temperature exceeding the 90th percentile, based on a three-decade historical baseline database (Hobday et al., 2016). In general, organisms have a lower capacity to overcome abrupt stress events rather than progressive ones. Thus, these extreme MHW may cause deleterious impacts on marine organisms that can result in shifts in species distributions and even local extinction (Easterling et al., 2000). The situation is predicted to worsen in the future with increasing evidence of more frequent, intense and longer-lasting MHW (Meehl and Tebaldi, 2004; Oliver et al., 2018; Darmaraki et al., 2019a). Indeed, a massive die-off of seagrass meadows has been reported after recent MHW, and in some cases had vast environmental consequences as the enormous amount of carbon dioxide stored in thousands of hectares of seagrass meadows were then released back to the atmosphere (Seddon et al., 2000; Arias-Ortiz et al., 2018).

As shown in terrestrial plants, epigenetic modifications and stress memory have the potential to provide responsive and adaptive mechanisms in seagrasses in order for them to withstand environmental changes (Davey et al., 2016; Duarte et al., 2018). As clonal plants, seagrasses provide a great opportunity to study the effects of epigenetics without concern about genetic variation. Nevertheless, our knowledge regarding the role of epigenetic modifications and stress memory remains unknown in seagrasses with only some experimental hints from transcriptomic studies (Marín-Guirao et al., 2017; Duarte et al., 2018; Marín-Guirao et al., 2019).

In an era of rapid global ocean changes, it is critical to better understand mechanisms driving seagrass thermal stress response in order to make timely decisions regarding seagrass conservation and management activities. Increasing our knowledge about the role of epigenetic modifications and stress memory can improve our recent predictions about the future of seagrasses (Marbà and Duarte, 2010; Jordà et al., 2012), enhancing our efforts to protect seagrasses worldwide. In this study, we simulated a scenario that will become more extreme and frequent in the future by conducting a two-heatwave experimental design for two Southern hemisphere seagrass species with different functional traits, *Posidonia australis* and *Zostera muelleri*. We hypothesized that plants pre-exposed to a stressful thermal event perform better and are less affected by subsequent heat stress than non-pre-heated plants. Plant responses were examined at different hierarchical levels including morphology, photo-physiology and gene expression in order to assess heat-stress induced priming effects on the two seagrass species. Regarding gene expression, special attention was paid to the response of methylation-related genes to explore the potential involvement of epigenetic modifications on seagrass heat-stress memory. A comparison between pioneer species with high morphological

plasticity (*Z. muelleri*) and climax species with more stable and long-lived characteristics (*P. australis*) could help us to forecast the persistence of more or less stable communities under the future climate change scenarios.

MATERIALS AND METHODS

Sample Collection

Fragments of *P. australis* and *Z. muelleri*, bearing several connected shoots were collected haphazardly at Port Stephens (PS) New South Wales (NSW), Australia (32°43′07.4″S 152°10′35.9′E) on the 19th of March 2019 and at Church Point (CP) NSW, Australia (33°38′46.8″S 151°17′11.9″E) on the 23rd of March 2019, respectively. Plant fragments were collected at a reciprocal distance >25 m in order to reduce the likelihood of sampling the same genotype twice. Both species were collected during low tide in shallow water (∼70 cm), then plant fragments were transported immediately to the seagrass mesocosm facility at the University of Technology Sydney (UTS). Environmental conditions including salinity and water temperature at PS and CP were measured at the same time as plant collection to mimic the natural conditions at the mesocosm facility at UTS. Water temperature was ∼25°C at both sites while the salinity was slightly higher at PS (34.1 ppt) than at CP (33.0 ppt). Rapid light curves were performed with a diving-PAM fluorometer (Walz GmbH, Germany) on three random plants at each site to define experimental light levels. These analyses showed that the saturating irradiance levels of plants in the field were approximately 350 μmol photons m^{-2} s^{-1} for both *P. australis* and *Z. muelleri* plants.

Experimental Design

Once at UTS, plant fragments of both species with a similar number of shoots (i.e., 8–10 shoots) were carefully selected, individually planted into plastic trays filled with mini pebbles and randomly allocated in tanks of the mesocosm facility (three fragments per tank). In total, six aquaria were used for each species, 60-L aquaria for *P. australis* and 40-L aquaria for *Z. muelleri*. For each species, three experimental treatments including control (CT), treatment 1 heatwave (1HW) and treatment 2 heatwave (2HW) were conducted in parallel. Thus, for each treatment, two aquaria were considered as experimental replicates while six trays (fragments) were treated as biological replicates. Each aquarium was equipped with an independent light source (Hydra FiftyTwo HD^{TM}, C2 Development, United States), two 55W-heaters and air and water pumps to maintain circulation and homogeneity of seawater temperature. For both species, the irradiance level was set at 350 μmol photons m^{-2} s^{-1} at canopy height according to the saturating levels of plants from the fields (mentioned above) with a 12 h:12 h light:dark period. Light cycle started from 7:30 a.m., with light levels progressively increasing to the maximum irradiance at 12:30 p.m. and kept for 2 h, before a progressive reduction until dark at 7:30 p.m. Water temperature was measured automatically every 30 min using iButton data logger (iButtonLink, United States) and manually checked twice a day using a digital thermometer (FLUKE 52II, United States). Throughout the experiment, purified water was added periodically to maintain the salinity level of 34 ppt and approximately 1/3 of seawater from each aquarium was renewed weekly to keep water quality consistent.

Water temperature was kept at 26°C (∼1°C above the temperature in natural conditions at the time of the experiment[1]) in all aquaria during a 2-week acclimation period (**Figures 1A,B**). Temperature was subsequently increased to 29°C (heating rate 1°C day^{-1}) in two aquaria of 2HW of each species and maintained for 6 days to simulate a MHW. Water temperature in these heated tanks was then reduced to control levels to allow heated plants to re-acclimate during a 1-week period before simulating a second, more intense and longer-lasting MHW (32°C for 9 days; heating rate 1°C day^{-1}). This second MHW was applied to four aquaria of each species, two pre-heated aquaria (2HW) and two non-pre-heated aquaria (1HW).

Chlorophyll *a* Fluorescence

The photophysiological response of *P. australis* and *Z. muelleri* plants was determined using a diving-PAM fluorometer following the methodology described elsewhere (Marín-Guirao et al., 2013). During the experiment, measurements were conducted on the second youngest leaf of five randomly selected plants from each treatment and each species at different time points along the course of the experiment (**Figure 2**): end of the first acclimation period – experiment started (T1); beginning of the first heatwave (T2); end of the first heatwave (T3); beginning of the re-acclimation period (T4); end of the re-acclimation period (T5); beginning of the second heatwave (T6) and end of the second heatwave – experiment ended (T7). Maximum quantum yield (*Fv*/*Fm*) of photosystem II (PSII) was measured on night dark-adapted plants (i.e., at 7 am, before start of light cycle) while the effective quantum yield of PSII ($\Delta F/Fm'$) measurement was determined on light-adapted plants at noon during the daily period of highest irradiance level. Non-photochemical quenching (*NPQ*) was calculated according to the method of Maxwell and Johnson (2000) to estimate the amount of photosynthetic energy lost as heat (i.e., photo-protection).

Plant Growth

Plant growth measurements were done by adopting the leaf marking method (Zieman, 1974). In the middle of the second acclimation period between both simulated heatwaves, five randomly selected plants of each treatment were marked just above the ligule. These samples were then collected at the end of the second heatwave (T7) for measuring leaf elongation (mm). Subsequently, newly grown leaf segments were dried at 70°C for 24 h and weighed to determine the growth as leaf biomass production (Dry weight).

Pigment Contents

Approximately 50 mm from the middle portion of the second youngest leaf of *P. australis* and the whole second youngest leaf of *Z. muelleri* was harvested from five randomly selected

[1]https://www.seatemperature.org/australia-pacific/australia/palm-beach.htm

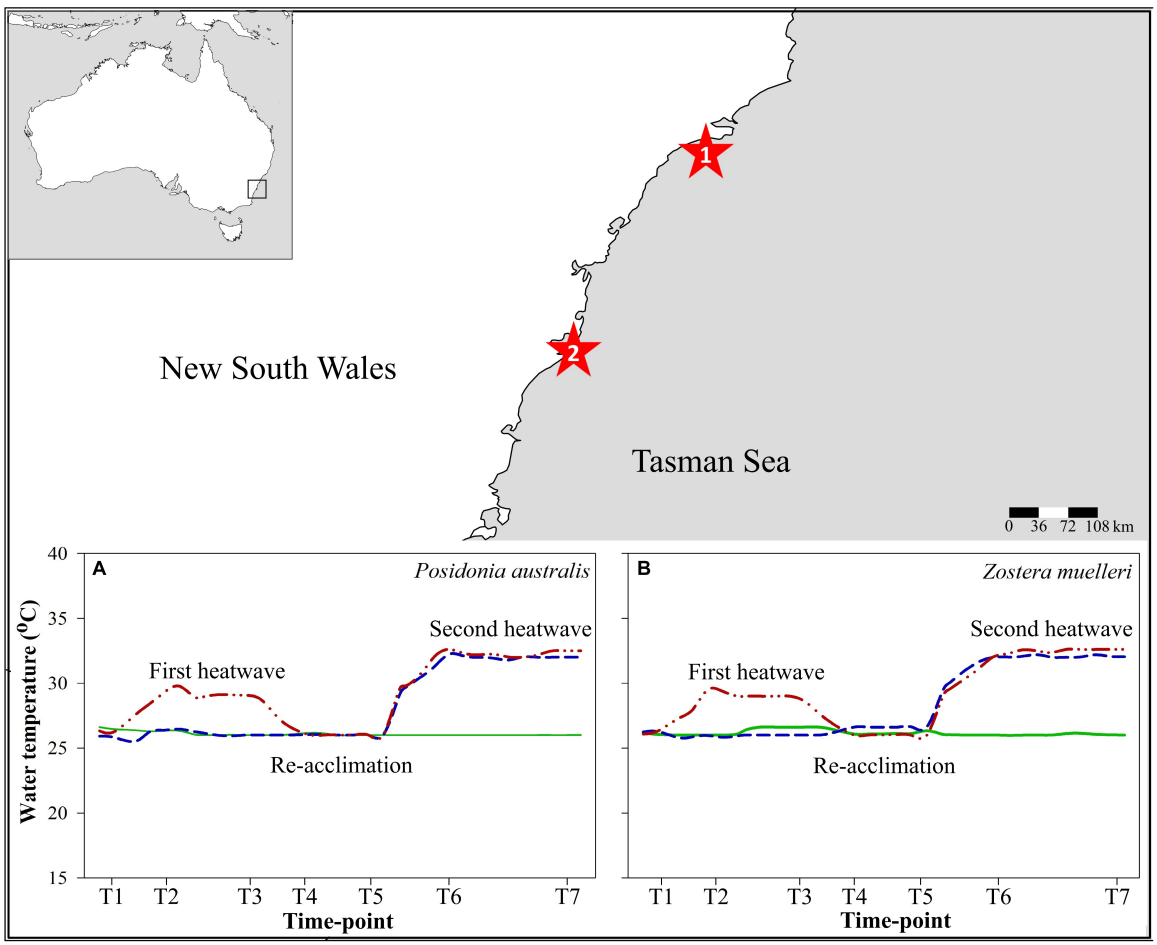

FIGURE 1 | Sample collection sites during low tides: (1) Collection site of *Posidonia australis* at Port Stephens, New South Wales, Australia, (2) Collection site of *Zostera muelleri* at Church Point, New South Wales, Australia. Thermal profile in experimental treatments during the course of the experiment **(A,B)**: Green continuous lines: control; Blue dashed lines: Treatment 1-heatwave (1HW) and Red dashed lines with dots: Treatment 2-heatwave (2HW).

plants of each treatment at the end of the experiment (T7) for analyzing pigments content. Collected leaf samples were cleaned of epiphytes and kept on ice before fresh weights were measured. Samples were homogenized in liquid nitrogen using pestles and mortars, transferred into 1.5 mL tubes containing 1 mL of 100% methanol and stored in complete darkness at 4°C for 8 h before centrifugation. Absorbance of 200 µL of obtained solution was read at 470, 652, 665, and 750 nm using a microplate reader (TECAN Infinite® M1000 PRO, Switzerland) for calculations of the chlorophyll *a*, chlorophyll *b* and total carotenoid concentrations using equations from Wellburn (1994) after converting microplate readings into 1cm cuvette readings following Warren (2008) as described in Tran et al. (2018). Finally, results were normalized to a milligram of fresh weight.

Quantitative Real-Time PCR (RT-qPCR)

Primer Design

Ten genes of interest (GOIs; **Table 1**) common to both species were chosen within three different categories including stress-related, photosynthesis-related and methylation-related genes.

Zostera muelleri GOIs were newly designed using *Z. muelleri* database from AquaticPlantsDB® (Sablok et al., 2018),[2] while housekeeping genes (HKGs) were taken from previous studies (Schliep et al., 2015; Pernice et al., 2016; Kim et al., 2018). For *P. australis*, however, no molecular resources are available to date, thus selected GOIs and HKGs were either newly designed or taken from previous studies on the congeneric species *P. oceanica*. Three photosynthesis-related genes (i.e., Photosystem II protein D1-psbA, Photosystem II protein D2-psbD and Rubisco large subunit-RBCL) and 4 HKGs were available in the literature (Serra et al., 2012; Dattolo et al., 2014; Marín-Guirao et al., 2016). The rest of the primers were designed using a *P. oceanica* transcriptome database available at the National Center for Biotechnology Information (NCBI) (Marín-Guirao et al., 2019).

Primers were designed using Primer3 v.0.4.0 (Koressaar and Remm, 2007; Untergasser et al., 2012) with the following default settings: primer lengths: 18–22 bp, product sizes:

[2]http://115.146.91.129/version3/index.php

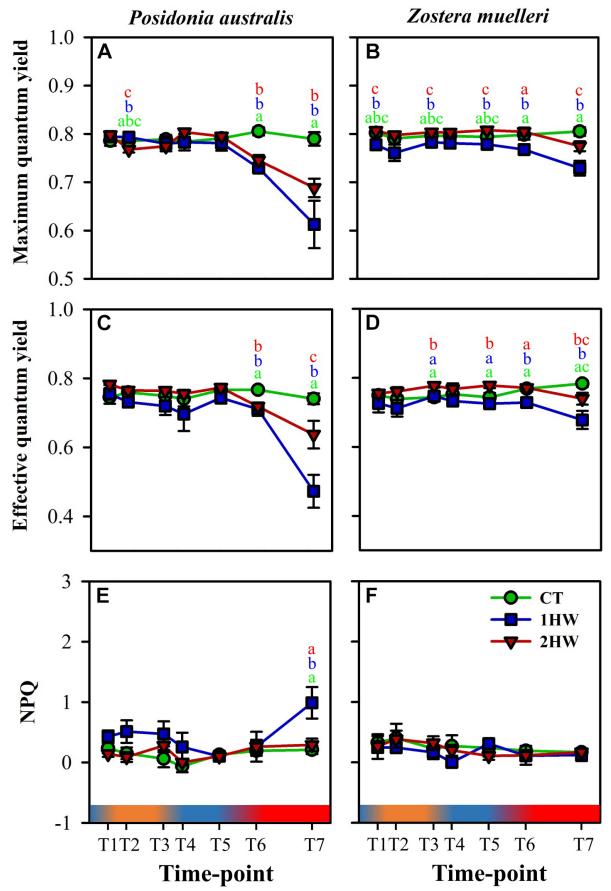

FIGURE 2 | Photo-physiological reponses from Posidonia australis and Zostera muelleri: **(A,B)** Maximum quantum yield (*Fv/Fm*) were measured on dark-adapted plants; **(C,D)** Effective quantum yield ($\Delta F/Fm'$) were measured on light-adapted plants and **(E,F)** Non-Photochemical Quenching (*NPQ*). CT: control, 1HW: 1 heatwave treatment, 2HW: 2 heatwaves treatment. At each time point, different colors correspond to different treatments (green – CT, blue – 1HW, and red – 2HW) and different letters (a-c) indicate significant differences between treatments [e.g. in **Figure 2A**: "a-green + b-blue + b-red" means CT ≠ 1HW = 2HW; Pair-wise comparison test, $p_{(perm)} < 0.05$]. Data are mean, ±SE, $n = 5$. Gradient bars present water temperature changes in treatment 2HW throughout the experiment.

100–200 bp and Tm = 59–61°C. Primers were validated for their specificities firstly by checking PCR amplification on agarose gel electrophoresis (i.e., only single band, similar size as designed) and secondly by checking the melting curve for each RT-qPCR run. RT-qPCR efficiencies were assessed via a series of cDNA dilutions of 384, 81, 27, 9, 3, and 1 ng using a linear regression model (Pfaffl, 2001). The efficiency of each primer pair was then calculated with the following equation: $E\ (\%) = (10^{-1/\text{slope}} - 1) \times 100$ (Radonić et al., 2004). Primers with efficiencies (E) within the range 90–110% and correlation coefficient >0.95 were used in the study (**Table 1**).

RNA Extraction and cDNA Preparation

Three leaf samples, targeted as a similar way for pigment content samples, were collected for RNA extraction at the end of each heatwave (T3 and T7). Epiphytes were carefully removed from plants and cleaned plant material was then immediately frozen in liquid nitrogen before being stored at −80°C until RNA extraction. PureLink™ RNA Mini Kit (ThermoFisher, United States) was used to extract total RNA from both species. For *Z. muelleri*, extraction was done by following the manufacturer's instructions. For *P. australis*, to minimize effects of phenolic compounds that can inhibit the extraction process, 2% (w/v) polyvinylpyrrolidone-40 (PVP) together with two glass beads were added to the lysis solution and vortexed at high speed at 4°C for 10 min, all other steps were completed by following the manufacturer's instructions. During the extraction of total RNA, PureLink™ DNase Set (ThermoFisher, United States) was added to eliminate genomic DNA. The total RNA quantity and quality were assessed with a NanoDrop spectrophotometer (ND-1000; NanoDrop Technologies, United States). Then, cDNA was synthesized from 500 ng of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, United States) according to the manufacturer's instructions. The resulting cDNA was diluted 1:20 prior to Reverse Transcription – quantitative Polymerase Chain Reaction (RT-qPCR) assays.

Gene Expression Analyses

A 5 μL-final volume RT-qPCR reaction including 2.7 μL of iTaq™ Universal SYBR® Green Supermix (BIO-RAD, United States), 0.3 μL of 10 pmol μL^{-1} primers and 2 μL of diluted cDNA was robotically prepared in a 384-well PCR plate (BIO-RAD, United States) via an Automated Liquid Handling Systems (EpMotion® 5075, Eppendorf, Germany). RT-qPCR assay was run in a Real-Time PCR Detection System (CFX384 Touch™, Bio-Rad) with the following conditions: 95°C for 10 min, followed by 45 cycles of 95°C for 30 s, 60°C for 30 s and 68°C for 30 s. A melting curve from 60 to 95°C was also included for each amplicon to check the specificity of each reaction.

All RT-qPCR reactions were performed in three technical replicates with three no-template negative controls. Additionally, three No Reverse Transcription (No-RT) controls were prepared for each primer's pair and included in each plate to ensure the absence effect of genomic DNA contamination (i.e., Cq value from No-RT sample was at least five cycles greater than the actual sample). Furthermore, an internal control assay was introduced in each plate to establish a reliable comparative result between different plates.

Data from RT-qPCR reactions were analyzed with Bio-Rad CFX Manager v3.1 software (BIO-RAD, United States) and normalized relative quantities of amplification were used to determine the changes in the gene expression level of GOIs as described in a previous study (Kim et al., 2018).

Before gene expression data analyses, three different algorithms were used to identify the best HKGs: NormFinder (Andersen et al., 2004), GeNorm (Vandesompele et al., 2002), and BestKeeper (Pfaffl et al., 2004).

Relative quantities of genes of interest (GOIs) were first normalized using the two best housekeeping genes selected from three different algorithms (**Supplementary Data**). Then, normalized data were used to determine gene expression levels of GOIs.

TABLE 1 | List of housekeeping genes and gene of interests used in this study.

Gene category	Gene name	Abbrev	Species	Forward primer (5′→3′)	Reverse primer (5′→3′)	Product size (bp)	E (%)	R^2	Accession number	Reference (note)
Stress-related	Heat Shock Protein 90	HSP90	Zm	GAGGGTTTGTGCAAGGTCAT	GTTGGCAGTCCACCCATACT	123	103.9	0.996	ZM251873	This study
			Pa	TCAAGGAGGTGTCACACGAG	CAGATGCTCCTCCCAGTCAT	134	109.8	0.997	PO008787	This study
	Catalase	CAT	Zm	AAGTACCGTCCGTCAAGTGG	CTGGGATACGCTCCCTATCA	169	100.5	0.999	ZM230093	This study
			Pa							Same as *Z. muelleri*
	Manganese superoxide dismutase	MSD	Zm	TTTTCGCCAAGAACAAAACC	TCTGCATGATCTCTCCGTTG	135	99.8	0.998	ZM212939	This study
			Pa	AATAATGCCGCTCAGCTTTG	ACCCAACCAGATCCAAACAG	176	98.0	0.994	PO035322	This study
Photosynthesis-related	Photosystem II protein D1	psbA	Zm	AAGCTTATGGGGTCGCTTCT	GTGCAGCAATGAAAGCGATA	134	100.4	0.999	ZM045788	This study
			Pa	GACTGCAATTTTAGAGAGACGC	CAGAAGTTGCAGTCAATAAGGTAG	136	100.9	0.999	KC954695	Dattolo et al. (2014)
	Photosystem II protein D2	psbD	Zm							Same as *P. australis*
			Pa	CCGCTTTTGGTCACAAATCT	CGGATTTCCTGCGAAACGAA	161	103.6	0.999	KC954696	Dattolo et al. (2014)
	Rubisco large subunit	RBCL	Zm	CCGAGACAACGGCTTACTTC	AGTCATCTCGCGTTCACCTT	175	100.1	1.000	ZM194765	This study
			Pa	GCTGCCGAATCTTCTACTGG	CACGTTGGTAACGGAACCTT	177	102.2	0.999	U80719.1	Marín-Guirao et al. (2016)
Housekeeping	Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	Zm	CGGTTACTGTAGCCCCACTC	CAAAGGCTGGGATTGGTTTA	79	100.8	0.992	Zoma_C_c6252	Kim et al. (2018)
			Pa	AGGTTCTTCCTGCTTTGAATG	CTTCCTTGATTGCTGCCTTG	138	110.3	0.998	GO347079	Serra et al. (2012)
	Elongation factor 1-alpha	Ef1A	Zm	AAGCAAAGGCGTCACTTGAT	TCTGCTGCCTTCTTCTCCTC	82	103.4	0.989	Zoma_C_c59090	Kim et al. (2018)
			Pa	GAGAAGGAAGCTGCTGAAATG	GAACAGCACAATCAGCCTGAG	214	107.2	0.997	GO346663	Serra et al. (2012)
	β-tubulin	TubB	Zm	GGACAAATCTTCCGTCCAGA	TCCAGATCCAGTTCCACCTC	185	102.8	0.995	Zoma_Contig120	Kim et al. (2018)
	Actin	Actin	Zm	TAAGGTCGTTGCTCCTCCTG	ACTCTGCCTTTGCAATCCAC	104	95.3	0.993	Zoma_ZMF02257	Kim et al. (2018)
	18S ribosomal RNA	18S	Pa	AACGAGACCTCAGCCTGCTA	AAGATTACCCAAGCCTGTCG	200	93.0	1.000	AY491942.1	Serra et al. (2012)
	Ubiquitin	UBI	Pa	CACCCTCGCTGACTACAACA	TTTCTCAGCCTGACGACCTT	195	97.2	0.998	GO347694	Serra et al. (2012)
Methylation-related	ProteinSet1/Ash2 histone methyl transferase complex subunit ash-2	ASH2L	Zm	CTCAGACCCCCAATTCTCAA	GTGGAAGAGACGACGGTGAT	153	100.3	0.994	ZM248014	This study
			Pa	CTATCCTGCTGCCTCCATGT	TCAACTGCACCTTCAACTCG	174	108.1	0.992	SRP126951	This study
	Histone-lysine N-methyltransferase setd3	SETD3	Zm	CGAACCTTCCTTTCTTGCTG	CCTCGGGTTGAGAATCAAAA	146	90.5	0.995	ZM228252	This study
			Pa	TGGGCTTGTGAACTGTGGTA	CGAATGATTGAGTCGTCCAG	200	103.9	0.949	SRP126951	This study
	Histone-lysine N-methyltransferase ATX2	ATX2	Zm	ATCCCGTGAATGTGGAGAAG	ATACCAGGCACCGTCGATAG	161	97.2	0.992	ZM254823	This study
			Pa	CCAGATACAAAGCTGCACCA	GCATTGTCATCCCCTTGAGT	170	103.1	0.993	SRP126951	This study
	Histone-lysine N-methyltransferase ATXR7 isoform X1	ATXR7	Zm	CAGAGGATCAATCCCTCCAA	CTTTGCCCGAACTCTTTCAG	138	102.0	0.990	ZM256759	This study
			Pa	CGAGTAGGGTCGAATGTGGT	ATCCATCCAGTCACACACGA	149	105.2	0.973	SRP126951	This study

Pa: Posidonia australis, Zm: Zostera muelleri; E: Efficiency (%); R^2: Calibration coefficient.

Statistical Analyses

One-way analysis of variance (ANOVA; statistical software SPSS v.20) was used to check for significant differences in plant growth and pigment content between treatments at the end of the second heatwave (T7). Since these parameters greatly differ between the two species, each species was analyzed independently. Prior to the analysis, Levene's test was used to check the homogeneity of variances and Shapiro–Wilk test was used to validate data normality. In case the parametric assumptions were not met, data were analyzed using Kruskal–Wallis test together with the Bonferroni correction for multiple tests (i.e., *P. australis*, Chl *b/a*, **Table 3**). A Tukey HSD *post hoc* test was applied whenever significant differences were determined.

Photo-physiological and gene expression results of GOIs were analyzed using Permutational Multivariate Analyses of Variance (PERMANOVA) on Primer 6 v.6.1.16 and PERMANOVA + v.1.0.6 software package (PRIMER-E Ltd) (Anderson et al., 2008). Analyses were performed on the resemblance matrices (created using Bray Curtis similarity) with a selected number of permutations of 9999. Within the analyses, treatment was treated as a fixed factor while time was treated as a random factor. Following, pair-wise test was performed to detect significant differences between treatments at each time point.

Principal component analyses (PCA) were also performed on normalized relative quantities of amplification of GOIs using the software PAST3 (Hammer et al., 2001) to determine responsive patterns to heat stress between treatment at each time point for gene expression data. Additionally, data from all measurements at T7 were analyzed all together using PCA to assess the difference in responses between the two seagrass species.

RESULTS

Photo-Physiological Responses

During the first heatwave (T2-T3), neither of the species showed significant differences in *Fv*/*Fm* between heated (2HW) and non-heated (CT and 1HW) plants (**Figures 2A,B**), evidencing the absence of accumulated heat-damage at the PSII level. In fact, the photochemical efficiency of PSII ($\Delta F/Fm'$) of heated plants was only slightly higher than that of control plants during this first heatwave (**Figure 2**), being significant only in *Z. muelleri* (CT = 1HW ≠ 2HW). The level of photo-protection (*NPQ*) of heated plants also showed no signs of alteration during this first warming exposure as seen by the lack of significant differences in *NPQ* between heated and control plants of both species (CT = 1HW = 2HW).

Contrarily, during the more intense and longer-lasting second heatwave (T6-T7), heated plants (1HW and 2HW) of both species experienced a significant reduction in their maximum and effective photochemical capacity of PSII (*Fv/Fm* and $\Delta F/Fm'$) with respect to controls (**Figures 2A–D**), that resulted in significant differences between treatments over time [$p_{(perm)} < 0.001$, **Table 2**]. However, this heat-induced photochemical reduction was generally higher in non-preheated (1HW) than in preheated (2HW) plants of both species, and we found significant differences between non-preheated plants versus controls and preheated plants at T6 for both *Fv/Fm* and $\Delta F/Fm'$ (**Figures 2B,D**; CT = 2HW ≠ 1HW). The differences between 1HW and 2HW plants were clear at T7. In *P. australis*, the second heatwave induced a 22% reduction in *Fv/Fm* and a 34% reduction in $\Delta F/Fm'$ of 1HW plants while the reductions were much smaller in 2HW plants (13 and 14%, respectively). Differences were significant in $\Delta F/Fm'$ (see **Figure 2C**, CT ≠ 1HW ≠ 2HW). Similarly, there was a significant reduction of 9% in *Fv/Fm* of *Z. muelleri*-1HW plants at T7, whereas there was only a slight reduction in *Fv/Fm* of 4% in *Z. muelleri*-2HW plants (**Figure 2B**, CT≠1HW≠2HW). We also observed a similar trend with $\Delta F/Fm'$ results from *Z. muelleri*. In respect to CT plants, the reduction in $\Delta F/Fm'$ in 1HW plants was more than double compared to that of 2HW plants (i.e., 14 and 6% respectively). Consequently, we found significant differences between plants from the two heating treatments (1HW and 2HW) as in case of *Fv/Fm* for *Z. muelleri* (**Figure 2B**; CT ≠ 1HW ≠ 2HW) and of $\Delta F/Fm'$ for *P. australis* (**Figure 2C**; CT ≠ 1HW ≠ 2HW).

TABLE 2 | PERMANOVA analysis performed on photo-physiological measurements assessing the effect of increased seawater temperature among different treatments overtime.

Species	Measurement	Source	df	SS	MS	Pseudo-F	$p_{(perm)}$	Unique perms
Posidonia australis	Maximum quantum yield	Time	6	537.87	89.646	12.632	**0.0001**	9946
		Treatment(Time)	14	516.3	36.879	5.1968	**0.0001**	9925
	Effective quantum yield	Time	6	1468	244.66	15.354	**0.0001**	9947
		Treatment(Time)	14	1497.5	106.97	6.7128	**0.0001**	9928
	NPQ	Time	6	713.35	118.89	3.3991	**0.0045**	9945
		Treatment(Time)	14	1084.3	77.448	2.2142	**0.0129**	9907
Zostera muelleri	Maximum quantum yield	Time	6	33.814	5.6357	3.5623	**0.0037**	9932
		Treatment(Time)	14	122.68	8.7628	5.5389	**0.0001**	9930
	Effective quantum yield	Time	6	36.221	6.0368	1.2295	0.2884	9938
		Treatment(Time)	14	264.19	18.871	3.8433	**0.0002**	9924
	NPQ	Time	6	203.36	33.893	1.0434	0.3953	9944
		Treatment(Time)	14	235.6	16.828	0.51807	0.9169	9918

Significant differences [p(perm) < 0.05] are in bold.

TABLE 3 | Results from One-way ANOVA analyses and Kruskal–Wallis test performed on plant growth and pigment content results.

Species	Measurement	Statistical analysis	df	*F*	*p*
Posidonia australis	Biomass	One-way ANOVA	2	8.130	**0.006**
	Leaf growth	One-way ANOVA	2	22.459	**0.000**
	Chl *a*	One-way ANOVA	2	3.698	0.056
	Chl *b*	One-way ANOVA	2	2.161	0.158
	Chl *b/a*	Kruskal–Wallis test	2		**0.007**
	Carotenoids	One-way ANOVA	2	1.301	0.308
Zostera muelleri	Biomass	One-way ANOVA	2	4.959	**0.027**
	Leaf growth	One-way ANOVA	2	11.473	**0.002**
	Chl *a*	One-way ANOVA	2	0.893	0.435
	Chl *b*	One-way ANOVA	2	0.041	0.960
	Chl *b/a*	One-way ANOVA	2	16.767	**0.000**
	Carotenoids	One-way ANOVA	2	0.795	0.474

Significant differences ($p < 0.05$) are in bold.

Regarding non-photochemical quenching (NPQ), *Z. muelleri* interestingly showed no significant differences [$p_{(perm)} = 0.9169$, Pseudo-$F = 0.5181$, **Table 2**] among three treatments throughout the whole experiment (**Figure 2E**). In contrast, *P. australis*-1HW plants significantly tripled their NPQ levels at T7 compared to CT and 2HW plants [**Figure 2F**, Treatment (Time): $p_{(perm)} < 0.001$, Pseudo-$F = 0.5181$, **Table 2**, CT = 2HW ≠ 1HW].

Plant Growth Responses

Increased temperatures during the second heatwave (32°C) significantly reduced leaf elongation and leaf biomass production of both preheated (2HW) and non-preheated (1HW) *P. australis* plants (**Figures 3A,C**; $p < 0.01$, **Table 3**). Growth reduction, however, was similar in 2HW plants (39%) and 1HW plants (40%).

In *Z. muelleri* plants, significant differences among treatments ($p < 0.05$, **Table 3**) were also detected for both leaf elongation and leaf biomass production measurements. During the second heatwave, leaf elongation rate decreased by 41% in 1HW plants while there was only a 16% reduction in the case of 2HW plants (**Figure 3B**; CT = 2HW ≠ 1HW). It is interesting to note that while leaf biomass production decreased by 38% in 1HW plants, 2HW plants accumulated 6% more biomass than the CT plants during the second heatwave (**Figure 3D**). This phenomenon led to a significant difference between 1HW vs. 2HW plants in terms of leaf growth (**Figure 3D**; CT = 1HW, 2HW; 1HW ≠ 2HW).

Pigment Content Responses

Chlorophyll *a* appeared as the most sensitive photosynthetic pigment to temperature increase among pigments measured at the end of the experiment, both in *P. australis* and in *Z. muelleri* (**Figures 4A,B**). Interestingly, 2HW plants were able to maintain their Chl *a* contents similar as in CT plants, while 1HW plants suffered a strong reduction (41 and 28% for *P. australis* and *Z. muelleri*, respectively). Via Tukey HSD *post hoc* test, we found a significant difference between 1HW plants and CT plants in *P. australis* (**Figure 4A**). Both Chl *b* and Carotenoids content (**Table 3**) from 1HW *P. australis* plants were further impacted by elevated temperature during the second heatwave when compared to those from 2HW plants (**Figure 4A**), although these differences were not statistically significant.

Temperature increase affected Chl *a* and Chl *b* contents differently of the two seagrass species, contributing to significant differences in Chl *b/a* ratios among experimental treatments ($p < 0.01$, **Table 3**). In *P. australis*, both 1HW and 2HW plants increased ∼13% of Chl *b/a* ratios in respect to the CT plants (**Figure 4A**). In contrast, only non-preheated (1HW) *Z. muelleri* plants increased their Chl *b/a* ratios (32% more than in CT plants) significantly, while preheated plants kept their Chl *b/a* ratios comparable to control levels (0.28 and 0.29 in CT and 2HW plants, respectively; **Figure 4B**).

Gene Expression Responses

All primers were tested in the two species and some of them successfully worked on both *P. australis* and *Z. muelleri* (i.e., psbD and CAT, **Table 1**), indicating the presence of conservative genomic regions between the two different seagrass species belonging to different genera.

In general, during the first heatwave (T3), 2HW plants from both species showed up-regulation of all analyzed GOIs with respect to plants under control temperature (CT and 1HW). The difference, however, was significant only for 3 and 6 genes in *P. australis* and *Z. muelleri*, respectively (**Figures 5A,C**).

At the end of the second heatwave (T7), all heated *P. australis* plants (1HW and 2HW) activated substantial molecular response to compensate with extreme temperature changes, with 80% of the GOIs tested showing significant up-regulation (**Figure 5B**). In *Z. muelleri*, while we observed a similar number of significantly affected genes at T3 and T7 (**Figure 5C**), the GOIs significantly regulated were different between the two time points. In both species, results from both T3 and T7 confirmed methylation-related genes were more sensitive to temperature increase than stress-related and photosynthesis-related genes. Details about statistical analysis results from each GOIs at T3 and T7 can be found in **Table 4**.

Methylation-Related GOIs

At T3, heated plants of both species (2HW) showed significant increased transcripts accumulation of ATX2 and ATXR7 (CT = 1HW ≠ 2HW). ASH2L was also highly up-regulated in heated plants although without significant differences among treatments (**Figures 5A,C**). We also found a significant upregulation of SETD3 in *Z. muelleri* heated plants during the first heatwave (**Figure 5C**).

At T7, most methylation-related GOIs showed significant up-regulations in 1HW and 2HW heated plants of both species (**Figures 5B,D**). Significant differences between 1HW and 2HW *P. australis* plants were found in ATX2 and ATXR7 (**Figure 5B**, 1HW > 2HW). *Z. muelleri* plants followed a similar trend, with 1HW plants showing higher gene expression levels than 2HW plants among all methylation-related GOIs with significant differences found for ASH2L and ATX2 (**Figure 5D**).

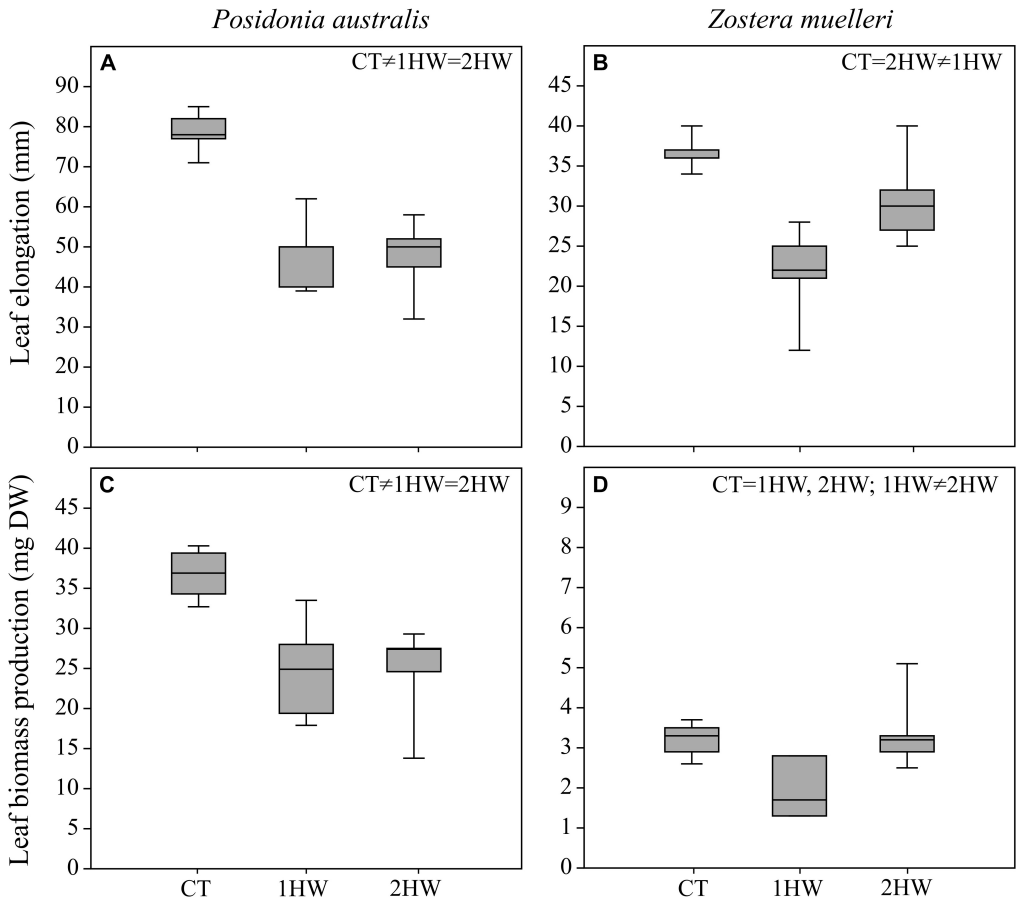

FIGURE 3 | Leaf elongation **(A,B)** and leaf biomass production (Dry weight; **(C,D)** from control (CT), non-pre-heated (1HW) and pre-heated (2HW) plants at the end of the second heatwave (T7). Tukey HSD *post hoc* results are shown on the top of the graphs (Significant difference means $p < 0.05$). Data are mean, $n = 5$, ±SE.

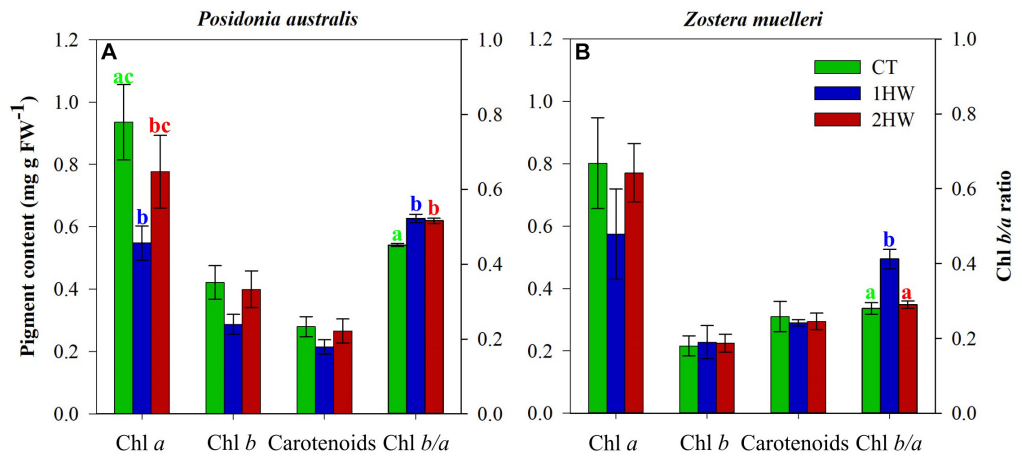

FIGURE 4 | Pigment relations at the end of the second heatwave (T7): Chlorophyll *a* (Chl *a*), Chlorophyll *b* (Chl *b*), Carotenoids and the Chlorophyll *b*/*a* molar ratio (Chl *b*/*a*) in *P. australis* **(A)** and Z. muelleri **(B)**. CT = control plants; 1HW = non-pre-heated plants; 2HW = pre-heated plants. Different letters (a–c; green letters correspond with CT, blue letters correspond with 1HW and red letters correspond with 2HW treatment) indicate significant differences ($p < 0.05$) among treatments as derived from Tukey HSD *post hoc* analyses. Error bars present ±SE, $n = 5$.

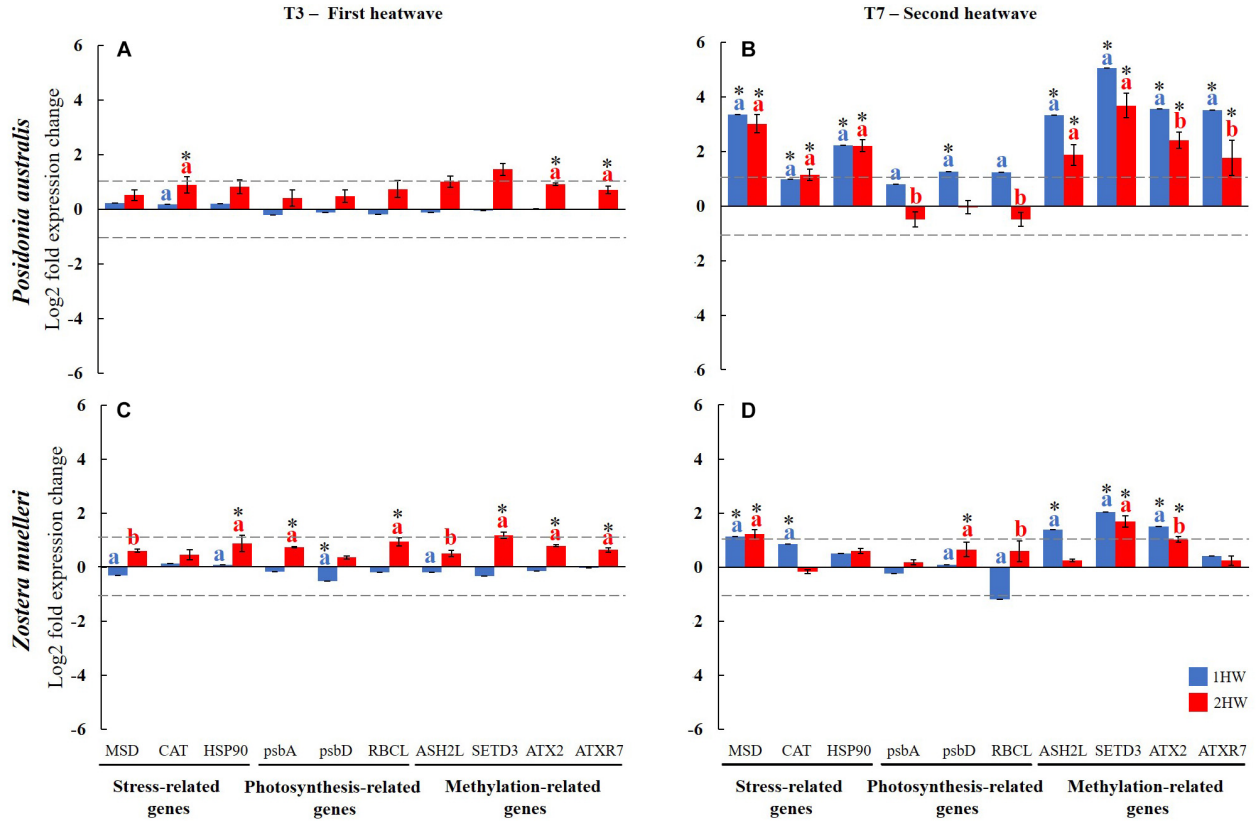

FIGURE 5 | Differential gene expression for GOIs at the end of the first (T3; left panels) and second heatwaves (T7; right panels), respectively. For *P. australis* **(A,B)** and *Z. muelleri* **(C,D)**. Data is expressed as log2 Relative Quantification versus the control group. Data are mean, ±SE, $n = 3$. Pair-wise results are presented on top of the column corresponding to significant difference between control and treatments (asterisk) or between the two treatments (letters), $p < 0.05$. 1HW: 1 heatwave plants; 2HW: 2 heatwave plants.

Stress-Related GOIs

At T3, positive changes were observed in all stress-related and photosynthesis-related GOIs, with significant up-regulations (CT = 1HW ≠ 2HW) detected in CAT from *P. australis* plants (**Figure 5A**) and in HSP90 from *Z. muelleri* plants (**Figure 5C**).

At T7, for *P. australis*, the three stress-related GOIs (i.e., MSD, CAT, and HSP90) showed similar and significant up-regulation in all heated plants (1HW and 2HW) (**Figure 5B**, CT ≠ 1HW = 2HW). In contrast, CAT showed a significant difference between the two categories of heated *Z. muelleri* plants (1HW > 2HW, **Figure 5D**).

Photosynthesis-Related GOIs

At T3, all photosynthesis-related GOIs showed up-regulations in heated (2HW) plants of both studied species, although significant differences (CT = 1HW ≠ 2HW) were only detected in *Z. muelleri* plants for psbA and RBCL (**Figure 5C**).

At T7, non-preheated *P. australis* plants (1HW) increased their levels of gene expressions significantly compared to CT plants (CT ≠ 1HW in psbD), while preheated-plants (2HW) maintained or even decreased the expression levels of those genes, resulting in significant differences between the two heated plants among all photosynthesis-related GOIs (1HW ≠ 2HW, **Figure 5B**). In contrast, in *Z. muelleri*, no significant difference was found between 1HW and 2HW plants in cases of psbA and psbD (1HW = 2HW, **Figure 5B**). Moreover, even if no significant difference was detected between CT versus heated plants (CT = 1HW = 2HW), RBCL was expressed differently between 1HW and 2HW plants. As a consequence, the expression levels of RBCL was significantly different between the two heated treatments at T7 (1HW ≠ 2HW).

Principal component analyses performed on gene expression results from both seagrass species demonstrated clearly that: (***a***) at T3, heated plants (2HW) were separated from non-heated plants (CT and 1HW) while (***b***) at T7, the two groups of plants experiencing heat stress (1HW and 2HW) were distant from CT plants, with 2HW plants showing more similarities to CT plants than to 1HW plants (**Figure 6**). PCA results also highlighted methylation-related genes were the main drivers differentiating 2HW plants at T3 and 1HW plants at T7. For instance, in *P. australis* at T3, ATX2 and ATXR7 together with CAT were the main drivers separating 2HW plants away from CT and 1HW plants along the PC1 *axis* responsible for 97.77% of this separation (**Figure 6A**). Whilst, in *Z. muelleri*, SETD3 and HSP90 mainly contributed to PC1, which was responsible for 86.42% of the separation between

TABLE 4 | PERMANOVA analysis performed on gene expression levels of GOIs from different treatments.

		Posidonia australis						*Zostera muelleri*					
GOI	**Source**	**df**	**SS**	**MS**	**Pseudo-F**	$p_{(perm)}$	**Unique perm**	**df**	**SS**	**MS**	**Pseudo-F**	$p_{(perm)}$	**Unique perm**
HSP90	Time	1	11370	11370	54.839	**0.0001**	9950	1	1367.2	1367.2	5.708	**0.023**	9946
	Treatment(Time)	4	9144.3	2286.1	11.026	**0.0001**	9928	4	2341.1	585.3	2.444	0.074	9952
CAT	Time	1	1654.8	1654.8	7.715	**0.0059**	9943	1	661.2	661.2	5.783	**0.032**	9932
	Treatment(Time)	4	3888.5	972.13	4.5323	**0.0052**	9944	4	2475.7	618.9	5.413	**0.009**	9945
MSD	Time	1	13097	13097	31.569	**0.0001**	9958	1	5659.8	5659.8	80.129	**0.000**	9956
	Treatment(Time)	4	11668	2917	7.0308	**0.0001**	9933	4	4383.4	1095.8	15.514	**0.000**	9937
psbA	Time	1	3337.6	3337.6	9.5367	**0.0009**	9939	1	1683.3	1683.3	9.690	**0.002**	9946
	Treatment(Time)	4	3082.5	770.63	2.2019	**0.0762**	9942	4	1797.0	449.2	2.586	**0.054**	9956
psbD	Time	1	6433.2	6433.2	23.064	**0.0001**	9930	1	446.1	446.1	2.997	0.099	9911
	Treatment(Time)	4	3889.9	972.47	3.4865	**0.0081**	9943	4	2147.8	536.9	3.607	**0.019**	9964
RBCL	Time	1	9830.1	9830.1	40.425	**0.0001**	9952	1	1219.8	1219.8	3.474	0.061	9948
	Treatment(Time)	4	5831.5	1457.9	5.9954	**0.0001**	9929	4	6060.9	1515.2	4.315	**0.004**	9938
ASH2L	Time	1	7703.7	7703.7	16.493	**0.0001**	9960	1	1789.8	1789.8	33.712	**0.000**	9945
	Treatment(Time)	4	12466	3116.6	6.6724	**0.0001**	9935	4	4312.0	1078.0	20.305	**0.000**	9954
SETD3	Time	1	8866.8	8866.8	13.863	**0.0001**	9953	1	217.0	217.0	1.685	0.196	9939
	Treatment(Time)	4	18997	4749.3	7.4254	**0.0001**	9923	4	10304.0	2576.0	19.997	**0.000**	9954
ATX2	Time	1	13600	13600	64.66	**0.0001**	9960	1	541.8	541.8	9.199	**0.002**	9953
	Treatment(Time)	4	14096	3523.9	16.754	**0.0001**	9942	4	5489.7	1372.4	23.301	**0.000**	9956
ATXR7	Time	1	5666.8	5666.8	14.48	**0.0001**	9951	1	128.4	128.4	1.680	0.226	9928
	Treatment(Time)	4	11148	2786.9	7.1212	**0.0001**	9942	4	1318.4	329.6	4.312	**0.015**	9950

Significant differences [p(perm) < 0.05] are in bold.

2HW plants with the other two groups (**Figure 6C**). At T7, in *P. australis* ATX2 and ATXR7 remained the strongest factors separating 1HW plants from 2HW and CT plants (**Figure 6B**) while in *Z. muelleri*, ASH2L together with ATX2 and SETD3 separated 1HW plants from CT and 2HW plants along PC2 (23.8%) (**Figure 6D**).

Principal component analyses results for both species and all analyzed plant variables at T7 showed similar results in both seagrass species with heated plants separated from control plants, reflecting the overall effects (i.e., molecular, physiological and organismal effects) of extreme temperature increase during the second heatwave (**Figure 7**). Nonetheless, preheated-plants (2HW) were closer to control plants than non-preheated ones, especially in the case of *Z. muelleri*. Additionally, control plants of both species were located within the same quadrat II of the PCA graph (**Figure 7**), in accordance with their higher photochemical capacity (Fv/Fm; $\Delta F/Fm'$) and pigments content (Chl *a* and carotenoids). In contrast to controls, heated plants of the two species were separated along PC1 *axis* (responsible for 61.61% of total variance; **Figure 7**), suggesting slight differences in the response of the two seagrass species to the experimental recurrent heatwave at T7.

DISCUSSION

This comparative experiment involving *P. australis* and *Z. muelleri* provided us with a unique opportunity to better understand the thermal tolerance of two contrasting functional types of seagrass species from the southern hemisphere. Results from molecular to organismal levels support the fast-growing - pioneer *Z. muelleri* to be more tolerant than the long-lived - climax *P. australis*. In addition, by including a two-heatwave experimental design, we demonstrated that pre-heated plants performed better during the more extreme second heatwave, suggesting that they might have acquired mild stress-induced traits during the first heatwave. These results provided the very first insight into thermal hardening in seagrasses. Furthermore, gene expression analyses supported a key role of methylation-related genes in the responses of these two seagrass species to thermal stress, suggesting the importance of epigenetic modifications on seagrass memory and response to changing environment.

Difference Between Climax Versus Pioneer Seagrass Species in Response to Thermal Stress

Photo-physiological results showed that both *P. australis* and *Z. muelleri* were more affected during the second heatwave (T6-T7) than during the first heatwave (T2-T3). This observation was expected since the second HW was more intense and longer-lasting than the first heatwave. On the other hand, the greater photochemical inhibition of heated-*P. australis* in comparison with heated-*Z. muelleri* (**Figure 2**), indicated interspecific differences in heat tolerance. Our photo-physiological results concur with previous studies on Mediterranean seagrass species (i.e., *Posidonia oceanica* and *Cymodocea nodosa*), showing the climax more stable species further suffer from negative effects of thermal stress rather than the fast-growing

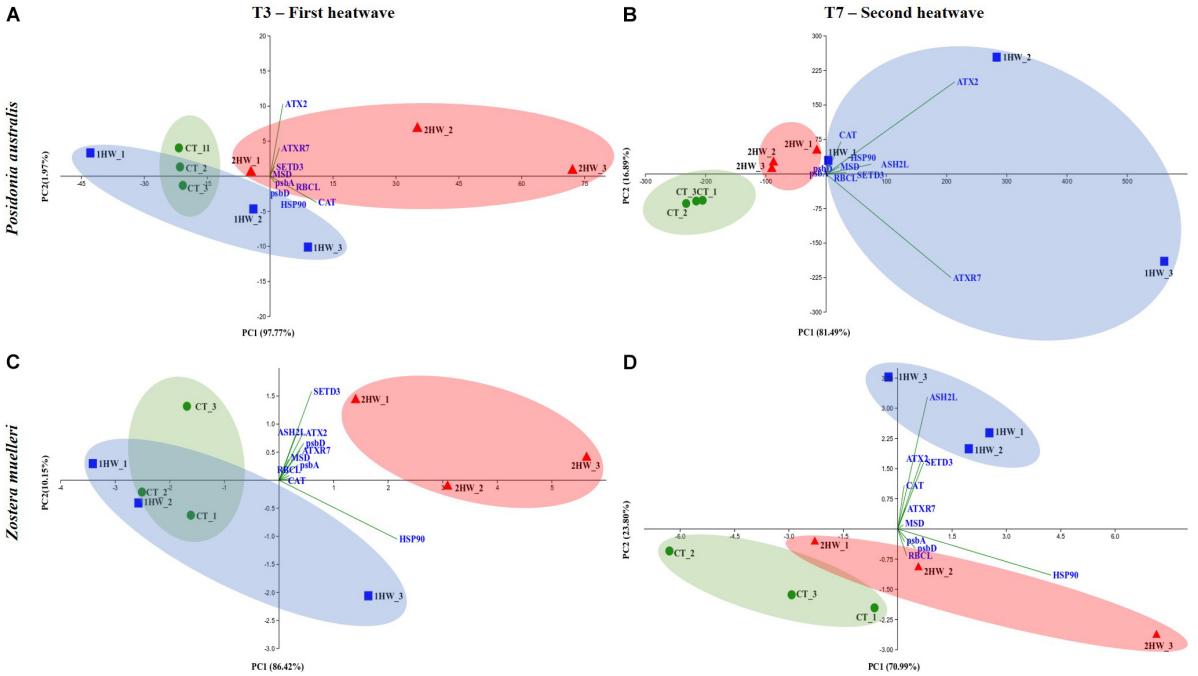

FIGURE 6 | PCAs conducted on gene expression data. **(A)** *Posidonia australis* at T3, **(B)** *P. australis* at T7, **(C)** *Zostera muelleri* at T3 and **(D)** *Z. muelleri* at T7. Different colors correspond to different treatments (Green circle = Control-CT, Blue square = Treatment 1-heatwave-1HW, Red triangle = Treatment 2-heatwave-2HW).

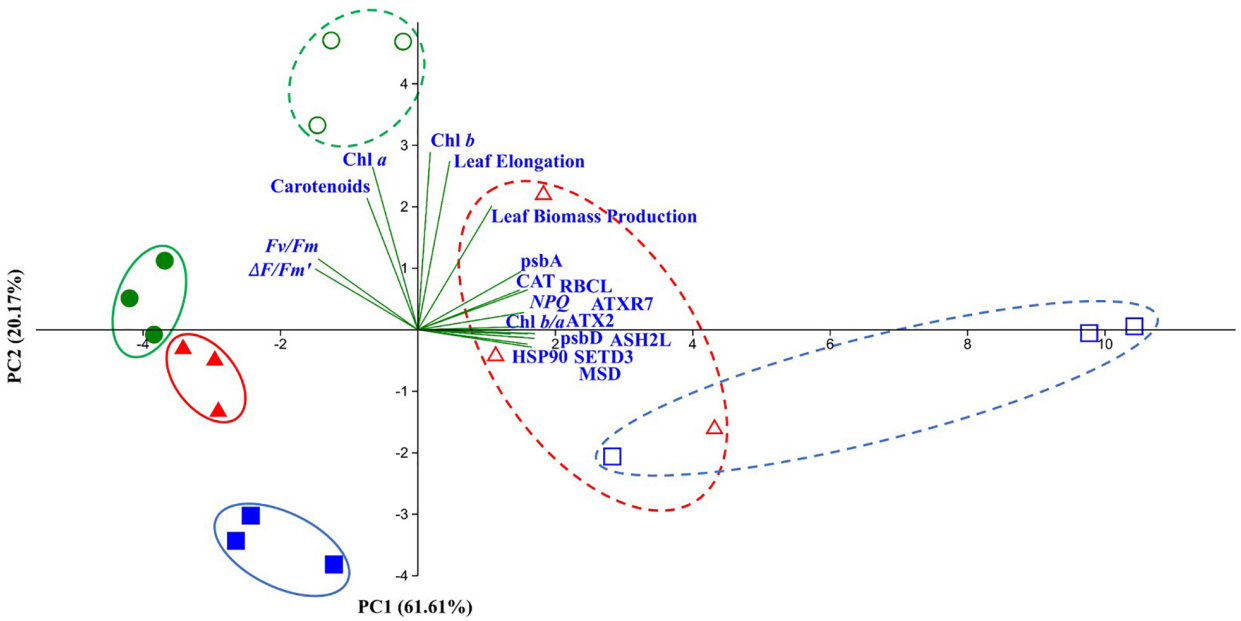

FIGURE 7 | PCA conducted on morphological, physiochemical and gene expression data at T7. Different colors and shapes correspond to different treatments (Green circle = Control-CT, Blue square = Treatment 1-heatwave-1HW, Red triangle = Treatment 2-heatwave-2HW) and species (filled = *Zostera muelleri*, un-filled = *Posidonia australis*).

pioneer species (Marín-Guirao et al., 2016, 2018). It is important to note that 1HW-*P. australis* activated Non-Photochemical Quenching (NPQ) machinery, a photo-protective mechanism commonly used by plants to overcome stressful conditions (Ashraf and Harris, 2013). Contrarily, neither 2HW nor 1HW-*Z. muelleri* changed their NPQ values during the

second heatwave. With the fact that 1HW-*Z. muelleri* suffered a significant reduction in both maximum quantum yield of PSII (*Fv/Fm*) and effective quantum yield capacities ($\Delta F/Fm'$) at T7 (**Figures 2B,D**), these results suggest that *Z. muelleri* plants went through a different pathway or initiated a different mechanism to protect their photosynthetic organelles from photo-damaging when exposed to heat stress.

In contrast to *Z. muelleri*, evidences of Chlorophyll *a* (Chl *a*) degradation were obtained for non-pre-heated *P. australis* plants at the end of the second heatwave. This reduction in pigments content was congruent with the greater photochemical alterations detected in *P. australis* during the second heatwave with regard to *Z. muelleri*. During the stressful condition, the degradation of Chl *a* might suggest that (***a***) Chl *a* was damaged by the higher temperature and/or (***b***) it is a response to modify the light harvesting capacity since changes in Chl *a* give a rise to changes in the Chl *b*/*a* ratio which is a proxy of PSII antenna size. Our results support previous work by York et al. (2013), showing a minor effect of temperature increase on modifying photosynthetic pigments in *Z. muelleri*. Interestingly, for *P. australis*, our results differed with the ones previously obtained for a closely related species from the same genus (i.e., the Mediterranean endemic seagrass *P. oceanica*) (Marín-Guirao et al., 2016, 2018). Marín-Guirao et al. (2018) did not find evidence of warming-induced pigment alterations after heat exposures of different intensity and duration from *P. oceanica* plants from different thermal origins. In contrast, in our study, we found negative effects of temperature increase on pigments content in *P. australis* with great reductions (especially in 1HW plants) in all pigment parameters. These contrasting findings could be explained by evolutionary and local adaptations that could also have played an important role in differentiating these two sister species (King et al., 2018).

Gene expression analyses provided more clues about the interspecific differences between the two species at the molecular level. As seen in many previous studies in seagrasses (Bergmann et al., 2010; Winters et al., 2011; Marín-Guirao et al., 2016, 2017; Tutar et al., 2017; Mota et al., 2018; Traboni et al., 2018; Nguyen et al., 2020), heat stress commonly yielded a high expression level of stress-related genes (e.g., HSP90, CAT) and photosynthesis-related genes (e.g., psbA and psbD). Similarly, we also detected significant up-regulation among our GOIs from the same categories during the second heatwave (T7) from both tested species. However, the differences in *P. australis* between heated and control plants were, in most cases, the double of the differences found in *Z. muelleri*. This could indicate that the applied thermal treatment induced a greater stress level to *P. australis* that, in consequence, required a stronger molecular response to compensate for the heat-stress experienced during the second heatwave.

Principal Component Analyses performed on all collected data at T7 (**Figure 7**), showed the differences in the response to heat stress between *P. australis* and *Z. muelleri.* Importantly, while photosynthetic-related factors (e.g., *Fv/Fm*, Chl *a*) were the main drivers differentiating *Z. muelleri*, the rest of measured parameters (e.g., GOIs, biomass) were responsible for *P. australis*.

All the differences from a molecular level, pigment contents to photo-physiology were translated to higher growth reductions in *P. australis* than in *Z. muelleri* as seen in **Figure 3**. These results clearly reflect the higher heat sensitivity of the climax species and are in agreement with previous studies in the Mediterranean, which have also shown greater growth reduction from heat stress for the climax *P. oceanica* compared to the pioneer *Cymodocea nodosa* (Olsen et al., 2012; Marín-Guirao et al., 2018; Savva et al., 2018). Together with previous studies, our study strongly demonstrates that the climax seagrass species (e.g., *P. oceanica* and *P. australis*) will likely suffer from ocean warming in the coming decades, while some pioneer species (e.g., *C. nodosa* and *Z. muelleri*) may be more tolerant and might even benefit from future-warmer oceans.

Thermal Priming Effects on Seagrasses

Our study provides, for the first time, some evidence for thermal priming effects in seagrasses. Looking at the photo-physiological results at the second heatwave, it is clear that 2HW plants had been primed during the first heatwave (**Figure 2**). From both tested seagrass species, *Fv/Fm* and $\Delta F/Fm'$ values were higher (significantly in some cases) in preheated plants (2HW) than in non-preheated ones (1HW). Studies from terrestrial plants (Smillie and Gibbons, 1981; Wang et al., 2014; Li et al., 2015) have similarly shown that primed plants had a higher photosynthetic rate in relation to the non-primed plants. Hence, our photo-physiological results strongly support priming effects on studied seagrass species from a photosynthetic point of view. Focusing on T7, while the 2HW-*P. australis* were able to keep their NPQ values similar to CT plants – indicating priming for the heatwave, in contrast, the 1HW-*P. australis* greatly increased their NPQ as a common photo-protective mechanism in stressed plants (Ashraf and Harris, 2013).

From a morphological perspective, we also detected significant differences between un-primed- (1HW) and primed-(2HW) *Z. muelleri* in terms of leaf elongation and leaf biomass production (**Figure 3**). For both parameters, 1HW-*Z. muelleri* suffered a significant reduction with respect to 2HW plants and CT plants as well. This indicated that 2HW plants were primed by the first heatwave, performed better during the second heatwave and were able to better maintain their growth as compared to that of the 1HW plants. Our results are similar to those from terrestrial plants (Wang et al., 2014) that also showed that primed *Triticum aestivum L.* maintained their biomass compared to un-primed plants during a more severe high-temperature stress. It is likely that the relatively slow growth rates of this climax species and the short marking time (i.e., growing period) compared to the pioneer species, did not allow for the detection of differences in growth between both heat treatments (1HW vs 2HW). As a result, we believe a longer growing period would be needed to detect a growth change.

In support of our hypothesis of thermal priming effects in seagrasses, large Chl *a* reductions were only detected in leaves of non-pre-heated plants (1HW). This becomes more obvious in the Chl *b*/*a* ratios of *Z. muelleri* at the end of the second heatwave (T7). While pre-heated 2HW plants kept their Chl *b*/*a* ratios similar to the controls, non-pre-heated 1HW plants experienced a significant increase in Chl *b*/*a* ratios as seen in previous

studies in terrestrial plants (Almeselmani and Viswanathan, 2012; Niu et al., 2017).

At the molecular level, there were more indications that priming had an effect on both species. This is indicated by a significantly lower expression level of some GOIs from 2HW plants compared to those from 1HW plants. In *P. australis* at T7, the expression levels of some methylation-related GOIs (i.e., ATX2 and ATXR7) and photosynthesis-related GOIs (i.e., psbD) were significantly higher in non-preheated plants (1HW) in comparison with preheated plants (2HW) and control plants (CT). Similarly, more evidence supporting the thermal priming hypothesis can also be found in stress-related GOIs (i.e., CAT) and methylation-related GOIs (i.e., ASH2L and ATX2) in heated *Z. muelleri*.

In addition, our PCA results at T7 (**Figures 6B,D**, **7**) further support the priming effects by showing, in both studied species, that 2HW plants were clustered with CT plants while 1HW plants were more separated away from those two former groups.

During the first heatwave (T3) the two species showed differences in gene expression. While a large amount of GOIs (i.e., 6/10) showed significant up-regulation in *Z. muelleri*, only 3 GOIs were significantly up-regulated in *P. australis*. An alternative to epigenetic modifications, the accumulation of protective molecules (i.e., HSPs) is also likely involved in facilitating a fast stress response and hence are also possible mechanisms underlying stress memory. At T3, only *Z. muelleri* activated HSP90 which is a well-known heat-protective molecule also involved in the heat stress response of different seagrasses (Marín-Guirao et al., 2016; Tutar et al., 2017; Mota et al., 2018; Traboni et al., 2018). Together, these differences between the two species suggest that *Z. muelleri* plants were, indeed, more prone to thermal priming and hence to acquire thermal tolerance after recurrent heat events than *P. australis* plants.

Our study also suggested the involvement of methylation-related genes or epigenetic modifications in response to thermal stress in seagrasses. Our results, indeed, confirmed recent transcriptomic discoveries in seagrasses showing the induction of genes involved in DNA and histone methylation, including our methylation-related GOIs (i.e., ATX2 and SETD3), in heated *P. oceanica* (Marín-Guirao et al., 2017, 2019). Among our methylation-related GOIs, ProteinSet1/Ash2 histone methyl transferase complex subunit ash-2 (ASH2L) and Histone-lysine N-methyltransferase ATX2 are known as being specifically involved in methylation and dimethylation at Lys4 of histone H3 (H3K4) (Wysocka et al., 2003; Patel et al., 2009). Methylation status of H3K4 has been shown to be involved in changing chromatin structure during environmentally-induced transcriptional memory (D'Urso and Brickner, 2017) and plant stress response via activating or silencing gene expression (Shanker, 2016). In addition, ATXR7 belongs to the Trithorax family proteins that connect with seasonal memory in plants (Iwasaki and Paszkowski, 2014). On the other side, Histone-lysine N-methyltransferase SETD3 is linked to H3K36 methyltransferase (Pontvianne et al., 2010; Suzuki et al., 2017) which in plants has been suggested to play an important role in development and stress responses (Huang et al., 2016). The regulations of the methylation-related GOIs in our study are consistent with previous work which highlighted the role of epigenetic modifications in seagrasses (Davey et al., 2016; Marín-Guirao et al., 2017; Duarte et al., 2018; Marín-Guirao et al., 2019) or in terrestrial plants (Chinnusamy and Zhu, 2009; Liu et al., 2015; Rey et al., 2016).

Future Perspectives

While our study demonstrates, for the first time, thermal priming effects on two seagrass species from the southern hemisphere, the duration of our experiment was relatively short in comparison to what the plants experience in their natural environment (i.e., marine heatwaves, see Hobday et al., 2016). For that reason, more ecologically relevant studies (e.g., Olsen et al., 2012; York et al., 2013) on stress memory in seagrasses are needed to confirm and broaden our findings. Moreover, considering that local adaptation could be responsible for many inter- and intra-specific differences among different species and different seagrass populations (Procaccini et al., 2007; King et al., 2018), together with the fact that we used only one population from each species, future studies clearly need to investigate more species and more populations in order to complete our knowledge on thermal priming effects on seagrasses.

Another point that should be considered in future studies is the importance of testing the length (duration) of the stress memory since the adaptive success of the species could be highly dependent on this factor. Recently, one study from the Baltic Sea has shown methylation patterns of *Zostera marina* changed under heat stress conditions and importantly, the seagrass did not return to pre-stress patterns after a 5.5-week recovery period (Jueterbock et al., 2019). This could explain why gene expression levels of methylation-related GOIs of 2HW plants were relatively lower than those from 1HW plants during the second heatwave in our experiment (**Figures 5B,D**). In terrestrial plants, stress memory has been predicted to last from several days or months (Iqbal and Ashraf, 2007; Rendina González et al., 2018). Together with Jueterbock et al. (2019), our study adds to the emerging knowledge of the length of thermal stress memory in seagrasses which could benefit from future studies to better understand stress memory duration in seagrasses. Also in this context, questions about the inheritance of stress memory in seagrasses deserve future efforts, especially when heat stress can induce and advance flowering in some seagrass species (Diaz-Almela et al., 2007; Blok et al., 2018; Ruiz et al., 2018; Marín-Guirao et al., 2019) as seen in many other plants (Wada and Takeno, 2010; Takeno, 2012, 2016). Heat stress-induced flowering/sexual reproduction can provide an "escaping" mechanism for seagrasses, allowing them to migrate to more favorable areas and/or stabilize the resilience of the plants' populations by increasing genetic diversity through sexual reproduction (Hughes and Stachowicz, 2004; Procaccini et al., 2007; Ehlers et al., 2008). Further to that, heat stress-induced flowering can also favor transgenerational memory of stress (Molinier et al., 2006; Boyko and Kovalchuk, 2010) in seagrasses, which could potentially secure the existence of threatened species in an era of rapid ocean change (Marín-Guirao et al., 2019).

In addition, the absolute degree and temporal stability of stress-memory demand special attention as priming could play an important role in stabilizing natural populations in the face of more frequent extreme heat events (Oliver et al., 2018; Darmaraki et al., 2019b). In fact, because heat stress often happens chronically in natural conditions, it could contribute to the maintenance of thermal stress memory (Bäurle, 2016) which benefits the resilience of seagrasses. This could partly explain the surprisingly weak effects of repeated heat events on natural populations. After the abrupt *P. oceanica* population decline reported after the 2006 heatwave (Marbà and Duarte, 2010; Jordà et al., 2012) no further mortality has been described after subsequent more intense and longer-lasting heatwaves in the Mediterranean occurred (e.g., 2012, 2015, 2017 see Darmaraki et al., 2019a).

In natural conditions, heat stress often does not occur alone, but in combination with multiple stressors (Gunderson et al., 2016). At this point it is also important to evaluate if heat acclimation and formation of heat-stress memory also prevent damage by other stressors, providing cross-stress memory and tolerance to current and future seagrass threats. Are heat-primed plants more tolerant also to other biotic and abiotic stress?

Controlled lab experiments need to be accompanied by field experiments and field observations after naturally occurring marine heatwaves. Conducting field experiments is often challenging, but new technological advances are promisingly allowing us to conduct more realistic mesocosm experiments and even conduct *in situ* experiments that simulate marine heatwaves (see Egea et al., 2019).

Lastly, although our results suggest the involvement of epigenetic modifications on stress memory in seagrasses, as broadly suggested in terrestrial plants (see reviews from Iwasaki and Paszkowski, 2014; Kinoshita and Seki, 2014; Latzel et al., 2016), the underlying mechanisms are yet to be revealed. Thus, future studies, exploring the mechanisms of stress memory in seagrasses are clearly needed.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the all datasets for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

HN, LM-G, MP, PR, and GP conceived and designed the experiment. HN, MK performed the experiment. HN analyzed the results. All authors wrote and reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00494/full#supplementary-material

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Morphological and Physiological Responses of *Enhalus acoroides* Seedlings Under Varying Temperature and Nutrient Treatment

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Seagrass meadows are declining globally. In Indonesia, 75% loss has been reported in the last 5 years. The decrease of the seagrass area is influenced by the simultaneous occurrence of many factors at the local and global scale, including nutrient enrichment and climate change. This study aims to find out how increasing temperature and nutrient enrichment affect the morphological, biochemical and physiological responses of *Enhalus acoroides* in the seedling phase, which has not previously been studied. To achieve these aims, a laboratory experiment of combined temperature and nutrient treatments was conducted using recently-germinated seedlings of *E. acoroides*. The results showed that the seedlings were tolerant to an extended exposure to the current ambient maximum temperature. Under higher temperature treatment, the seedlings were observed to increase in aboveground size traits (e.g., number of leaves, leaf length, biomass, and leaf area), as well as in belowground traits, such as root length. The results in this study also showed that the initial seed size matters for morphological responses. On the contrary, nutrient responses of seedlings were practically absent, suggesting they could rely on internal reserves. Interaction between both factors was limited, with the exception of low temperature and high nutrient treatment, in which the AG:BG ratio and leaf elongation rate increased. Fluorescence parameters were not influenced by any of the water treatments. The results in this study suggest that *E. acoroides* seedlings rely energetically in the reserves within the seedling and that increasing temperature might result in faster seedling development, although no interactions with other organisms were tested. This is of importance when studying the resilience capacity of this species and when restoration attempts are planned, as a faster root development would provide a faster stabilization in the sediment and the survival of the whole plant.

Keywords: tropical seagrass, Indo-Pacific, traits, growth, nutrient content, photosynthesis, carbohydrates, Indonesia

INTRODUCTION

Seagrasses are marine flowering plants that are globally distributed and can form dense meadows in shallow water coastal environments (Duarte, 1991, 2001; Duarte and Cebrián, 1996; Short et al., 2007). They are key ecosystem engineers and the only submerged marine species with both above- (AG) and below-ground (BG) tissues that sustain multiple ecosystem services and functions. Seagrasses fuel food webs by supplying a combination of food and shelter to various macro flora and fauna, including commercially important fish species, sustain high rates of primary production, filter coastal waters by capturing particles and dissolved nutrients, participate in nutrient cycling, and provide coastal protection by attenuating waves and stabilizing sediments by their singular BG tissues (Hemminga et al., 1991; Duarte and Cebrián, 1996; Hemminga and Duarte, 2000; Duarte, 2001; Cullen-Unsworth and Unsworth, 2013; Tuya et al., 2014; Weitzman et al., 2015). Seagrass meadows also help to mitigate climate change by the capture and storage of organic carbon ("blue carbon"), reducing CO_2 concentrations in seawater (Fourqurean et al., 2012; Macreadie et al., 2019). Despite their global significance the attention paid to seagrass meadows is much lower than other coastal ecosystems (Cullen-Unsworth et al., 2014).

Seagrasses are also one of the most threatened ecosystems due to their presence in coastal areas (Orth et al., 2006; Burkholder et al., 2007), where they are declining globally, with a loss of 29% from 1876 to 2006 (Waycott et al., 2009). Many factors have been identified as the reason for this decrease, both at the local and global scale, including nutrient enrichment and climate change (Orth et al., 2006; Waycott et al., 2009). Regional losses may be even higher in the tropics, such as in Indonesia, where 75% loss has been reported in the last 5 years (Unsworth et al., 2018). Tropical ecosystems are biodiversity hotspots, with the tropical Indo-Pacific bioregion hosting 24 of the approximately 60 seagrass species (Short et al., 2007). As seagrass traits and functions sustaining different ecosystem services and the stressors that affect them are species-specific, multi-specific assemblages have a greater probability of containing a greater functional diversity, but also greater losses (Duarte, 2000).

Human development and associated activities (e.g., agriculture, fish and seaweed aquaculture) have triggered land use changes and urbanization which have led to an increase in the concentration of nutrients and sediments into the coastal waters. This nutrient increment exceeds the nutrient cycling capacity of the system, increasing the organic carbon supply, and ultimately leading to eutrophication processes that are characterized by algal blooms, epiphyte growth, anoxic conditions in the sediments and, eventually, biodiversity loss and seagrass death (Lee et al., 2006; Khan and Mohammad, 2014). The effects of nutrient enrichment depend on species-specific features, such as nutrient uptake capacity, the level of nutrient surplus that can vary from moderate to severe, and on local physical conditions, such as currents and tides. Eutrophication has been identified as one of the most important factors affecting productivity, community carbon dynamics, and seagrass growth, and is one of the major threats confronting coastal ecosystems. Direct effects occur through stability of physiological mechanisms (Burkholder et al., 1992; Bird et al., 1998; Brun et al., 2002; Invers et al., 2004; Touchette and Burkholder, 2007) causing increased nutrient uptake ability (Viana et al., 2019), nutrient imbalance (Li et al., 2019), changes in morphological indices (Mvungi and Pillay, 2019), changes in growth (Terrados et al., 1999a), changes in sexual reproduction (Duarte et al., 1997), or direct ammonium toxicity (Van Katwijk et al., 1997). Indirect effects of nutrient inputs occur through blooming algae which cause light depletion or nutrient competition (Duarte, 1995; Short et al., 1995; Moore and Wetzel, 2000; Nixon et al., 2001; Burkholder et al., 2007), through the ecological role of herbivores due to modifications in palatability and plant defenses against herbivory (Tomás et al., 2015; Jiménez-Ramos et al., 2017; Marco-Méndez et al., 2017; Campbell et al., 2018; Hernán et al., 2019), or through oxygen depletion in sediments (Terrados et al., 1999b). Therefore, the effects of nutrients on seagrass responses range from no impacts to positive or negative impacts. Tropical systems are oligotrophic and naturally nutrient limited; therefore, even small amounts of nutrient additions might rapidly increase competence among primary producers.

Climate change may also impact seagrasses due to rising mean water temperatures and more frequent and lengthy heat waves (Marbà et al., 1996; Short and Wyllie-Echeverria, 1996). Impacts of temperature increase can be detected at the plant level and have been found to benefit the growth, biomass (Masini et al., 2001), flowering (Diaz-Almela et al., 2007), or photosynthetic rates (Campbell et al., 2006) of several seagrass species. But if elevated temperature rises above a threshold, or persist for longer periods of time, effects could be detrimental and result in community structure damages by causing impacts on seagrass metabolism and nutrient uptake ability (Lee et al., 2007; Moore and Short, 2007; Collier and Waycott, 2014). This can eventually lead to die off if extreme temperatures persist (Cambridge et al., 1986; Bulthuis, 1987; Short and Neckles, 1999; York et al., 2013). Recent experiments suggest that heat waves may enhance the autotrophic metabolism of seagrass communities in contrast to other previous research suggesting solely negative effects (Egea et al., 2019). Therefore, temperature is of crucial importance in determining seagrass metabolism, growth and survival. While climate change has not yet significantly impacted seagrass areas in Indonesia (Unsworth et al., 2018), it is essential to take in consideration that tropical seagrasses are growing closer to their photosynthetic and physiological limits in comparison to temperate seagrasses, making them highly vulnerable to rising temperature thresholds caused by climate warming (Tewksbury et al., 2008).

The ecosystem functions performed by seagrasses are consequences of their physiological, biochemical and morphological traits. Therefore, even though changes in seagrass traits could be seen as positive for individual seagrass plants (i.e., increasing growth or photosynthetic rate), it could also lead to changes in their functions and imbalances in their biotic and abiotic interactions, negatively affecting the ecosystem services they perform. For example, it could lead to changes in hydrodynamic conditions or sedimentation rates (Fonseca et al., 2019) which affect the distribution of organisms within the

canopies, and therefore, biodiversity (González-Ortiz et al., 2014; Jiménez et al., 2019; Meysick et al., 2019). Seagrass responses to changes in nutrient and temperature conditions can be measured by changes in their trait values which are often used as indicators of environmental stress in coastal management. Different traits have been identified as indicators of seagrass stress such as nutrient inputs, temperature or shading (Lee et al., 2007; Martínez-Crego et al., 2008; De los Santos et al., 2016; Roca et al., 2016). But seagrass plants are rarely affected by just one variable and identifying the effect of single stressors is a challenge in natural seagrass meadows. The interaction between stressors is now viewed as a crucial issue, and it is suggested that single-factor experiments are not adequate for assessing the effects of several disturbances on coastal marine ecosystems (Wernberg et al., 2012; Todgham and Stillman, 2013; Ontoria et al., 2019). In this way, laboratory experiments under controlled conditions might help to isolate the effects on plant trait variability of single and multiple stressors. These physiological experiments are also needed in order to make predictions about seagrass resilience or tolerance to future climate scenarios. The combined impacts of rising temperature and increased nutrient loading has been studied in adult species of *Zostera* spp. and *Cymodocea nodosa* (Touchette and Burkholder, 2002; Touchette et al., 2003; Kaldy, 2014; Jiménez-Ramos et al., 2017; Mvungi and Pillay, 2019; Ontoria et al., 2019), but, as far as we know, no studies were carried out in seagrass early life stages. There are few works on the combined effects of other stressors in seagrass seedlings but none in tropical species (Hernán et al., 2016; Alexandre et al., 2018; Pereda-Briones et al., 2018, 2019; Yue et al., 2019).

Enhalus acoroides is a tropical seagrass with a high tolerance to environmental changes such as temperature and nutrients (McMillan, 1984; Terrados et al., 1999a), and therefore, changes in its morphological, biochemical or physiological traits can be used as indicators to increasing temperature and varying nutrient fluctuations. *E. acoroides* is also an ecosystem engineer which, by altering the physical and chemical properties of the environment, can facilitate the presence of species that otherwise would be absent. The opportunity of colonizing new habitats and the genetic diversity provided by sexual reproduction could make seagrass populations more resistant to the current changing scenario. In addition, due to the highly variable flower production and low success of seedling establishment, sexual reproduction and seedling stages are critical phases in the life of seagrasses (Bewley and Black, 1994; Schupp, 1995; Peterson and Baldwin, 2004). However, there is a lack of research conducted on *E. acoroides* seeds and seedlings in order to understand their response to various environmental changes. This is of importance as seagrass restoration programs could be based on adult seagrass transplantation or on generative techniques. Planting seedlings is a cost-efficient method for large-scale seagrass meadow restoration. However, the main limitation of seedling establishment programs is the low seedling survival rate observed due to unsuitability of environmental conditions (Ambo-Rappe et al., 2019). Therefore, the study of seedling trait responses under different environmental conditions, such as temperature or nutrient enrichment, on this early-life phase may enhance future restoration and conservation management plans of these threatened ecosystems.

This study aims to assess the morphological, physiological and biochemical trait responses of the seedling stage of *E. acoroides* to increased temperature and nutrient enrichment. Furthermore, the results of this study will provide important information and serve as a reference to predict the effects of temperature changes, as a proxy for climate change conditions, and nutrient enhancement on seagrass survival. To achieve these aims, a laboratory experiment was conducted using seedlings of *E. acoroides* under the combination of increased temperature and nutrient enrichment. We hypothesized that seedlings of *E. acoroides* might be tolerant to rising temperatures and that nutrient enrichment would increase their growth performance, causing synergetic effects under higher temperature.

MATERIALS AND METHODS

Collection and Maintenance of Seagrass Seeds

Fruits of *E. acoroides* were collected on mid-January 2017 on the southwest side of Barrang Lompo Island, South Sulawesi, Indonesia (S 5°03'05, E 119°19'37), where *E. acoroides* is abundant at a depth range of 1–3 m. Highest nitrate concentrations in the area range between 0.1 and 0.6 μM, while phosphate concentrations range between 0.12 and 0.14 μM (Kegler et al., 2018). Annual temperature range in the dry season varies in this area between 26 and 32°C (Teichberg et al., 2018). During seed collection, we measured 28–32°C during mid-day. The ripe seagrass fruit was opened, packed in a Styrofoam box with wet breathable polyester fiber sheets, and then transported to the Marine Experimental facilities (MAREE) at the Leibniz Centre for Tropical Marine Research (ZMT) in Bremen (Germany) in <24 h. Once at the MAREE, seeds were planted directly in polypropylene trays previously filled with silicate sediment of at least 10 cm depth.

All trays with seeds were kept in 250-l aquaria filled with low nutrient artificial sea water (ASW) (Red Sea Salt, Red Sea Europe Company) under controlled conditions of light irradiance (200 ± 30 μmol photons m^{-2} s^{-1}), temperature (26 ± 1°C) and salinity (35 PSU) for a 1 week acclimation phase until the root and some leaf growth was observed (at which point they entered the seedling stage). The photoperiod of the fluorescent lights was 12:12 h light:dark cycle.

Experimental Design and Setup

We conducted a full-factorial experiment combining two water temperatures (26 and 31°C) representing the minimum and maximum temperatures within the home region that seagrasses are currently exposed to, and two nutrient treatments (low nutrient concentrations of 2 μM of NH_4NO_3 and 0.1 μM KH_2PO_4 and high nutrient concentrations of 20 μM of and 1 μM of NH_4NO_3 and KH_2PO_4). This yielded in 4 experimental treatments: low temperature and low nutrient concentrations, low temperature and high nutrient concentrations, high temperature and low nutrient concentrations, and high temperature and high nutrient concentrations. The experiment

was conducted under laboratory conditions in an indoor flow-through system at the MAREE (ZMT, Bremen) with 24 individual aquaria with 29 × 13 × 30 cm dimensions and 10 l volume (**Figure 1A**). Seedlings (consisting of visible cotyledon, seeds and no roots at the beginning of the experiment) were categorized as small (diameter between 0.6 and 1.0 cm), medium (diameter between 1.1 and 1.5 cm) and large (diameter between 1.6 and 1.7 cm). One seedling of each size was distributed in each aquarium, making a total of three seedlings per aquarium. The aquaria additionally contained adult seagrasses from 3 different species (Viana et al., in prep).

The target temperature values were obtained by placing aquaria in larger experimental tanks (ETs) of 250 l that acted as water baths maintaining a constant water temperature. Six aquaria were placed in 4 different ETs following a split-plot experimental design with nutrient treatments nested within the 4 ETs set at the two temperatures (**Figure 1A**). There was no interaction between aquaria that acted as replicates ($n = 6$). Water temperature was controlled in each ET by using heaters (EHEIM) connected to an individual electronic system that was continuously regulating the temperature of the water bath by digital controllers and individual temperature probes (±0.2°C). Air pumps were also placed in each ET to ensure water movement of the water bath. The light was provided by LED lamps (Hydra Fifty-two HD, AquaIllumination®, Iowa), 2 lamps were placed at the same height at the top part of each ET, providing 200 ± 20 μmol m^{-2} s^{-1} of light (measured at each aquarium to ensure homogeneous irradiance). Light was set on a 12:12 h light:dark photoperiod with sunrise and sunset simulation. Transparent PVC lids were placed on each ET to reduce water evaporation.

High and low nutrient ASW solutions were individually supplied to each of the 24 aquaria from two different water reservoirs of ∼115 l each, with either high or low nutrient concentrations using a 24-channel peristaltic pump (ISMATEC, Germany). The flow from both water reservoirs was maintained at a constant rate at ∼5.8 l d^{-1} ensuring total water renovation inside each aquarium every ∼1.5 days. Water reservoirs were manually emptied from any remaining water and refilled with fresh ASW every other day. Nutrients to the water reservoirs were added in a previously dissolved form from stock solutions of NH_4NO_3 and KH_2PO_4 (Merck, Germany). Once in the water reservoir, ASW was gently mixed and an air pump was placed in each water reservoir to ensure further aeration and mixing. Air pumps were placed in each aquarium to ensure water aeration and mixing by moving water from the bottom to the top. Water constantly overflowed from the aquaria to the water bath of the ETs ensuring water renewal. At the same time, ETs were drained of the surplus water flowing out of the aquaria. Algae were removed from the blades of the plants throughout the experiment but not from the rest of the aquaria.

For the experiment to begin, the temperature was increased in 2 random ETs from 26 to 31°C at 1°C d^{-1} while the other 2 ETs remained at the initial temperature of 26°C. Once the desired temperatures were stable in all ETs, the nutrient enrichment began. From that moment, the experiment lasted for approximately 1 month (January 20th to February 22nd, 2017).

Water Sampling

Water parameters, including pH, temperature and salinity, were monitored three times per week during the treatment phase with a multi parameter probe (WTW Multiprobe). During the experiment the temperature inside aquaria was also continuously monitored by Hobo loggers (Onset, MA, USA) placed in one random aquarium of each ET ($n = 4$). Water samples were taken every week from the two water reservoirs and random aquaria of each treatment ($n = 4$ each week) for DIN (dissolved inorganic nitrogen, NH_4^+, NO_x^- and NO_2^-); silicate and phosphate. Water samples were sampled with a syringe, immediately filtered (0.45 μm pore size) in pre-rinsed polyethylene bottles and frozen (−20°C). Analysis was performed using a continuous flow injection analyzing system (Skalar SAN++-System) following Grasshoff et al. (1999).

At the end of the experiment, water samples from all aquaria were collected and immediately filtered for chlorophyll *a* and *b* (Chl-*a* and -*b*) measurement. Water was filtered under constant pressure onto pre-combusted (5 h, 450°C) Whatman GF/F filters. Filters for Chl-*a* and -*b* analysis were stored at −20°C. Pigments were extracted from the filters in 8 ml of 96% ethanol in glass vials placed for 5 min at 80°C and subsequently placed in a rotor at room temperature in the dark for approximately 24 h. Extracts were subsequently centrifuged at 5,000 rpm for 20 min. Chl-*a* and -*b* samples were determined in a photometer Shimadzu UV-1700.

Seedlings Morphological and Physiological Traits

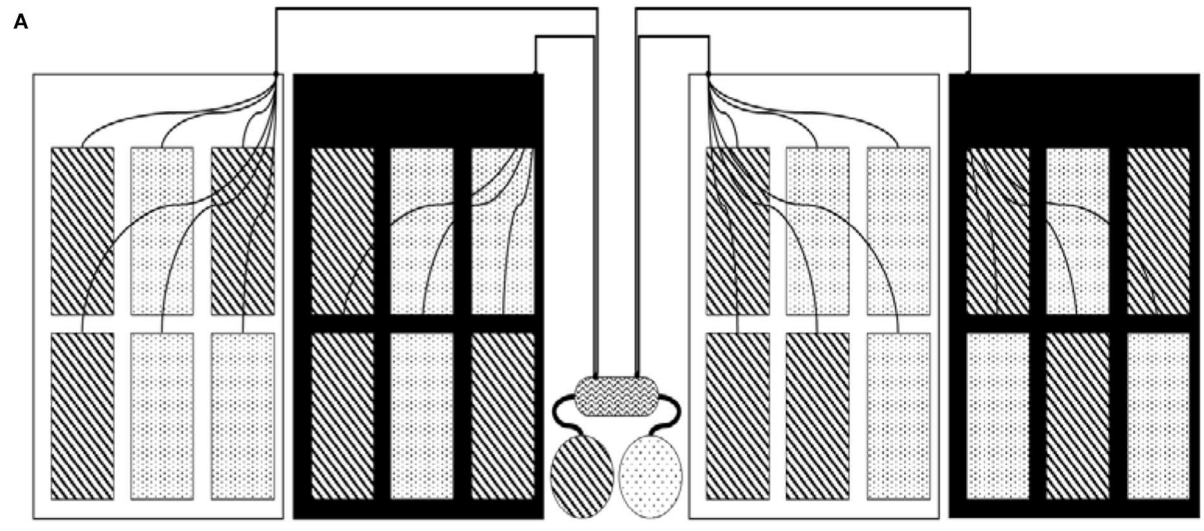
At the end of the experiment, seedlings were removed from the aquaria and the morphological measurements on each plant were first performed. Afterwards, plants were carefully separated with a glass slide into the different parts: leaves (for fluorescence measurements and nutrient content), seeds and roots (for nutrient content). While morphological and fluorescence measurements were individually performed on the three seedlings, the nutrient content was analyzed in the pooled material of the three seedlings from each aquarium. Samples of the separated plant were gently cleaned with distilled water to remove any sediment or epiphytes and subsequently frozen at −80°C until analysis.

Morphological Traits

Seagrass morphological traits were determined by measuring the length, width and number of leaves per seedling, the length and number of roots, the height and diameter of seeds and the biomass of leaves, seeds and roots. AG (blades) and BG (roots) biomass was also determined (±0.01 g).

Growth Rates

Seedling growth rate measurements were done using the leaf marking method (Short and Duarte, 2001). At the beginning of the experiment, leaves were perforated close to the seed using a pin. At the end of the experiment, the length from the seed to the mark of each leaf was measured. Leaf growth rates were obtained by dividing the elongation (distance from the base to the mark) with the number of days since the seagrass leaves



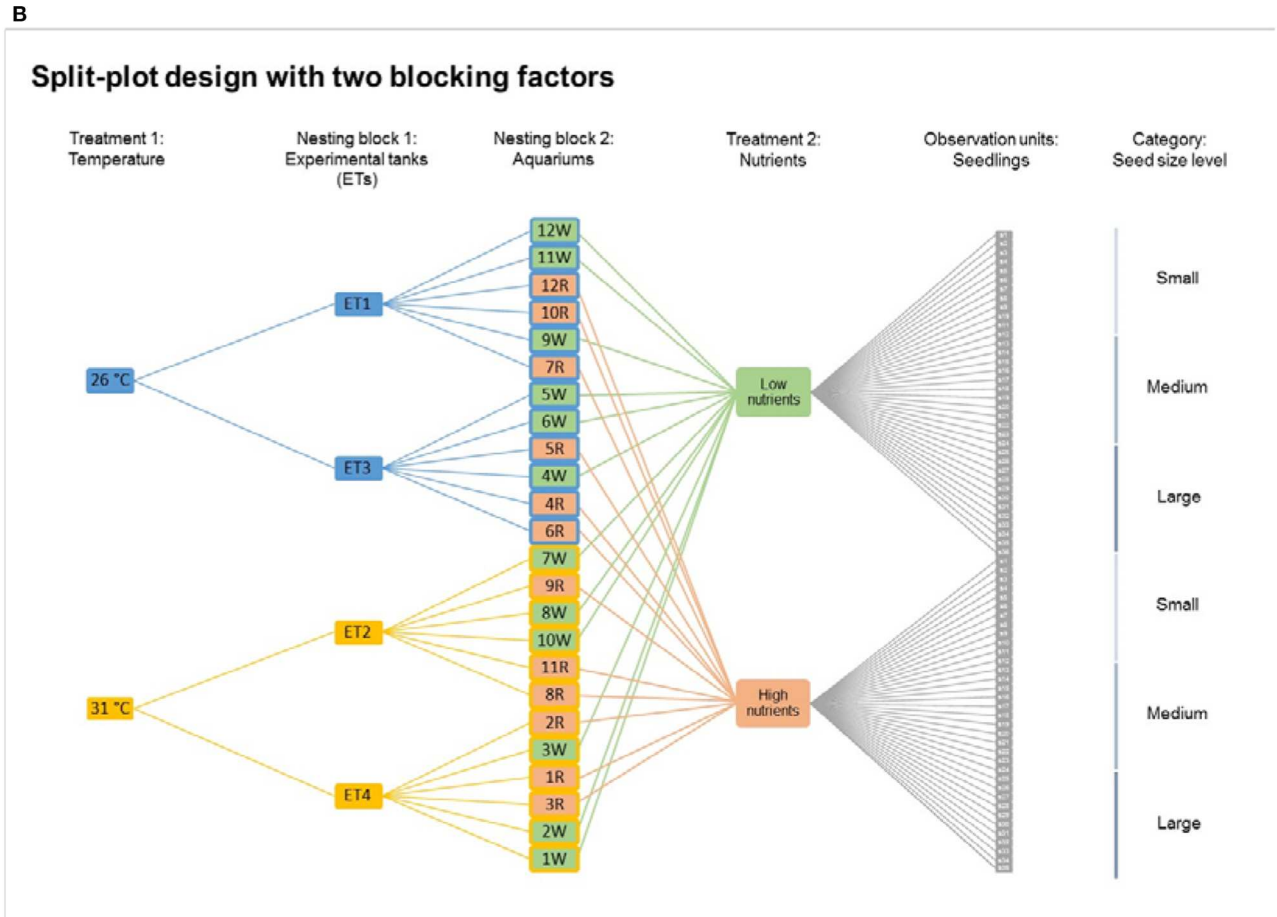


FIGURE 1 | (A) Nutrient and temperature split-plot experimental design. Artificial seawater was individually supplied to aquaria ($n = 24$) from reservoirs with either high nutrient (dot boxes/circle) or low nutrient artificial seawater (strip boxes/circle) using a peristaltic pump. Six aquaria were placed in each experimental tank (ET) that acted as a water bath maintaining a constant temperature of either 26°C (white boxes) or 31°C (black boxes), with no interaction between aquaria that acted as replicates ($n = 6$). **(B)** Experimental split-plot design with two blocking factors. Temperature and nutrient treatments are the fixed effects (explanatory variables) in the model, and they are fully crossed (nutrient treatments are not nested by temperature treatments). Temperature, as it groups ETs and aquaria, is a group level predictor. Nutrients, as it groups the observation units (seedlings) is a data level predictor. ET and aquaria are nesting variables, this means that ET nests the aquaria and the aquaria nests the observation units (seedlings). The seedlings are also further classified as large, medium and small.

were marked. Surface area (SA) was calculated with the following Equation (1).

$$SA\ Growth\ rate\ (cm^2 d^{-1}) = \frac{distance\ from\ the\ base\ to\ the\ mark \times leaf\ width}{number\ of\ days} (1)$$

Photosynthetic Performance

A PAM-2500 (Walz, Germany) was used for the measurement of the fluorescence of the seagrass through rapid light curves (RLC). The optical cable of the PAM was attached with leaf clips to the second leaf of the seedling, above the meristem, and at 3 mm distance from the tissue. The leaves were dark adapted for 5 min before measurement.

The RLC consisted of 12 saturating light pulses (separated by 30 s intervals), increasing the photosynthetic active radiation (PAR) between pulses until 2,001 μmol photons m^{-2} s^{-1}. From the data of the RLC, several parameters were calculated.

Light saturation coefficient (Ek) and the slope of the light limited part of the curve (Alpha) were calculated using the package Phytotools (Silsbe and Malkin, 2015) with the R software (R Core Team, 2019) following the model of Jassby and Platt (1976). The maximum light utilization efficiency or maximum quantum yield was calculated following Equation (2) (Genty et al., 1989).

$$Maximum\ quantum\ yield = \frac{(Fm - Fo)}{Fm} \quad (2)$$

Where F_m is the maximum dark-adapted fluorescence and F_o is the minimum dark-adapted fluorescence. The relative electron transport rate (rETR) was calculated for each step of the curve following Equation (3) (Sakshaug et al., 1997).

$$rETR = \frac{Fm' - F'}{Fm'} \times \frac{PAR}{2} \quad (3)$$

Where Fm' is the light adapted maximum fluorescence and F' the fluorescence yield at a particular light level. From the rETR values, maximum rETR (rETRmax) was calculated as the inflection point of the fitted rETR curve.

Nutrient Content

Leaf, seed and root nitrogen (N) and carbon (C) content (%N and %C, respectively) was analyzed on previously dried (60°C, 48 h) and powdered seagrass tissue samples. Aliquots of the samples were weighed into tin capsules using an analytical scale prior to analysis (Euro EA3000 Elemental Analyzer).

Non-structural Carbohydrate (NSC) Content

The concentrations of soluble sugars (sucrose) and starch were measured on leaf and seed material that was previously freeze-dried (48 h) and ground to a fine powder. Sucrose was extracted from plant tissue by heating (80°C) in 95% EtOH. The ethanol extracts were subsequently evaporated bubbling the samples with N_2, and the remaining residues were dissolved in deionized water for sucrose analysis. Starch was extracted during 24 h from the ethanol-insoluble residue in 0.1 N NaOH. The sucrose and starch concentrations were determined spectrophotometrically (486 and 640 nm, respectively) using an F200-Pro TECAN© plate reader. Resorcinol and anthrone assays were used for sucrose and starch determination, respectively, and sucrose was used as the standard for the calibration curve (Yemm and Willis, 1954; Huber and Israel, 1982). Results were reported in glucose equivalents g^{-1} DW. Current testing of this method has shown that NaOH extracts both starch and cellulose which can confound the results. Regarding the sucrose determination, this method only determines ketoses (as fructose) so we are ignoring the other component of sucrose, glucose, underestimating the final concentrations (M. Birkicht, personal communication).

Statistical Analysis

The experiment followed a split-plot design with three nesting factors (Schielzeth and Nakagawa, 2013). The two main factors (temperature and nutrient treatments) had two levels each (26 and 31°C, and low and high nutrient concentrations, respectively), which were fully crossed.

We used permutational multivariate analysis of variance (PERMANOVA) (Anderson et al., 2008) to analyze the data. The statistic to test the null hypothesis of no differences in the position of the group centroids in the space of the chosen dissimilarity measure is the pseudo F-statistic. The fixed effects in the model were temperature and nutrient treatments, the seedling size and their interactions, together with the nesting factors temperature, ET and Aquarium. Three seedlings were nested in each aquarium; six aquaria were nested in four ETs, and two ETs were nested within each temperature treatment. Seedlings, with 3 levels of sizes, were evenly distributed between aquaria (**Figure 1B**). Seedling size and Aquarium were not included as factors in the model for biochemical trait analysis because seedling samples within the same aquaria were pooled. Data was scouted for outliers, which were identified as data exceeding 1.5 times the interquartile range of variation of the dataset. Outliers were eliminated from the model when they did not allow for meeting the model assumptions. Afterwards, we calculated the dissimilarity matrix using the Euclidean dissimilarity measure for all continuous variables and the Bray-Curtis dissimilarity measure for the count variable (number of leaves). The assumptions of exchangeability of permutable units and homogeneity of multivariate dispersion were tested before analysis. When the homogeneity of multivariate dispersions was not met, the data were transformed (square root, log or inverse) and the dissimilarity matrix was recalculated. Once the assumptions were met, the model selection was performed with the Akaike Information Criterion (AIC). The model with the lowest AIC was chosen with three extra rules: The nesting variables and seedling size, and the interaction Temperature*Nutrient treatment was never dropped from the model to avoid pseudo-replication and because it was part of the hypothesis, respectively. If the final model had no significant interaction with seedling size, these interactions were dropped for simplification. The statistical analysis in the variable number of roots could not be performed due to presence of zeros in the data, which does not allow for the calculation of the Bray-Curtis dissimilarity matrix. Starch concentration in seeds was not analyzed as homogeneity of multivariate dispersion assumptions

could not be met. Water parameters (DIN, phosphate, and Chl-*a* and -*b*) and temperature in aquaria were compared using PERMANOVA, while DIN and phosphate concentrations in water reservoirs were analyzed using a two-way ANOVA.

We used R software to perform the analysis (R Core Team, 2019) with the adonis2 function of the package "vegan" (Oksanen et al., 2019). Temperature, nutrient, seed size, their interactions and the nesting structure (Temperature:ET:Aquarium) were the fixed effects in the model. The permutational unit for the model was the aquarium with 999 permutations, which is the recommended minimum number to test at an alpha-level of 0.05 (Manly, 1997).

RESULTS

Experimental Water Chemistry and Trophic Conditions Within the Aquaria

Water temperature was nearly constant throughout the experiment and within the target temperatures (PERMANOVA, $P < 0.01$) (**Table 1**). Nutrient concentrations in the two main water reservoirs that continuously provided ASW to the aquaria were within the target concentrations throughout the experiment and were significantly different between the nutrient treatments (two-way ANOVA, $P < 0.001$). Once in the aquaria, however, nutrients were rapidly taken up resulting in low inorganic nutrient concentrations in all treatments regardless of the inputs (PERMANOVA, $P > 0.05$). In fact, some of the concentrations measured were not included in the analysis as they were below the quantification limit. Chl-*a* concentrations in the water column were higher in the high nutrient treatments (2.6–5.9 $\mu g\ l^{-1}$) than in the low nutrient treatments (2–2.1 $\mu g\ l^{-1}$) and significantly higher in the high temperature and high nutrient treatment (5.91 $\mu g\ l^{-1}$); Chl-*b* concentrations also increased in the high temperature and high nutrient treatment although no significant concentrations were observed among treatments (PERMANOVA, $P > 0.05$). Therefore, the different treatments effectively changed trophic conditions within aquaria, as indicated by the increased Chl-*a* and Chl-*b* concentrations, as well as other algal blooms which were observed in the high nutrient treatments. Although the abundance of these other microorganisms could not be quantified, they were observable by naked eye and could be felt as a slimy layer on the aquaria and some seagrass leaves. They also formed fluffy masses with a slimy feel which disintegrated when attempts were made to capture them. Therefore, even though nutrient concentration parameters in aquaria were low, other observable parameters suggested eutrophic conditions were occurring in the high nutrient treatments. These symptoms were especially noticeable when high nutrients were combined with high temperature, leading to a greater growth of epiphytic algae. The salinity, pH and silicate values were constant across all treatments.

Seedling Traits

Seagrass seedling morphological traits and biochemical and physiological traits are shown in **Tables 2**, **3**. Results of the PERMANOVA analyses are shown in **Tables 4**, **5** for morphological and biochemcial and physiological traits respectively.

Seedling Morphological Traits

Seagrass seedling morphological traits showed a greater response to temperature changes with fewer traits affected by nutrient enrichment (**Table 2**). Leaf traits showed the greatest differences relative to root traits, especially under different temperature treatments (**Table 4**). The high temperature and low nutrient treatment had 5 leaves, while all other treatments had 4 leaves per seedling. Maximum leaf length was the highest in the high temperature treatments, particularly when combined with high nutrients. This also had an impact on leaf SA and AG biomass, although significant interactions were not observed. The only seed trait that was significantly influenced by temperature was the diameter. Seed height and biomass were not significantly affected by any of the treatments (**Tables 2, 4**).

All seedlings had two roots except for the low temperature and high nutrient treatment that just had one root (**Table 2**).

TABLE 1 | Experimental water quality parameters.

		Treatments			
	n	**26°C + Low nutrient**	**31°C + Low nutrient**	**26°C + High nutrient**	**31°C + High nutrient**
DIN (μM) [R]	4	5.66 ± 0.51		22.40 ± 0.98	
PO_4^- (μM) [R]	4	0.19 ± 0.01		1.01 ± 0.04	
Water temperature (°C)	25*	26.25 ± 0.05	31.01 ± 0.07	26.28 ± 0.03	31.01 ± 0.05
	3735‡	26.47 ± 0.003	31.13 ± 0.004	26.22 ± 0.004	31.48 ± 0.002
Salinity	25	35.43 ± 0.12	35.39 ± 0.10	35.33 ± 0.07	35.39 ± 0.11
pH	12	8.47 ± 0.02	8.39 ± 0.02	8.67 ± 0.03	8.59 ± 0.02
DIN (μM)	5-10	0.62 ± 0.33	0.88 ± 0.44	0.64 ± 0.31	1.25 ± 0.01
PO_4^- (μM)	5-10	0.15 ± 0.01	0.14 ± 0.02	0.14 ± 0.00	0.14 ± 0.01
Si (μM)	5-10	0.71 ± 0.07	1.15 ± 0.22	0.91 ± 0.20	0.85 ± 0.06
Chl-*a* ($\mu g\ l^{-1}$)	24	2.07 ± 0.53	2.07 ± 0.53	2.59 ± 0.98	5.91 ± 2.41
Chl-*b* ($\mu g\ l^{-1}$)	24	0.36 ± 0.07	0.36 ± 0.07	0.42 ± 0.18	0.88 ± 0.43

Water temperature (°C) data correspond to the multiprobe (*) and Hobo loggers (‡) measurements. DIN (Dissolved inorganic nitrogen as the sum of NH_4^+, NO_x^- and NO_2^-) and phosphate (PO_4^-) concentrations in water reservoirs (R) and random aquaria are shown. Values are given as means (±SE).

TABLE 2 | Morphological traits (mean ± SE, $n = 6$) of *Enhalus acoroides* seedlings in the four different temperature (Temp) and nutrient treatments at the end of the experiment.

Traits	Treatments		
	Temp	Low nutrient	High nutrient
N° of leaves seedling^{-1}	26°C	4 (±0.13)	4 (±0.31)
	31°C	5 (±0.18)	4 (±0.35)
Maximum leaf length (cm)	26°C	1.79 (±0.17)	1.97 (±0.25)
	31°C	3.45 (±0.33)	4.19 (±0.33)
Leaf width (cm)	26°C	0.35 (±0.01)	0.33 (±0.01)
	31°C	0.38 (±0.03)	0.37 (±0.01)
Leaf SA (cm^2)	26°C	3.40 (±0.3)	3.44 (±0.32)
	31°C	7.86 (±0.98)	9.34 (±0.78)
AG biomass (g FW)	26°C	0.19 (±0.02)	0.18 (±0.02)
	31°C	0.28 (±0.03)	0.31 (±0.02)
Seed diameter (cm)	26°C	1.03 (±0.03)	1.05 (±0.04)
	31°C	1.13 (±0.07)	1.17 (±0.07)
Seed height (cm)	26°C	1.14 (±0.03)	1.11 (±0.03)
	31°C	1.14 (±0.07)	1.21 (±0.09)
Seed biomass (g FW)	26°C	0.63 (±0.04)	0.68 (±0.04)
	31°C	0.73 (±0.05)	0.76 (±0.07)
N° of roots	26°C	2 (±0.25)	1 (±0.06)
	31°C	2 (±0.21)	2 (±0.15)
Maximum root length (cm)	26°C	2.92 (±0.42)	2.43 (±0.21)
	31°C	5.9 (±0.2)	5.62 (±0.27)
BG biomass (g FW)	26°C	0.12 (±0.02)	0.05 (±0)
	31°C	0.25 (±0.04)	0.21 (±0.04)
Ratio AG:BG	26°C	2.48 (±0.68)	7.53 (±3.16)
	31°C	1.30 (±0.16)	2.11 (±0.28)
Ratio BG:Seed	26°C	0.16 (±0)	0.08 (±0.02)
	31°C	0.33 (±0.05)	0.27 (±0.04)
Ratio AG:Seed	26°C	0.30 (±0.02)	0.25 (±0.03)
	31°C	0.40 (±0.02)	0.43 (±0.03)
Total biomass (g FW)	26°C	0.95 (±0.08)	0.88 (±0.07)
	31°C	1.23 (±0.13)	1.28 (±0.13)

SA, Surface area; AG, above-ground tissues; BG, below-ground tissues.

TABLE 3 | Biochemical and physiological traits (mean ± SE, $n = 6$) of *Enhalus acoroides* seedlings in the four different temperature (Temp) and nutrient treatments at the end of the experiment.

Traits	Treatments		
	Temp	Low nutrient	High nutrient
Leaf elongation rate (cm d^{-1})	26°C	0.01 (±0.002)	0.02 (±0.001)
	31°C	0.03 (±0.003)	0.03 (±0.003)
SA growth rate (cm^2 d^{-1})	26°C	0.11 (±0.01)	0.11 (±0.01)
	31°C	0.26 (±0.03)	0.31 (±0.03)
Maximum quantum yield	26°C	0.73 (±0.01)	0.70 (±0.03)
	31°C	0.75 (±0.01)	0.67 (±0.05)
rETRmax	26°C	16.11 (±2.28)	15.43 (±2.28)
	31°C	14.54 (±0.77)	15.10 (±2.38)
Alpha	26°C	0.70 (±0.01)	0.66 (±0.03)
	31°C	0.72 (±0.01)	0.66 (±0.04)
Ek	26°C	8.83 (±1.91)	8.54 (±3.06)
	31°C	8.41 (±1.23)	8.89 (±1.13)
Leaf C (%DW)	26°C	31.84 (±0.37)	30.96 (±0.32)
	31°C	30.90 (±0.53)	30.62 (±0.32)
Seed C (%DW)	26°C	35.00 (±0.21)	33.98 (±0.68)
	31°C	33.11 (±1.16)	33.65 (±0.36)
Root C (%DW)	26°C	23.12 (±1.74)	27.26 (±0.56)
	31°C	24.4 (±2.23)	23.62 (±2.81)
Leaf N (%DW)	26°C	2.29 (±0.02)	2.2 (±0.04)
	31°C	2.26 (±0.05)	2.26 (±0.07)
Seed N (%DW)	26°C	1.32 (±0.11)	1.28 (±0.06)
	31°C	1.03 (±0.07)	1.15 (±0.07)
Root N (%DW)	26°C	1.84 (±0.17)	2.08 (±0.2)
	31°C	1.38 (±0.15)	1.16 (±0.12)
Leaf C:N ratio	26°C	13.9 (±0.18)	14.1 (±0.21)
	31°C	13.71 (±0.15)	13.59 (±0.34)
Seed C:N ratio	26°C	27.35 (±2.14)	26.67 (±1.22)
	31°C	32.73 (±1.88)	29.79 (±2.02)
Root C:N ratio	26°C	12.69 (±0.59)	13.55 (±1.14)
	31°C	17.90 (±0.67)	20.24 (±1.46)
Leaf sucrose (sucrose eq g^{-1} DW)	26°C	35.85 (±4.41)	45.39 (±2.84)
	31°C	49.13 (±2.77)	48.30 (±2.50)
Seed sucrose (sucrose eq g^{-1} DW)	26°C	88.42 (±10.60)	69.09 (±14.40)
	31°C	33.46 (±5.47)	43.22 (±3.62)
Leaf starch (sucrose eq g^{-1} DW)	26°C	7.23 (±1.63)	7.83 (±0.78)
	31°C	11.93 (±1.91)	12.66 (±1.45)
Seed starch (sucrose eq g^{-1} DW)	26°C	11.56 (±1.03)	15.95 (±2.70)
	31°C	13.06 (±1.27)	9.63 (±1.23)

Maximum root length and root biomass were significantly higher in the high temperature treatments (**Table 4**). Overall, the total seedling biomass was the highest in the high temperature treatments (**Figure 2),** and there was an effect of the initial seed size (**Table 4**). Seed biomass was the highest, with no differences among treatments, followed by AG, and lowest in BG biomass, which showed a significant decrease in low-temperature treatments (**Table 4**). Seed size also had an effect on almost all biomass traits, BG:seed ratio and number of leaves (**Table 4**). The AG:BG biomass was the only morphological trait that significantly varied both with temperature and nutrients, with the highest ratio observed in the low temperature and high nutrient treatment, and the lowest ratio with high temperature and low nutrient treatment (**Tables 2**, **4**). Also, significant differences were found in the ratio between AG:seed biomass and BG:seed biomass with highest ratios under the high temperature treatments. This trait was also the only morphological measurement that showed an interaction between temperature and nutrient treatments.

Seedling Physiological and Biochemical Traits

Leaf elongation rate was significantly higher in the high nutrient low temperature treatment (0.02 cm d^{-1}, **Tables 3**, **5**). With the exception of biomass traits, we found that initial seed size did not matter in seedling physiological responses, growth and photosynthetic parameters.

Photosynthetic parameters used to measure the relative photosynthetic performance of the seedlings showed little differences across treatments (**Figure 3**, **Tables 3**, **5**). No photoinhibition was observed under any of the treatments as

TABLE 4 | Results of the permutational analysis of variance (PERMANOVA) of the effects of temperature and nutrient treatments on the morphological traits of *Enhalus acoroides* seedlings included in **Table 2,** with the exception of number of roots.

Traits		df	SS	R-squared	Pseudo-F	*P-value*
N° leaves seedling^{-1}	Temperature	1	0.164	17.202	18.028	**0.001**
	Nutrient	1	0.004	0.467	0.490	0.510
	Seed size	2	0.015	1.573	0.824	0.455
	Temperature*Nutrient	1	0.002	0.187	0.196	0.695
	Nutrient*Seed size	2	0.053	5.604	2.937	**0.049**
	Temperature:ET:Aquarium	20	0.313	32.982	1.728	**0.058**
	Residual variability	44	0.399	41.985		
Max leaf length	Temperature	1	33.970	47.845	68.991	**0.001**
	Nutrient	1	1.393	1.962	2.829	0.109
	Seed size	2	1.835	2.584	1.863	0.180
	Temperature*Nutrient	1	0.700	0.986	1.421	0.249
	Temperature:ET:Aquarium	20	10.453	14.723	1.062	0.444
	Residual variability	46	22.649	31.900		
Leaf width	Temperature	1	6.527	9.194	6.300	**0.015**
	Nutrient	1	0.299	0.422	0.289	0.594
	Seed size	2	5.077	7.150	2.450	0.091
	Temperature*Nutrient	1	0.076	0.107	0.073	0.777
	Temperature:ET:Aquarium	20	11.359	15.998	0.548	0.929
	Residual variability	46	47.662	67.129		
Leaf SA	Temperature	1	29.332	41.313	44.792	**0.001**
	Nutrient	1	0.473	0.667	0.723	0.418
	Seed size	2	2.699	3.802	2.061	0.140
	Temperature*Nutrient	1	0.867	1.222	1.325	0.241
	Temperature:ET:Aquarium	20	7.505	10.570	0.573	0.921
	Residual variability	46	30.123	42.427		
AG biomass	Temperature	1	14.839	22.148	17.626	**0.002**
	Nutrient	1	0.084	0.125	0.100	0.758
	Seed size	2	4.904	7.319	2.912	**0.050**
	Temperature*Nutrient	1	1.076	1.605	1.278	0.272
	Temperature:ET:Aquarium	19	9.896	14.770	0.619	0.899
	Residual variability	43	36.202	54.032		
Seed diameter	Temperature	1	4.511	6.353	4.904	**0.026**
	Nutrient	1	0.251	0.353	0.272	0.586
	Seed size	2	4.827	6.798	2.624	0.085
	Temperature*Nutrient	1	0.032	0.046	0.035	0.846
	Temperature:ET:Aquarium	20	19.069	26.857	1.037	0.454
	Residual variability	46	42.311	59.593		
Seed height	Temperature	1	0.416	0.586	0.487	0.490
	Nutrient	1	0.055	0.077	0.064	0.805
	Seed size	2	2.395	3.373	1.399	0.271
	Temperature*Nutrient	1	0.897	1.264	1.049	0.310
	Temperature:ET:Aquarium	20	27.876	39.263	1.629	0.072
	Residual variability	46	39.360	55.437		
Seed biomass	Temperature	1	1.771	2.644	1.611	0.207
	Nutrient	1	0.382	0.571	0.348	0.566
	Seed size	2	6.238	9.310	2.837	0.075
	Temperature*Nutrient	1	0.021	0.031	0.019	0.892
	Temperature:ET:Aquarium	19	11.315	16.888	0.542	0.928
	Residual variability	43	47.273	70.556		

(Continued)

TABLE 4 | Continued

Traits		df	SS	R-squared	Pseudo-F	*P-value*
Max root length	Temperature	1	29.864	42.663	45.703	**0.001**
	Nutrient	1	0.111	0.159	0.171	0.672
	Seed size	2	3.155	4.508	2.414	0.115
	Temperature*Nutrient	1	0.356	0.508	0.544	0.458
	Temperature:ET:Aquarium	20	7.110	10.157	0.544	0.926
	Residual variability	45	29.404	42.006		
BG biomass	Temperature	1	18.659	26.280	27.083	**0.001**
	Nutrient	1	0.678	0.955	0.984	0.349
	Seed size	2	7.049	3.929	5.116	**0.011**
	Temperature*Nutrient	1	0.606	0.854	0.880	0.373
	Temperature:ET:Aquarium	20	12.316	17.347	0.894	0.590
	Residual variability	46	31.692	44.636		
Ratio AG:BG	Temperature	1	7.837	13.750	10.418	**0.003**
	Nutrient	1	4.448	7.804	5.912	**0.018**
	Seed size	2	0.789	1.383	0.524	0.628
	Temperature*Nutrient	1	0.203	0.356	0.270	0.605
	Temperature:ET:Aquarium	19	18.897	33.152	1.322	0.233
	Residual variability	33	24.826	43.555		
Ratio BG:Seed	Temperature	1	20.217	30.174	31.709	**0.001**
	Nutrient	1	0.861	1.286	1.351	0.246
	Seed size	2	7.188	10.728	5.637	**0.008**
	Temperature*Nutrient	1	1.277	1.906	2.003	0.147
	Temperature:ET:Aquarium	19	10.041	14.987	0.829	0.665
	Residual variability	43	27.416	40.919		
AG:Seed ratio	Temperature	1	20.678	30.863	31.457	**0.001**
	Nutrient	1	0.107	0.160	0.163	0.678
	Seed size	2	0.926	1.382	0.704	0.502
	Temperature*Nutrient	1	2.973	4.437	4.523	**0.032**
	Temperature:ET:Aquarium	19	14.049	20.969	1.125	0.370
	Residual variability	43	28.266	42.188		
Total biomass	Temperature	1	8.619	12.864	10.194	**0.003**
	Nutrient	1	0.085	0.127	0.100	0.752
	Seed size	2	8.243	12.303	4.875	**0.014**
	Temperature*Nutrient	1	0.110	0.164	0.130	0.711
	Nutrient*Seed size	2	6.267	9.353	3.706	**0.040**
	Temperature:ET:Aquarium	19	9.011	13.449	0.561	0.905
	Residual variability	41	34.666	51.740		

P-values are in bold when significant differences were observed (≤0.05) SA, Surface area; AG, above-ground tissues; BG, below-ground tissues.

shown by the RLCs (**Figure 3**). From the curve fit parameters, only maximum quantum yield values were significantly higher under high temperature treatments (**Table 5**).

The photosynthetic performance of the seedlings was affected by the nested blocking variables, as reflected by the significant effect they have on rETRmax, alpha and Ek. Due to the high variability between enclosures, any effect of the temperature and nutrient treatments may have been confounded, and therefore we cannot draw any conclusion about the influence of these factors on *Enhalus* seedlings.

Values of %C did not show any significant difference within any of the seedlings parts, leaves, seeds or roots (**Tables 3**, **5**). In contrast, %N of the seeds and roots was significantly lower in the high-temperature treatments, but did not change in leaves. Therefore, C:N ratio of leaves showed no significant differences, while C:N ratio in seeds and roots were the highest under the high temperature and low nutrient treatment (**Table 3**).

Concentrations of NSC in the leaves were significantly higher in high temperature treatments (**Table 5**). Although both types of NSC, sucrose and starch, showed significant differences in the leaves among treatments ($P < 0.05$), concentrations of sucrose in the seeds showed the opposite trends. The lowest concentrations of sucrose in seeds were observed in the high temperature treatments, while starch concentration in the seeds was the lowest in the high temperature and high nutrient treatment and the highest in the low temperature and high nutrient treatment.

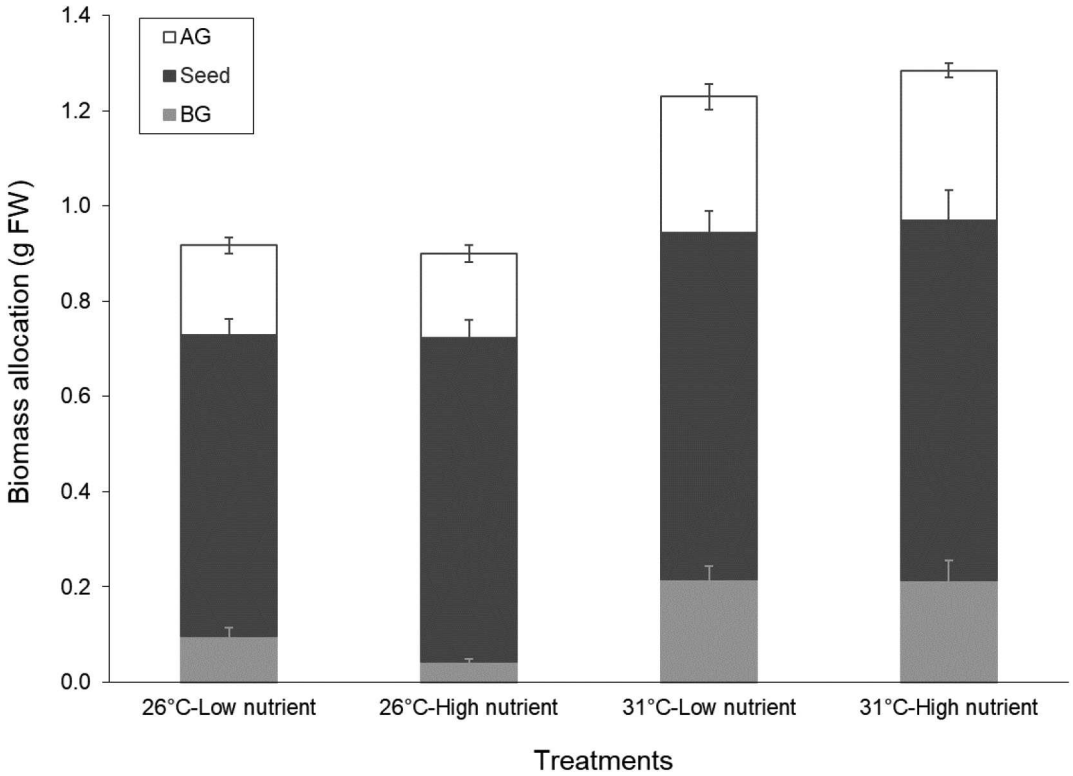


FIGURE 2 | Allocation of above-ground (blades, AG), seed, and below-ground (roots, BG) biomass in the seedlings of *Enhalus acoroides* in the four different temperature and nutrient treatments at the end of the experiment. Values are mean ± SE ($n = 6$). Statistical results are shown in **Table 4**.

DISCUSSION

Overall, we found that *E. acoroides* seedlings were highly tolerant to an extended exposure to high temperature within the reported tolerance limits for adult individuals of this species with enhanced development of new tissue. There were no signs of seedling mortality nor stress response during the experiment under any of the treatments. This indicated that the initial development of the seedling from a seed of this species showed resilience under future average temperature increases expected to occur under climate change impacts. Additionally, increased temperature stimulated rapid development and growth of both AG and BG tissues compared to ambient temperature. In contrast, nutrient enrichment did not increase growth performance of *E. acoroides* seedlings, suggesting that they rely on internal nutrient and energy stores during this stage. Multiple stressor experiments are now highlighted as the necessary steps for predicting the consequences under future scenarios, as interactions between stressors could be synergistic, additive or antagonistic (Gunderson et al., 2016). In our experiment, however, the effects on most traits showed no strong interactions, indicating a lack of synergistic or antagonistic effects under combined stressors. The combined treatments, therefore, can be considered additive, in which nutrient effects were generally lacking and did not add further to the temperature effect.

Seedling Responses to Increased Temperature

The increase in size and quantity of the majority of morphological and physiological traits of the leaves and roots under increasing temperature indicates that this abiotic factor may be an important driver of seedling development of tropical species at the seedling phase. This is in contrast to previous studies on subtropical and temperate seagrass species that showed negative effects on seedling performance under increasing temperatures (Abe et al., 2009; Niu et al., 2012; Guerrero-Meseguer et al., 2017; Pereda-Briones et al., 2019). The seedlings of *Zostera japonica*, for example, survived up to 29°C, but died at temperatures above 30°C in a temperature tolerance study (Abe et al., 2009). In *Zostera marina* seedlings, growth was inhibited at temperatures higher than 30°C, and photochemical pigments were negatively affected at 25°C, a temperature that is 8 to 9°C higher than the optimum temperature for this species (Niu et al., 2012). The opposite effect of rising temperatures between our study and other studies could be due to different reasons, such as the inter-species differences to responses to temperature changes. Unlike *Z. marina,* which is a temperate species, *E. acoroides* is tropical, with a higher temperature range in its native distribution. Meanwhile, positive effects of temperature have also been observed during germination processes in some subtropical seagrass species, such as *Ruppia sinensis* collected from northern China (Gu et al., 2018). Such effects could also be positive in

TABLE 5 | Results of the permutational analysis of variance (PERMANOVA) of the effects of temperature and nutrient treatments on the biochemical and physiological traits of *Enhalus acoroides* seedlings included in **Table 3,** with the exception of starch concentration in seeds.

Traits		df	SS	R-squared	Pseudo-F	*P-value*
Leaf elongation rate	Temperature	1	27.285	38.429	51.351	**0.001**
	Nutrient	1	2.942	4.144	5.537	**0.024**
	Seed size	2	0.911	1.283	0.857	0.406
	Temperature*Nutrient	1	0.827	1.164	1.556	0.214
	Temperature:ET:Aquarium	19	14.594	20.555	1.373	0.178
	Residual variability	43	24.441	34.425		
SA growth rate	Temperature	1	15.632	22.656	18.276	**0.001**
	Nutrient	1	1.608	2.331	1.880	0.205
	Seed size	2	0.749	1.085	0.438	0.647
	Temperature*Nutrient	1	0.170	0.246	0.199	0.681
	Temperature:ET:Aquarium	20	13.205	19.137	0.772	0.734
	Residual variability	44	37.636	54.545		
Max quantum yield	Temperature	1	7.554	12.589	11.683	**0.003**
	Nutrient	1	0.088	0.146	0.136	0.689
	Seed size	2	1.104	1.840	0.854	0.434
	Temperature*Nutrient	1	0.013	0.022	0.021	0.889
	Temperature:ET:Aquarium	17	26.673	44.455	2.427	**0.018**
	Residual variability	38	24.569	40.948		
rETRmax	Temperature	1	0.001	0.002	0.001	0.969
	Nutrient	1	0.000	0.000	0.000	0.989
	Seed size	2	1.377	2.295	0.639	0.546
	Temperature*Nutrient	1	1.491	2.486	1.384	0.255
	Temperature:ET:Aquarium	17	16.188	26.981	0.884	0.589
	Residual variability	38	40.941	68.236		
Alpha	Temperature	1	1.739	2.898	2.605	0.136
	Nutrient	1	1.071	1.786	1.605	0.210
	Seed size	2	2.331	3.886	1.747	0.184
	Temperature*Nutrient	1	0.029	0.048	0.044	0.832
	Temperature:ET:Aquarium	17	29.466	49.110	2.597	**0.012**
	Residual variability	38	25.363	42.272		
Ek	Temperature	1	0.004	0.007	0.005	0.966
	Nutrient	1	0.015	0.025	0.020	0.890
	Seed size	2	2.173	3.621	1.393	0.237
	Temperature*Nutrient	1	0.053	0.088	0.068	0.800
	Temperature:ET:Aquarium	17	28.129	46.882	2.122	**0.035**
	Residual variability	38	29.626	49.376		
Leaf C	Temperature	1	2.7266	11.855	3.971	0.059
	Nutrient	1	2.2771	9.9	3.316	0.078
	Temperature*Nutrient	1	0.6059	2.634	0.882	0.351
	Temperature:ET	2	5.0314	21.876	3.664	**0.050**
	Residual variability	18	12.3591	53.735		
Seed C	Temperature	1	1.8067	8.212	2.592	0.121
	Nutrient	1	1.6922	7.692	2.427	0.132
	Temperature*Nutrient	1	0.4675	2.125	0.671	0.425
	Temperature:ET	2	6.1823	28.101	4.434	**0.039**
	Residual variability	16	11.8513	53.869		
Root C	Temperature	1	0.2007	1.004	0.228	0.663
	Nutrient	1	2.8388	14.194	3.217	0.080
	Temperature*Nutrient	2	3.7103	18.552	4.205	0.058
	Temperature:ET	2	0.0153	0.077	0.009	0.995
	Residual variability	15	13.2349	66.174		

(Continued)

TABLE 5 | Continued

Traits		df	SS	R-squared	Pseudo-F	*P-value*
Leaf N	Temperature	1	0.1031	0.448	0.114	0.741
	Nutrient	1	1.0645	4.628	1.177	0.284
	Temperature*Nutrient	1	1.2696	5.52	1.404	0.274
	Temperature:ET	2	4.2825	18.62	2.368	0.121
	Residual variability	18	16.2802	70.784		
Seed N	Temperature	1	6.3413	27.571	12.495	**0.005**
	Nutrient	1	0.3072	1.335	0.605	0.459
	Temperature*Nutrient	1	0.8496	3.694	1.674	0.205
	Temperature:ET	2	6.3667	27.681	6.272	**0.008**
	Residual variability	18	9.1353	39.719		
Root N	Temperature	1	11.4762	49.896	21.096	**0.001**
	Nutrient	1	0.001	0.005	0.002	0.976
	Temperature*Nutrient	1	1.25	5.435	2.298	0.118
	Temperature:ET	2	0.4809	2.091	0.442	0.660
	Residual variability	18	9.7918	42.573		
Leaf C:N ratio	Temperature	1	2.7546	11.976	2.624	0.124
	Nutrient	1	0.0478	0.208	0.046	0.830
	Temperature*Nutrient	1	0.5742	2.496	0.547	0.471
	Temperature:ET	2	0.7293	3.171	0.347	0.690
	Residual variability	18	18.8941	82.148		
Seed C:N ratio	Temperature	1	5.2276	22.729	8.125	**0.009**
	Nutrient	1	0.9534	4.145	1.482	0.257
	Temperature*Nutrient	1	0.3696	1.607	0.574	0.466
	Temperature:ET	2	4.868	21.165	3.783	**0.042**
	Residual variability	18	11.5814	50.354		
Root C:N Ratio	Temperature	1	14.4959	63.026	41.555	**0.001**
	Nutrient	1	1.0544	4.584	3.023	0.098
	Temperature*Nutrient	1	0.2246	0.977	0.644	0.415
	Temperature:ET	2	0.9459	4.113	1.356	0.296
	Residual variability	18	6.2791	27.301		
Leaf sucrose	Temperature	1	9.59	20.404	11.396	**0.004**
	Nutrient	1	1.423	3.028	1.691	0.210
	Temperature*Nutrient	1	0.001	0.001	0.001	0.980
	Temperature:ET	2	0.644	1.371	0.383	0.684
	Residual variability	18	35.342	75.196		
Seed sucrose	Temperature	1	10.062	21.408	11.757	**0.003**
	Nutrient	1	0	0	0.000	0.992
	Temperature*Nutrient	1	0.022	0.047	0.026	0.876
	Temperature:ET	2	0.972	2.068	0.568	0.581
	Residual variability	18	35.944	76.477		
Leaf starch	Temperature	1	17.129	36.446	24.453	**0.001**
	Nutrient	1	0.096	0.204	0.137	0.696
	Temperature*Nutrient	1	0.157	0.334	0.224	0.630
	Temperature:ET	2	0.197	0.419	0.140	0.864
	Residual variability	18	29.421	62.598		

P-values are in bold when significant differences were observed (≤0.05).

recently germinated seeds, such as the ones used in our study. On the other hand, selected target temperatures of the different experimental studies could draw different conclusions. As long as the temperature is not increased above the thermal tolerance of the species in question, higher physiological performance in terms of growth and photosynthesis are more probable. It is possible that seedlings in this study were still within their thermal niche, as adult *E. acoroides* plants grow naturally in

seawater within a temperature range of 24 to 33°C (Agawin et al., 2001). More specifically, average seawater temperature at mid-day recorded in Barrang Lompo, Spermonde Archipelago (*E. acoroides* fruit collecting site) during December to January was 29.3 ± 0.3°C (Artika et al., 2019), and 31.5 ± 0.1°C during April to May (Ambo-Rappe, 2014). Our study did not intend to find the optimal temperature for growth of *E. acoroides* seedlings, but rather to test the effect of increasing temperature under different nutrient regimes. Further research is required to determine the thermal niche and optimum thermal regime for these seedlings, as well as temperatures in which thermal stress is observed.

The combined enhancement of the various morphological leaf trait responses, including the increase in number of leaves, maximum leaf length, leaf SA and AG biomass, confirmed the positive growth response of AG tissue of the seedlings observed under increasing temperature, specifically the higher leaf elongation rates under high temperature. Root trait responses including the increase in number of roots per seedling, maximum root length, and BG biomass, additionally supported the positive growth response of BG tissue to increasing temperature. In combination, we suggest that these morphological traits can be used as indicators of either healthy or suboptimal *E. acoroides* seedling development under climate related effects. Overall, the strategy of seedlings also showed that under lower temperature (which is suboptimal for the tropical species), more energy goes for development of AG biomass first and less biomass allocation to BG tissues (see **Figure 2**). This was contrary to biomass accumulation under high temperature treatments, where AG and BG tissues were equally supported, suggesting that temperature plays a role in determining energy resource allocation in tropical seagrass seedling development. This finding is interesting in the context of climate change, specifically with respect to possible pole-wards migration. Many tropical species ranges are moving to higher latitudes as average water temperatures rise. These include animals and plants (Doney et al., 2011), and *E. acoroides* could be expected to migrate in a similar manner to mangroves (Osland et al., 2016). However, this energy budget allocation pattern might be a limiting factor because root development is key when establishing and maintaining a seagrass meadow. Less BG development under temperatures at the lower range of tolerance (even seasonally) could make otherwise suitable habitat difficult to colonize, especially as hydrodynamic forces (e.g., wave action) tend to increase in the subtropics and temperate regions compared to the tropical/equatorial region. The slow root development under high nutrient levels could further impede seedling establishment in cooler waters with natural or man-made eutrophic conditions.

Additionally, seed size of the seedlings matters for morphological responses; this has been widely observed in terrestrial plants (e.g., Kennedy et al., 2004) and just in *Posidonia australis* seagrass seedlings (Glasby et al., 2014). This could be related with the positive relation found between starch and nutrient contents and seedling size (Delefosse et al., 2016). In our study, the decrease in %N and sucrose content of the seed also indicates that growth and biomass allocation was supported by internal nutrient and energy stores found in the seed. This has been previously observed in temperate seagrass seedlings under different stressors, such as CO_2 enrichment, invasive algae or temperature (Hernán et al., 2016; Guerrero-Meseguer et al., 2017; Pereda-Briones et al., 2019).

Seedling Response to Increased Nutrients

Our results show that nutrient affected the AG:BG ratio and leaf elongation rate of *E. acoroides* seedlings only at the low temperature treatment. This suggests that the initial development of the seedling phase of *E. acoroides* does not depend much on the availability of external nutrients, at least on these newly germinated seedlings.

While it seems that seedlings of some opportunistic species, namely *C. nodosa* or *Amphibolis antarctica*, take nutrients from the water (Paling and McComb, 1994; Alexandre et al., 2018), other studies show that seagrass seedlings in some other species, including *Posidonia oceanica*, use internal resources on the early stages of development (Balestri et al., 2009). Moreover, despite this capacity for nutrient uptake, the responses in terms of growth and enhanced morphological features are variable, even within the same species (Zarranz et al., 2010; Pereda-Briones et al., 2018). Different factors including environment, availability of phosphate and its balance with nitrogen, seedling's age, or the seed size have been discussed as the reason for the variability of responses (Balestri et al., 2009; Delefosse et al., 2016; Alexandre et al., 2018).

This last feature, seed size, is related with the absolute quantity of nutrients in the seeds (Delefosse et al., 2016). In this way, *E. acoroides* seedlings have common features with other persistent species such as *Posidonia* sp., as seeds are bigger in size compared to other fast-growing species (Orth et al., 2007). Therefore, the ability of *E. acoroides* to produce big nutrient-rich seeds, as also observed in other persistent species like *P. oceanica*, may be a strategy to allow prolonged seedling development in oligotrophic environments (Balestri et al., 2009). In this way, studies with persistent seagrasses showed that seedling growth was more dependent on seed nutrient reserves rather than the external nutrient additions (Statton et al., 2014).

In this study, the eutrophic conditions in the high nutrient treatment aquaria are confirmed by the enhanced microalgae growth and the significant effect on some adult seagrass biochemical traits, including higher leaf nitrogen, free amino acid content or enhanced leaf SA (Viana et al., in prep). These adult seagrasses, namely *Thalassia hemprichii* and *Cymodocea serrulata*, have greater SA and potentially higher absolute uptake rates than seedlings, therefore differences between them could be expected. Moreover, the former rely mainly on external nutrient concentrations for growth (Viana et al., 2019), contrary to recently germinated seedlings which can use resources stored in the seed (Balestri et al., 2009). Competition for nutrients among microalgae, seagrass adults and seedlings, however, cannot be discarded, but was not directly measured in this study. Nevertheless, enhanced leaf elongation and AG:BG ratio, plus evidence of former studies for persistent seagrasses and the seed features of *E. acoroides* suggest that it is very likely that the seedlings in this study rely primarily on nutrient reserves in the seeds.

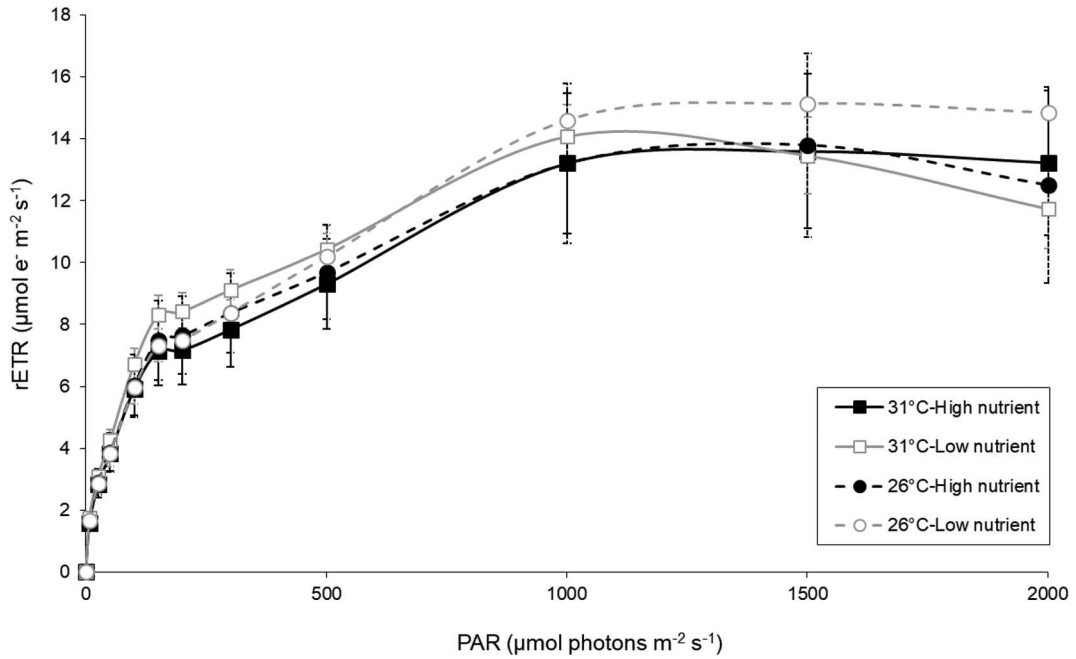


FIGURE 3 | Rapid light curves. Relative electron transport rate (rETR) as a function of photosynthetic active radiation (PAR) on leaves of *Enhalus acoroides* seedlings in the four different temperature and nutrient treatments at the end of the experiment. Values are mean ± SE (n = 5–6).

The findings of this study are the first data available on *Enhalus* seedling response to nutrients, and, as far as we know, is the first study among tropical seagrass seedlings. With the exception of a few studies on seedlings from temperate seagrasses in which no positive effects on physiology, growth, survival or photosynthetic potential were observed with nutrients (Glasby et al., 2014; Alexandre et al., 2018), most studies on nutrient-enrichment effects have been done on adult stages of seagrasses with mixed results. For example, while nutrient enrichment often has an effect on seagrass tissue nutrient content (e.g., Ontoria et al., 2019; Viana et al., in prep), it does not always have a significant effect on leaf length, leaf width and seagrass production, as has been shown in *Thalassia testudinum* (Heck et al., 2000). *Z. marina*, on the other hand, has been found to survive and stay healthy under high nutrient enrichment for 2 weeks, as under natural conditions this species is acclimated and often exposed to high nutrient concentrations (Kaldy, 2014). Other studies that have reviewed seagrass responses to nutrients show that a number of environmental factors, such as sedimentary characteristics or water velocity, and intraspecific characteristics, such as seagrass leaf SA, influence nutrient limitation in seagrasses (Short, 1987; Lee et al., 2007).

Our results are in line with previous studies which did not observe interactive effects of both factors on adult temperate seagrass plants (Touchette and Burkholder, 2002; Kaldy, 2014; Moreno-Marín et al., 2018; Mvungi and Pillay, 2019; Ontoria et al., 2019) and tropical seagrass plants (Viana et al., in prep). In these studies, the combination of effects of nutrient enrichment of the water column and increasing temperature did not show clear effects on seagrass plants. In contrast, other previous studies showed that morphological traits, such as leaf length, growth or number of leaves per shoot, were the most variable traits under the influence of both factors (Bintz et al., 2003; Mvungi and Pillay, 2019). Interactive effects on the response in the %N of the leaves were only observed in *Z. marina* (Moreno-Marín et al., 2018). Otherwise, interactive effects were observed when temperature was combined with other nutrient sources such as labile organic C in the sediment in *C. nodosa* (Ontoria et al., 2019). Therefore, there is still a limited interaction between temperature and nutrient enrichment.

Although nutrient effects may be considered as positive on seagrass individual traits, many studies of nutrient enrichment on seagrasses address the negative indirect effects of eutrophication. This is supported by the fact that enrichment of nutrients can cause algae blooms which will reduce light and contribute to the decline of seagrass (McGlathery, 2001). In our experiment, nutrient treatments led to higher chlorophyll concentrations in the water. However, no significant negative effects of the eutrophication in our tanks were found on seedling morphology and physiology, indicating that light was not limiting seedling growth. This is furthermore supported by the lack of an effect on the photosynthetic performance across treatments, as shown by the fluorescence data (see **Table 3**). This implies that initial seedling development is not light or nutrient limited. Actually, while rETRmax values for adult *E. acoroides* individuals fall within 45–200 µmol e^- m^{-2} s^{-1} (Jiang et al., 2014; Moreira-Saporiti et al., in prep) values in our study are 3- to 8-fold lower (**Figure 3**). This suggests that photosynthesis in *E. acoroides* seedlings might develop later, as observed in experiments with *C. nodosa* (Alexandre et al., 2018).

Ecological Implications

In addition to being a critical stage (Ambo-Rappe and Yasir, 2015) for further seagrass development, the seedling stage response to climate change and nutrient enrichment has been little studied compared to adult seagrasses (Touchette and Burkholder, 2002; Kaldy, 2014; Moreno-Marín et al., 2018; Mvungi and Pillay, 2019; Ontoria et al., 2019). Overall, as far as we know, even less data is available on seagrass seedling responses in tropical species. This is the first study to suggest that the seedling stage of a tropical seagrass may be tolerant to and even positively affected by an extended exposure to the current ambient maximum temperature. This implies that extended exposure to warmer temperatures such as those close to the maximum as that expected under climate change, will not affect seedling survival. To further test temperature tolerance of tropical seagrass seedlings, however, we would need to carry out experiments under a higher range of temperatures (above 32°C). Additionally, we showed that increased nutrient inputs may play a less important role in seedling growth response during its initial growth phase, due to internal nutrient reserves in the seed. As nutrient enrichment has direct and indirect effects, the seedling performance under more persistent eutrophication processes, during which organic matter concentration increases in the sediment or light deprivation happens, should be addressed. We should also consider further studies on the effect of increasing temperature and nutrients on reproductive outputs of the adult plants, including number of fruits, number of seed per fruit, and the size on the fruit and the seed, as well as energy reserves of the seed. All these factors combined will influence long-term seagrass resilience to climate related and local stressors, as well as restoration programs based on seedling establishment. This experiment provides interesting results but there are still a large number of gaps and the need to continue researching how factors related to global change affects the success in seedlings of seagrass. This information would help in future management plans and recovery.

Moreover, this is the first step in studying the combined impact of temperature and nutrients on seagrass seedlings. In the natural environment, biotic and abiotic interactions with the other elements in the ecosystem might also change (Brodeur et al., 2015; Hernán et al., 2017; Pereda-Briones et al., 2019) and, therefore, need to be considered in combination with these stressors. Variations in seedling traits due to temperature and nutrients might have an influence in their functions, and therefore in a number of seagrass ecosystem services, as has been observed to happen with other stressors (Hernán et al., 2016, 2017). Additionally, climate change not only will bring higher average temperatures but also more frequent and adverse events, such as heat waves, that might affect seagrass seedlings differently than smaller increments of higher constant temperature exposure (Guerrero-Meseguer et al., 2017).

Enhanced growth under sub-lethal temperatures at the seedling phase could improve the resilience capacity of *E. acoroides* under natural heat stress processes or in restoration attempts. Higher temperatures will enhance a faster AG and BG development providing further root development. This is critical for stabilization into sediment, and the survival of the whole plant in the following life stages. This step, when the anchoring capacity of the seedling happens, is the bottleneck to seagrass further development (Ambo-Rappe and Yasir, 2015). The ability of seeds to germinate and remain attached to ever-changing sediments within the marine environment is a critical factor in the establishment of new populations and the ongoing survival of pre-existing genetically diverse populations.

Combining morphological, biochemical, physiological, and life history traits allowed us to better understand the effects of environmental stressors on ecological functioning and survival of seedlings, and the influence on future seagrass community dynamics. Further study of seedling and adult plant traits of other tropical seagrass species is important to filling gaps of knowledge of tropical seagrass ecology and future consequences of environmental change on this critical ecosystem.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

MT and IV designed the experiment. SA, AM-S, and IV carried out the experiment and processed the samples. AM-S ran the statistical analysis. SA, MT, and RA-R wrote a first version of the manuscript. All authors made significant contributions to the manuscript and critically revised the different versions of the manuscript.

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The Tropical Seagrass *Halophila stipulacea*: Reviewing What We Know From Its Native and Invasive Habitats, Alongside Identifying Knowledge Gaps

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Halophila stipulacea is a small tropical seagrass, native to the Red Sea, Persian Gulf, and the Indian Ocean. It invaded the Mediterranean Sea 150 years ago as a Lessepsian migrant, but so far has remained in insulated, small populations across this basin. Surprisingly, in 2002 it was reported in the Caribbean Sea, where within less than two decades it spread to most of the Caribbean Island nations and reaching the South American continent. Unlike its invasion of Mediterranean, in the Caribbean *H. stipulacea* creates large, continuous populations in many areas. Reports from the Caribbean demonstrated the invasiveness of *H. stipulacea* by showing that it displaces local Caribbean seagrass species. The motivation for this review comes from the necessity to unify the existing knowledge on several aspects of this species in its native and invasive habitats, identify knowledge gaps and develop a critical strategy to understand its invasive capacity and implement an effective monitoring and conservation plan to mitigate its potential spread outside its native ranges. We systematically reviewed 164 studies related to *H. stipulacea* to create the "*Halophila stipulacea* database." This allowed us to evaluate the current biological, ecological, physiological, biochemical, and molecular knowledge of *H. stipulacea* in its native and invasive ranges. Here we (i) discuss the possible environmental conditions and plant mechanisms involved in its invasiveness, (ii) assess the impact of *H. stipulacea* on native seagrasses and

ecosystem functions in the invaded regions, (iii) predict the ability of this species to invade European and transoceanic coastal waters, (iv) identify knowledge gaps that should be addressed to better understand the biology and ecology of this species both in its native and non-native habitats, which would improve our ability to predict *H. stipulacea*'s potential to expand into new areas in the future. Considering the predicted climate change scenarios and exponential human pressures on coastal areas, we stress the need for coordinated global monitoring and mapping efforts that will record changes in *H. stipulacea* and its associated communities over time, across its native, invasive and prospective distributional ranges. This will require the involvement of biologists, ecologists, economists, modelers, managers, and local stakeholders.

Keywords: *Halophila stipulacea*, alien species, invasiveness, Red Sea, Mediterranean Sea, Caribbean Sea, climate change, predictions

INTRODUCTION

Seagrass meadows represent one of the most valuable ecosystems on Earth, with an estimated value of \$ 2.8 10^6 yr^{-1} km^{-2} (Costanza et al., 2014). As "ecosystem engineers," they provide crucial ecological services, including sequestering and storing "blue" carbon from the atmosphere and oceans, nutrient cycling, sediment stabilization, and formation of essential habitats for economically important marine species (Bloomfield and Gillanders, 2005; Orth et al., 2006; Fourqurean et al., 2012). Loss of seagrasses, recorded worldwide, entails the loss of primary productivity, the devastation of associated biological communities, reduction of local fishing grounds, and increased coastal erosion (Orth et al., 2006). Runoff of nutrients and sediments that reduce water quality and light penetration, increases in water temperatures, alongside longer, and more frequent heatwaves, have been identified as major threats to seagrass meadows (Waycott et al., 2009; Marbà and Duarte, 2010; Jordà et al., 2012; Oliver et al., 2018; Savva et al., 2018). Particularly for Mediterranean and Caribbean marine ecosystems, a new threat to native seagrass species could be the ongoing expansion of the invasive seagrass *H. stipulacea* (Buckley and Csergo, 2017).

Halophila stipulacea (Forsskål and Niebuhr) Ascherson (order Alismatales, family Hydrocharitaceae) is a dioecious, small tropical seagrass (**Figures 1**, **2**), native to the Red Sea, the Persian Gulf and Indian Ocean (Den, 1970; Spalding et al., 2003; Mejia et al., 2016). *H. stipulacea* has become an invader in two major biogeographic areas: (i) the eastern and southern Mediterranean (Lipkin, 1975a,b; Gambi et al., 2009; Sghaier et al., 2011), and (ii) the eastern Caribbean island nations (Willette and Ambrose, 2012; Vera et al., 2014; Scheibling et al., 2018; **Figure 4**).

There is a clear difference between these two invasions. In the Mediterranean, many invasion sites were recorded over the last 150 years (**Figure 4A**), but the areas occupied by *H. stipulacea* in each site, have remained small and highly restricted. In contrast, in the Caribbean, the invader has occupied vast areas and has done so rapidly (**Figure 4B**).

Studies from the Caribbean have demonstrated the invasiveness of *H. stipulacea* by showing that it physically displaces native Caribbean seagrass species (e.g., *Syringodium filiforme*, *Halophila decipiens*, and *Halodule wrightii*), entailing changes in the Caribbean's seagrass landscapes.

Like many non-native species that have become highly invasive in the Mediterranean over the past decades (Rilov and Galil, 2009; Zenetos et al., 2012), there is a possibility that the non-native *H. stipulacea* might become increasingly invasive in the Mediterranean. There are initial indications of this already occurring in some sites within the Mediterranean, where the native *Cymodocea nodosa* has been replaced by the invasive *H. stipulacea* (Sghaier et al., 2014), hinting that the dynamics of this invasion in this region may be changing.

The ongoing "tropicalization" of the Mediterranean basin, with its waters becoming saltier and warmer (Bianchi and Morri, 2003; Borghini et al., 2014), accompanied by the recent doubling of the Suez Canal (Galil et al., 2015), may cause less favorable conditions for native seagrasses.

Despite the capability of *Posidonia oceanica* plants to acclimate to temperature changes (e.g., Marín-Guirao et al., 2017, 2019), it has been predicted that even under a relatively mild greenhouse-gas emissions scenario, the endemic *P. oceanica* will face functional extinction by the middle of this century (Jordà et al., 2012). As the conditions may be worsening for native Mediterranean seagrasses (Jordà et al., 2012), they may become more favorable to tropical seagrass species. Indeed, the potential threat posed by this rapidly spreading plant is serious and has resulted in the inclusion of *H. stipulacea* in the "100 Worst Invasive Alien Species in the Mediterranean" (Lowe et al., 2000).

This review presents the current biological, ecological, physiological, biochemical, and molecular knowledge of *H. stipulacea* from both its native and invaded ranges. This combined knowledge allowed us to (i) discuss the possible environmental conditions and plant mechanisms involved in the two different invasions, (ii) assess the impact of *H. stipulacea* on native seagrasses and ecosystem functions in the invaded regions, and (iii) predict the ability of this species to invade European and transoceanic Atlantic coastal waters.

FIGURE 1 | General features of the tropical seagrass *Halophila stipulacea*. Shown are typical **(A,B)** shallow (3–6 m) and **(C,D)** deeper (20–50 m) meadows growing in the native habitat of the northern tip of the GoA (Eilat, Israel), where *H. stipulacea* grows in extensive meadows or within neighboring local coral reefs. Shown are also examples of plants growing in the invasive habitat of the Mediterranean **(E,F)** where *H. stipulacea* plants grow intermixed with native Mediterranean temperate seagrasses such as *Cymodocea nodosa* and *Posidonia oceanica* (Dream café site, Limassol, Cyprus). Photos were taken by Gidon Winters **(A–C,F)**, Yoni Sharon **(D)** and Yuval Sapir **(E)**. All photos in this figure have been obtained with permission from the original copyright holders.

SYSTEMATIC REVIEW PROTOCOL—*H. STIPULACEA* SEARCH CRITERIA

For this review, the search words "*Halophila stipulacea*" were entered into Google Scholar (accessed 14/03/2017-01/10/2019). The selected studies were all available online, in English, and a very few in Italian (due to the relatively abundant number of studies on *H. stipulacea* in the Mediterranean Sea in Italian waters). Downloaded studies were from published peer-reviewed journals, proceedings of scientific symposiums, published books, one M.Sc. thesis, and in rare occasions technical reports from academic institutions that were published as reports but not as scientific papers. Studies had to be easily downloadable (as opposed to publications on Google scholar that were not accessible via several platforms). Studies had to be focused specifically on *H. stipulacea* (general studies that just mentioned *H. stipulacea* by the way, were excluded) and usually included the species name in the title. All articles reviewed concerned geographical distribution, invasiveness, ecological, physiological, and biotic and abiotic interactions studies of the species itself. In addition, to account for older references that may not have been available through the literature search, the reference lists of each article was also checked and added to the database if considered to be relevant. We also updated the database with our own unpublished articles. Resulting articles were downloaded

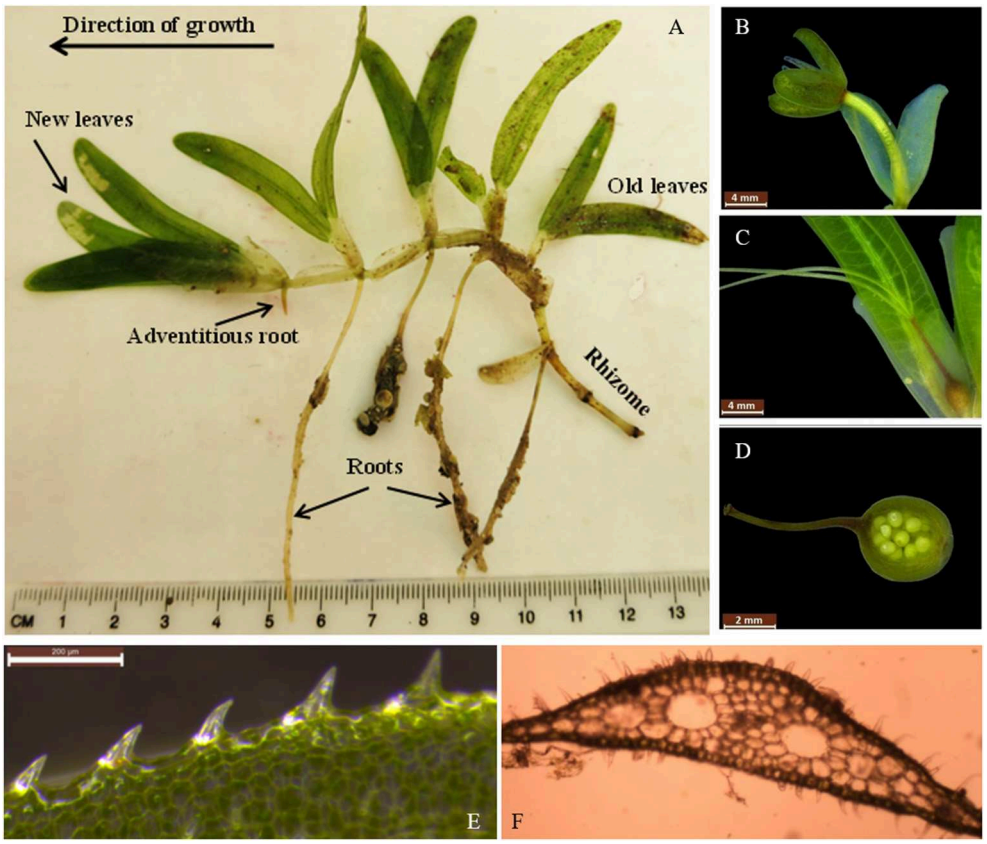

FIGURE 2 | Characteristic morphology of the tropical seagrass *Halophila stipulacea*. **(A)** Shown are rhizomes (smooth with long internodes and leaf scars at stem base), roots (covered by small hairs, could be sand-or gravel-binding), and shoots (each carrying two linear leaf blades that contain mid and branched veins). (B-D) Flowers and fruits of *H. stipulacea*: shown are mature male **(B)** and female **(C)** flowers, alongside seeds within a cut fruit (D). Magnification (x10) showing that leaf margin is serrated **(E)** and minute trichomes may be present on one side of the leaf surface **(F)**. Photos were taken by Gidon Winters **(A,E,F)** and Hung Manh Nguyen **(B–D)**. **(C)** and **(D)** were adapted from Nguyen et al., 2018. All photos in this figure have been obtained with permission from the original copyright holders.

into Endnote to create a "*H. stipulacea* database" containing a total of 164 studies (**Table S1**; **Figure S1**).

Studies were categorized into regions in which studies were performed: Red Sea (R), Mediterranean Sea (M), Indian Ocean (I), Arabian Gulf (A) or the Caribbean Sea (C). Within each region, each study was assigned a reference label. Labels were spatially displayed using QGIS (https://qgis.org/) on maps pinpointing the precise/approximate location described in each study (447 sites; **Table S1**, **Figures 3**, **4**, **Supplementary Material S1**). For uniformity, coordinates were converted into decimal degrees (D.ddd) in World Geographic System 84 coordinates (WGS84). Published studies with only a general location (e.g., the coast of Bahrain; Naser, 2014) were discriminated from those with precise localization (**Table S1**, **Figures 3**, **4**).

Studies within the "*H. stipulacea* database" (**Table S1**) were evaluated and classified according to their region, publication period (**Figure 5A**), and the general topic of study (study category 1): physiology, ecology, distribution, links with humans, or other (**Table S1**, **Figure 5B**). Studies were further assigned into more specific subcategories (study category 2; **Table S1**), such as sexual reproduction, grazing, mapping, etc. For this, a word cloud was generated using www.wordart.com (wordart.com/create; accessed 13/12/2019) to graphically display the diversity and frequency of the specific topics of research (study category 2) of the entire "*H. stipulacea* database" (**Table S1**). The wordlist is provided in **Table S1**.

To quantitatively assess the envelope of environmental conditions in which *H. stipulacea* exists in native and invasive regions, publications were searched for information associated with the abiotic conditions in described study sites—minimal/maximal depths, salinity, sediment characteristics, irradiance, minimal/maximal sea surface temperatures. Plant-related parameters including *H. stipulacea*'s horizontal growth rates, leaf production rates, per cent cover, and characteristics of sexual reproduction, were collated and compared across all regions (**Table 1**, **Supplementary Material S1**).

RESULTS

Geographical Distribution of Studies on *H. stipulacea*

The distribution of published studies on *H. stipulacea* from its native habitat (**Figure 3**) reveals that most studies were

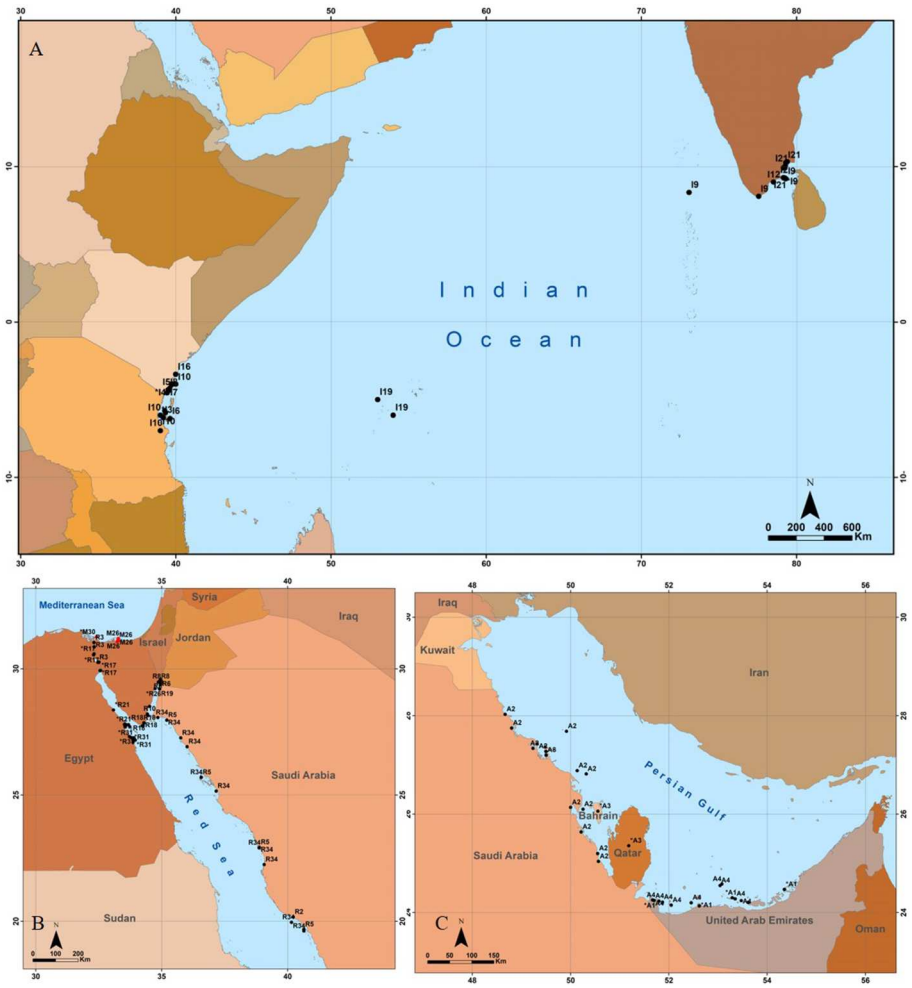

FIGURE 3 | Geographic distribution of published studies on *Halophila stipulacea* from its native habitat (black color). Shown are known records for the Indian Ocean **(A)**, Red Sea **(B)**, and Persian Gulf [**(C)**; numbers for each site refer to the appropriate source (**Table S1**)]. Original coordinates were converted into decimal degrees (D.ddd) in World Geographic System 84 coordinates (WGS84).

concentrated in just a few points in each biogeographic region. For example, in the 2,250 km long Red Sea, known for vast areas of *H. stipulacea*, most studies originate from the northern tip of the Gulf of Aqaba (GoA; **Figure 3B**). Studies from the Arabian Gulf stem only from the southern part of the Gulf, with not even one published study from Iranian shoreline (some 1,000 km long; **Figure 3B**). In the vast Indian Ocean (**Figure 3A**), all published studies originate from Tanzania, Kenya, or southern India.

The distribution of published studies on *H. stipulacea* from its historical invaded habitat in the Mediterranean Sea (**Figure 4A**) reveals a large number of studies, most of which are reported from the northeastern corner of this basin (Cyprus, Greece, and Turkey; Lipkin et al., 2003), with the most western record coming from Tunisia (Sghaier et al., 2011, 2014). Together with recent reports from Sicily (Gambi et al., 2018), these western locations potentially confirm the beginning of a westward migration of local populations in a rapidly warming Mediterranean Sea (Georgiou et al., 2016). The fact that *H. stipulacea* was recorded along nearly all shores of the eastern Mediterranean basin, but not along the Israeli and most of Libya's shorelines, is probably due to different reasons. In Libya, given the species' distribution in the surrounding nearby areas (Sghaier et al., 2011), we suspect that the absence of *H. stipulacea* in most of Libya is probably due to the lack of extensive monitoring data and underreporting (Badalamenti et al., 2011) rather than true absence. For the Israeli Mediterranean shoreline, the absence of *H. stipulacea* might be related to the fact that along this ∼190 km there are no natural shallow protected bays that would allow the development of seagrasses.

The distribution of published studies on *H. stipulacea* from its new invaded range in the Caribbean (**Figure 4B**) demonstrates that *H. stipulacea* has expanded there rapidly; starting from its first finding in Grenada in 2002 it has expanded fast, both northwards and westwards (reviewed by Willette et al., 2014; discussed below).

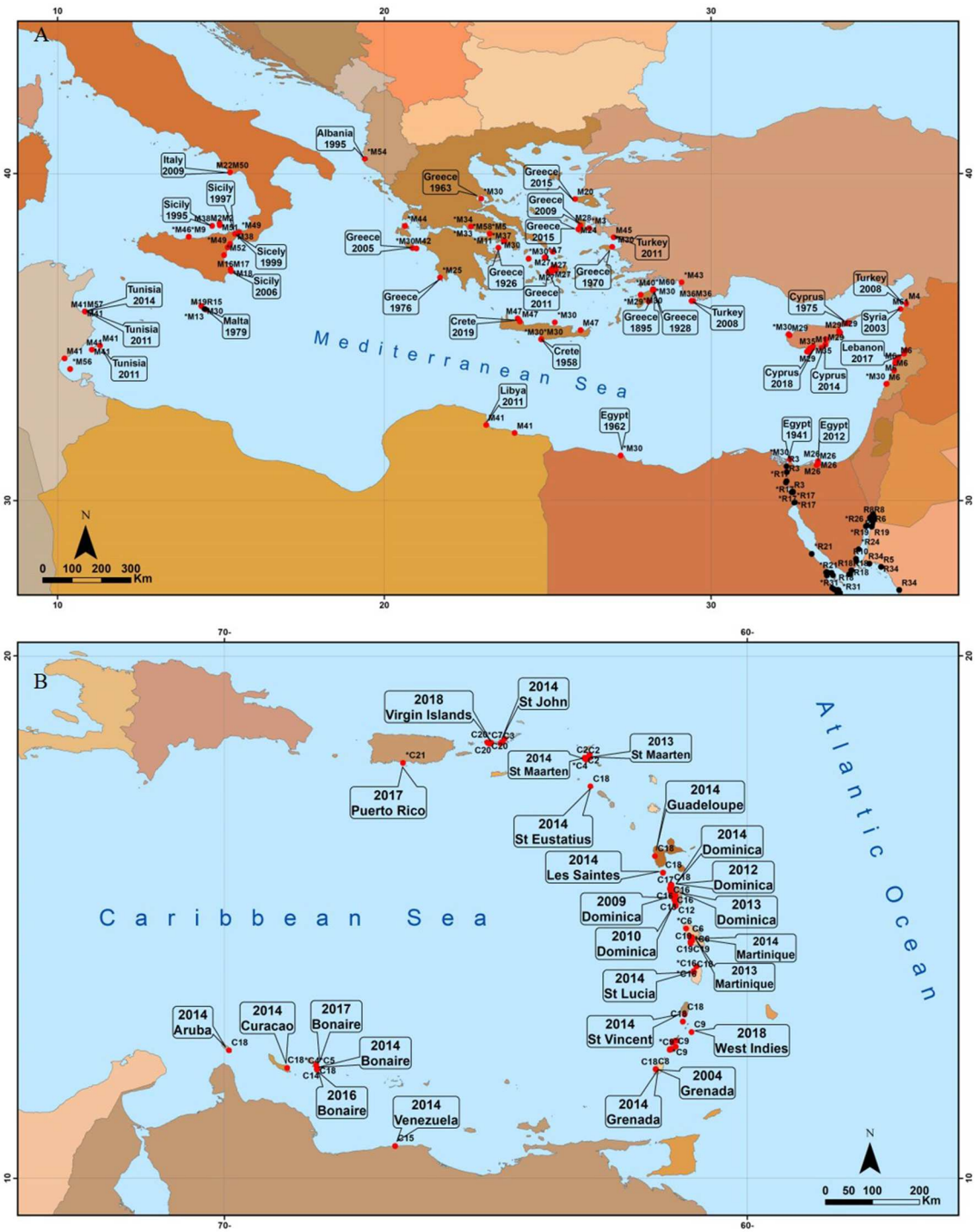

FIGURE 4 | Geographic distribution of published studies on *Halophila stipulacea* from its historical and more recent invaded habitats (red color) in the Mediterranean **(A)** and Caribbean **(B)** Seas. Numbers for each site refer to the appropriate source (**Table S1**). Labels refer to the year of the first report of *H. stipulacea* by location (**Table S1**). Original coordinates were converted into decimal degrees (D.ddd) in World Geographic System 84 coordinates (WGS84).

Regional and Topical Focus of Published Studies

The summary of published studies (**Table S1**) highlights continuous research efforts (spanning over more than 40 years) on different aspects of *H. stipulacea*, both in its native Red Sea habitat (33% of studies) and in its historical invaded area in the Mediterranean Sea (35% of studies; **Figure 5A**). While *H. stipulacea* is also native to the Indian Ocean and Arabian Gulf

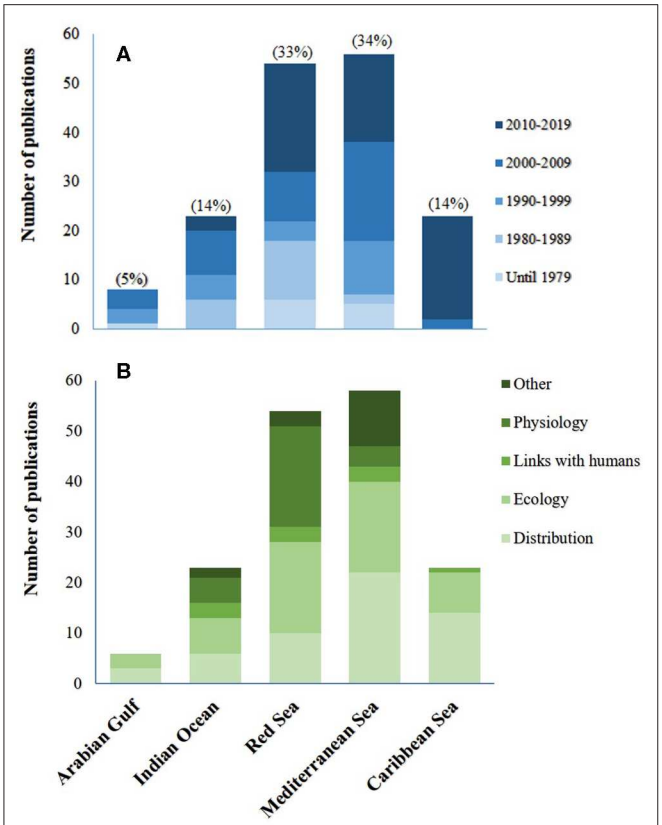

FIGURE 5 | (A) Number of publications per decade and region (based on **Table S1**). **(B)** Number of publications according to the paper's general field of study (category 1—physiology, ecology, distribution, links with humans, or other), per region.

(**Figure 4**), our review points to the relatively few studies, and thus a large gap of knowledge, in these two regions (**Figure 5A**). This is of particular concern in the Arabian Gulf which accounts for only 4% of the studies on *H. stipulacea*. On the other hand, the results confirm the growing research interests in the Caribbean (14% of *H. stipulacea* studies), where, in less than two decades, there are as many publications as in the much larger native habitat in the Indian Ocean (14% of total studies).

Categorizing the *H. stipulacea* data set (**Table S1**) into regions and according to their main area of research (category 1; **Figure 5B**), revealed that, across all regions, studies on distribution and ecology of *H. stipulacea* were numerous. However, the Arabian Gulf and the Caribbean regions lack studies on the physiology, links with humans and genetics/eco chemistry ("other" study category), in comparison with the diversity of *H. stipulacea* studies from the Red and Mediterranean Seas. These results indicate an "evolution" of topics of study, starting with distribution record, and with time, including other topics as ecology, physiology, and eventually links with humans (**Figure 5B**).

The generated word cloud (**Figure 6**) shows that across the entire "*H. stipulacea* database" (164 published studies; **Table S1**), the most frequent areas of research are ecology, habitat surveys and physiology. This word cloud identifies specific gaps of knowledge with relatively few studies on associated fish and epibiotic communities, ecosystem services, and on conservation and management of *H. stipulacea,* highlighting necessary future attention in these fields.

Summarizing the main environmental and ecological parameters published for *H. stipulacea* across its native and non-native ranges (**Table 1**) demonstrates that *H. stipulacea* grows in a wide range of depths (1–70 m), salinities (24–70 PSU), temperatures (17–42°C) and substrates (**Table 1**). It is interesting to observe how in its non-native habitats, sexual reproduction is less frequent. This summary also highlights some gaps of knowledge of its ecology in the Arabian Gulf and the Indian Ocean, despite these regions being much of *H. stipulacea*'s native habitat.

DISCUSSION

H. stipulacea as an Invader

Researchers have been debating on what characteristics make alien species successful invaders (see e.g., Williamson and Fitter, 1996; Kolar and Lodge, 2001; Sol et al., 2012). In principle, the most basic attributes include high reproductive capacity (sexual and/or asexual), wide phenotypic plasticity, high dispersal ability (e.g., extended planktonic duration) and strong competitive ability. Furthermore, the receiving environment should, in theory, exhibit "invadable" characteristics such as elevated level of disturbance for the native species, availability of empty niches, low level of biotic resistance, and high availability of resources (Olyarnik et al., 2009).

The uniformity of conditions in the receiving environment is also important. The variability of environmental conditions in the Mediterranean and Caribbean Seas are different. The Mediterranean exhibits a wider range of temperature and salinity values from the south-eastern Levant corner characterized by salty, warm, and fast-warming waters (Rilov, 2016; Ozer et al., 2017) to the coolest and less salty parts of the Adriatic Sea (Russo et al., 2012). Conversely, the conditions are much more uniform in the Caribbean and Red Seas, which apparently are ideal for the growth and spread of *H. stipulacea* (Georgiou et al., 2016).

Spreading of *H. stipulacea* in the Mediterranean Sea

The invasion history (timeline) and distributional spread of *H. stipulacea* in the Mediterranean and Caribbean Seas (**Figure 4**) show contrasting patterns. In the Mediterranean, *H. stipulacea*'s invasion followed the opening of the Suez Canal in 1869, with the first meadow reported only 25 years later in Rhodes (Fritsch, 1895; **Table S1**), making it one of the first successful Lessepsian migrants (Lipkin, 1975a,b). By 1926, well-established meadows were reported from Rhodes (Issel, 1928; **Figure 4A**). After that, it was recorded along the coasts of Greece, Egypt, Malta, Cyprus and Lebanon (Den Hartog, 1970; Lipkin, 1975a,b; Van der Velde and Den Hartog, 1992), followed by a northward expansion into Turkey and Albania (Alpinar, 1987; Kashta and Pizzuto, 1995) and a westward expansion toward Malta and the Ionian coast of Sicily (Lanfranco, 1970; Biliotti and Abdelahad, 1990). The

TABLE 1 | Summary of main environmental and ecological parameters published for *Halophila stipulacea* across its entire distribution (see **Table S1** for full details).

Parameters	Red Sea (native)	Arabia Gulf (native)	Indian Ocean (native)	Mediterranean (historical invasive)	Caribbean (new invasive)	References
Depth (min. – max. m)	0.5–70	1–14.5	1.5–37	0–27	0.2–32	Lipkin, 1979; Price and Coles, 1992; Procaccini et al., 1999a; Kamermans et al., 2002; Milchakova et al., 2005; Sharon et al., 2009, 2011a,b; Katsanevakis, 2011; Maréchal et al., 2013; Winters et al., 2017
Salinity (PSU or PPT)	38.8–47.5	42–70	24–56	36–39	36.9–37.9	Aleem, 1980; Coppejans et al., 1992; Price and Coles, 1992; Kenworthy et al., 1993; Malea, 1994; Debrot et al., 2012; Naser, 2014; Georgiou et al., 2016; Anton et al., 2018
Substrate characteristics	Silt, sand, rubble. Grain size: 125 μm–1 mm	Sand, mud	Sand, mud, silt	Sand, rubble, and dead seagrass mats	Sand, silt, and coral rubble substrate	Aleem, 1984; Coppejans et al., 1992; Pereg et al., 1994; Kamermans et al., 2002; Naser, 2014; Mejia et al., 2016; Rotini et al., 2017
Solar Irradiance kWh m^{-2} day^{-1}	2.8–8.39			2.21–7.7	4.57–6.92	Al-Salaymeh, 2006; Al-Sayed, 2013
Sea surface temperatures (Min - Max) (°C)	21–27°C	17–31°C	23–42°C	14–28°C	21–28°C	Robinson, 1973; Mahalingam and Gopinath, 1987; Coppejans et al., 1992; Price and Coles, 1992; Naser, 2014; Shaltout and Omstedt, 2014; Willette et al., 2020;
Growth rate (cm day^{-1})	0.16–1.12		2.7	0.35–0.5	0.5–6.7	Wahbeh, 1984; Angel et al., 1995; Marbà et al., 2002; Willette and Ambrose, 2009; Georgiou et al., 2016
Leaf production (mg day^{-1})	0.02–3.51		10.8			Angel et al., 1995; Marbà et al., 2002
Percent cover (%)	3–100		0.2–100	10.6–27.8	1–95	De Troch et al., 2001; Gab-Alla, 2001; Kamermans et al., 2002; Steiner et al., 2010; Winters et al., 2017; Scheibling et al., 2018; Nguyen et al., 2020b; Beca-Carretero et al., 2020
Sexual reproduction characteristics	Both genders are common (May–September). Fruits common (August–October).		Flowers and fruits observed in February	Female flowers and fruits are rare but at certain sites, female flowers could be abundant	Absence of female flowers or seed pods	Lipkin, 1975c; Procaccini et al., 1999a; Malm, 2006; Vera et al., 2014; Chiquillo et al., 2018; Nguyen et al., 2018, 2020b; Beca-Carretero et al., 2020

first report of *H. stipulacea* in the western Mediterranean was in 1995 off Vulcano (Sicily; Acunto et al., 1995), followed by reports from the southern coast of Italy, Libya, and Tunisia (Gambi et al., 2009; Sghaier et al., 2011, 2014). By now *H. stipulacea* has spread throughout most of the eastern and southern Mediterranean Sea (Lipkin, 1975a,b; Procaccini et al., 1999b; Gambi et al., 2009, 2018; Sghaier et al., 2011, 2014; Nguyen et al., 2018; **Figure 4A**). Based on these records, the spread of the invasive *H. stipulacea* in the Mediterranean can be considered old, slow, and highly punctuated in space. The species spread rate across the Mediterranean Sea over these 120 years is roughly 12 km yr^{-1} (Georgiou et al., 2016) which is very low compared to the 300 km yr^{-1} expansion of other invasive macrophytes in this region (Lyons and Scheibling, 2009; Mineur et al., 2015).

Within the Mediterranean, sightings have mostly been limited to locations near ports and marinas. Meadows sizes have been from relatively minute (e.g., 16 m^2; Gambi et al., 2009) to large (e.g., 0.2 ha; Sghaier et al., 2014). These invasion dynamics suggest that the main vectors for the introduction and further spread were shipping activities (it was first recorded in a port and all subsequent locations were also ports and marinas). Although *H. stipulacea* was categorized as one of the worst invasive species in the Mediterranean (Lowe et al., 2000; Streftaris and Zenetos, 2006), these observations suggest, in fact, a relatively limited "invasion success" in this region, as can also be inferred from the limited number of studies reporting competitive displacement of native seagrasses by the alien *H. stipulacea* (Williams, 2007; Tsiamis et al., 2010). However, reports on competitive displacement do exist. Sghaier et al. (2014) showed that a large (0.2 ha) patch of *H. stipulacea* in Cap Monastir Marina (eastern Tunisian coast) grew to cover more than 2.2 ha in only 4 years, and, in the process, displaced 50% of the native *Cymodocea nodosa*. It is also possible that this transition is not driven by competitive exclusion, but by natural (or human-driven) reduction of the native which freed areas for colonization by the alien seagrass.

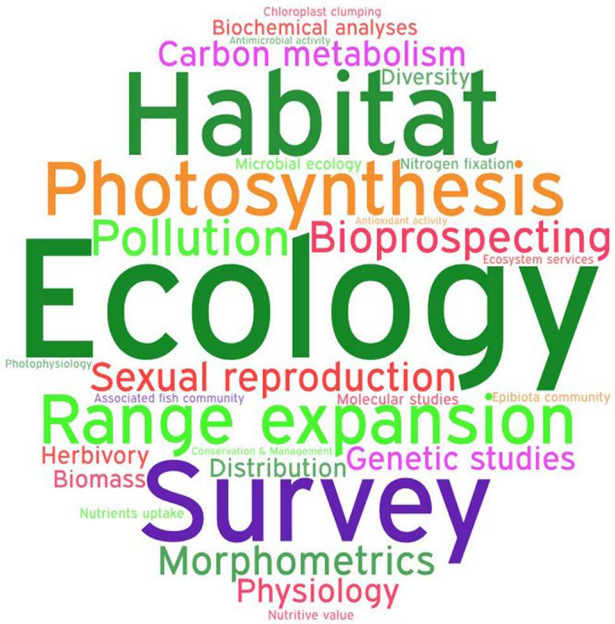

FIGURE 6 | Word cloud showing the diversity of the specific topics of research on *Halophila stipulacea* (category 2 in **Table S1**, **Figure 5B**). The size of each word indicates the relative frequency of the research topic.

It has been observed that *H. stipulacea* populations in the Mediterranean are ephemeral (Chiquillo et al., 2018), with meadows shrinking in winter, and expanding in summer (Nguyen et al., 2020a; Procaccini, pers. comm.). Concordantly, the Mediterranean temperatures fluctuate rapidly between 14°C in the winter and 29°C in the summer. Similarly, growth rates vary from 0.5 cm d^{-1} during the summer and dropping to a minimum of 0.06 cm d^{-1} in the winter (Georgiou et al., 2016).

However, water temperatures in the Mediterranean Sea do not seem to be a limiting factor for this species' survival and expansion, as growth rates in the Mediterranean are overall greater (0.35–0.5 cm d^{-1}; Georgiou et al., 2016), than in its native Red Sea (maximum growth rate 0.206 cm d^{-1}; Wahbeh, 1984). These differences may be related to other intrinsic properties that may act to control the spread and growth of *H. stipulacea* in the Mediterranean (Shaltout and Omstedt, 2014).

Spreading of *H. stipulacea* in the Caribbean

The introduction of *H. stipulacea* into the Caribbean is believed to have been unintentionally caused mainly by recreational vessels traveling from the Mediterranean to the Caribbean (Ruiz and Ballantine, 2004). In contrast to the invasion history and distributional spread of *H. stipulacea* in the Mediterranean (**Figure 4A**), the Caribbean invasion by *H. stipulacea* (**Figure 4B**) is young (<17 years) and rapid.

H. stipulacea has been in the Caribbean for at least 17 years. This seagrass was first reported growing as a 30 m^2 monospecific bed in bare sand in Flamingo Bay, Grenada, in 2002 (Ruiz and Ballantine, 2004). Five years later it was recorded 350 km to the north on Dominica, covering an area of 22 ha (Willette and Ambrose, 2009). Since then, reports on *H. stipulacea* found on other eastern Caribbean islands and along the Venezuelan coastline have been published almost yearly (Vera et al., 2014; Willette et al., 2014; Ruiz et al., 2017; **Figure 4B**).

The regional spread of *H. stipulacea* in the Caribbean is likely due to a combination of storm-induced redistribution, inter-island vessel transit, and near-shore fishing activities (Willette and Ambrose, 2012; Willette et al., 2014). Fragments of *H. stipulacea* were shown to survive for days in the water column, settle, and take root (Willette et al., 2020). Smulders et al. (2017) showed all *H. stipulacea* fragments tethered above the sediment rooted within 10 days. On average, these fragments added 0.9 new shoots d^{-1} (Smulders et al., 2017), approximately twice the rate reported for *H. stipulacea* in the Mediterranean Sea (Georgiou et al., 2016).

Halophila stipulacea fragments are released during the removal of wooden and metal fish traps commonly used by fishermen in the eastern Caribbean. Fish trap removal from *H. stipulacea* beds generated fragments 72% of the time, with each trap creating on average 11 fragments consisting of multiple shoots (Willette and Ambrose, 2012). Furthermore, these traps are often moved across bays and are not regularly cleaned from fouling organisms, including seagrass fragments (Willette, pers. observations), thus facilitating local dissemination of *H. stipulacea*.

In the Caribbean, *H. stipulacea* grows at depths between 0.2 and 32 m (Maréchal et al., 2013; van Tussenbroek et al., 2016) and is often reported in harbors and ports but is also found in bays and along open coastlines (Willette et al., 2014). The seagrass has been reported to grow on a range of substrates, including sand, mud, and coral rubble (Steiner et al., 2010; Willette et al., 2014). Much of the Caribbean landscape that *H. stipulacea* has expanded into consists of bare sand, including sand "halos" and the margins of coral reefs, where other seagrasses usually do not grow (Steiner and Willette, 2015a). These sand "halos" and coral reef margins are maintained by the grazing activities of reef-associated invertebrates and fish (Randall, 1965; Valentine and Heck, 2005).

The loss of the sand "halos" and colonization of *H. stipulacea* in these areas suggest that *H. stipulacea*'s growth rate is faster than local grazers can consume and, that it is able to utilize the available bare sand as its niche habitat (Steiner and Willette, 2015a). Indeed, in the Caribbean *H. stipulacea* has a rapid and wide range of lateral rhizome expansion rates, ranging between 0.5–6.7 cm d^{-1} (Willette and Ambrose, 2009; Willette et al., 2020; **Table 1**). Failure of reef-associated organisms to maintain these sand "halos" may also indicate lower herbivory pressure on *H. stipulacea* compared to other native Caribbean seagrass species (e.g., *S. filiforme*; Muthukrishnan et al., in review).

In the Caribbean, *H. stipulacea* is typically reported growing in monospecific beds or as understory in the much taller *S. filiforme* and *Thalassia testudinum* but also described growing in mixed meadows with *H. decipiens* (Willette et al., 2014). van Tussenbroek et al. (2016) observed that *H. stipulacea* grew at different densities depending on the nitrogen (N) content of its substrate—low shoot densities in substrates with low N content (<7%), and dense, thick mats in areas with high N content (>11%).

In the Caribbean, as the density of *H. stipulacea* increases, it sends out lateral rhizomes that grow between shoots of the native *S. filiforme*, enfolding and eventually (within months) displacing it by monopolizing its space (Willette and Ambrose, 2012; Steiner and Willette, 2015b). Over a 5-year period in Dominica, Steiner and Willette (2015b) documented a dramatic gain in seagrass cover, from 316 ha to 773 ha, attributing this increase to the expansion of *H. stipulacea* beds. They found that *S. filiforme*'s distribution decreased by 150 ha, while in parallel the cover of *H. stipulacea* increased by 649 ha, mainly through colonization of bare sand and notably by physically displacing beds of *S. filiforme*. Likewise, *H. stipulacea* replaced *H. wrightii* at depths >4 m, while *H. decipiens* was entirely eliminated from the surveyed sites. Similarly, Smulders et al. (2017) used fixed location surveys and observed a significant decrease in *T. testudinum* cover, from 53 to 33%, and a significant increase in *H. stipulacea* from 6 to 20% in Lac Bay, Bonaire between 2011 and 2015. *S. filiforme* maintained a consistently low coverage over this period. Shifts illustrating a decrease in native seagrass coverage and the concurrent increase in *H. stipulacea* have also been quantified or anecdotally reported from Carriacou, Martinique, St. Thomas, St. John, and Curaçao (Maréchal et al., 2013; Willette et al., 2014; Scheibling et al., 2018; Engelen, pers. observation). These studies indicate *H. stipulacea*'s contribution toward the transformation of the Caribbean seagrass species composition, leading to a major change in the Caribbean's seagrass landscape (Steiner and Willette, 2015b).

Compared with the relatively old (<120 years) and limited "invasion success" in the Mediterranean (discussed above), the fast and far-reaching spread in the Caribbean, along with the competitive exclusion of several native species (Steiner and Willette, 2015b), suggest a highly "successful" invasion by *H. stipulacea* (Ruiz et al., 2017). Understanding the differences between these two invasions is crucial for being able to predict the potential mechanism of *H. stipulacea*'s success in its new invaded habitats.

H. stipulacea in Its Native and Invaded Ranges: From Individuals to the Ecosystem

The vegetative and some of the reproductive morphology of *H. stipulacea* from its native habitats have been described before (Lipkin, 1975c; El Shaffai, 2016; Nguyen et al., 2018). Key morphological features of *H. stipulacea* from its native habitats include short stems, each carrying two leaves, linear leaf blades (>6 cm long and 0.8–1.0 cm wide) that contain a clear mid vein with branched cross veins (**Figure 2A**). The leaf margin is serrated and minute trichomes may be present on one side of the leaf surface (**Figure 2E**). *H. stipulacea*'s rhizome is smooth with long internodes (1–4 cm) and is covered by leaf scars at the stem base (El Shaffai, 2016). Roots are usually shallow and are covered by small hairs and, depending on the substrate, they could be sand- or gravel-binding (Den Hartog, 1970; Kuo and McComb, 1989). Structurally, *H. stipulacea* has not been reported to differ between non-native and native ranges.

Halophila stipulacea reproduces both sexually (through seeds) and asexually (i.e., fragmentation or vegetative rhizome growth) in its native range (Malm, 2006; Nguyen et al., 2018). While the importance of sexual recruitment in seagrass populations is assumed to be generally low (Rasheed, 1999), small seagrass species such as *H. stipulacea* are thought to be more sexually fecund than larger seagrass species (Kenworthy, 2000; Malm, 2006). In terms of sexual reproduction, *H. stipulacea* belongs to a rare group of plants (only ∼5% of angiosperms; Charlesworth, 2002) that are dioecious, meaning that there is a separation between male and female individuals.

Morphologically, male (staminate) and female (pistillate) flowers are both inconspicuous (Ackerman, 2000). The almost translucent perianth consists of three tepal lobes (Lipkin, 1975c; Chiquillo et al., 2018; **Figure 2B**) that, for female flowers, are fused into a 6 mm perianth-tube enclosing an inferior ovary (Kaul, 1968), three carpels, three styles, and three stigmas (Simpson, 1989; **Figure 2C**). The perianth of male flowers encloses a sessile stamen and three dark-colored anthers (Lipkin, 1975c; Pettitt, 1981; **Figure 2B**). Male flowers release trinucleate pollen in four mucilaginous strands (Pettitt, 1981) which may fertilize female flowers to form seed capsules (4–6 mm) containing 0.2 mm diameter seeds (**Figure 2D**). Ripe seed capsules (**Figure 2D**) detach from the mother plant (**Figure 2C**) and float on the water surface for some hours before seeds are dispersed, the latter of which do not float (Malm, 2006).

Sexual reproduction seems to vary across its native and non-native ranges. In the former, the flowering season lasts 4–5 months (May-Sep), with flowering events producing both staminate (male) and pistillate (female) flowers (Den Hartog, 1972; Lipkin, 1975c; Malm, 2006; **Figures 2C–E**). In the northern Red Sea where this species is native, flowering occurs annually (Malm, 2006; Nguyen et al., 2018) and the female/male sex ratio (F/M) is strongly biased toward female flowers (Malm, 2006). In the Mediterranean, the flowering of *H. stipulacea* is much less common. Flowers in that region were first reported by Politis in Greece in 1926, while fruits were first reported from Cyprus in 1967 (Lipkin, 1975a), ∼73 years after the Lessepsian migration. Typically, only male flowers were observed in the Mediterranean region (Procaccini et al., 1999a; Gambi et al., 2009) and it was assumed that (a) clonal propagation might be the dominant reproductive mode in the Mediterranean Sea (Procaccini et al., 1999a; Chiquillo et al., 2018), and/or that (b) the introduction of *H. stipulacea* into the Mediterranean was of male genotypes only, which spread clonally; or alternatively, that (c) female flowers were unable to develop under the Mediterranean environmental conditions (Gambi et al., 2009). However, in 2012, Gerakaris and Tsiamis (2015) reported on the presence of mature seed capsules (female plants) in the Chios (Greece). More recently, Nguyen et al. (2018) found adjacent female and male flowers in the Mediterranean (Cyprus) and confirmed that sexual reproduction was indeed taking place; Nguyen et al. (2018) reported that sex ratios, however, were male-dominated in the invaded sites as opposed to the female-dominated native habitats.

In the northern GoA, where *H. stipulacea* is the native dominant seagrass species, reproduction starts in May and ends by the beginning of September (with <5% of plants flowering by mid-September Malm, 2006; Nguyen et al., 2018). Although we don't have any data on the exact beginning of the reproductive

season in the Mediterranean (highlighting another knowledge gap), it seems that it lasts much longer into the autumn, with Nguyen et al. (2018) showing that some 23% of *H. stipulacea* invasive plants in Cyprus, were still reproducing in Mid-October.

Flowering in the Caribbean appears to be even less common than in the Mediterranean, although the lack of reports on *H. stipulacea* female flowers and fruits could be a consequence of past limited survey efforts. The first flowering report in the Caribbean found only male flowers occurring in Venezuela (Vera et al., 2014), 12 years after *H. stipulacea* was initially observed in this region (Ruiz and Ballantine, 2004). The fact that since then, reports on flowering of invasive *H. stipulacea* in the Caribbean have only found male flowers (e.g., Chiquillo et al., 2018) suggests that introductions of *H. stipulacea* in this region have, so far, included only male plants of this dioecious seagrass, or that local conditions are somehow preventing the appearance/survival of female flowers. If female flowers were to be found in invasive Caribbean populations, this may have important implications for the future dispersal, survival, and maintenance of invasive populations in this region.

Traits in Its Native Range: The Red Sea and the Indian Ocean

In its native range in the Indian Ocean and the Red Sea (where it was originally described; Forsskål and Niebuhr, 1775; Lipkin, 1975b), *H. stipulacea* is one of the most widespread seagrasses (Wahbeh, 1984; Price and Coles, 1992). In the Arabian Gulf, it co-occurs with the fast-growing *Halodule uninervis* and *Halophila ovalis* (Phillips et al., 2002; Campbell et al., 2015c). Recent records from the central and southern Red Sea have shown that, in some sites (e.g., Umluj, Jazan), it forms mixed meadows with *H. uninervis, H. ovalis, Syringodium isoetifolium, Thalassodendron ciliatum*, and *Thalassia hemprichii* (Qurban et al., 2019). On the other hand, *H. stipulacea* in the northern GoA (northern Red Sea) usually forms mono-specific meadows (Al-Rousan et al., 2011) both in shallow and deep environments (1–50 m depth; Sharon et al., 2011b; Winters et al., 2017), although even here it has been reported to mix with *H. uninervis* (Al-Rousan et al., 2011).

Along the Indian and eastern African coasts, *H. stipulacea* is markedly less documented (Jagtap, 1991; De Troch et al., 2001; Kamermans et al., 2002). In Madagascar and Kenya, its existence is rare and poorly documented, but it was reported at depths beyond all other local seagrass species (De Troch et al., 2001). Reproductive female and male flowers were observed off the Kenyan coasts (Pettitt, 1981) but, altogether, the presence of *H. stipulacea* seems to be scarcer there than in the Red Sea.

Although the general morphological features of *H. stipulacea* have been described before (e.g., Den Hartog, 1970; see also **Figure 2**), in its native areas, it displays high morphological and biochemical plasticity in response to temporal and spatial environmental gradients. For example, in the GoA *H. stipulacea* produced new leaves at intervals of 5–12 days depending on the season, resulting in an estimated leaf turnover of 64.8 days (Wahbeh, 1984). Studies in the GoA pointed out significant variability in leaf density and biometry, with a lower number of leaves and larger leaf area in winter relative to the number of leaves and leaf area in summer (Beca-Carretero et al., 2020). There was a marked increase in leaf descriptors such as length, width, and leaf area with depth, which would allow for better light capturing at depth (i.e., Lipkin, 1979; Rotini et al., 2017). Also, internode lengths varied from 11.2 cm in deeper areas (30 m) compared to 6.8 cm at intermediate depth (17 m) (Schwarz and Hellblom, 2002). Similar observations have been widely documented for other seagrass species (Short and Duarte, 2001; Olesen et al., 2015).

Accompanying these structural leaf changes, biochemical variations with depth were also observed, with higher photosynthetic pigment concentrations (chlorophyll a, chlorophyll b, carotenoids) recorded at deeper areas, allowing to optimize light-capturing at dim irradiances (Lee et al., 2007; Rotini et al., 2017). Indications of biochemical plasticity also included temporal and spatial adjustments of *H. stipulacea*'s leaf phenol content, with significant reductions at increasing depth/reduced light (Mejia et al., 2016; Rotini et al., 2017). A significantly higher phenol content was also found in winter than in summer months (Beca-Carretero et al., 2020), suggesting that this species is better protected from herbivory during certain seasons.

Interestingly, total fatty acid (TFA) content and composition were found to vary at different depths (6–21 m; Beca-Carretero et al., 2019). There was a high capacity to accumulate significantly more TFAs from shallow [6 m depth, 1.2% of dry weight (DW)] to deep areas (21 m, 1.6% of DW). These differences were mainly related to the synthesis of polyunsaturated fatty acids (PUFAs), which promote the fluidity of the chloroplast membranes, as well as electron transport in the photosystems, thus improving optimal photosynthetic responses.

Carbon (C) content in *H. stipulacea* from the GoA varied from 18 to 37% for leaves and 25.5–34.4% of DW for rhizomes/roots; while the N content ranged from 0.8 to 1.7% in leaves and 0.31–1.62% of DW in roots/rhizomes (Wahbeh, 1984; Schwarz and Hellblom, 2002; Beca-Carretero et al., 2020). Overall, the C content remained rather stable over seasons and depths, whereas the N content changed significantly across seasons and depths (Beca-Carretero et al., 2020). The low levels of nitrogen observed in *H. stipulacea* in native areas (<1.8% of DW) indicated a marked nutrient limitation in those marine environments (Duarte, 1992). Lipid accumulation in leaves (5.1–16.7% of DW) and rhizomes (27.2–3.4% of DW) varied significantly from season to season, with higher levels observed in spring (Wahbeh, 1984).

Working in the GoA, Beca-Carretero et al. (2019) recently assessed the total content of fatty acids (TFA) in *H. stipulacea*, and showed that the TFA content in *H. stipulacea* leaves (1.4 $\pm$ 0.2 mg g^{-1} DW) was comparable to seagrass species at similar latitudes (Nichols and Johns, 1985; Hanson et al., 2010). However, further analysis revealed an unusually high content of PUFA (66.0% of TFA), more similar to seagrass species inhabiting higher latitudes, and thus colder regions (e.g., 64.0% of TFA in *Zostera noltii*) than tropical or subtropical species, including *H. ovalis* (48% of TFA) (Viso et al., 1993; Hanson et al., 2010; Beca-Carretero et al., 2018). Lipid composition of the thylakoid membrane partially determine the thermal tolerance of primary

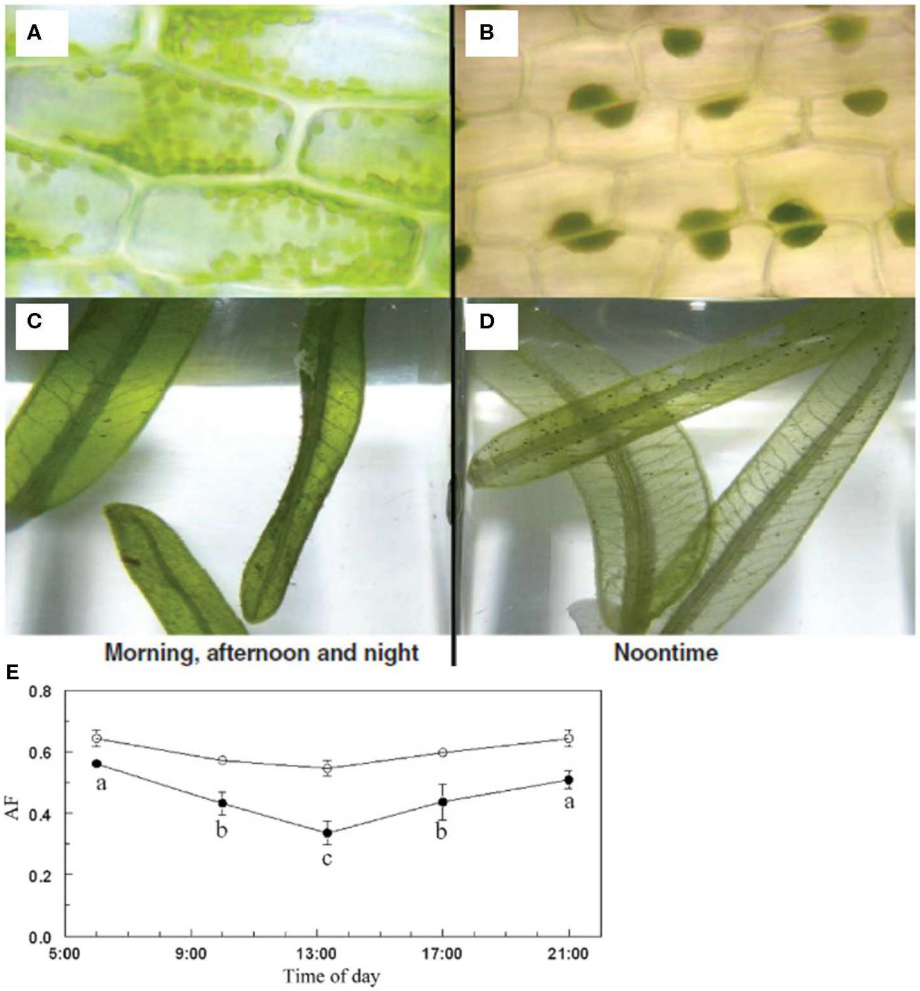

FIGURE 7 | Chloroplast clumping in *Halophila stipulacea*. Microscope pictures **(A,B)** and whole-leaf pictures **(C,D)** of *H. stipulacea* leaves growing in low and high light environments. The chloroplasts (residing mainly in the epidermis) are diffused throughout the cells' cytoplasm at low irradiance **(A,C)**, thus leaves appear dark green. In contrast, the chloroplast clump together **(B,D)** in shallow high-light growing plants, thus leaves appear more transparent. The chlorophyll content per leaf area is the same in **(A–D)**, but the difference in color is due to clumping of the chloroplasts, entailing less light-absorption and more photoprotection (from Beer et al., 2014). **(E)** Daily changes in absorption factor (AF, $n = 10$) of *H. stipulacea* grown on a sunlit water table under shading nets at ∼150 (open circles) and ∼450 (closed circles) μmol photons m^{-2} s^{-1} during midday. Significant differences ($p < 0.01$, one-way ANOVA) along the day are indicated with different letters (adapted from Sharon and Beer, 2008; Beer et al., 2014). Photos taken by Yoni Sharon **(A–D)**. All photos in this figure have been obtained with permission from the original copyright holders.

producers (i.e., Nishida and Murata, 1996), consequently, this physiological characteristic of *H. stipulacea* might partially explain its capacity to survive to winter temperatures (∼14–16°C) in the Mediterranean Sea.

Plasticity in Photosynthetic Responses to Irradiance

H. stipulacea features a unique way of adapting to various irradiances by its ability to perform the so-called chloroplast clumping. This phenomenon was first described by Drew (1979); he observed that leaves of *H. stipulacea* from high-irradiance intertidal southern Sinai (Red Sea) became pale during midday, and then turned darker green from dusk until the following morning. Microscopy (performed in the field) revealed that the paleness of the leaves was due to clumping of chloroplasts to one part of the cytoplasm of each epidermal (i.e., photosynthesizing) cell.

Chloroplast clumping (Sharon and Beer, 2008; **Figures 7A–D**) could be induced by growing *H. stipulacea* in high midday irradiance of 450 μmol photons m^{-2} s^{-1} (ca. ¼ of full sunlight), whereas no such clumping occurred in a shaded midday irradiance of 150 μmol photons m^{-2} s^{-1} (Sharon and Beer, 2008). The chloroplast clumping resulted in leaf optical changes, with a decrease in absorbance and an increase in transmittance, causing a decrease in the absorption factor (AF) of the leaves from ∼0.6 to ∼0.3 (Sharon and Beer, 2008; **Figure 7E**). This has implications on photosynthetic measurements by pulse-amplitude modulated (PAM) fluorometry since electron transport rates (ETR) are a direct function of AF (Beer et al., 1998, 2014). Another important consequence of such chloroplast

clumping is that it provides *H. stipulacea* the potential to adapt to a large spatial and temporal variation in irradiances, e.g., along depth gradients, seasons, and localities (as well as diurnally). This was demonstrated when *H. stipulacea* ramets in the GoA were transplanted from shallow (8 m with ∼400 μmol photons m^{-2} s^{-1} during midday) to deeper (33 m, ∼35 μmol photons m^{-2} s^{-1} during midday at the low-light season) areas, and vice versa, along a continuous meadow, and the photosynthetic properties of the leaves were followed for 2 weeks using *in situ* PAM fluorometry. It was found that both maximal photosynthetic rates at light saturation of photosynthesis (P_{max}) and the onset of saturating light (I_k) acclimated in the transplanted plants within 1 week to values similar to the control plants (i.e., plants that grew naturally at the corresponding depths and were moved within the same depth; Sharon et al., 2009). Similarly, chlorophyll levels in the leaves of the transplanted ramets closely reflected values from the control plants. Thus, plants were able to photo-acclimate rapidly to both increased and decreased irradiances.

In a follow-up study, chloroplast clumping in *H. stipulacea* plants also occurred in response to high UV irradiance (Sharon et al., 2011a). Hence, it seems that chloroplast clumping protects the leaves' photosynthetic machinery by shielding one another from potentially harmful irradiances, including UV light. While some chloroplasts in the periphery of the clump "sacrifice" themselves and become photodamaged, most chloroplasts benefit from the clumping mechanism thus allowing the species to survive in shallow intertidal and high-light exposed waters. Conversely, the intracellular spreading of chloroplasts in the leaf surface of deep-water seagrasses allows for maximum light capture in the light-limited environment.

While the clumping phenomena are rare and have so far not been documented in other seagrasses, there are few reports on its existence in the terrestrial plant literature (e.g., Kondo et al., 2004; Yang et al., 2011).

An additional feature that could support photosynthesis and growth in dim-light environments (e.g., in deep waters) is the apparent ability to change photosystem II (PSII) to photosystem I (PSI) ratios under extremely low irradiances. At the ∼50 m depth limit of *H. stipulacea* in the northern Red Sea, the PSII:(PSII + PSI) ratio was ∼0.4 compared to the ∼0.6 for *H. stipulacea* in shallow environments (Sharon et al., 2011b). This is an apparent adaptation to both the low light (∼100 μmol photons m^{-2} s^{-1} at midday in summer) and blue-shifted irradiance spectrum prevailing at these depths.

So, what are the light requirements for maintenance of positive net photosynthetic rates to sustain growth in *H. stipulacea*? Being rooted, with a considerable part of their biomass underground, seagrasses, in general, have a higher light requirement than both phytoplankton (0.1–1% of surface light) and macroalgae (1–2% of surface light), with the dogma for a "typical" seagrass surface irradiance requirement is ∼10% (Duarte, 1991). However, given that *H. stipulacea* is thin-leaved (a large proportion of the leaf consists of only two layers of photosynthesizing epidermal cells) and the root/shoot ratio is low, this seagrass may need much less light. A good estimate is ∼5% of surface light (i.e., ∼100 μmol photons m^{-2} s^{-1}) as derived from the irradiance measured at its ∼50 m depth limit in the northern Red Sea on a sunny summer's day (Sharon et al., 2011b).

The photosynthetic traits that were described here for *H. stipulacea* are unique among seagrasses (although they might be shared with other *Halophila* species, e.g., *H. ovalis*; Beer et al., 2002; Phandee and Buapet, 2018). These traits, together with an efficient Ci-acquisition system, undoubtedly play a role in *H. stipulacea*'s adaptability to various environments and its apparent rapid acclimation to changing conditions. This might be one reason for its invasiveness into habitats where it was recently introduced. What we do not know is how these photosynthetic abilities may influence its competitiveness with other seagrasses and marine macrophytes. Pursuing research into the degree by which the special photosynthetic traits of *H. stipulacea* contribute toward invasiveness is thus recommended (e.g., can blocking the chloroplast clumping in *H. stipulacea* influence its competitiveness with other seagrasses? Yang et al., 2011).

Plasticity in Sources of Inorganic Carbon

Halophila stipulacea is not only highly adaptable to various irradiances, but also features very efficient inorganic carbon (Ci) acquisition mechanisms. These carbon concentrating mechanisms (CCM) consist of either a bicarbonate (HCO_3^-) transporter localized within the outer membranes of the photosynthesizing cells, or a carbonic anhydrase (CA)-catalyzed extracellular enzyme (within the cell wall) for the conversion of HCO_3^- to CO_2; both are assisted by proton pumps acting outwards from the cells (Beer et al., 2002). Since HCO_3^- is the major Ci source in seawater, either of these mechanisms (or both together) may confer high photosynthetic rates to *H. stipulacea*.

ABIOTIC AND BIOTIC CONDITIONS IN THE NATIVE AND INVADED RANGES

Abiotic Conditions

It has been suggested that the invasiveness of *H. stipulacea* might be attributed to it being highly adaptive to a wide range of abiotic conditions, including light intensities (Sharon et al., 2009, 2011b), water temperatures (Angel et al., 1995; Georgiou et al., 2016) and salinities (Por, 1971, reviewed by Gambi et al., 2009; Oscar et al., 2018).

Salinity Tolerance

Halophila stipulacea is known as a euryhaline species because of its wide range of salinity tolerance (Den Hartog, 1970; Por, 1971; Oscar et al., 2018). Salinity is a major environmental component that can influence the growth, function, structure and distribution of seagrasses (Montague and Ley, 1993; Salo et al., 2014). Although it is assumed that the first establishments of *H. stipulacea* in the Mediterranean were directly from ships (Lipkin, 1975b), tolerance to the hypersaline waters of the Suez enabled this euryhaline seagrass species to become very abundant in the canal (Fox, 1926; Aleem, 1979; Gab-Alla, 2001) and in the same way, also made it possible to thrive in the less saline waters of the Mediterranean (Lipkin, 1975b). Changing conditions, such as the ongoing increase in water temperature and salinity associated with the tropicalization of the Mediterranean Sea (Bianchi and

Morri, 2003; Borghini et al., 2014) can potentially restructure seagrass communities, where species with lower salinity and temperature tolerance range can possibly disappear (Zieman et al., 1999; Rudnick et al., 2005).

The only known study investigating salinity tolerance of *H. stipulacea* at the cellular level has shown that the epidermal concentrations of Na^+ and Cl^- were lower than in the surrounding seawater, indicating the existence of some ion exclusion mechanisms (Beer et al., 1980). Additionally, this study also showed that carbon-fixing enzymes were able to function in the presence of intra-cellular salt concentrations *in vitro*, which is an important adaptive mechanism to salinity variations. Detailed experiments exploring the thresholds of *H. stipulacea*'s hyper- and hypo-salinity tolerance need to be conducted and combined with niche models in order to predict if salinity is a limiting factor for the spread of this species (see for example Oscar et al., 2018; Gamliel et al., 2020).

Water Temperatures

Differences in other abiotic factors among the various geographic basins of *H. stipulacea* do not seem strong enough to justify the observed differences in the growth rates and the occurrence of sexual reproduction (**Table 1**). For example, SSTs differ greatly between the Mediterranean and the Red Sea, while irradiance is relatively similar. However, SSTs and irradiance in the Red Sea and the Caribbean are relatively similar. Alternatively, the differences in the occurrence of sexual reproduction may be related to the dynamics of the different introductions (the Mediterranean and Caribbean Seas).

Experimentally, Georgiou et al. (2016) showed that *H. stipulacea* from Cyprus is functional at most Mediterranean temperatures (from 10 to 30°C). While Georgiou et al. (2016) did not test the functionality of *H. stipulacea* beyond 30°C, it was expected to thrive within the warming waters of the western and northern Mediterranean (Georgiou et al., 2016). Based on experiments with plants from one of its invaded locations in the eastern Mediterranean (Limassol, Cyprus), Georgiou et al. (2016) suggested that summer maxima in the Levant are indeed beyond the optimal conditions for growth.

In a recent experimental study (Nguyen et al., 2020b), native (Eilat, northern GoA), and invasive (Limassol, Cyprus, eastern Mediterranean Sea) *H. stipulacea* populations were subjected to a 2-week heatwave (29 and 32°C) in a controlled microcosm experiment. While invasive plants remained largely unaffected after the heatwave, native plants experienced reduced fitness and biochemical and photo-physiological parameters. These results not only point out the differences in the thermal tolerance among populations but also suggest a rapid adaptation (or a previous selection, as happens in ballast waters) by the invasive population to the ongoing warming of the Mediterranean Sea. This indicates that high temperatures in the Levant may not be a limiting factor for the presence of the alien seagrass in the region, although longer exposure might be more detrimental for this population.

Substrate

In terms of substrate, *H. stipulacea* can grow in different sediment types, ranging from fine sand/mud to coarse gravel-sand, and even in patches between coral heads (Jacobs and Dicks, 1985; Angel et al., 1995; Mejia et al., 2016; **Figure 4**). In disturbed areas, such as the oil-polluted waters of Saudi Arabia, *H. stipulacea* was the most abundant amongst other seagrass species, highlighting its capacity to survive in contaminated and unfavorable environments (Kenworthy et al., 1993).

Nutrient Uptake

In terms of nutrient uptake, a recent study in the GoA reported a limited capacity of *H. stipulacea* for nitrate uptake, but high capacity and efficiency for ammonium, a trait common to other seagrass species (Cardini et al., 2018). Noticeably, this species exhibited an unusual high capability for N uptake under N-limited environmental conditions, potentially due to a high capacity for N_2 fixation and ammonium production of its associated diazotrophic epiphytes. This may represent an asset for *H. stipulacea* when interacting and competing for resources with other seagrass species (Cardini et al., 2018). In its invaded ranges, *H. stipulacea* displayed also a limited capacity to use nitrate, which may restrict growth and survival in areas where the availability of ammonium, the preferred nitrogen source of the species, becomes infrequent or non-existent (Alexandre et al., 2014). On the other hand, the equal capacity and efficiency of leaves and roots for ammonium uptake may contribute to the dispersion of the species in sites where nutrients are available both in the water and sediment.

Biotic Conditions

Seagrasses and seagrass meadows are in general considered one of the most productive and complex systems on a worldwide scale (Den Hartog, 1970). *H. stipulacea* beds support a wide diversity of algal and animal communities in both their native and invaded ranges (De Troch et al., 2001, 2003; Tsirika and Haritonidis, 2005; Di Martino et al., 2007; Willette and Ambrose, 2012; Scheibling et al., 2018). In its native range in the Indian Ocean, studies of seagrass-associated fauna and flora are scarce (Aleem, 1979; De Troch et al., 2001, 2003; section Associations of *H. stipulacea* With Other Organisms in Its Native Ranges), whereas such studies in its invaded ranges in the Caribbean and the Mediterranean are more abundant (see sections Associations of *H. stipulacea* With Other Organisms in the Mediterranean and Association of *H. stipulacea* With Other Organisms in the Caribbean Sea).

Seagrasses host highly diversified microbial communities (Bagwell et al., 2002; Hamisi et al., 2009; Cúcio et al., 2016; Mejia et al., 2016; Rotini et al., 2017) that are known to form a singular entity or holobiont, in line with what has been suggested for corals (Rosenberg et al., 2007). In the "holobiont" framework, the associated microbial communities may influence the health, performance and resilience of the hosts (Taylor et al., 2007; Zilber-Rosenberg and Rosenberg, 2008; Rout et al., 2013; Coats and Rumpho, 2014; Singh and Reddy, 2014). Likewise, the host condition may shape the structure and the diversity

of the microbial communities (Meron et al., 2011; Campbell et al., 2015a,b; Marzinelli et al., 2015; Martin et al., 2018). Unfortunately, microbiome research in seagrasses is still at its infant stage, far less than microbial studies in sponges and corals. The great metabolic variability of microbes, made available to host plants, calls for further studies aimed at investigating plant-microbes interactions and their functional outcomes, including ecological resilience and invasive capacity.

Associations of *H. stipulacea* With Other Organisms in Its Native Ranges

In its native range, *H. stipulacea* leaves and rhizomes were found to be almost devoid of epibionts across different seasons (Aleem, 1979). Macroalgae like *Turbinaria* spp. and *Caulerpa* spp. were found to occasionally co-occur with *H. stipulacea* in the Red Sea (Jacobs and Dicks, 1985). Among the marine fauna associated with *H. stipulacea* meadows, De Troch et al. (2001, 2003) reported high diversity of harpacticoid copepods off the coast of Kenya where a deep and mixed bed of *H. stipulacea* and *S. isoetifolium* exists.

Very few studies have been conducted on *H. stipulacea*-associated fish communities, however, a fish feeding experiment at the Kenyan coast showed higher feeding preference for pioneering, short-lived, species such as *C. rotundata*, *S. isoetifolium,* and *H. stipulacea* over "climax," long-lived, species such as *Enhalus acoroides* and *Thalassodendron ciliatum* (Mariani and Alcoverro, 1999). In the GoA, our knowledge of fish associated with local *H. stipulacea* meadows is limited to the study of Khalaf et al. (2012) that did not find any *H. stipulacea*-dedicated fish species.

In terms of mega grazers, dugongs have been sighted in the Red Sea (Egyptian and Saudi Arabian coast; Preen, 1989), grazing heavily on *H. stipulacea* meadows (e.g., in Abu Dabab and Marsa Alam, Egypt). Studies have shown that dugongs prefer "pioneer" seagrasses (Preen and Marsh, 1995), especially those of the genera *Halophila* and *Halodule*. While it seems important to quantify these dugong-*Halophila* interactions, its frequency in most of the native habitat of *H. stipulacea* meadows is unknown, and reports are anecdotal. This represents an important gap of knowledge in the regions where *H. stipulacea* is native.

Other studies in the GoA focused on the interactions between local *H. stipulacea* and invertebrates. The collector urchins (*Tripneustes gatilla*) were found to graze heavily on *H. stipulacea* (Hulings and Kirkman, 1982), while *Operculina ammonoides* was the dominant epiphytic foraminifera on leaves (Oron et al., 2014). In the northern GoA,next generation sequencing (NGS) studies on *H. stipulacea*-associated microbial communities showed differences across sites and plant compartments (aboveground compartment, i.e., leaves; belowground compartment, i.e., roots and rhizomes), providing an "environmental fingerprint." In addition to these differences, these studies also found the existence of a "core microbiome" consisting of bacteria that were always present, shared across sites, and independent of the depth or location (Mejia et al., 2016; Rotini et al., 2017). This hints toward the existence of a functional relationship between *H. stipulacea* and these shared microbes, as in the framework of the "holobiont theory" (Rosenberg et al., 2007; Zilber-Rosenberg and Rosenberg, 2008). When comparing *H. stipulacea* at different sites (Mejia et al., 2016), this "core microbiome" was composed of the phyla Proteobacteria and Planctomycetes, representing more than 70% of the Operational Taxonomic Units (OTUs) shared on both leaves and roots/rhizomes. Within this phylum, Alphaproteobacteria, Gammaproteobacteria, and Deltaproteobacteria were the most abundant classes: on the leaves, Alphaproteobacteria was the dominant class across all stations (68% of the community), while on the roots/rhizomes no single dominant class was found. Nevertheless, along the gradient, all the sites had a higher number of unique OTUs (i.e., "environmental fingerprint" bacteria), than shared ones, with only 7% of the OTUs shared among different meadows (i.e., "core microbiome"). The microbial diversity in *H. stipulacea* may contribute to its adaptiveness and may aid its colonization and expansion into new territories. This could be particularly important considering that rhizosphere-associated microbial communities are known to persist on the roots and rhizome segments established in new environments (Coats and Rumpho, 2014; Cúcio et al., 2016).

Associations of *H. stipulacea* With Other Organisms in the Mediterranean

Surprisingly, regarding the associations of *H. stipulacea* with other organisms, we seem to know much more from studies in its invaded habitats compared with studies in its native habitats. In the Mediterranean, numerous species of macroalgae associated with *H. stipulacea* have been reported (Alongi et al., 1993; Rindi et al., 1999; Di Martino et al., 2006), with up to 30 species (mostly Rhodophyta) found in meadows of the Catania harbor (Alongi et al., 1993). The presence of the epiphytic rhodophyte *Chondria pygmaea* in the Mediterranean is noteworthy (Garbary and Vandermeulen, 1990), raising the possibility of co-migration with its host, *H. stipulacea,* from the Red Sea (Cormaci et al., 1992). Di Martino et al. (2006) studied temporal variations in the algal assemblage within an *H. stipulacea* meadow in Syracuse, eastern Sicily, where 110 species, mostly epiphytic Rhodophyta, were found. Nevertheless, Rindi et al. (1999) stated that, in comparison with other Mediterranean seagrass meadows, *H. stipulacea* has a qualitatively and quantitatively poor epiphytic flora, with the distinct absence of encrusting coralline algae. The fast turnover rate of *H. stipulacea* leaves was hypothesized to be the main reason for this scarcity (Rindi et al., 1999).

Cancemi et al. (1994) and Acunto et al. (1997) described the animal communities associated with *H. stipulacea* in eastern Sicily, Italy. Mollusca, Amphipoda, and Decapoda were the most abundant groups in Taormina, Province of Messina (Cancemi et al., 1994), while Polychaeta, Crustacea, and Mollusca were the dominant macrozoobenthos at Vulcano island (the Aeolian Islands, Sicily; Acunto et al., 1997). The fish assemblages associated with *H. stipulacea* were mainly characterized by the presence of sparids, labrids, and benthic gobiids (Di Martino et al., 2007). Gambi et al. (2009) also observed a school of *Sarpa salpa* in a small patch of *H. stipulacea* at 5 m depth but found no signs of direct grazing on its leaves. It is likely that the observed fish grazed on the leaf epiphytes or upon the small

macroalgae interspersed between the *H. stipulacea* shoots. In general, fish assemblages associated with *H. stipulacea* meadows in the Mediterranean were related to the stable structure of the meadow throughout the year and not with shoot density (Di Martino et al., 2007).

Despite the invasiveness of *H. stipulacea* in various parts of the world and the established role of the associated microbes, there is hardly any information on *H. stipulacea* microbiomes in its invaded range. Two recent, yet unpublished, studies in the eastern Mediterranean Sea (Limassol, Cyprus; Conte et al., unpublished), highlighted the influence of the environment on the epiphytic microbial community structure but, at the same time, the capability of *H. stipulacea* to host a diverse microbial community that may contribute to its invasiveness.

Association of *H. stipulacea* With Other Organisms in the Caribbean Sea

In the Caribbean, *H. stipulacea* has been reported growing with a range of native and non-native Caribbean marine organisms. Native Chlorophyta algal species, namely *Caulerpa* spp., *Penicillus pyriformis*, *Penicullus* sp., *Udotea cyathiformis,* and *Ulva intestinalis,* have all been found growing with *H. stipulacea* (Steiner and Willette, 2010; Maréchal et al., 2013; Willette et al., 2014). Additionally, *Parvocaulis exiguus*, an Indo-Pacific green alga potentially introduced by ships, was collected in *H. stipulacea* beds in St. Eustatius (Maréchal et al., 2013; Steiner and Willette, 2015b). Mats of unidentified cyanobacteria and dinoflagellates have also been reported growing on top of *H. stipulacea* beds, yet the cause or impact of these mats is unknown (Maréchal et al., 2013; Steiner and Willette, 2015b).

Epifaunal invertebrates occurring on the blades of *H. stipulacea* in the Caribbean include representative ascidians, annelids, crustaceans, molluscs, and nematodes (Ortea et al., 2012; Willette and Ambrose, 2012; Scheibling et al., 2018). Larger sessile and benthic invertebrates have also been recorded within *H. stipulacea* beds, including native *Strombus gigas* (Gastropoda), *Pinna carnea* (Bivalvia), *Astichopus multifidus* (Holothuroidea), *Oreaster reticularis* (Asteroidea) as well as other ascidians, crustaceans, and echinoderms (Willette et al., 2014; Scheibling et al., 2018). The seagrass-grazing urchin *Tripneustes ventricosus* is often found in *H. stipulacea* beds (Willette et al., 2014); however, densities of this sea urchin are less than half of that found on native *T. testudinum* (Scheibling et al., 2018). The dense growth form of *H. stipulacea* beds does seem to benefit the feeding strategy of *O. reticularis*, a native Caribbean Sea star whose populations have been decimated elsewhere in the region due to seagrass loss (Scheibling et al., 2018). Ferry et al. (2017) reported the presence of the Indo-West Pacific crab *Charybdis hellerii* in the island of Martinique, where more than 90% of the specimens found were exclusively on dense beds of *H. stipulacea* (roughly 0.37 crabs m^{-2}). The absence of *C. hellerii* on bare sand, coral, and mixed beds of seagrass was attributed to the presence of predators on native substrates. Thus, *H. stipulacea* may provide a refuge for this introduced crab to thrive in the Caribbean.

Seagrass beds form essential fish habitats in the Caribbean, serving as nurseries for juvenile fish and shelter and foraging grounds for larger fish (Nagelkerken et al., 2001). Thus, the impact of *H. stipulacea* on native fish is of particular ecological and resource management interest. Using local fish trap methods, Willette and Ambrose (2012), reported significantly larger average fish sizes, and slightly higher fish abundance and species richness on non-native *H. stipulacea* compared with native *S. filiforme* beds. This difference in fish abundance and species richness can be attributed to the significantly higher fish prey abundance (namely crustaceans) on *H. stipulacea*. Juvenile fish, however, were twice more abundant on native *S. filiforme* than on *H. stipulacea,* which in part, could be attributed to the latter's much shorter canopy height and thus lower sheltering provision (Willette and Ambrose, 2012). Olinger et al. (2017) conducted an intensive field study focusing on juvenile fish abundances in meadows of *H. stipulacea* and native seagrasses along St. Thomas (U.S. Virgin Islands). Overall, fish diversity was higher among native seagrasses and over sand than on *H. stipulacea* in the bays examined. Nocturnal carnivores, however, showed higher abundance in *H. stipulacea* meadows in contrast to the low abundance of diurnal carnivores and herbivores in the same area, indicating different habitat preferences for different trophic species (Olinger et al., 2017).

Working in Lac Bay, Bonaire, Caribbean Netherlands, Becking et al. (2014a) found that fish abundance was almost half in *H. stipulacea* meadows compared with that measured in meadows dominated by the native *T. testudiunum*, in addition to significant differences in the composition of fish species assemblage between the two meadows—Pomacentridae, Mullidae, and Sphyraenidae were present in *T. testudinum* meadows but absent in the invaded meadows. Becking et al. (2014a) estimated that future expansion and/or persistence of *H. stipulacea* could possibly result in a diminished nursery function of certain fish species in Lac Bay.

Lastly, southern stingrays, *Dasyatis americana*, and the sharptail snake-eel *Myrichthys breviceps* have been reported foraging among *H. stipulacea* beds (Willette et al., 2014; Scheibling et al., 2018), as has the green turtle *Chelonia mydas* (Becking et al., 2014b; Christianen et al., 2019). Yet, studies on the interactions between *H. stipulacea* and these marine megafaunas are limited, hence warrant further examination.

Available information on the *H. stipulacea*'s microbiome in the Caribbean has shown that across bays of Curaçao island there were large distinctions between the below and above ground *H. stipulacea* compartments and that microbial communities within roots and rhizomes (i.e., the below ground compartment) also differed from the microbial communities found in local sediments (Stuij, 2018). This distinction suggests that *H. stipulacea* selects and cultures specific microbial communities within its roots and rhizomes. Microbial communities associated with *H. stipulacea* across five bays in Curaçao did not show strong spatial differentiation, in contrast to the site differentiation demonstrated in the northern GoA (Mejia et al., 2016). In Curaçao, where microbial communities were compared among different seagrass species, the microbial communities associated with *H. stipulacea* were highly diverse and specific to *H. stipulacea* but differentiation between below- and above-ground tissue-associated microbiomes was the smallest of the three seagrasses investigated (Stuij, 2018). Sulfur and nitrogen cycling bacterial OTUs were abundant and widespread for all seagrasses including *H. stipulacea*, suggesting a strong shared functionality among

host species-specific microbiomes. Despite, or because, of its recent arrival in the Caribbean, the only study available on microbial communities associated with *H. stipulacea* seems to suggest that *H. stipulacea* microbial communities perhaps did not suffer a bottleneck effect, and its high diversity and species specificity may contribute to *H. stipulacea*'s proliferating potential. Clearly much more research in this area is required, preferably combining descriptive and experimental approaches covering micro to global scales.

DEVELOPING MOLECULAR AND "OMIC" TOOLS FOR STUDYING *H. STIPULACEA*

The ability of *H. stipulacea* to establish itself first in the Mediterranean and later in the Caribbean makes it an attractive model species for reconstructing its potentially complex history of introductions and studying tolerance and resilience to different environmental conditions at the molecular level (Sakai et al., 2001; Lee, 2002; Davey et al., 2016).

Developing Molecular Tools for Studying the Genetic Diversity of *H. stipulacea*

First genetic diversity studies of *H. stipulacea* employed sequence data of single DNA regions or multi-locus markers that do not allow a precise estimation of population genetic parameters. Ruggiero and Procaccini (2004) found no differentiation in the ITS rDNA regions between *H. stipulacea* from the Red Sea (native) and Mediterranean (invasive) populations, suggesting that *H. stipulacea* populations in the Mediterranean originated from the Red Sea (Ruggiero and Procaccini, 2004). This type of molecular marker, however, could not infer whether the introduction occurred once or at multiple times. Interestingly, the same study found a high degree of intra-individual variability in the ITS region, suggesting a high rate of sexual recombination and a slow rate of concerted evolution in the genotypes analyzed. Recent results on the caryology of Mediterranean and the Red Sea individuals of *H. stipulacea* exclude the existence of polyploidy as a possible cause for the observed intra-individual variability (Gargiulo et al., 2018). Conversely, Varela-Álvarez et al. (2011) found no ITS intra-individual nucleotide diversity in Turkey. The first extensive population recorded in the western Mediterranean basin (i.e., Vulcano Island, Sicily, Italy) has been analyzed by means of randomly amplified polymorphic DNA (RAPD) markers, and high genetic diversity was found together with a clear genetic distinction between shallow and deep stands of the same population (Procaccini et al., 1999a).

The use of more polymorphic and reliable markers would allow addressing ecological questions related to the reproductive and spreading mode as well as track, with more precision, the origin of the invasions.

Developing "Omic" Tools for Studying *H. stipulacea's* Tolerance and Resilience to Stress at the Molecular Level

Seagrasses belong to four/five different families in the subclass Alismatidae (Les et al., 1997). *H. stipulacea* belongs to the family Hydrocharitaceae, that evolved together with the other major clades 40–78 Mya (Olsen et al., 2016). Seagrass species belonging to different families have different genome size. The genome size of *H. stipulacea* has been assessed in samples collected from Eilat, northern GoA and it was 12.26 picogram in size (∼5.9 Gb; Gargiulo et al., 2018). The value is 2, 6 and 30 times higher than the genome size of *P. oceanica, Z. muelleri* (∼900 Mbp) and *Z. marina* (∼202.3 Mbp), respectively (Procaccini pers. comm. for *P. oceanica*; Cavallini et al., 1995).

Z. muelleri and *Z. marina* (Zosteraceaae) are the only two seagrass species for which the complete genome is available at the moment (Lee et al., 2016; Olsen et al., 2016). Although this could represent a potential source of information to scan for the presence of genes that can relate to the *H. stipulacea* plasticity and invasiveness, the phylogenetic distance between Hydrocharitaceae and Zosteraceaae strongly reduces the power of such analysis. The availability of the *H. stipulacea* genome would represent an imperative step toward explaining its invasiveness and plasticity.

Understanding the response of *H. stipulacea* to changes in abiotic factors will facilitate our prediction of the further expansion of this species. One way of comprehending ecological traits is to combine phenotypic and physiological assessments with transcriptomic and their equivalent metabolic pathways (Exadactylos, 2015). With the emergence of molecular profiling and "omics" techniques in seagrass biology (Procaccini et al., 2007; Mazzuca et al., 2013; Davey et al., 2016), the ability to investigate plant responses to biotic and abiotic factors has become more feasible. Recent studies have focused on the response to light, increased water temperature, salinity, and high CO_2 levels at the transcriptomic and proteomic levels. These studies have revealed new insights into mechanisms applied by seagrasses to survive under various abiotic stresses (Franssen et al., 2012; Kong et al., 2014; Piro et al., 2015a,b; Kumar et al., 2017; Marín-Guirao et al., 2017; Procaccini et al., 2017). While most of these studies have been performed on the temperate seagrass species *Z. marina, P. oceanica*, *C. nodosa*, *Z. muelleri,* and *Z. noltii,* there are no reports of any of such study on the tropical *H. stipulacea* (see Nguyen et al., 2020a).

Although metabolomics is not so much explored in seagrasses (see Gu et al., 2012; Hasler-Sheetal et al., 2016), it holds great potential in combination with transcriptomics and proteomics, in understanding responses to biotic/abiotic stress (Buapet, 2017). Reprogramming of the metabolome under various stresses such as heat (Gu et al., 2012), anoxia (Hasler-Sheetal et al., 2015) and light (Hasler-Sheetal et al., 2016) in seagrasses like *Z. marina* and *Z. noltii* were observed. Identifying and studying the regulation of primary and secondary metabolites in *H. stipulacea* will provide essential insights into the adaptive mechanisms of this seagrass to changing abiotic conditions, significantly increasing our ability to predict the further expansion of this species.

Epigenetic variation is often an important prerequisite and has also been known to facilitate the survival of invasive species in new environments (Schrey et al., 2012; Richards et al., 2017). The extent and the form of such epigenetic plasticity can be an advantage of invasive species over indigenous species

(Stachowicz et al., 2002; Chown et al., 2007; Kleynhans et al., 2014).

An interesting area to look into is the shifting in methylation patterns in DNA, i.e., epigenetic variation in response to biotic and abiotic factors (Ardura et al., 2017). Several studies have recently begun to link phenotypic plasticity with changing methylation patterns both in animals and plants (Kardong, 2003; Bossdorf et al., 2008; Zhang et al., 2013) with recent work also on seagrasses (Jueterbock et al., 2019; Ruocco et al., 2019). Genomic tools like whole-genome bisulfite sequencing and ChIP-Seq (to study histone modification) might help to further explain the invasive capability of *H. stipulacea* as has already been shown in studies of other invasive species, such as in populations of marine invertebrate (Ardura et al., 2017) and insect pests (Jones et al., 2018).

In summary, a combination of metabolomics, proteomics, transcriptomics, and epigenomic studies, in combination with physiological, biochemical, and other more classic indicators (Roca et al., 2016), could provide a holistic view of how *H. stipulacea* responds to abiotic and biotic stress and in turn help our understanding of this seagrass' invasive capabilities.

NATIVE AND INVADED RANGES: WHAT IS COMING NEXT?

Given the widespread of *H. stipulacea* in its invaded ranges, it is important to understand its potential for future range expansions. This can be done using species distribution models (SDMs) that typically correlate species occurrences with environmental layers (Guisan and Thuiller, 2005; Elith and Leathwick, 2009; Kearney and Porter, 2009). These models are developed using the knowledge on the current distribution of the studied species (the realized niche) which can be potentially projected in space or time to forecast the species distributions within the invaded ranges within a time frame (Fitzpatrick and Hargrove, 2009; Gallien et al., 2010). Applying SDMs for *H. stipulacea* (Gamliel et al., 2020) using mean annual bottom temperature and net primary productivity as environmental predictors revealed some interesting patterns (**Figure 8**). The main result was the striking differences in the predicted suitability of the Mediterranean Sea to support *H. stipulacea* when the model is based on the native (**Figure 8A**) vs. invaded (**Figure 8B**) range occurrences. When using the native range occurrences only, the Mediterranean Sea climate appears to be very marginal for this species. However, when using the invaded Mediterranean range occurrences, the entire Mediterranean seems to provide an adequate climate for *H. stipulacea*. Thus, the climatic niche as estimated from the native range does not represent the full physiological potential of this species (Parravicini et al., 2015). Only after the invasion, when the species may be enjoying reduced biological constraints (the "biotic release" hypotheses) is the full climatic affinity of the species exposed. This means deducing the climatic constraints on *H. stipulacea* invasion using native range SDMs may severely underestimate invasion potential. Indeed, SDMs work best when the species-realized niches are representative of their fundamental niche, shaped by the underlying physiological constraints. However, if the fundamental and realized niches diverge, correlative SDMs may be far less accurate in predicting the future distribution of the species (Elith et al., 2010; Parravicini et al., 2015).

One way to overcome this limitation is to directly model the fundamental niche, e.g., using physiology. Physiological models take into account the biological mechanism behind the species distribution, and thus can be used more confidently for forecasting the response into novel environmental conditions such as in the invaded ranges (Kearney et al., 2010; Cheaib et al., 2012). The simplest form of these models utilizes a physiological threshold, such as temperature, to predict species future distributions. However, complex physiological models require substantial data on the relationship between the specific environmental conditions and species performance (Buckley et al., 2011; Cheaib et al., 2012).

A promising direction is to combine physiological estimates of species performance and correlative SDMs (Woodin et al., 2013; Martínez et al., 2015; Talluto et al., 2016). Such models may provide more robust forecasts of species distributions in novel climates. Gamliel et al. (2020) used a recently proposed a Bayesian approach that combines SDMs with physiological data (*sensu* Talluto et al., 2016) to forecast the distribution of *H. stipulacea*. The physiological data included *H. stipulacea*'s change in leaf area at different temperatures (Georgiou et al., 2016), which was used to calculate a temperature response curve. This data was then used as a prior for the coefficients relating environmental predictor to species occurrences within an SDM. RCP (Representative Concentration Pathway) scenarios were used to make predictions for 2100, based on the CCSM45 (Community Climate System Model 4), HadGEM2-ES (Hadley Centre Global Environmental Model 2), and MIROC55 (Interdisciplinary Research on Climate 5) climatic models. Surprisingly, the incorporation of the physiological data did not change the present and predicted future (2100) distribution of this species within the Mediterranean (**Figure 8**). This likely reflects the wide temperature tolerance of the species (Georgiou et al., 2016). In contrast, the hybridization of SDMs with reproductive window phenology of the invasive seaweed *Sargassum muticum* did strongly affect the distribution projections of the species under future climate change scenarios.

The results of this modeling exercise suggest that to accurately predict the potential for range expansion of *H. stipulacea,* as well as its response to climate change, it may be necessary to move beyond both correlative SDMs and simple combination of SMDs with physiology. For example, model performance may be enhanced by using more sophisticated models that incorporate physiological data for other environmental variables beyond temperature (e.g., salinity, turbidity, etc.) as well as phenological information. Conventional SDMs may also be improved by careful selection of occurrences, background data and predictors (Mainali et al., 2015). Further improvements may be achieved with including data on dispersal ability, reproductive features (e.g., reproductive periods, reproductive timing; Chefaoui et al., 2019) and biotic interactions such as competition, predation or facilitation, which are also likely to impact future distributions

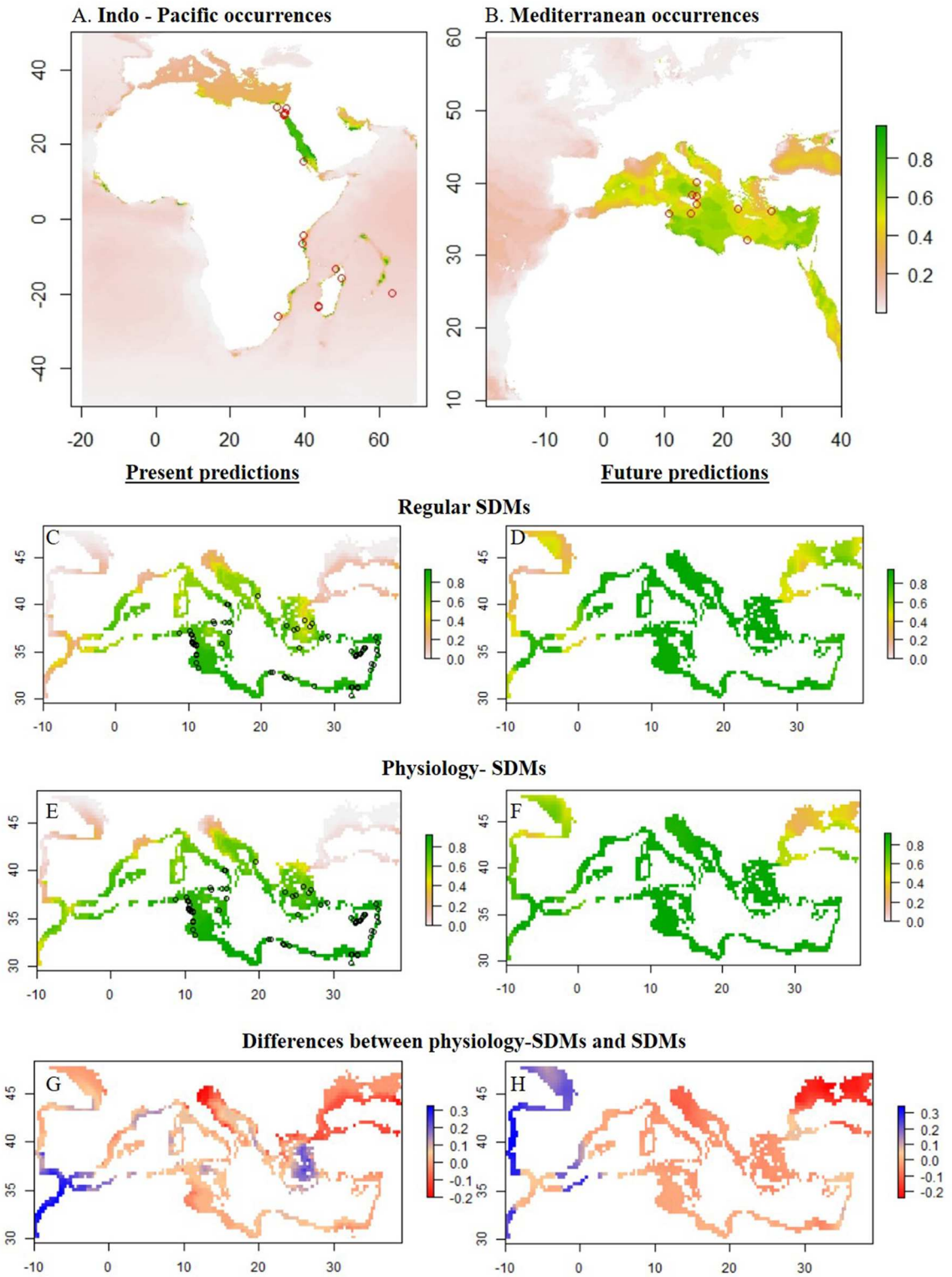

FIGURE 8 | *Halophila stipulacea* distribution models based on MaxEnt showing relative habitat suitability based solely on **(A)** native Indo-Pacific and **(B)** invasive Mediterranean occurrences. Predicted habitat suitability of *H. stipulacea* in the Mediterranean Sea under current environmental conditions (left; **C,E,G**) and forecasted under future (2100) environment (right; **D,F,H**). Models include either regular SDMs **(C,D)** or combined physiology-SDMs **(E,F)**. Lower panels **(G,H)** show the difference between the physiology-SDMs **(E,F)** and the regular SDMs **(C,D)**. Blue colors **(G,H)** indicate regions where physiology-SDMs predict high occurrence probability than regular SDMs. Figures adapted from Gamliel et al. (2020).

(Gilman et al., 2010; Kissling et al., 2012; Zarnetske et al., 2012; Wisz et al., 2013; Record et al., 2018).

CLOSING THE KNOWLEDGE GAPS

The motivation for this review comes from the risk of *H. stipulacea* becoming invasive worldwide. This is a risk that has not been identified so far—at least in terms of research efforts and funding priorities. This species has high plasticity, characteristics typical of an *r*-strategist species and thus has the potential to become an invader in a wide range of environmental conditions. Indeed, with the recent doubling of the Suez Canal (Galil et al., 2015, 2017) and the ongoing tropicalization and warming of the Mediterranean (Bianchi and Morri, 2003; Borghini et al., 2014), a process that is happening even faster in the eastern Mediterranean (Ozer et al., 2017), *H. stipulacea* could potentially become more prevalent in these waters in the coming years. This is even more probable considering that conditions in the Mediterranean Sea are becoming less favorable for its temperate, native, seagrass species (Jordà et al., 2012; Chefaoui et al., 2018; Marín-Guirao et al., 2018; Savva et al., 2018) and more welcoming of tropical species (Sghaier et al., 2014; Georgiou et al., 2016; Gerakaris et al., 2020). The traits that make *H. stipulacea* amenable to invasiveness include rapid horizontal growth and leaf turnover rates (Wahbeh, 1984; Willette and Ambrose, 2012), tolerance to a wide range of environmental conditions, including salinity (Oscar et al., 2018), light (Sharon et al., 2009, 2011a,b), and temperatures (Georgiou et al., 2016). This species has the ability to grow from very small fragments (Willette et al., 2020). It is capable of maintaining high-density meadows in both high and relatively low nutrient levels (Beca-Carretero et al., 2020) in different types of sediments (from soft mud to the nutrient-limited carbonate sediments). It has physiological plasticity (e.g., an efficient Ci-acquisition system, changes in leaf area, chloroplast clumping), and, probably, is capable of interacting with many different microbial species.

At least for the Caribbean, it seems that the invasive *H. stipulacea* with its short leaves and relatively smaller roots, cannot replace all the traits and services provided by the native Caribbean seagrass species that it displaces (e.g., wave attenuation and protection from storms, habitat complexity and use of meadows as fish nurseries), potentially entailing changes to the economic and social benefits that seagrasses provide in this region (reviewed by Viana et al., 2019).

The aim of this review was to evaluate the existing knowledge on the biological, ecological, physiological, biochemical, and molecular traits of *H. stipulacea* in its native and invaded habitats. This framework allowed us to (i) compare traits and environmental conditions across basins, (ii) discuss the possible environmental conditions and plant mechanisms involved in its invasion, (iii) assess the impact of *H. stipulacea* on native seagrasses and ecosystem functioning in the invaded regions, and (iv) predict the ability of this species to invade European and transoceanic coastal waters.

This review has also allowed us to identify several knowledge gaps, highlighted throughout the text, that need to be addressed in the future: The ecological interactions between *H. stipulacea* meadows and grazers (e.g., from small species up to dugongs) have been investigated mostly in the Caribbean, but we know very little about fish associated with *H. stipulacea* meadows in the Red and Mediterranean Seas. We know little about the functional role of the associated microbiome—do they contribute to the invasive success of their host? We lack data on the genetic diversity and connectivity of *H. stipulacea* populations. For these knowledge gaps, developing "omic" tools would be of particular relevance. We lack data on thermal tolerance of *H. stipulacea* populations (that could be collected from mesocosm experiments, modeling or *in situ* long term data) and how these compare with other neighboring seagrass or other species (e.g., corals and sponges). For this, the use of new technologies and innovative approaches (e.g., mesocosm common garden experiments, isotopic, biochemical, ecological, and molecular markers) will be mandatory. We need better niche models, accurate SDMs or climate envelope distribution models—these could help in predicting future expansions of *H. stipulacea*'s distributions (e.g., what regions and ecological niches are likely to be invaded?) and the impacts of such changes (can we even control such expansions?). The word cloud highlights that we know little about ecosystem services directly associated with *H. stipulacea* in both native and invaded habitats. We need to compare reproductive seasons (timing, duration, female/male ratios) among different sites in both native and invaded ranges.

Our review identifies regions for which we have even larger knowledge gaps—we know very little about populations of *H. stipulacea* in many parts of its native range. Similarly, we lack studies on seasonal changes in Mediterranean populations, where quantitative data dealing temporal changes of *H. stipulacea* don't exist. Finally, we conclude that a coordinated mapping of *H. stipulacea* and permanent monitoring efforts are needed across native and invaded distribution areas. The issues at stake entail the involvement of biologists, ecologists, modelers, managers, and local stakeholders. In the current scenarios of climate change and exponential human pressure on coastal areas, long-term monitoring is needed to record changes in *H. stipulacea* over time with associated communities to contextualize current observations in native (Red Sea), invaded (Mediterranean and Caribbean Seas), and prospective distributional ranges.

From the perspective of future management efforts in regions where *H. stipulacea* might become invasive, we do not believe it would be possible to remove newly discovered plants—unless on a very small scale (<10 m^2). Due to its rapid clonal growth, prolonged survival as fragments, and its ability to regrow from small fragments (Smulders et al., 2017; Willette et al., 2020), we need to take into account that if *H. stipulacea* plants are pulled out, tiny fragments can survive and settle in other places. Perhaps, more efforts should be placed on prevention of loss of native seagrasses. We know that when native seagrass disappears, *H. stipulacea* can rapidly colonize the available area (especially in the Caribbean). But if native seagrass is still there, this probably is not that easy for *H. stipulacea*. Indeed, this was demonstrated by a study by Steiner and Willette (2015b) in Dominica, where so long as *S. filiforme* had a cover of $<45\%$ it was able to resist invasion by *H. stipulacea* (*S. filiforme* "strongholds"), but if *S. filiforme* was

below this, there was space for *H. stipulacea* to come in and *S. filiforme* eventually was displaced (Steiner and Willette, 2015b). The comparison of the invasiveness of *H. stipulacea* in protected vs. unprotected MPAs has yet to be done, but the mere ban of fishing in seagrass meadows located in Caribbean island nations might help slowing invasiveness of *H. stipulacea* since it has been shown that wooden and metal fish traps commonly used by fishermen in the eastern Caribbean facilitate local dissemination of *H. stipulacea* (Willette and Ambrose, 2012; discussed above). While protecting native seagrasses from global warming is difficult, they can be protected from local stressors such as physical damage (e.g., anchoring), and more importantly from eutrophication. While setting up marine protected areas (MPAs) has become a fundamental strategy in marine conservation, their effectiveness on seagrass meadows has been relatively less studied (reviewed by Alonso Aller et al., 2017). Seagrass MPAs in tropical areas were shown to increase the temporal stability of seagrass-associated fish communities, which in turn enhanced herbivory followed by enhanced seagrass growth rates (Alonso Aller, 2018). However, MPAs were not able to protect seagrasses from land-use effects, highlighting the importance of coupling seagrass conservation with land-based management. Indeed, both Björk et al. (2008) and Waycott et al. (2009) have identified nutrient inputs as the number one threat to seagrass ecosystems worldwide. Thus, in parallel to mapping and monitoring changes in areas where *H. stipulacea* meadows have already invaded, it might be more important, in areas where this species has not yet completely overtaken native seagrasses, to apply improved land-based management strategies that would reduce potential eutrophication and prevent loss of water quality, stressors that would enhance such invasiveness.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

GW and GR initiated the Euro Marine workshop that kicked off this review. GW led the writing and editing helped by IV. TA-G, BV, and BM led the review of published studies (**Table 1**, **Table S1**). RS-T prepared the maps. All authors contributed to writing sections: SB (responses to light, carbon sources), PB-C (biochemistry), LM, AE, and AR (microbial studies), MO (tolerance to salinity), GR (invasiveness, *H. stipulacea* as an invader), JB and IG (modelling), AA (nutrient uptake), AA and GP (*H. stipulacea* in the Mediterranean), DW, AE, and KC (*H. stipulacea* in the Caribbean), MO and GP (developing omic tools). SB, DW, IV, and AR helped with editing final versions.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2020.00300/full#supplementary-material

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The Seagrass Methylome Is Associated With Variation in Photosynthetic Performance Among Clonal Shoots

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Evolutionary theory predicts that clonal organisms are more susceptible to extinction than sexually reproducing organisms, due to low genetic variation and slow rates of evolution. In agreement, conservation management considers genetic variation as the ultimate measure of a population's ability to survive over time. However, clonal plants are among the oldest living organisms on our planet. Here, we test the hypothesis that clonal seagrass meadows display epigenetic variation that complements genetic variation as a source of phenotypic variation. In a clonal meadow of the seagrass *Zostera marina*, we characterized DNA methylation among 42 shoots. We also sequenced the whole genome of 10 shoots to correlate methylation patterns with photosynthetic performance under exposure to and recovery from 27°C, while controlling for somatic mutations. Here, we show for the first time that clonal seagrass shoots display DNA methylation variation that is independent from underlying genetic variation, and associated with variation in photosynthetic performance under experimental conditions. It remains unknown to what degree this association could be influenced by epigenetic responses to transplantation-related stress, given that the methylomes showed a strong shift under acclimation to laboratory conditions. The lack of untreated control samples in the heat stress experiment did not allow us to distinguish methylome shifts induced by acclimation from such induced by heat stress. Notwithstanding, the co-variation in DNA methylation and photosynthetic performance may be linked *via* gene expression because methylation patterns varied in functionally relevant genes involved in photosynthesis, and in the repair and prevention of heat-induced protein damage. While genotypic diversity has been shown to enhance stress resilience in seagrass meadows, we suggest that epigenetic variation plays a similar role in meadows dominated by a single genotype. Consequently,

conservation management of clonal plants should consider epigenetic variation as indicator of resilience and stability.

Keywords: DNA methylation, ecological epigenetics, clonality, heat stress, seagrass, *Zostera marina* (eelgrass)

INTRODUCTION

Genetic variation is considered key to long-term survival of populations (Bijlsma and Loeschcke, 2012), and is recognized as a key for the conservation and restoration of biological diversity (Lande, 1988; Spielman et al., 2004; Laikre et al., 2009). In contrast, lack of genetic variation is regarded as an evolutionary dead-end (Lynch and Lande, 1998) but clonal growth challenges the expected relationship between genetic diversity and long-term survival. For example, conservation management follows the rough guideline that within a population >1,000 genetically different individuals must mate randomly to avoid inbreeding depression, and retain evolutionary potential (Frankham et al., 2014). Yet, roughly 40% of all plants can reproduce asexually (Tiffney and Niklas, 1985), mostly by clonal growth where parental genotypes (genets) grow vegetative modules (ramets) that often remain connected *via* underground stolons or rhizomes.

Although clones benefit from resource sharing, niche specialization, and rapid vegetative growth (Liu et al., 2016), they are predicted to survive only for short periods, and in stable environments (Silvertown, 2008). Asexual reproduction is assumed to lead to slow rates of genetic evolution, and the lack of DNA repair mechanisms afforded by meiosis (Muller's ratchet, mutational meltdown) (Muller, 1964; Gabriel et al., 1993; Lynch et al., 1993). Despite asexual reproduction, many of our most important crops are clones (McKey et al., 2010), including banana, garlic, hops, potatoes, and turmeric, as well as many of the earth's most invasive and oldest plants. For example, genets of Palmer's oak (*Quercus palmeri*) and seagrass (*Posidonia oceanica*) are estimated to be older than 10,000 years (May et al., 2009; Arnaud-Haond et al., 2012). Thus, long-term survival appears not to rely solely on sexual reproduction.

Somatic mutations can create a certain level of genetic diversity, and may explain some evolutionary potential of clonal organisms (Whitham and Slobodchikoff, 1981; Loxdale et al., 2003; Lushai et al., 2003; Reusch and Boström, 2011). For example, ~7,000 single nucleotide polymorphisms (SNPs), 597 in coding regions, and 432 non-synonymous, distinguish ramets of a large Finnish clone of the seagrass *Zostera marina* (Yu et al., 2020). To set this in perspective, 139,321 biallelic SNPs were reported in coding regions among four populations of the same species (Jueterbock et al., 2016). The degree to which epigenetic variation can contribute to phenotypic heterogeneity in ecologically relevant traits, independently from the underlying genetic variation, is a key question in assessing its contribution to stress tolerance, and long-term survival of clonal organisms.

The definition of epigenetics is currently heavily debated (Ptashne, 2007; Greally, 2018). Here, "epigenetics" implies molecular variations that do not alter the DNA sequence but have the potential to change gene expression, and include non-coding RNAs (ncRNAs), histone modifications, and DNA methylation (Bossdorf et al., 2008). DNA methylation is, from an evolutionary perspective, the most relevant epigenetic mechanism because it can be independent from genetic variation (Bossdorf et al., 2008; Schmitz et al., 2013; Kilvitis et al., 2014), and transgenerationally stable (Boyko et al., 2010; Verhoeven et al., 2010; Ou et al., 2012; Bilichak et al., 2015; Williams and Gehring, 2017). DNA methylation involves the addition of a methyl-group to the C5 position of a cytosine in DNA sequence motifs (CG, CHG, and CHH in plants, where H stands for A, C, or T) (Kilvitis et al., 2014). Depending on sequence context, methylation can be associated with gene activation or silencing (Bossdorf et al., 2008; Niederhuth and Schmitz, 2017). While CG methylation in gene bodies often correlates with increased gene expression, methylation in promoters and repeat regions, such as transposable elements (TEs), silences expression (Feng et al., 2010; Seymour et al., 2014; Dubin et al., 2015; Bewick and Schmitz, 2017; Zhang et al., 2018).

The methylome, or set of DNA methylation modifications in an organism's genome, can change spontaneously at a rate of 2.5×10^{-4} to 6.3×10^{-4} methylation polymorphisms per CG site per generation, which is about 7×10^4 higher than the genetic mutation rate of base substitutions per site per generation (Schmitz et al., 2011; van der Graaf et al., 2015). That methylome variation can enhance productivity, and pathogen resistance, has been shown in *Arabidopsis thaliana* plant populations (Latzel et al., 2013). Moreover, methylation variation explained the rapid invasive success of the Japanese knotweed (*Fallopia japonica*) by facilitating differentiation in response to new habitats despite decreased genetic variation (Richards et al., 2012). This suggests that methylation variation complements genetic variation as a source of phenotypic variation in plant populations deprived of genotypic diversity (Gao et al., 2010; Richards et al., 2012; Verhoeven and van Gurp, 2012; Latzel et al., 2013; Zhang et al., 2013; Vanden Broeck et al., 2018).

Unlike genetic variants, methylation variants can also switch state directly in response to the environment (Dowen et al., 2012) and, if stable enough, establish a molecular memory that can be involved in stress priming. Under clonal growth, DNA methylation patterns are expected to be more stably inherited than under sexual reproduction (Verhoeven and Preite, 2014), because clonal growth circumvents epigenetic reprogramming during gameto- and embryogenesis. Although stable transmission across asexual generations has been shown for environment-specific phenotypes and DNA methylation patterns in clonal plants (Verhoeven et al., 2010; Verhoeven and van Gurp, 2012; Vanden Broeck et al., 2018; Verhoeven et al., 2018), a link between methylation variation and fitness-related traits has yet only been demonstrated in non-clonal plants (Latzel et al., 2013). Thus, while epigenetic mechanisms have been suggested to

contribute to clonal plant success (Douhovnikoff and Dodd, 2014; Verhoeven and Preite, 2014; Dodd and Douhovnikoff, 2016; Latzel et al., 2016), empirical evidence is virtually lacking.

Clonal propagation is especially well-developed in aquatic plants (Barrett, 2015). Seagrasses, the only plants to inhabit the marine world, form the foundational basis of some of the most productive and highly diverse coastal marine ecosystems on the planet, and are essential for the health and abundance of economically exploited marine species (Costanza et al., 1997; Larkum et al., 2006; Orth et al., 2006). Ecosystem services are worth more than € 25,000 ha^{-1} $year^{-1}$ (Costanza et al., 2014), including nursery grounds, habitat and food for fish and invertebrates, protection of the coastline from erosion, carbon sequestration of up to 186 g C m^2 yr^{-1} (Fourqurean et al., 2012; Duarte et al., 2013), and nutrient fixation (Orth et al., 2006; Procaccini et al., 2007).

Over the last decades, losses of seagrass ecosystems have been documented worldwide due to increasing anthropogenic stressors such as invasive species, sediment and nutrient runoff, dredging, aquaculture, rising sea levels, and global warming (Orth et al., 2006; Chefaoui et al., 2018). Losses are expected to accelerate under projected global temperature increase, as ocean warming is considered the most severe threat among climate change factors (Repolho et al., 2017; Duarte et al., 2018) and seagrass meadows have tracked temperature changes in the past (Olsen et al., 2004). How accurate these shifts and losses can be predicted depends on our knowledge of drivers of adaptive potential, including epigenetic diversity (Duarte et al., 2018).

In this study, we characterize the functional relevance of epigenetic variation for heat stress resilience in the seagrass *Zostera marina*. *Z. marina* is the most widely distributed seagrass in the northern hemisphere, inhabiting highly contrasting habitats from sub-arctic to sub-tropical waters (Green and Short, 2004; Olsen et al., 2004; Boström et al., 2014). Few plants display such dramatic range in clonal diversity as *Z. marina* (Reusch et al., 2000; Olsen et al., 2004). Its clonal architecture varies from genetically diverse meadows with high levels of sexual reproduction, to meadows composed of a single large clone due to exclusive vegetative reproduction (Reusch et al., 1999; Olsen et al., 2004; Becheler et al., 2010; Reusch and Boström, 2011). Clonality of *Z. marina* peaks in the Baltic Sea, where large clones were estimated > 1,000 years old (Reusch et al., 1999). These meadows display remarkable phenotypic plasticity and persistence in time under drastic changes in ice cover, salinity, and temperature (Reusch et al., 1999), and under perturbations that represent environmental stress predicted elsewhere for the future (e.g. high temperatures) (Reusch et al., 2018). Moreover, extension of a single seagrass clone in space over ca. 160 × 40 m, and a water depth ranging from 1.5 to 4.5 m with an environmental gradient in light, sedimentation, wind exposure, and ice scour, strongly suggests niche differentiation among ramets of a single clone (Reusch et al., 1999). Thus, clonal meadows (such predominated by a single genet) confound experimental results showing a positive effect of genotypic (i. e. clonal) richness on the productivity and stress resilience of *Z. marina* (Hughes and Stachowicz, 2004; Reusch et al., 2005; Reusch and Hughes, 2006; Ehlers et al., 2008; Hughes et al., 2008). In other words, if stress resilience and tolerance would rely strongly on genotypic diversity, clones would not be able to disperse widely in space or survive for such long time periods.

The resilience, longevity, and adaptive potential of clonal seagrass meadows remain unknown without a fundamental analysis of their epigenetic variation and its ecological relevance. Therefore, this study tests the hypothesis that variation in DNA methylation can promote functional phenotypic diversity and, thus, may explain how clonal seagrass meadows can persist over millennia.

Specific objectives were to: 1) Characterize DNA methylation variation in an ancient clonal meadow of *Z. marina* (>1,000-years-old); and 2) Identify the functional role of this variation in photosynthetic performance—specifically how photosynthetic performance under benign and stressful temperatures is linked to DNA methylation variation independently from underlying somatic mutations.

MATERIALS AND METHODS

Sampling and Cultivation

In June 2015, we sampled seagrass shoots from a clonal meadow (Reusch et al., 1999) in the Baltic Sea (Åland Islands, 60°09′50.4″N, 19°31′48.1″E) (**Figure 1**) by collecting two to three shoots attached to the same rhizome (ramets) every 3 meters along a 250 m transect (**Supplementary Table S1**). Along the transect running perpendicular to the shore, water depth increased from 0.5 to 3 m. Shoots were transported in seawater-filled cooling boxes to the field station at Nord University, Norway. The most mature leaf of 42 shoots (field transect samples), randomly chosen from the two to three connected ramets, was flash frozen in liquid nitrogen for subsequent DNA extraction (**Supplementary Table S1**). Thus, recorded DNA methylation patterns are expected to reflect the average state of methylation of a mature leaf, along which global DNA methylation has been shown to vary with tissue age in the seagrass Posidonia oceanica (Ruocco et al., 2019b).

Ten ramets of the same genet (**Supplementary Table S1**), sampled at the same time and from the same transect as the field samples, were individually replanted in plastic containers (10 × 15 cm) filled with sand from the sampling site, placed in a 1,280-L aquarium at 15°C (corresponding to field temperature), and illuminated with a 16:8 h light–dark cycle, under light-saturating conditions (200-220 μmol m^{-2} s^{-1},OSRAM Fluora, 150 W) (Dennison and Alberte, 1982; Alcoverro et al., 1999). Seawater at 5.5 PSU, corresponding to the salinity at the collecting site, was obtained by mixing freshwater and natural filtered seawater at 32 PSU. The water, filled to a 40-cm level, was kept in constant motion with airstones. Once a week, 50% of the water was renewed after removing epiphytic algae.

Heat Stress Experiments

After two weeks of acclimation, the 10 clonal ramets were exposed to heat stress (**Supplementary Table S1**) in a climate chamber (Fitotron, weisstechnik), where they were distributed in three aquaria (60 L, 3–4 ramets in each aquarium) filled with

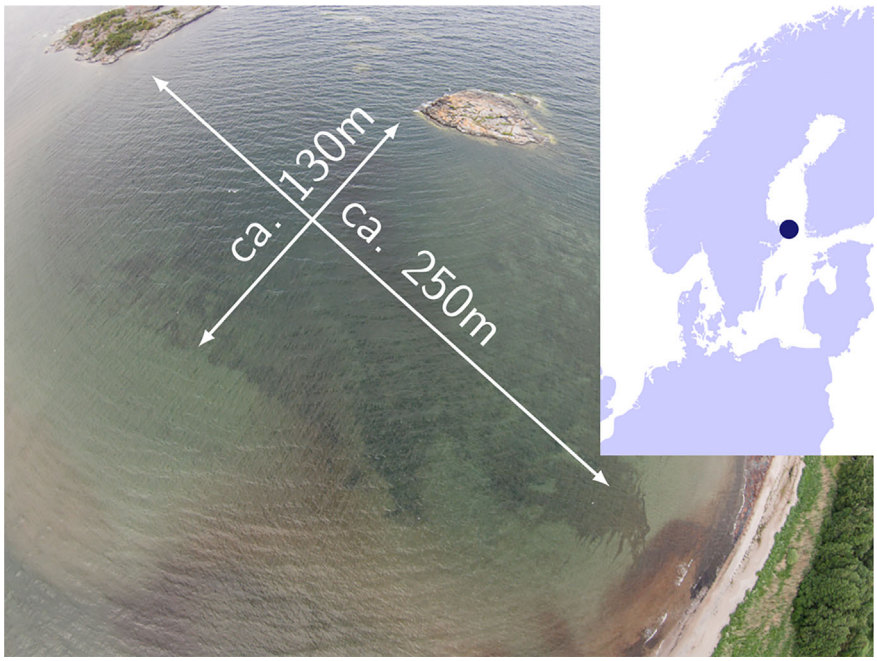

FIGURE 1 | Baltic Sea sampling site of a > 1,000 years old clonal meadow where 42 *Zostera marina* shoots were collected along a 250 m transect.

aerated brackish water (5.5 PSU) to 30 cm, of which 50% was weekly renewed. Light was kept at 240 µmol m^{-2} s^{-1} and a 16:8-h light:dark cycle. The temperature in the climate chambers was increased with a daily increment of 3°C from 15°C to 27°C, which can be lethal for *Z. marina* (Greve et al., 2003) and exceeds maxima of sea surface temperatures recorded in the Baltic Sea by ca. 2°C (Reusch et al., 2005), but is likely to be reached in the Baltic Sea during future summer heat waves. After three weeks at 27°C, the temperature was decreased by a daily increment of 3°C from 27°C to 15°C, after which all shoots were returned to the 1,280 L aquarium at 15°C for a recovery period of 5.5 weeks where they were exposed to the same conditions as under acclimation.

At three time points (control, stress, recovery), one entire mature (outer-most) leaf of each shoot was excised and flash-frozen in liquid nitrogen. Thus, the methylation pattern of each sample represents the average methylation state across young and mature tissues, which have been shown to vary in their epigenetic heat-stress response in the seagrass *Posidonia oceanica* (Ruocco et al., 2019a). Control samples were collected at 15°C on the day before the temperature was increased. Stressed samples were collected at 27°C on the day before the temperature was decreased. Recovery samples were collected after the 5.5-week recovery period at 15°C.

Photosynthetic Performance

At control, stress, and recovery time points, we measured for each shoot (two measurements for each of the two inner leaves) the increase in chlorophyll *a* fluorescence upon illumination after a 10 min dark period (OJIP curve) (Bussotti et al., 2010), with a PAM-Fluorometer (FluorPen FP100, Photon Systems Instruments) using a saturating pulse of 75% light intensity at 455 nm. From the measurements, we extracted the photosynthetic performance index PiABS (Strasser et al., 2000), reflecting the functionality of photosystem II (PSII) and photosynthetic performance in general (Živčák et al., 2008; Bussotti et al., 2010; Stefanov et al., 2011). PiABS combines three parameters: 1) the density of reaction centers, 2) the efficiency of electron transport beyond Q_A at the onset of illumination, and 3) the probability for an absorbed photon to reach the reaction center in PS II. PiABS is calculated as: $PiABS = ((1 - F_0/F_M)/(M_0/V_J)) \times ((F_M - F_0)/F_0) \times ((1 - V_J)/V_J)$. Here, F_0 is the minimum fluorescence intensity in a dark adapted leaf when all reaction centers are open (all quinone acceptors are oxidized), F_J is the fluorescence intensity at 2 ms illumination, F_M is the maximum fluorescence intensity when all reaction centers are closed (all quinone acceptors are reduced), V_J is the relative variable fluorescence at 2 ms calculated as $V_J = (F_J F_0)/(F_M F_0)$, and M_0 reflects the initial slope of fluorescence kinetics, and is calculated as $M_0 = 4 \times (F_{300\ \mu s} F_0)/(F_M F_0)$ (Živčák et al., 2008). We tested for correlation of PiABS values (mean values per shoot) between time points using a two-sided Pearson's product moment correlation test in R v3.4.4 (R Core Team, 2019).

DNA Extraction

All flash-frozen tissue was freeze-dried for one day, then stored at −80°C until DNA extraction. DNA was extracted with the HP Plant DNA mini kit (Omega Bio-Tek, protocol version May 2013) from ≥ 5 mg of lyophilized leaf tissue after grinding with a mixer-mill (Retsch MM 400) in 2 mL Eppendorf tubes with tungsten beads supplied (60 s at 30 Hz). We added 10 µl beta-mercaptoethanol at step 2, and equilibrated the columns at step 8 of the standard protocol. The extracted DNA was eluted in 2 ×

100 µl EB buffer (Qiagen), cleaned, and concentrated with the Clean and Concentrator-5 kit (Zymo Research, protocol v1.2.1) using 15,000 g for all centrifugation steps, and finally eluted in 2 × 30 µl EB Buffer (Qiagen) at 60°C.

Clonality

In order to identify ramets belonging to the same genet, we genotyped each shoot for seven microsatellite loci (ZosmarGA2, -GA3, -GA6, -CT3, -CT12, -CT19, -CT20) (Reusch and Boström, 2011). PCR was performed using a Veriti 96-Well Thermal Cycler (Applied Biosystems, Life Technologies) in two 10-µl multiplex reactions containing 2 µl cleaned genomic DNA, and 1× AccuStart II PCR ToughMix (Quanta bio). One multiplex reaction contained forward and reverse primers GA2, GA3, and GA6 at 0.5 µM each, and ran at 94°C for 4 min, followed by 30 cycles of 94°C for 60 s, 55°C for 90 s, and 72°C for 90 s, and a final extension at 72°C for 10 min. The other multiplex reaction contained forward and reverse primers CT3 (0.5 µM), CT12 (0.3 µM), CT19 (0.3 µM), and CT20 (0.3 µM), and ran at 94°C for 3 min, followed by 35 cycles of 94°C for 60 s, 57°C for 60 s, and 72°C for 60 s, and a final extension at 72°C for 10 min.

DNA fragment lengths were determined on an ABI 3500xl Genetic Analyzer from 1 µl of 1:99 diluted PCR products mixed with 8.9 µl of HiDi Formamide (Life Technologies), and 0.1 µl of Gene Scan 500 LIZ Size Standard (Life Technologies) after 5 min denaturation at 95°C. Alleles were called with the GeneMapper v4.1 Software (Applied Biosystems, Thermo Fisher Scientific). The shoots were assigned to multi-locus genotypes using the R package "RClone" (Bailleul et al., 2016).

Whole Genome Sequencing, SNP Detection, and Genetic Distance in Experimental Samples

In order to detect SNPs resulting from somatic mutations in the 10 heat-stressed ramets, genomic DNA libraries were prepared according to the TruSeq DNA PCR-Free (Illumina) protocol, and sequenced on one Illumina HiSeq 3/4000 lane (2 × 150 bp) at the Norwegian Sequencing Centre (University of Oslo, Norway). Raw reads (25.7 million to 44.3 million per library, **Supplementary Table S2**, NCBI BioProject PRJNA575339) were quality-checked with FastQC v0.11.8[1] to control for aberrant read base content, length distribution, duplication, and over-representation. We used TrimGalore! v0.6.0[2] to remove adapter sequences with a stringency of 3 bp overlap, and low-quality bases with a Phred score Q < 20 (99% base call accuracy). The high-quality reads (25.5 to 44.0 million per library, **Supplementary Table S2**) were mapped to the *Z. marina* genome v2.1 (Olsen et al., 2016) with BWA v0.7.17 (Li and Durbin, 2009). Read duplicates were removed with MarkDuplicatesSpark within GATK v4.1.4.1 (Auwera et al., 2013). SNPs were called with HaplotypeCaller, followed by CombineGVCFs and GenotypeGVCFs within GATK v4.1.4.1 (Auwera et al., 2013).

[1] http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

[2] https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/

Before filtering SNPs, we excluded indels, non-variant sites, and alternate alleles not present in any genotypes from the vcf file with SelectVariants within GATK v4.1.4.1 (Auwera et al., 2013). This set of 759,407 raw SNPs was reduced to 105,443 SNPs after hard-filtering with vcffilter from vcflib (Garrison, 2020) with thresholds that were based on density plots drawn with ggplot2 (Wickham, 2016): QualByDepth (QD < 15.0), FisherStrand (FS >12.0), RMSMappingQuality (MQ < 38), MappingQualityRankSumTest (MQRankSum < −1.5), ReadPositionRankSumTest (ReadPosRankSumand < −4.0), and Depth (DP > 4000.0, in order to remove SNPs potentially caused by genome duplication). Subsequently, we used VCFtools v0.1.15 to remove genotypes with genotype quality < 30 (–minGQ 30) or depth < 20 (–minDP 20), and to remove SNPs with more than 2 alleles (–min-alleles 2 –max-alleles 2), with a minor allele frequency of 0.01 (–maf 0.01), and with any missing genotype (–max-missing-count 0). From the remaining 15,508 high-quality SNPs (**Supplementary File S1**) we excluded all that shared the same genotypes among all 10 heat-stressed shoots, as these reflected genetic differences only to the reference genome. The remaining 1,079 SNPs (**Supplementary File S2**) were used to estimate Euclidean genetic distances among the 10 shoots using the R package vcfR v1.9.0 (Knaus and Grünwald, 2017) and the *dist* function of the R package "stats" v3.6.9 (R Core Team, 2019). We tested for correlation between genetic and physical distance among the 10 heat-stressed shoots with Mantel tests in the R package "vegan" v1.4-2 (Oksanen et al., 2019), using 1,000 permutations, and the Pearson's product moment correlation method.

Methylome Characterization

Sequencing libraries were prepared according to the MethylRAD protocol (Wang et al., 2015) with few adjustments. MethylRAD is a genome-reduction method based on the methylation-dependent restriction enzyme FspEI that targets fully methylated CCGG and CCWGG motifs, thus capturing methylation in CG and CHG sequence contexts. MethylRAD has the potential to reveal genome-wide DNA methylation patterns that are consistent with those generated from Whole Genome Bisulfite Sequencing (Wang et al., 2015). First, sense and anti-sense oligos of adapters A1 and A2 (**Supplementary File S3**) were annealed in 10 µl containing 10 µM of each oligo (Eurofins), 10 mM Tris HCl (Thermo Fisher), 50 mM NaCl (Thermo Fisher), and 1 mM EDTA (Thermo Fisher). Library preparation began with digestion of 100 ng cleaned genomic DNA at 37°C for 4 h in 15 µl containing 4 U FspEI (NEB), 1× CutSmart Buffer (NEB), and 30× Enzyme Activator Solution (NEB). Digestion was verified on a TapeStation 2200 with a D1000 ScreenTape. Second, adapters were ligated to the digested fragments over night at 4°C in 26 µl containing 13 µl digestion solution, 0.1 µM each of two annealed adapters, 1× T4 ligase buffer (NEB), 1.5 µM ATP (NEB) and 1040 U of T4 DNA ligase (NEB). Ligation products were amplified in 20-µl reactions containing 7 µl ligated DNA, 0.05 µM of each primer (P1 and P2, **Supplementary File S3**), 0.3 mM dNTP, 1× Phusion HF buffer (NEB) and 0.4 U Phusion high-fidelity DNA polymerase (NEB). PCR was conducted using a Veriti 96-Well Thermal

Cycler (Applied Biosystems, Life Technologies) with 16 cycles of 98°C for 5 s, 60°C for 20 s, 72°C for 10 s, and a final extension of 5 min at 72°C. The target band (approx. 100 bp) was extracted from a 2% E-Gel (Thermo Fisher). For multiplex sequencing, shoot barcodes were introduced by means of PCR. Each 20 µl PCR reaction contained 12 µl of gel-extracted PCR product, 0.2 µM of each primer (P3 and index primer, **Supplementary File S3**), 0.3 mM dNTP, 1× Phusion HF buffer (NEB) and 0.4 U Phusion high-fidelity DNA polymerase (NEB). PCR was conducted with the same PCR cycling program outlined above. PCR products were purified using AMPURE XP beads (Beckman Coulter) using a 1.8:1 volume ratio of beads to product, and a final elution in 22 µl EB buffer (Qiagen). The purified fragments were sequenced on an Illumina NextSeq 500 (1 × 75 bp) using three high-output flow-cells: The 10 experimental samples were sequenced on a single flow-cell, while the field transect samples were split over two other flow-cells (detailed in **Supplementary Table S3**).

The sequenced reads were quality-trimmed with TrimGalore! v0.4.1[3] by removing the adapter sequences with a stringency of 3 bp overlap, low-quality bases with a Phred score Q < 20, and the terminal 2 bp from both ends in order to eliminate artifacts that might have arisen at the ligation position. Quality was checked with FastQC v0.11.8[4] to control for aberrant read base content, length distribution, duplication and over-representation.

The high-quality reads were mapped with SOAP v1.11 (Li et al., 2008) to 628,255 *in silico* predicted MethylRAD tags that were extracted from the *Z. marina* genome v2.1 from ORCAE (Sterck et al., 2012) with the custom python script *InSilicoTypeIIbDigestion.py*[5]. For mapping, we allowed for two mismatches, filtered reads with >1 N, and used a seed size of 8 bp. Based on the uniquely mapped reads, we counted the coverage of each methylated site for each shoot using htseq-count (v0.7.2). Methylation calls were retained only for sites with ≥ 2× coverage, which reduced the false-positive rate from 1.10% to 0.23% for CG sites and from 2.50% to 0.89% for CHG sites. False-positive rates were estimated as the percentage of methylation sites supported by at least two reads in the generally unmethylated chloroplast genome. For each shoot, raw counts were normalized to reads-per-million by dividing reads per site through the total number of reads per shoot library, times one million.

The methylation sites were annotated with the v2.1 gff3 file from ORCAE (Sterck et al., 2012), and separated into genes, intergenic regions, and TEs. TEs were located in both genes and intergenic regions, and contained repeats classified by RepeatModeler[6] as rnd-1/2/3/4/5 families, referring to the series of processing rounds of the *de novo* TE family identification program. The methylation sites were further separated into such containing CG and CHG recognition sites. Sequence contexts for all 628,255 *in silico* predicted MethylRAD tags are listed in **Supplementary Table S4**.

[3] https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/
[4] http://www.bioinformatics.babraham.ac.uk/projects/fastqc/
[5] http://marinetics.org/2017/04/11/REdigestions.html
[6] http://www.repeatmasker.org/RepeatModeler/

Methylation Variation Between the Field Transect Shoots

In order to describe the level of intra- and inter-clonal epigenetic distance among the 42 transect shoots (of which shoot 27 belonged to a different genet than the 41 other ramets, **Supplementary Table S1**), we calculated the Euclidean distance between shoots based on their coordinates in the 2-dimensional PCA (Principal Component Analysis) plot using the *dist* function of the R package "stats" v3.6.0 (R Core Team, 2019). PCA was done on reads-per-million for all shoots with the PCA function of the R package "FactoMineR" (Lê et al., 2008). We tested for correlations between epigenetic and physical distance among the 41 ramets with Mantel tests using the R package "vegan" v1.4-2 (Oksanen et al., 2019) with 1,000 permutations, and the Pearson's product moment correlation method. *P*-values were adjusted according the Benjamini-Hochberg method (Benjamini and Hochberg, 1995).

Correlation Between Methylome Variation and Photosynthetic Performance

For the 10 experimental samples, we tested for correlations between epigenetic distance and photosynthetic performance difference (PiABS values) among shoots, while controlling for genetic distance with Partial Mantel tests using the function *partial.mantel.test* of the R package "ncf" v1.2.9 (Lê et al., 2008). Only significant correlations ($p<0.05$, adjusted for multiple comparisons according to (Bjornstad, 2020) in R (R Core Team, 2019)) with coefficients $R > 0.65$, when controlled for genetic distance, were considered strong enough to be reckoned as biologically linked. The same analysis ran Mantel tests for correlation between genetic and epigenetic distance, and between genetic distance and photosynthetic performance difference.

Methylome Shift Under Experimental Conditions

The methylome of the 10 heat-stressed shoots was characterized under control, stress, and recovery conditions (3 shoots died from the stress), and compared with the methylome of field transect samples with PCA on reads-per-million data using the PCA function of the R package "FactoMineR" (R Core Team, 2019). Epigenetic distances between the samples were estimated for each sequence context (gene, intergene, TE, each in CG and CHG regions, respectively) as the Euclidean distances in the 2-dimensional PCA plot using the *dist* function of the R package "stats" v3.6.9 (Lê et al., 2008).

Differential Methylation Analyses

To estimate the number of sites that changed in methylation state from control to stress and recovery conditions, we applied differential methylation analyses using the R package "edgeR" 3.20.9 (R Core Team, 2019) within the "SARTools" pipeline v1.6.6 (Robinson et al., 2009). Read counts were normalized using a trimmed mean of M-values (TMM) between each pair of samples (Varet et al., 2016). All samples taken under control, stress, and recovery served as replicates for the three different conditions. Methylation levels were considered significantly

increased (hyper-methylated) or decreased (hypo-methylated) when Benjamini-Hochberg adjusted *p*-values (Robinson and Oshlack, 2010) fell below 0.05.

To identify how the methylome differed between samples of high and low photosynthetic performance, we used differential methylation analyses using the R package "edgeR" 3.20.9 (Benjamini and Hochberg, 1995) within the "SARTools" pipeline v1.6.6 (Robinson et al., 2009). Read counts were normalized using a trimmed mean of M-values (TMM) between each pair of samples (Varet et al., 2016). Differential methylation analysis was done only for conditions and sequence contexts where a positive correlation was found between photosynthetic performance differences and epigenetic distances. For control conditions, we compared methylation levels in genes (CG regions) between the two samples of highest photosynthetic performance (79.1, and 13.2) and the two samples of lowest photosynthetic performance (63.1 and 59.2, **Figure 2**). For recovery conditions, we compared methylation levels in intergenic TEs (CHG regions) between the two samples of highest photosynthetic performance (79.1 and 17.1) and the two samples of lowest photosynthetic performance (57.1 and 59.2). Methylation levels were considered significantly different between shoots of high and low photosynthetic performance, when Benjamini-Hochberg adjusted *p*-values (Robinson and Oshlack, 2010) fell below 0.05.

For differentially methylated sites within gene bodies, we tested with Fisher's exact tests for enrichment of gene ontology terms of biological processes with the R package "topgo" (Benjamini and Hochberg, 1995) using Fisher's exacts tests. GO terms were obtained from the v2.1 *Zostera* genome annotation from the ORCAE database (Alexa and Rahnenführer, 2010). To reduce redundancy in the significantly enriched GO terms (p-values < 0.05), we calculated "sim rel" scores (Sterck et al., 2012) (Allowed similarity=0.5), based on the *A. thaliana* GO-term database, using the REVIGO web server (Schlicker et al., 2006).

RESULTS

Methylome Characterization and Variation Among the Clonal Transect Shoots

On average, 74 million high-quality reads were obtained per sequencing library (*Methylome Characterization* section, ranging from 0.7 to 151 million (**Supplementary Table S3**). DNA raw reads are accessible from NCBI under BioProject number PRJNA575339. On average, 35% of the high-quality reads mapped to the *in silico* digested *Z. marina* genome, and 11% mapped uniquely (annotated reads-per-million in **Supplementary File S4**). In total, 144,420 sites were methylated (covered by at least two reads) across all transect shoots. Across all transect shoots, 84,640 methylated CG sites and 59,780 methylated CHG sites were detected, which represents 59% and 41% of all methylated sites, respectively (**Supplementary Table S5**). About 23% of all methylated sites fell in gene bodies (of which 41% in TEs), and 77% in intergenic regions (of which 42% in TEs) (**Figure 3**). In gene bodies, 67% of the methylated sites fell in CG regions, in intergenic regions only 56%.

Based on their multi-locus microsatellite genotype, 41 of 42 shoots sampled along the transect (except sample 27 in **Supplementary Table S1**) belonged to the same genet (*Clonality* section; **Supplementary Table S6**). Methylome variation between these 41 clonal ramets (*Methylation Variation between the Field Transect Shoots*) exceeded the methylome shift over the course of the heat-stress experiment (*Methylome Shift Under Experimental Conditions* section; **Figure 4**). Epigenetic distances between the 41 ramets of one genet and the single ramet of the other genet was not higher than among the 41 clonal ramets (*Methylation Variation between the Field Transect Shoots* section; **Figures 5A, B**, **Supplementary Table S5**). Epigenetic distances were generally lower in CHG than in CG sequence contexts, and lowest in TEs in gene regions (**Figures 5A, B**). Epigenetic distances were not significantly correlated with physical distances in any sequence

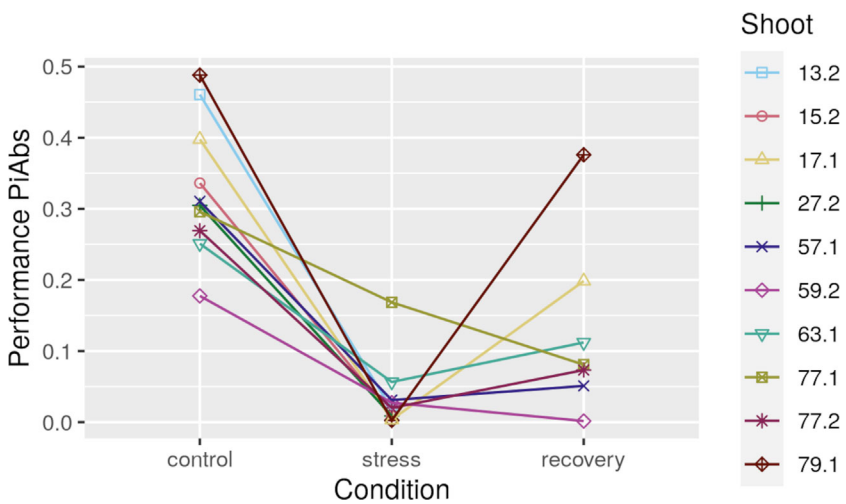

FIGURE 2 | Photosynthetic performance (PiAbs, absolute values) for all ten heat-stressed *Zostera marina* shoots at control, stress, and after recovery. Shoots 13.2, 15.2, and 27.2 did not recover from the stress.

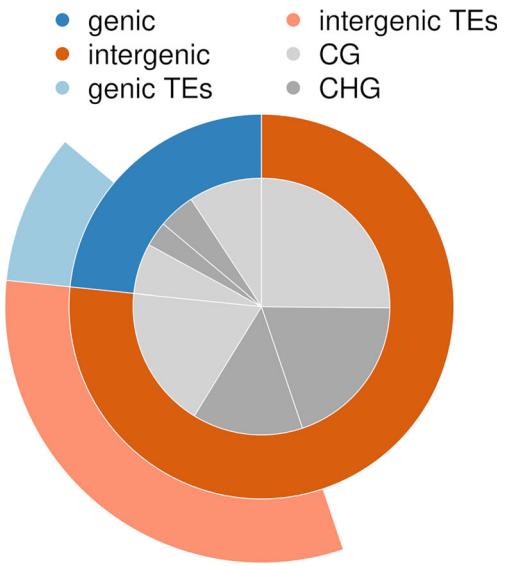

FIGURE 3 | Proportion of methylated sites detected in genes, intergenic regions, and transposable elements (TEs), separated by CG and CHG sequence contexts.

context (*Methylation Variation between the Field Transect Shoots* section; **Supplementary Table S7**).

Genetic Variation Among Heat-Stressed Shoots

Based on whole genome sequences of the 10 heat-stressed shoots, we identified 15,508 high-quality SNPs (*Whole Genome Sequencing, SNP Detection, and Genetic Distance in Experimental Samples* section; **Supplementary File S1**). That all 10 shoots shared the same heterozygous state in 14,429 (93%) of all SNPs, and shared the same multi-locus microsatellite genotype (*Clonality* section; **Supplementary Table S6**), suggests that they were clones having originated from one ancestral zygote (**Supplementary Figure S1**) (Supek et al., 2011).

Despite being clonal, the 10 shoots differed in 1,079 SNPs resulting from somatic mutations (*Whole Genome Sequencing, SNP Detection, and Genetic Distance in Experimental Samples* section). Based on these SNPs, Euclidean genetic distances ranged from 9 to 35 (frequency distribution in **Supplementary Figure S2**), and were not significantly correlated (*Whole Genome Sequencing, SNP Detection, and Genetic Distance in Experimental Samples* section) with physical distances between shoots (R=0.18, p=0.12).

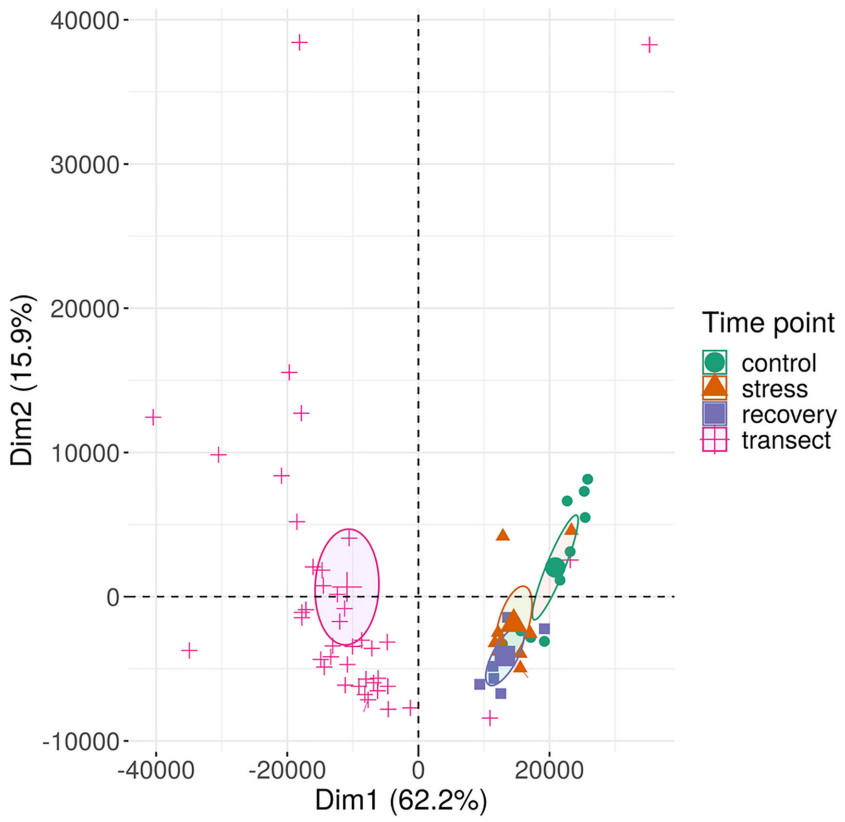

FIGURE 4 | Methylation variation of field transect samples and of experimental samples of *Zostera marina* along the first two principal components. The samples (small symbols) are plotted along the first two principal components (Dim) based on methylation profiles across all sequence contexts. Circles represent 95% confidence intervals around group means (large symbols). Bracketed numbers represent the percentage of explained variation.

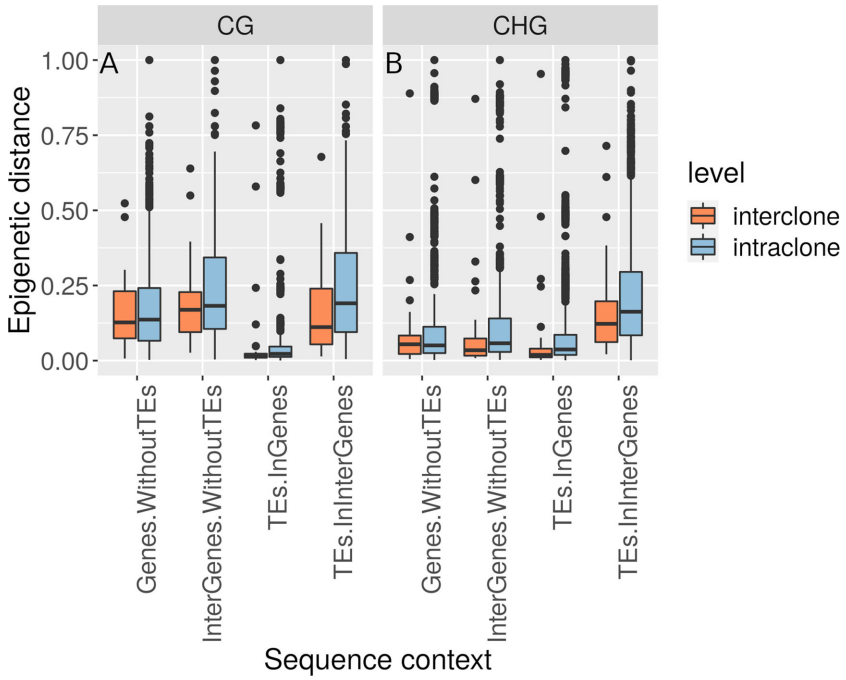

FIGURE 5 | Boxplot of relative epigenetic distances (distances divided by the maximum distance for each sequence context) showing the median, upper and lower quartiles (25th and 75th percentiles, box margins), minimum and maximum values falling within 1.5 X the interquartile range above and below the box (ends of the vertical lines), and all outlying points individually. Epigenetic distances in **(A)** CG and **(B)** CHG regions were comparable within ramets of the same genet (intraclone) and between ramets of different genets (interclone) of *Zostera marina* across all sequence contexts.

Link Between Epigenetic Distance and Photosynthetic Performance Changes Under Heat Stress

Photosynthetic performance (PiABS, *Photosynthetic Performance* section) declined in all 10 shoots under heat stress (**Figure 2**, **Supplementary Table S8**, raw data in **Supplementary Table S9**). Seven of the 10 shoots recovered from the heat stress, yet photosynthetic performances did not reach pre-stress levels. Photosynthetic performances under control and recovery conditions were positively correlated (adjusted $p<0.05$, $R=0.93$, **Figure 6**, **Supplementary Table S10**).

Genetic distances correlated moderately ($0.4<R<0.6$, adjusted $p<0.05$) with photosynthetic performance differences in recovered samples, and with epigenetic distances (*Correlation between Methylome Variation and Photosynthetic Performance* section) in some sequence contexts of stressed and recovered samples (**Figure 6**, **Supplementary Table S10**). Nevertheless, epigenetic distances correlated strongly ($R>0.65$, adjusted $p<0.05$) with photosynthetic performance differences even after controlling for genetic distance based on 1,079 SNPs: 1) epigenetic distances among control samples in CG gene body regions correlated with photosynthetic performance differences prior to stress, and after recovery (stress resilience, **Figure 6**, **Supplementary Table S10**). However, some of the samples, including one that performed well before the stress (sample 13.2 in **Figure 6**), did not recover from the heat stress (*Photosynthetic Performance* section). Epigenetic distances among recovered samples in CHG regions of intergenic TEs correlated with photosynthetic performance differences after recovery (**Figure 6**, **Supplementary Table S10**).

Methylome Changes in the Course of the Heat-Stress Experiment

Over the course of the heat-stress experiment, methylation patterns in all sequence contexts changed and did not return to but instead diverged further from control (pre-stress) patterns during the recovery period (*Methylome Shift Under Experimental Conditions* section; **Figure 7** for all sequence contexts combined, **Supplementary Figure S3** for the different sequence contexts, **Supplementary File S5** listing annotated reads-per-million for each sample). More sites became hyper- (increased in methylation) than hypo-methylated (decreased in methylation) in the course of the experiment (*Differential Methylation Analyses* section 2.11; **Figures 8A, D**). Methylation levels differed significantly at 437 sites between control and stress conditions (257 hyper- and 180 hypo-methylated), at 1788 sites between control and recovery conditions (1141 hyper-, and 647 hypo-methylated), and only at 39 sites between stress and recovery conditions (18 hyper-, and 21 hypo-methylated, **Supplementary Table S11**). After recovery, CG methylation had changed more strongly in gene body regions, and CHG methylation in intergenic regions (**Figures 8B, E**).

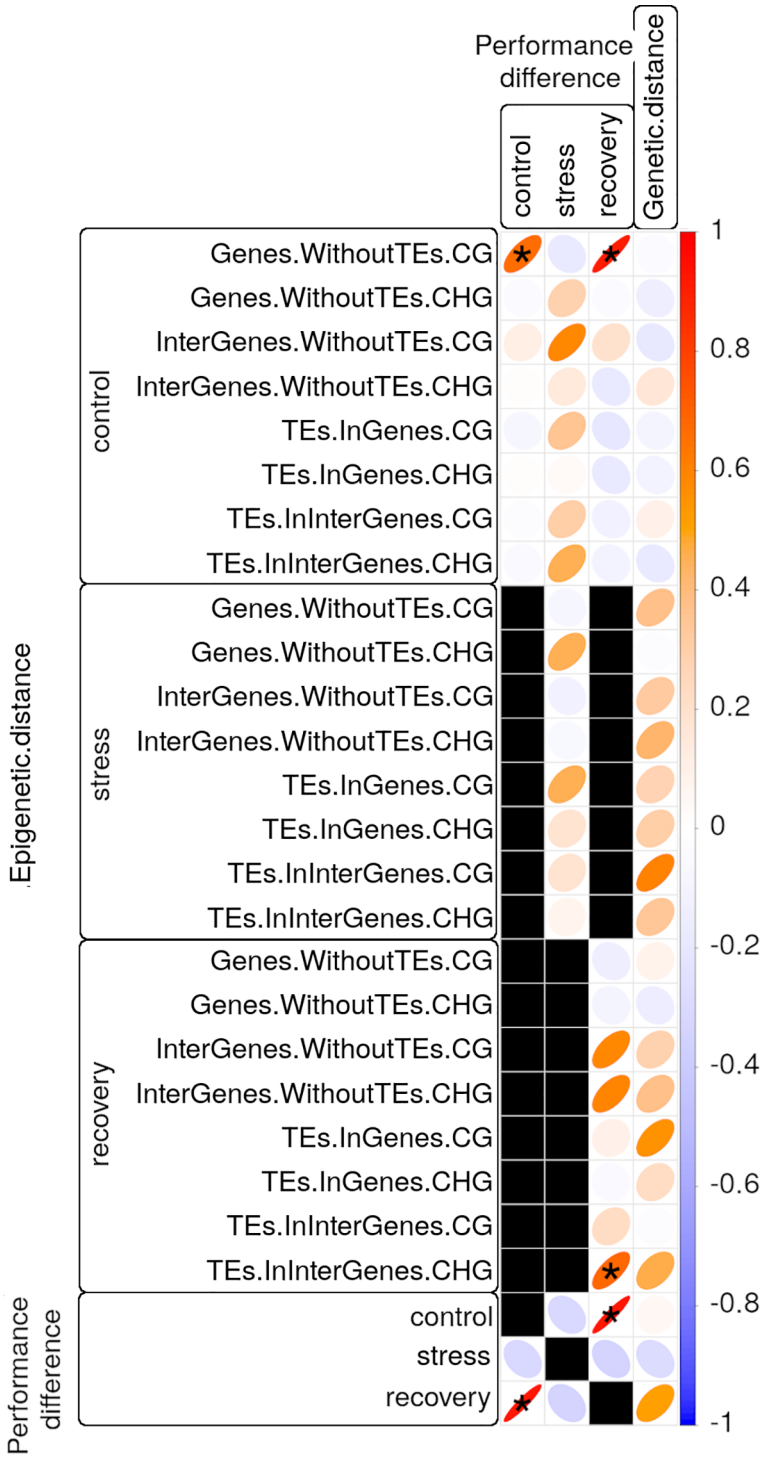

FIGURE 6 | Correlation matrix of photosynthetic performance (PiABS) differences (first three columns) or of genetic distance (last column) among clonal *Zostera marina* shoots with epigenetic distances (first 24 rows) or with photosynthetic performance differences at control, stress, or recovery conditions (last three rows). All correlations between photosynthetic performance differences and epigenetic distances were controlled for genetic distances. Black squares represent untested correlations that were considered biologically not meaningful. Pearson product-moment correlation coefficients R are encoded by the color gradient explained in the bar on the right end, and by the shape of the ellipses. Narrow ellipses represent stronger correlations as compared with wide ones. Asterisks highlight strong (R>0.65) and significant (adjusted p<0.05) correlations. CG and CHG: sequence contexts of the methylated cytosine; TE: Transposable element.

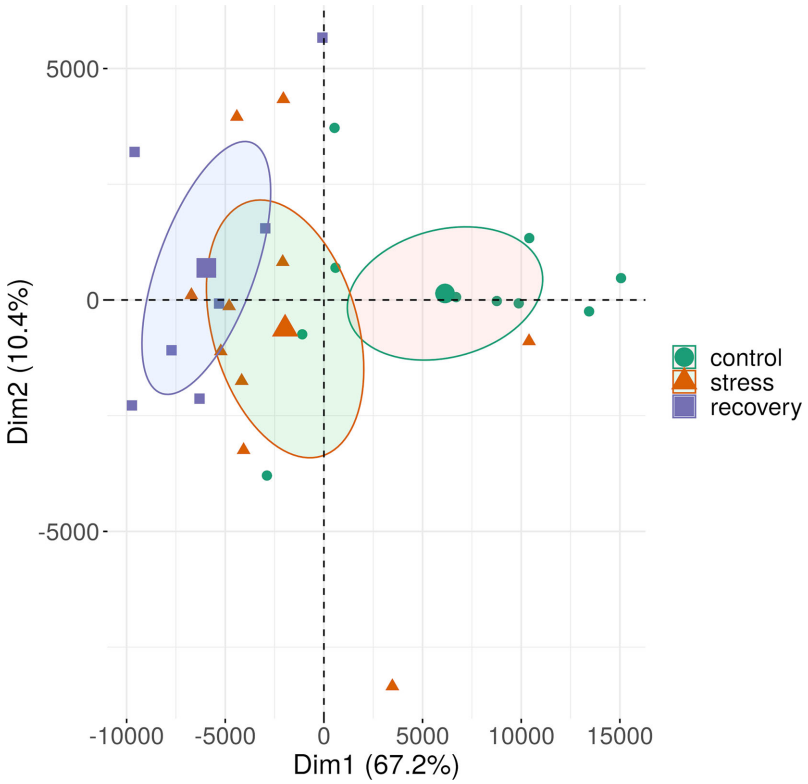

FIGURE 7 | Methylation patterns of all sequence contexts in shoots of a *Zostera marina* over the course of the stress experiment. See Supplementary Figure S3 for methylation shifts separated by sequence context. The samples (small symbols) are plotted along the first two principal components (Dim) based on methylation profiles across all sequence contexts. Circles represent 95% confidence intervals around group means (large symbols). Bracketed numbers represent the percentage of explained variation.

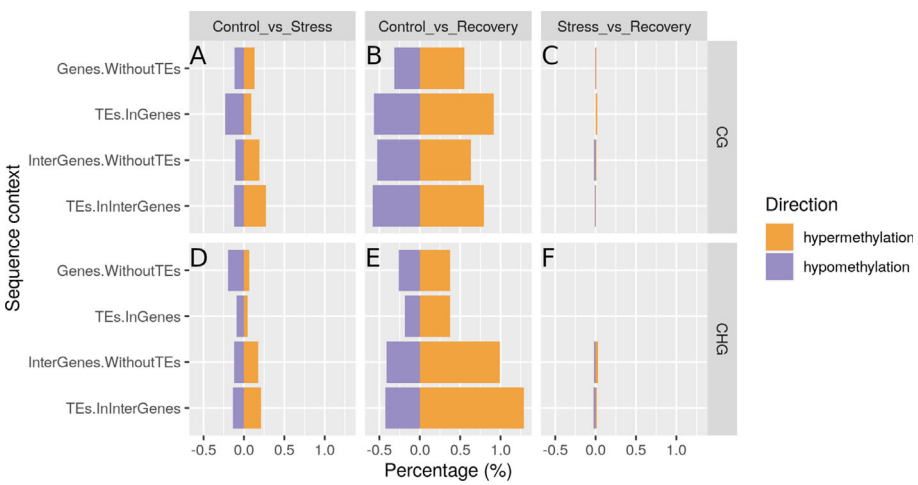

FIGURE 8 | Hyper- and hypo-methylated sites in CG **(A–C)** and CHG **(D–F)** regions in stressed vs. control samples **(A, D)**, recovered vs. control samples **(B, E)**, and in recovered vs. stressed samples **(C, F)**. Hyper-methylation refers to higher methylation, hypo-methylation to lower methylation in samples taken during stress as compared with samples taken before stress **(A, D)** in samples taken after recovery as compared with samples taken before stress **(B, E)**, and in samples taken after recovery as compared with samples taken during stress **(C, F)**. TE, transposable element.

After recovery, methylation increased (*Differential Methylation Analyses* section) in gene bodies with functions including DNA transcription and replication (GO:0006353, GO:0006261, GO:0006270); catabolism of misfolded proteins (GO:0006515), gamma-aminobutyric acid (GABA) (GO:0009450, GO:0009448), and neurotransmitter (GO:0042133, GO0042135, GO:0001505); as well as amino acid metabolism (GO:0009072, GO:0009073, GO:0009063) (**Supplementary Table S12**). DNA methylation had decreased in gene bodies with functions including transmembrane transport of cations, ions, and protons (GO:0006811, GO:0055085, GO:0006810, GO:0034220, GO:0006812, GO:0098655, GO:0098660, GO:0015672, GO:0099132, GO:0015991, GO:0098662, GO:0099131, GO:0015988, GO:1902600); transport of ammonium and phospholipids (GO:0072488, GO:0015696, GO:0015914, GO:0006869); localization of lipids and organelles (GO:0051179, GO:0051234, GO:0010876, GO:0051640); exocytosis (GO:0006887, GO:0006904); and secretion (GO:0032940) (**Supplementary Table S12**).

Differential Methylation Between Shoots of High and Low Photosynthetic Performance

Recovered shoots of high and low photosynthetic performance presented too similar methylation patterns to allow for detection of many regions showing significant differentiation in methylation levels (*Differential Methylation Analyses* section). Only four CHG sites in intergenic TEs were hyper-methylated in recovery samples of high photosynthetic performance (**Supplementary Table S13**). In contrast, 90 CG gene body sites were hyper-methylated (1 hypo-methylated) in control samples of high photosynthetic performance (**Supplementary Table S13**). Enriched biological processes in the 90 hyper-methylated CG sites included "light harvesting in photosystem I" (GO:0009768, GO:0009765) and "protein folding" (GO:0006457, GO:0042026, GO:0006515) (**Supplementary Table S14**).

DISCUSSION

Plant genets persisting >1,000 years challenge the positive correlation between genetic variation retained through recombination, and long-term survival. Our study shows for the first time that ramets of the same seagrass genet display DNA methylation variation that is independent from underlying genetic variation, and associated with phenotypic variation in the fitness-related trait of photosynthetic performance under experimental conditions. To what degree this association could have been affected by transplantation-induced stress responses in the methylome remains unknown, given the strong methylome shift under acclimation to laboratory conditions. The lack of untreated control samples in the heat stress experiment did not allow us to distinguish methylome shifts induced by acclimation from such induced by heat stress. Nevertheless, our findings support the hypothesis (Yu et al., 2020) that methylation variation, *via* variation in gene regulation, compensates potential costs of clonal reproduction (Dodd and Douhovnikoff, 2016), and contributes to the long-term survival of clonal seagrass meadows by increasing variation in ecologically relevant traits that cannot be simply explained by the underlying somatic genetic variation.

Methylome Variation of Functional Relevance

Methylome variation among ramets of the same genet resulted either from random epimutations or from microscale variations in the environment because its correlation with physical distance between shoots was weak. By sampling entire leafs composed of young and mature tissue, we aimed to standardize variation in tissue maturity among shoots, since tissue maturity can affect methylation patterns (Lynch et al., 1993; Lynch and Lande, 1998). None of the shoots had been reproductive at the time of sampling, so that variation in reproductive status is unlikely to explain the recorded methylome variation among shoots (Ruocco et al., 2019b).The change in depth of 3 meters and, thus, gradual changes in environmental factors along the sampled transect may not have been extreme enough to impose a result. The disagreement between methylome variation and transect position may also result from recent uprooting and re-settling of some shoots. This was suggested to explain disagreement between genetic similarity of clonal shoots and their transect position in another clonal meadow of *Z. marina* (Marín-Guirao et al., 2019), and would further explain the absence of correlation between genetic and physical distance in our heat-stressed ramets. In such case, the re-settled shoots would display a methylome shaped by distant environmental conditions.

Epigenetic distance was comparable at the intra- and inter-clonal level at all sequence contexts (**Figures 5A, B**). It is important to note that the inter-clonal comparisons relied on 41 shoots of one genet versus a single shoot of another genet. That we could not identify more genets in our samples supports the finding that this meadow is dominated by a single genotype (Reusch et al., 1999). It is unlikely that this genet could belong to the same genet as the other 41 shoots with a somatic mutation explaining its different microsatellite genotype at locus ZosmarCT19 (heterozygous with a 147bp fragment, and not homozygous for the 150bp fragment as the other shoots). This microsatellite locus had been developed as part of a set of seven less variable loci to discriminate genets, in contrast to other hypervariable microsatellite loci that allow to identify somatic mutations (Yu et al., 2020). However, the inter-clonal comparisons have to be taken with caution as it remains unknown how different the methylation profile of one or more additional genets from the same meadow could be.

The seagrass methylome is flexible and responds directly to environmental change, given its strong change from field transect samples to acclimated control samples within two weeks (**Figure 4**). Three factors may explain why the shift in methylation patterns appears stronger in response to acclimation to laboratory conditions than in response to heat stress (**Figure 4**). First, several environmental factors had likely changed from field to lab conditions, including light intensity, and nutrient composition of the water. The wider variation in methylation profiles among field samples than among lab samples may mirror microscale variations in the environment and appears

to have converged to more similar methylation patterns under controlled and uniform laboratory conditions. Although the applied heat stress (27°C) was strong, only temperature had changed over the course of the experiment, potentially requiring less changes in methylation state as compared with lab acclimation. Second, field and acclimation epigenotypes differed already before the acclimation. Thus, the methylation changes from field to lab conditions can not only be ascribed to changes in environmental conditions but also to intrinsic differences between the epigenotypes. This applies to a lower extent to the experimental samples, because the same epigenotypes had been sampled throughout the experiment. Third, since we had not run technical replicates on the three sequencing flow-cells, part of the methylome shift under acclimation may have been due to flow-cell batch effects. All experimental samples had been run on a different flow cell than the field transect samples, which had been run on two other flow-cells (**Supplementary Table S3**). Although the 95% confidence intervals of methylation patterns of the field transect samples from different flow-cells did not overlap, the patterns were still more similar between all field samples than between field and experimental samples (**Supplementary Figure S4**). This does not alter the conclusion that there has been a shift in methylation patterns from field to lab conditions, because it is supported by two independent sequencing runs. However, this shift may be weaker than shown in **Figure 4**, due to sequencing batch effects.

Methylome variation in CG gene regions predicted photosynthetic performance (**Figure 6**) and, thus, is likely functionally relevant for this fitness-relevant trait. Methylation variation did not predict photosynthetic performance under acute heat-stress, given that the photosynthetic performance of the different epigenotypes converged to low and highly similar photosynthetic performance values under stress (**Figure 2**). Although difficult to prove (Reusch and Boström, 2011), the correlations between methylome and photosynthetic performance differences before and after the heat-stress may be causal, given that control samples of high photosynthetic performance showed hyper-methylated CG sites in gene bodies with relevant functions: "light harvesting in PSI" (GO:0009768), "protein folding" (GO:0006457), "protein refolding" (GO:0042026), and "misfolded or incompletely synthesized protein catabolic process" (GO:0006515) (**Supplementary Table S14**). While increased light harvesting can enhance photosynthetic performance, the functions related to protein folding can prime against heat stress when increased methylation is associated with expression of the underlying genes and, thus, with accumulation of protective molecules like heat-shock proteins. Heat shock proteins are involved in repair and prevention of heat-induced protein damage (Crisp et al., 2016), and that can facilitate a fast stress response. Indeed, while genes are generally repressed by methylated promoter regions (Feder and Hofmann, 2002), genes are often activated by CG gene body methylation (Lisch, 2009) that prevents aberrant expression from intragenic promoters (Zhang et al., 2006; Schmitz et al., 2013; Yang et al., 2014; Dubin et al., 2015; Bewick and Schmitz, 2017; Niederhuth and Schmitz, 2017). Accordingly, in the seagrass *Posidonia oceanica* hyper-methylation was associated with transcriptionally active leaf segments (Takuno and Gaut, 2012; Neri et al., 2017), and appears to be a common response to stress (Greco et al., 2012, 2013; Ruocco et al., 2019a). Thus, *via* association with gene expression, the observed methylome variation in control samples may putatively create ecologically relevant phenotype variation that predicts photosynthetic performance. However, it is important to note that the methylome variation in control samples does not reflect the methylome variation present in the field but may have already been shaped by transplantation-related stress, given the strong methylome shifts under acclimation to laboratory conditions. It is also important to note that the methylome of a high-performing shoot does not always protect from stress, given that one of the highest-performing samples (shoot 13.2 in **Figure 2**) did not recover from the heat stress.

Only four CHG sites in intergenic TEs were differentially methylated between the recovered samples of high and low photosynthetic performance (**Supplementary Table S13**). This appears to contradict the correlation between methylation patterns and photosynthetic performance. However, especially the samples of the high-performance group (79.1 and 17.1) showed a big difference in photosynthetic performance values between themselves (**Figure 2**) and, thus, were likely also too different in methylation patterns to allow for the detection of sites being differently methylated as compared with the samples of the low-performance group.

Although our study was based on clonal ramets that presented identical seven-locus microsatellite genotypes, the 10 heat-stressed ramets varied genetically at 1,079 SNPs generated by somatic mutations. Epigenetic differentiation between populations can be tightly linked to genetic differentiation (Ruocco et al., 2019b). Accordingly, trans-acting SNPs appeared to explain the correlation between gene body methylation and latitude in *Arabidopsis* (Herrera and Bazaga, 2010; Gáspár et al., 2019). In contrast, part of the correlation between DNA methylation with ecological factors in landscape epigenomic studies on non-model plant species could not be predicted from the observed underlying patterns of genetic relatedness (Dubin et al., 2015). Genetic variation could also not explain the association between epigenetic variation and phenotypic variation (leaf, petiole and functional traits) in natural populations of the perennial herb *Helleborus foetidus* (Schulz et al., 2014; Foust et al., 2016; Gugger et al., 2016), and in clonal populations of the introduced clonal herb *Hydrocotyle vulgaris* (Medrano et al., 2014). In agreement, the correlation between methylome variation and photosynthetic performance in our study could not be predicted from the underlying genetic variation. This suggests that differences in performance among ramets in clonal plants can be explained not exclusively by somatic mutations (Wang et al., 2020) but at least partly by independent differences in their methylome. Independent from genetic variation was also the methylome shift in the experimental samples that provides potential for temporally stable phenotypic change at time scales unattainable by somatic mutations. Particularly across mitotically grown generations, epigenetic patterns can be expected to be more faithfully

inherited than across sexual generations, because clonal growth circumvents epigenetic reprogramming during meiosis and embryogenesis (Santelices et al., 2018; Simberloff and Leppanen, 2019; Yu et al., 2020). Thus, our results provide a first indication that DNA methylation variation provides a layer of ecologically relevant phenotypic variation that is independent from genetic variation in clonal seagrass meadows.

Methylome Shift Over the Course of the Heat-Stress Experiment

Methylation profiles changed over the course of the heat stress experiment with a clear shift from control to stress and recovery profiles (**Figure 7**). Interestingly, methylation profiles were highly similar between the stress and recovery phase as shown by the overlap of the 95%-confidence intervals around the group means (**Figure 7**, **Supplementary Figure S3**), and the low number of differentially expressed sites between stress and recovery samples (**Figures 8C, F**, **Supplementary Table S11**). Nevertheless, recovery profiles showed an apparently stronger divergence from the control profiles (**Figures 8B, E**) than the stress profiles did (**Figures 8A, D**). This shift in methylation profiles may be due to different processes that likely acted in parallel.

A first explanation is that part of the recorded methylome shift was likely induced by heat stress, given that the recovered shoots showed hyper-methylation and, thus, potentially constitutive upregulation (Zhang et al., 2006; Schmitz et al., 2013; Yang et al., 2014; Dubin et al., 2015; Niederhuth and Schmitz, 2017) of genes involved in the functions of catabolism of misfolded proteins (GO:0006515) (**Supplementary Table S12**). This suggests increased investment in the breakdown of heat-denatured proteins, a common response to heat-stress (Feder and Hofmann, 2002). This function was also hyper-methylated in control samples of highest photosynthetic performance (**Supplementary Table S14**), that also recovered better from the stress (although some samples had died, **Figure 2**). Heat-responsive DNA methylation changes in plants appear not to show a consistent trend across different species, and little is yet known about their functional role (Liu et al., 2015). In *Brassica rapa*, heat-responsive DNA methylation was shown to be associated with differential expression of genes involved in RNA metabolic processing, and in heat stress signal transduction (Liu et al., 2018). Stress signal transduction may also have been affected by the experimental methylation changes in *Z. marina* as enriched biological processes in recovered shoots included the regulation of transmembrane transport of ions and protons (GO:0006811, GO: 0055085, GO:0006810, GO:0034220, GO:0006812, GO:0098655, GO:0098660, GO:0015672, GO:0099132, GO:0015991, GO:0098662, GO:0099131, GO:0015988, GO:1902600), and neurotransmitter levels (GO:0042133, GO0042135, GO:0001505) (**Supplementary Table S12**). Experimental removal of DNA methylation, e.g. using zebularine or 5-Azacytidine (Griffin et al., 2016), or the targeted change of methylation patterns, e.g. *via* CRISPR (Xu et al., 2016), will ultimately allow us to identify the relationship between methylation changes under stress and adaptive phenotypic changes.

However, an alternative explanation for the shift in methylome profiles over the course of the experiment is the continued acclimation of seagrass shoots to laboratory conditions. In order to correlate methylome differences with photosynthetic performance differences between the epigenotypes, we had to choose a longitudinal sampling design in which we followed single shoots/epigenotypes over the course of the experiment. To reduce the effect of acclimation on methylome profiles, we allowed for a 2-week acclimation period before the start of the experiment, but can not rule out that the methylome shift from field to lab conditions (**Figure 4**) was completed after this time period. Moreover, although we had planted the shoots in soil from the sampling site and observed continuous growth of the shoots, we cannot exclude the possibility that the dilution of natural seawater with freshwater may have resulted in lower nutrient contents as compared with field conditions, which may have induced a stress factor in addition to the applied heat. Most likely, heat-stress, acclimation, and nutrient depletion have contributed in parallel to the recorded methylome shift and can not be disentangled in this study. Thus, future studies need to run a cross-sectional sampling design with unstressed samples as control treatment in parallel to a longitudinal sampling design that traces methylome changes in single epigenotypes, in order to distinguish methylome changes induced by transplantation to laboratory conditions from such induced exclusively by the applied heat-stress.

If the recorded methylation shift over the experiment has been primarily a response to the applied heat-stress, the similarity in methylation profiles between stress and recovery phases can be interpreted as the formation of an epigenetic stress memory (Crisp et al., 2016). Continued divergence of methylation profiles from stress to recovery conditions (**Figures 8B, D**) agrees with increasing divergence of gene transcription profiles from heat-stress to recovery conditions in a Danish *Z. marina* population (Franssen et al., 2011). We speculate that gene expression changes, and other molecular mechanisms involved in the heat-stress response, could have triggered additional methylation changes after the stress was removed (e.g. Secco et al., 2015). A stress-memory lasting longer than 5 weeks, would be long enough to potentially heat-harden the same generation of previously exposed shoots. In agriculture, hardening/priming of seeds is a long-standing practice to enhance crop resistance to environmental challenges, including hot, cold, dry, or saline conditions, or pathogen infections (Ibrahim, 2016; Wojtyla et al., 2016; Pawar and Laware, 2018). Priming refers to the plants' ability to acquire a stress memory that enhances performance under second stress exposure by responding faster, stronger, or in response to a lower threshold as compared with naïve plants (Balmer et al., 2015; Lämke and Bäurle, 2017). Molecular mechanisms involved in forming a stress memory include stalled RNA polymerase II, storage of chemical signaling factors, accumulation and phosphorylation of transcription factors, and epigenetic mechanisms such as microRNAs, histone modifications, and DNA methylation (Iwasaki and Paszkowski, 2014; Crisp et al., 2016; Hilker et al., 2016; Gallusci et al., 2017; Lämke and Bäurle, 2017). Heat-priming has only very recently been described in seagrasses (Nguyen et al., 2020). *Zostera muelleri* and *Posidonia australis* both performed better under a second heat-wave, in terms of photosynthetic capacity, leaf growth, and chlorophyll *a* content, when they had been previously exposed to a first heat-wave as compared with naïve

controls (Nguyen et al., 2020). This could explain why no mortality was reported for the seagrass *Posidoinia oceanica* after intense and long-lasting heat-waves in 2012, 2015, and 2017 (Darmaraki et al., 2019), although it had suffered high mortality rates after the 2006 heatwave ((Marbà and Duarte, 2010), as discussed in Nguyen et al. (2020). However, whether the experimental shift in methylation patterns in *Z. marina* may be involved in heat-priming can only be answered *via* exposure to a second heat-wave.

Conclusions and Perspectives

Our study suggests that DNA methylation is functionally relevant for photosynthetic performance, independent from underlying somatic mutations. In seagrass meadows composed of several genotypes, stress resilience, growth, and associated invertebrate species diversity is enhanced by genotypic variation (Hughes and Stachowicz, 2004; Reusch et al., 2005; Ehlers et al., 2008). In clonal meadows, epigenetic variation may play a similar role in the potential to secure function and resilience not only of *Z. marina* plants, but also of the entire associated ecosystem.

Due to anthropogenic stressors, nearly one-third of global seagrass area has disappeared over the last 100 years, and the rate of loss accelerated from ca. 1% yr^{-1} before 1940 to 7% yr^{-1} since 1990 (Waycott et al., 2009). At the same time, rising temperatures open up new thermally suitable habitat in the Arctic (Krause-Jensen and Duarte, 2014). How fast and far warm-temperate and subarctic range edges will move polewards depends on the ability of seagrass to rapidly acclimate and adapt to rising temperatures and other environmental changes (Duarte et al., 2018). Thus, future studies are needed to assess the adaptive value and transgenerational stability of the epigenetic stress response, and to compare the ability to build up epigenetic variation between seagrass meadows composed of a single or multiple clones, as well as between range center *versus* edge populations.

The functional role of methylation variation in plant genets is not only of fundamental interest but also of applied interest for management programs of clonal organisms designed to assess evolutionary potential and population stability, and to minimize the loss of biodiversity. Our results can be relevant for restoration of seagrass ecosystems that largely depends on the success of replanted shoots to overcome natural variability/stress (Suykerbuyk et al., 2016; van Katwijk et al., 2016). Given that 40% of all plant species can reproduce clonally (Tiffney and Niklas, 1985), our findings are further important to other fields, such as invasion biology and crop breeding strategies (Bilichak and Kovalchuk, 2016) of clonal plants.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

GH (project leader) and AJ were planning the project and designing the experiments. AJ, CB, IS, and GH collected the shoots. AJ analyzed the data and wrote the manuscript. AJ, MK, AD, JC, and IS performed the DNA extraction, library preparation, and sequencing. SA-H, JO, and YP were involved in data interpretation. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.571646/full#supplementary-material

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Species-Specific Trait Responses of Three Tropical Seagrasses to Multiple Stressors: The Case of Increasing Temperature and Nutrient Enrichment

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Seagrass meadows are declining globally. The decrease of seagrass area is influenced by the simultaneous occurrence of many factors at the local and global scale, including nutrient enrichment and climate change. This study aims to find out how increasing temperature and nutrient enrichment affect the morphological, biochemical and physiological responses of three coexisting tropical species, *Thalassia hemprichii*, *Cymodocea serrulata* and *Halophila stipulacea*. To achieve these aims, a 1-month experiment under laboratory conditions combining two temperature (maximum ambient temperature and current average temperature) and two nutrient (high and low N and P concentrations) treatments was conducted. The results showed that the seagrasses were differentially affected by all treatments depending on their life-history strategies. Under higher temperature treatments, *C. serrulata* showed photo-acclimation strategies, while *T. hemprichii* showed decreased photo-physiological performance. In contrast, *T. hemprichii* was resistant to nutrient over-enrichment, showing enhanced nutrient content and physiological changes, but *C. serrulata* suffered BG nutrient loss. The limited response of *H. stipulacea* to nutrient enrichment or high temperature suggests that this seagrass is a tolerant species that may have a dormancy state with lower photosynthetic performance and smaller-size individuals. Interaction between both factors was limited and generally showed antagonistic effects only on morphological and biochemical traits, but not on physiological traits. These results highlight the different effects and strategies co-inhabiting seagrasses have in response to environmental changes, showing winners and losers of a climate change scenario that may eventually cause biodiversity loss. Trait responses to these stressors could potentially make the seagrasses weaker to cope with following events, due to BG biomass or nutrient loss. This is of importance as biodiversity loss in tropical seagrass ecosystems could change the overall effectiveness of ecosystem functions and services provided by the seagrass meadows.

Keywords: ***Cymodocea serrulata*, *Thalassia hemprichii*, *Halophila stipulacea*, morphology, storage, photophysiology, nutrient allocation, interactive effect**

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INTRODUCTION

Seagrass meadows are valued for the ecosystem services they provide that have been recorded in an increasing number of studies (Costanza et al., 1997; Kirsch et al., 2002; Romero et al., 2006; Fourqurean et al., 2012; Ondiviela et al., 2014; Dewsbury et al., 2016; Nordlund et al., 2016). Despite their global significance and them being threatened by multiple anthropogenic stressors that affect their biodiversity and functioning (Lu et al., 2018) the attention paid to seagrass meadows is much lower than other coastal ecosystems, such as coral reefs (Unsworth et al., 2019).

The most prominent stressor of seagrasses generated locally is cultural eutrophication caused by increased loading of nutrients from human activities (Beiras, 2018). During the last decades, losses in seagrass meadows have been documented worldwide, especially in quiet, and poorly flushed estuaries where nutrient loads are intense and frequent (Burkholder et al., 2007). Contrary to temperate ecosystems, in the oligotrophic tropical environments, seagrass productivity is mainly limited by nutrients and not by light irradiance (Short, 1987); therefore, nutrient inputs could result in drastic changes in diversity loss (Kamermans et al., 2002). As seagrasses are the only submerged marine angiosperms, they can access both nutrients from water column and pore water. Thus, below-ground (BG) tissues play a key role in taking up different nutrient sources to meet *in situ* demands and translocating them along the plant (Viana et al., 2019a). This is especially important in the tropics as nitrogen (N) is constantly available in the pore water at higher concentrations than in the water column, and leaf turnover is constant throughout the year, whereas BG tissues have lower turnover rates (Duarte, 1991). Seagrasses are able to take advantage of nutrient pulses by increasing the enzymatic activity in response to uptake and assimilation and storing the incorporated nutrients (Viana et al., 2019a). In this way, nutrient additions can result in higher growth rates, primary productivity or nutrient content (Duarte, 1990; Agawin et al., 1996; Terrados et al., 1999; Ferdie and Fourqurean, 2004). However, if eutrophication persists, changes in biotic or abiotic interactions might occur, including algal blooms that increase nutrient competition and light deprivation, alterations in top-down regulation because of changes in leaf palatability, or increasing organic matter in the sediments that might create anoxic environments for benthic organisms (Burkholder et al., 2007). All these changes may ultimately affect species fitness and survival.

Although nutrient over-enrichment has been one of the main drivers of seagrass mortality worldwide, other abiotic factors, including temperature, play an important role in species distribution and survival. Temperature is one of the main drivers of biochemical reactions, significantly affecting growth, photosynthesis, sexual reproduction and survival (Durako and Moffler, 1985; Bulthuis, 1987; Lee et al., 2007; Xu et al., 2016; Zayas-Santiago et al., 2020). Temperature also affects seagrass nutrient content, as lower carbon (C) and N concentrations and higher C:N ratios have been observed at increasing temperatures (Kaldy, 2014; Mvungi and Pillay, 2019; Ontoria et al., 2019b). While moderate elevations in temperature might be positive for seagrass performance (Artika et al., 2020), the individual fitness is lost once the thermal optimum range is exceeded often leading to increasing mortality rates (Collier and Waycott, 2014). Tropical seagrasses usually grow at their upper optimal temperature limit, therefore changes in mean seawater temperatures might be critical (Koch et al., 2013). Changes in seagrass population and community structures have already been observed in areas affected by climate change (Jordà et al., 2012; Kendrick et al., 2019; Beca-Carretero et al., accepted) and will become more relevant under future climate change. Therefore, the knowledge accumulated on the effects of these individual stressors through field and laboratory experiments on temperate and tropical seagrasses is very extensive (see as example Bulthuis, 1987; Burkholder et al., 1994; Campbell et al., 2006; Winters et al., 2011; Collier and Waycott, 2014; Li et al., 2019).

In coastal systems, stressors rarely occur alone in the environment and, when acting together, their effects can be synergistic, additive or antagonistic (Todgham and Stillman, 2013; Gunderson et al., 2016; Stockbridge et al., 2020), although synergistic effects are most likely to occur when the stress events happen simultaneously or in quick succession (Gunderson et al., 2016). Accordingly, local anthropogenic impacts of human development, such as eutrophication, can combine with indirect consequences of climate change, including sea surface temperature or CO_2 enrichment, causing even more dramatic consequences of future scenarios than initially predicted by single-factor experiments. As a result, the rates of change in seagrass ecosystems are faster than those experienced in their evolutionary history and may occur too fast to allow seagrasses to adapt (Orth et al., 2006; Waycott et al., 2009). Therefore, the interaction between stressors is now viewed as a critical issue, and it is suggested that single-factor experiments are not adequate for assessing the effects of several disturbances on coastal marine ecosystems (Wernberg et al., 2012; Todgham and Stillman, 2013; Ontoria et al., 2019b). In the last years, an increasing number of papers aiming to understand cumulative impacts of stressors have exponentially increased (Gunderson et al., 2016; Adams et al., 2020; Stockbridge et al., 2020), and more empirical data on the effects of the interaction of increasing temperature and nutrient over-enrichment at an individual level has been obtained (Touchette and Burkholder, 2002; Bintz et al., 2003; Touchette et al., 2003; Burnell et al., 2013; Kaldy, 2014; Kaldy et al., 2017; Egea et al., 2018; Moreno-Marín et al., 2018; Mvungi and Pillay, 2019; Ontoria et al., 2019b). Nevertheless, responses depend on their local adaptation and life history traits (Tuya et al., 2019; Anton et al., 2020) are species-specific, and to our knowledge, there is very limited information about the combined effects of these two stressors in any tropical seagrasses species (Artika et al., 2020).

The study of the response of seagrass individual traits, namely biochemical, morphological, and physiological traits, serve as early indicators of environmental change (Roca et al., 2016) before population level responses, such as changes in shoot density, biomass and species composition or biodiversity loss, are detected. This approach has been used in a wide number of studies to detect rapid responses (within weeks) to different stressors (Lee et al., 2007; Roca et al., 2016; Bertelli and Unsworth, 2018, and previously cited references). One important

limitation of trait-based responses is that different stressors could cause the same effect, for instance, lower rhizome carbohydrate content is both observed after nutrient over-enrichment or reduced light exposures (Roca et al., 2016). More recent studies that simultaneously assess different plant individual traits have highlighted the importance of testing the responses at different levels of organization in the plants (Bertelli and Unsworth, 2018; Mvungi and Pillay, 2019). Moreover, a deeper understanding of the species-specific responses to stressors and their interaction is important as different combinations of seagrass traits may sustain different ecological functions that upscale to the ecosystem level (Barbier et al., 2011). Therefore, even though changes in seagrass traits could be seen as positive at an individual plant level (i.e., increasing photosynthetic rate) they could potentially change their related functions, negatively affecting the sustained ecosystem services (Burnell et al., 2013; Jiménez-Ramos et al., 2017; Soissons et al., 2018).

Tropical areas are seagrass biodiversity hotspots, gathering most of the 60 seagrass species that exist worldwide (Short et al., 2007). *Thalassia hemprichii* and *Cymodocea serrulata* are widely distributed in the Indo-Pacific bioregion. These two species have large blades and slow shoot turnover, especially *T. hemprichii* (Duarte, 1991). They both form persistent mixed or monospecific meadows that sustain food webs, including commercially important species (de la Torre-Castro et al., 2014). *Halophila stipulacea* is a tolerant species native to the Indo-Pacific bioregion that has colonized both the Mediterranean and Caribbean Seas (Winters et al., 2020). It has a smaller size than most seagrasses, so it frequently grows in sand patches or in the edges of bigger seagrass meadows, but it can also grow mixed with other macrophytes or form large monospecific meadows (Boudouresque et al., 2009; Sghaier et al., 2011). It presents faster shoot turnover and growth than the two other species (Duarte, 1991). All three species have been observed to tolerate different trophic conditions (Van Tussenbroek et al., 2016; Mwaura et al., 2017; Thomsen et al., 2020; Teichberg et al., *in preparation*) and adverse maximum temperatures (Campbell et al., 2006; Georgiou et al., 2016; Pedersen et al., 2016; Collier et al., 2017; George et al., 2018; Anton et al., 2020; Nguyen et al., 2020; Winters et al., 2020; Beca-Carretero et al., accepted). Furthermore, *T. hemprichii, C. serrulata* and *H. stipulacea* show differences in their life-history traits representing permanent, opportunistic and colonizing strategies, therefore different resistance and responses to stressors might be expected (O'Brien et al., 2018).

This study aims to find out how increasing temperature, nutrient over-enrichment, and the combination of both factors affect the morphological, biochemical and physiological responses of three common tropical Indo-Pacific seagrasses. To achieve this aim, combined temperature and nutrient enrichment laboratory experiments were conducted, and the responses in trait values of the three selected species were measured after 1 month under four different treatment combinations. We hypothesized that the combination of both factors will cause interactive (synergistic or antagonistic) responses in the three species, and these responses will be species-specific according to their life-history traits. The results of this study will provide important information on how the combined effects of climate change and nutrient enrichment will shape tropical seagrass meadows and their responses.

MATERIALS AND METHODS

Collection and Maintenance of Seagrasses

Thalassia hemprichii, *C. serrulata* and *H. stipulacea* were collected in the dry season 2016 from different areas of a seagrass meadow located in the western coast of Zanzibar, Tanzania (6°7′43″S, 39°10′47″E). The selected meadows are within a shallow area (0.5–4 m depth) situated in the East coast of Changuu Island, located approximately 5 km northwest of Stone Town. This island is an uninhabited coral rock outcrop, although is a touristic spot due to its turtle zoo and snorkeling trips to the fringing reef surrounding the seagrass meadows. Low nutrient concentrations were observed in both the water column (NO_3^- + NO_2^-: 0.13 ± 0.01 μM, NH_4^+: 0.67 ± 0.05 μM, PO_4^{3-}: 0.42 ± 0.07 μM), and in pore water at 5 cm below the sediment surface (NO_3^- + NO_2^-: 0.42 ± 0.07 μM, NH_4^+: 1.36 ± 0.37 μM, PO_4^{3-}: 0.74 ± 0.11 μM). Therefore, this area is relatively pristine with the highest relative cover of seagrasses and the highest water quality within the sites included in our study in Zanzibar Archipelago (Teichberg et al., *in preparation*). Temperature in the seagrass meadows varied between 26.4 and 28.4°C with mean values of 27.30 ± 0.08°C during daytime (Teichberg et al., *in preparation*). Rhizomes with approximately 5 to 6 shoots were collected for each species from different areas around the meadow to avoid collecting shoots from the same individual plant. Seagrasses were packed in paper tissues dampened with seawater, placed inside plastic bags and transported within 48 h to the Marine Experimental facilities (MAREE) at the Leibniz Centre for Tropical Marine Research (ZMT) in Bremen (Germany). Once at the MAREE, seagrasses were replanted in polypropylene trays filled with marine carbonate substrate of at least 10 cm depth. Acclimation took place in 300-l aquaria with a recirculation system mimicking as best as possible the original natural conditions of the seagrass plants. The collected seagrasses co-inhabited the aquaria with fish, hermit crabs, sponges and natural rocks providing nutrient recycling to low nutrient (NO_3^-: 2.8 ± 1.5 μM, NH_4^+: < 0.3 μM, PO_4^{3-}: < 0.002 μM) artificial seawater (ASW), and, therefore, avoiding any extra nutrient addition. The fluorescent lights (200 ± 30 μmol photons m^{-2} s^{-1}) were regulated to a photoperiod of 12:12 h, and temperature (26 ± 1°C) and salinity (35) acclimation conditions were similar to the average values observed in native meadows, providing the seagrasses time to recuperate and acclimate to the aquaria, which based on our experience, is approximately 3 months.

Experimental Design and Setup

We conducted an experiment with a nested split-plot design (Schielzeth and Nakagawa, 2013) to study the effects of nutrient over-enrichment, elevated temperature and their interaction on the performance of adult individuals of the three tropical seagrass species *T. hemprichii, C. serrulata* and *H. stipulacea*.

The experiment was carried out in the same experimental setup as Artika et al. (2020). We applied low and high levels of temperature, 26°C (LT) and 31°C (HT), respectively, and low and high levels of nutrients by adding 2 μM NH_4NO_3 + 0.1 μM KH_2PO_4 (LN) and 20 μM NH_4NO_3 + 2 μM KH_2PO_4 (HN), respectively. This resulted in 4 experimental treatments: low temperature and low nutrient concentrations (LT + LN) considered as the control, low temperature and high nutrient concentrations (LT + HN), high temperature and low nutrient concentrations (HT + LN), and high nutrient concentration and high temperature (HT + HN). Experimental temperature treatments were selected based on lower and higher average temperatures in the area. Nutrient concentrations were selected on nutrient concentrations in impacted seagrass meadows in the area, as well as in the sewage effluent in Stone Town (Zanzibar) (Teichberg et al., *in preparation*) and the nutrient concentrations found in Changuu Island, the relatively pristine site were seagrasses were collected.

The experiment was conducted under laboratory conditions in an indoor system in the MAREE (ZMT, Bremen) with a total of 24 glass aquaria (29 × 13 × 30 cm dimensions) of 10 l volume. Each aquarium was considered as a replicate, and each of the 4 treatments had 6 replicate aquaria (see experimental design in Artika et al., 2020).

The experimental temperatures were obtained by placing aquaria in larger (250 l) experimental tanks (ETs) that acted as water baths maintaining a constant experimental water temperature. Six aquaria were placed in 4 different ETs with nutrient treatments nested within the 4 ETs set at the two temperatures and with no interactions among aquaria. Water bath temperature was controlled in each ET by heaters (EHEIM, Germany) connected to an individual electronic system that was continuously regulating the temperature (± 0.2°C) by digital controllers and individual temperature probes. Air pumps were placed in each ET to ensure water movement of the water bath. The light (200 ± 20 μmol photons m^{-2} s^{-1}) was provided by 2 LED lamps (Hydra Fifty-two HD, AquaIllumination®, Iowa) at the top of each ET and placed at the same height. A photoperiod of 12:12 h light:dark was set with sunrise and sunset simulation. Transparent PVC lids were placed on each ET to lower water evaporation.

The experimental setup was a flow through system that took water from two water reservoirs (∼115 l each) with either high or low nutrient ASW solutions. To achieve the experimental nutrient concentrations, previously dissolved stock solutions of NH_4NO_3 and KH_2PO_4 (Merck, Germany) were added to each water reservoir. Once in the water reservoir, the solution was gently mixed with fresh ASW and an air pump was placed in each water reservoir to ensure further aeration and mixing. Water reservoirs were manually emptied from any remaining water and refilled with fresh ASW every other day. Equal water flow was assured to all aquaria by using a 24-channel peristaltic pump (ISMATEC, Germany) that maintained a constant flow rate (4 ml min^{-1}) to each aquarium. Water constantly overflowed from the aquaria to the water bath of the ETs ensuring constant water renewal inside the aquaria and, at the same time, ETs drained the surplus water. In each aquarium, an air pump ensured water aeration and mixing by moving water from the bottom to the top.

After the 3-month acclimation period, seagrasses with several ramets (i.e., iterating modular plant units) and no apparent damage or epiphyte cover were selected. Three selected ramets (here forward referred to as plant) of each species were cut and carefully planted in silicate sediment (∼7 cm depth) together with 3 seedlings of *Enhalus acoroides* (Artika et al., 2020) in the experimental setup previously described, where they were acclimated one more month before the start of the treatments. *T. hemprichii* and *C. serrulata* plants consisted of one shoot of 2–4 leaves, roots and a small portion of rhizome (3.6 ± 0.2 cm long). Plants of the same rhizome were randomly distributed along the treatments. *H. stipulacea* was divided in fragments of rhizome with 3–5 shoots and 8.8 ± 0.6 cm long.

After the acclimation period, temperature was elevated by increasing 1°C day^{-1} until reaching the 31°C in two random ETs. The other two ETs remained at the acclimation temperature of 26°C. Once the target temperatures were stable, nutrient addition started, and from that moment the experiment started. Plants were exposed to the selected experimental treatments for approximately 1 month (January 20th to February 22nd 2017) to ensure a response of the selected individual plant traits (Lee et al., 2007; McMahon et al., 2013; Roca et al., 2016).

Water Monitoring and Sampling

Water pH, temperature and salinity were monitored every other day during the acclimation and experimental phase with a multi-parameter probe (WTW Multiprobe). Salinity was adjusted by adding distilled water when necessary to aquaria and water reservoirs. Hobo loggers continuously monitored water temperature inside one of the aquaria in each ET (n = 4). The electronic system within the ETs continuously measured the temperature of all the water baths. Water was sampled every week from the two water reservoirs and random aquaria of each treatment (n = 4 each week) for silicate, phosphate, and dissolved inorganic N (DIN), as the sum of NH_4^+, NO_3^- and NO_2^-. Water was sampled with a syringe, immediately filtered (0.45 μm pore size, Whatman GF/F filters) in pre-rinsed polyethylene bottles and frozen (−20°C). Analysis was performed using a continuous flow injection analyzing system (Skalar SAN^{++}-System) following Grasshoff et al. (1999). The measuring procedure had a relative standard deviation < 3.5% with reference to the linear regression of an equidistant 10-point calibration line from NIST standards.

Detached leaves of the seagrasses were removed from the aquaria every day, and epiphytes growing on the blades of the seagrasses were removed weekly. Microalgae growing in the chamber walls, however, were not removed.

At the end of the experiment, the sediment N and C content were analyzed by drying the homogenized samples in a forced air oven at 60°C until constant dry weight (DW), ground to a fine powder with mortar and pestle, and weighed into tin capsules prior to analysis using Euro EA3000 Elemental Analyzer. Water samples from all aquaria were sampled for chlorophyll *a* (Chl-*a*) and *b* (Chl-*b*) and suspended particulate matter (SPM) measurements. Water was immediately filtered under constant

pressure onto pre-combusted (5 h, 450°C) and pre-weighed Whatman GF/F filters (0.45 μm pore size). Filters for SPM analysis were dried at 50°C and filters for Chl-*a* and *b* analysis were stored at −20°C. Concentrations of SPM were determined by weighing the dried filter, subtracting the weight of the empty filter and dividing it by the respective volume of water filtered. Pigments were extracted from the filters in 8 ml of 96% ethanol in glass vials heated for 5 min at 80°C, covered with aluminum foil, and placed in a rotor at room temperature for approximately 24 h. Extracts were subsequently centrifuged at 5000 rpm for 20 min. Chl-*a* and *b* samples were determined in a photometer Shimadzu UV-1700.

Measurement of Seagrass Traits

Different biochemical, morphological and physiological individual-level traits were selected based on their quick response times observed under other single effect experiments of nutrient over-enrichment or temperature in order to better understand the combined effect of these factors (Campbell et al., 2006; Lee et al., 2007; Leoni et al., 2008; Martínez-Crego et al., 2008; Pedersen et al., 2016; Roca et al., 2016).

At the end of the experiments, seagrasses were removed from the aquaria and the morphological measurements on each plant were performed. Afterward, plants were carefully separated with a glass spatula into the different parts: leaves (for fluorescence measurements, nutrient content, and free amino acids content, FAAs), rhizome (for nutrient content, and non-structural carbohydrate content, NSC), and roots (for nutrient content) in *T. hemprichii* and *C. serrulata* plants. Plant tissues of the three plants of each species within each aquarium were pooled together for nutrient analysis. In *H. stipulacea* plants, due to the limited plant material, rhizome and root material were pooled, and FAAs in leaves were not measured. Moreover, samples from two aquaria of this latter species had to be pooled for fluorescence measurements and nutrient content analysis.

Biochemical Traits

Nitrogen and carbon content

To assess whether the experimental water column N enrichment affected internal nutrient storage and allocation, we measured the final N and C contents of different plant tissues. *T. hemprichii* and *C. serrulata* plants were divided into leaves, rhizome and roots; while *H. stipulacea* individuals were divided into leaves (representing the AG compartment) and rhizome and roots (representing the BG compartment). Samples were dried at 50°C in a forced air oven until constant DW, ground to a fine powder with mortar and pestle, and weighed (1.42 ± 0.02 mg) into tin capsules using an analytical scale prior to analysis using Euro EA3000 Elemental Analyzer.

Free amino acid content in leaves

The FAAs were extracted from ∼50 mg fresh weight (FW) of leaves of *T. hemprichii* and *C. serrulata* grounded material (FastPrep®-24 Instrument) during 60 min at room temperature by adding 4 ml of 0.05 N HCl. The supernatant (5 min, 10,000 *g*) was filtered through 0.2 μm CA-filters into glass vials and stored at −20°C for posterior FAAs composition and concentration analysis using an ion-exchange liquid chromatography for hydrolyzed samples (Biochrom 30). For simplicity, only the total FAA concentration (sum of 14 FAAs for these samples: ALA, ARG, ASP, GLU, GLY, HIS, ILE, LEU, MET, PHE, THR, TYR, SER and VAL) were considered in this study. However, relative differences among FAAs can also be identified (**Supplementary Table 1**).

Non-structural carbohydrate content in rhizomes

The concentration of sucrose and starch were measured on rhizome material of *T. hemprichii* and *C. serrulata* and in the BG compartment (rhizome and roots) of *H. stipulacea*. We followed a modified protocol from Salo and Pedersen (2014). The samples were frozen (−80°C) and freeze-dried for 48 h. Soluble sugars, namely sucrose, were extracted from ground plant tissue by boiling in 96% ethanol. The ethanol extracts were evaporated and the residues were dissolved in deionized water for sucrose analysis. Starch was extracted from the ethanol-insoluble residue in 1 N NaOH for 24 h. The sucrose and starch concentrations of the extracts were determined spectrophotometrically (wavelengths 486 and 640 nm, respectively) using resorcinol and anthrone assays, respectively, with sucrose as a standard (Yemm and Willis, 1954; Huber and Israel, 1982). Results were reported in sucrose equivalents g^{-1} DW. Current testing of this method has shown that NaOH extracts not only starch, but also cellulose, which can confound the results. Regarding the sucrose determination, this method only determines ketoses (as fructose) so we are ignoring glucose, the other component of sucrose, therefore underestimating the final concentrations (M. Birkicht, personal communication). Despite its drawbacks, this method has been frequently used in other studies and allows for direct comparisons of the data.

Morphological Traits

Morphological measurements were individually performed in each of the three plants of each species within the different aquaria. The measurements included leaf morphometrics (length, width and surface area, SA), sheath length (or petiole for *H. stipulacea*), root length, and internode length (IL) (just for *H. stipulacea*).

Physiological Traits

Growth rates

Leaves of *T. hemprichii* and *C. serrulata* were double-pinned in parallel just above the sheath at the beginning of the experiment for leaf growth measurements following the method by Short and Duarte (2001). However, leaf growth and turnover were higher than expected during the experiment, and no leaves with pinning remained at the end of the experiment. Therefore, the growth was estimated by collecting the detached leaves found daily in each aquaria. Leaf growth (cm d^{-1}) was measured as the distance from the base of the detached leaf to the place where the pins were, divided by the days when the leaf was sampled. Only leaves detached during the last week of the experiment were considered. We tried to measure *H. stipulacea* growth by marking the rhizome, however, no label was found by the end of the experiment, and no optional method of growth estimation was possible with this species.

Photosynthetic variables

The photosynthetic performance of the seagrasses was measured through pulse amplitude modulated (PAM) chlorophyll fluorescence using rapid light response curves (RLCs) generated by the PAM-2500 chlorophyll fluorometer (Walz, Germany). RLCs were performed above the meristem of the second leaf of the three plants of each aquarium for *T. hemprichii* and *C. serrulata*. For *H. stipulacea*, 3 measurements were performed at the base of the leaf, close to the petiole, and plants of two aquaria of each treatment had to be pooled. The basal portion of the leaf was chosen since it represents similar distances from the surface (and thus from the light source) among plants with different leaf lengths, thus minimizing variability within plants and species (Winters et al., 2011). A clip was attached to the leaf and helped to hold the optical cable of the PAM at 3 mm distance from the tissue and to dark adapt the tissue during 5 min. Leaves were maintained in a petri dish with some ASW during the dark adaptation and the measurements.

The first quantum yield measurement was performed in the absence of actinic light (dark-adapted effective quantum yield, Y_o; Saroussi and Beer, 2007), after which the RLC consisted of 12 saturating light pulses (separated by 30s intervals), increasing the photosynthetic active radiation (PAR) between pulses until 2000 μmol photons m^{-2} s^{-1}. Each step lasted 10 s and was followed by a measurement of the effective quantum yield ($\Delta F/F_m$') (Ralph and Gademann, 2005). From the data of the RLC, light saturation coefficient (E_k) and the slope of the light limited part of the curve (Alpha) were calculated using the package Phytotools (Silsbe and Malkin, 2015) following the model of Jassby and Platt (1976) under the R software (R Core Team, 2019).

The maximum light utilization efficiency or maximum quantum yield of PSII was calculated following equation by Genty et al. (1989) [$F_v/F_m = (F_m\text{-}F_o)/F_m$], where F_m is the maximum dark-adapted fluorescence and F_o is the minimal fluorescence from a dark-adapted sample.

The relative electron transport rate (rETR) was calculated for each step of the curve following equation by Sakshaug et al. (1997), [rETR = $(F_{m'}\text{-}F'/F_{m'})$*(PAR/2)], where $F_{m'}$ is the light adapted maximum fluorescence and F' the fluorescence yield at a particular light level. From the rETR values, maximum rETR ($rETR_{max}$) was estimated as the inflection point of the fitted rETR curve.

Statistical Analysis

The experiment followed a split-plot design with three nesting factors (Schielzeth and Nakagawa, 2013). The two main factors (temperature and nutrient) had two fully crossed levels each (LT and HT, and LN and HN respectively). Two ETs were nested within each temperature treatments, six aquaria were nested within each ET and three plants of each species within each aquaria.

We used permutational multivariate analysis of variance (PERMANOVA) (Anderson et al., 2008) to analyze the data of each species. The Pseudo F-statistic was used to test the null hypothesis of no differences in the position of the group centroids in the space of the chosen dissimilarity measure. The fixed effects in the model were temperature and nutrient treatments, and their interactions, together with the nesting structure of temperature, ET and Aquarium. The factor Aquarium was not included in biochemical trait analysis because tissues from the different plants within aquaria were pooled. For *H. stipulacea* data, the factors Aquarium or ET were not included in biochemical measurements analysis because tissues from different aquaria were pooled. Data were scouted for outliers, which were identified as data exceeding 1.5 times the interquartile range of variation of the dataset. Outliers were only eliminated from the model when they did not allow to meet the model assumptions. We calculated the Euclidean dissimilarity matrix for all variables, as they were continuous. The assumptions of exchangeability of permutable units and homogeneity of multivariate dispersion were tested before analysis. When the homogeneity of multivariate dispersions was not met, the data were transformed (square root, or log) and the dissimilarity matrix recalculated. The homogeneity of multivariate dispersion assumption for ET grouping could not be met for leaf C:N ratio and Alpha values in *T. hemprichii*.

Water parameters in aquaria (DIN, phosphate, SPM, and Chl-*a* and *b*) were compared using PERMANOVA while DIN and phosphate concentrations in water reservoirs were analyzed using a two-way ANOVA. Temperature, nutrient, their interactions and the nesting structure (Temperature:ET:Aquarium) were the fixed effects in the model. The permutational unit for the model was the aquarium with 999 permutations, which is the recommended minimum number to test at an alpha-level of 0.05 (Manly, 1997). Comparisons were considered significant when P was ≤ 0.05. We used R software to perform the analysis (R Core Team, 2019) with the adonis2 function of the package "vegan" (Oksanen et al., 2019).

Pearson correlation analysis was applied in order to investigate potential relationships between biochemical, morphological and physiological traits. Data from aquaria of all treatments were combined to generate statistically independent means for each aquarium (without error), resulting in statistically independent replicate measurements ($n = 24$, except of *H. stipulacea*, $n = 12$). This statistical analysis was performed with SPSS (IBM SPSS Statistics for Windows v.24, Armonk, NW, United States).

RESULTS

Experimental Conditions

The four different treatments, combining two nutrient and temperature levels, changed the conditions under which the seagrasses were grown. Water temperature in the aquaria was shown to be constant both during the day, with no sharp variations when light was absent (data not shown), and through the experimental period, while showing significant differences between temperature treatments (PERMANOVA, $P < 0.01$) (**Table 1**). DIN and phosphate concentrations in the two main water reservoirs were within the target concentrations throughout the experiment and were significantly different between the HN and LN treatments (two-way ANOVA, $P < 0.001$). However, once in the aquaria, nutrients were rapidly taken up, resulting in low inorganic nutrient concentrations in all treatments regardless of the treatment (PERMANOVA, $P > 0.05$). In fact, some of the concentrations measured were

TABLE 1 | Experimental seawater parameters (mean ± SE) and number of measurements taken (n) during the experimental period in each of the four treatments (LT, low temperature; LN, low nutrient; HT, high temperature; HN, high nutrient).

		Treatments			
	n	LT + LN	HT + LN	LT + HN	HT + HN
DIN (μM)^R	4	5.66 ± 0.51		22.40 ± 0.98	
PO_4^- (μM)^R	4	0.19 ± 0.01		1.01 ± 0.04	
Water temperature (°C)	25*	26.25 ± 0.05	31.01 ± 0.07	26.28 ± 0.03	31.01 ± 0.05
	3735‡	26.47 ± 0.003	31.13 ± 0.004	26.22 ± 0.004	31.48 ± 0.002
Salinity	25	35.43 ± 0.12	35.39 ± 0.10	35.33 ± 0.07	35.39 ± 0.11
pH	12	8.47 ± 0.02	8.39 ± 0.02	8.67 ± 0.03	8.59 ± 0.02
DIN (μM)	5	0.62 ± 0.33	0.88 ± 0.44	0.64 ± 0.31	1.25 ± 0.01
PO_4^- (μM)	5	0.15 ± 0.01	0.14 ± 0.02	0.14 ± 0.00	0.14 ± 0.01
Si (μM)	5	0.71 ± 0.07	1.15 ± 0.22	0.91 ± 0.20	0.85 ± 0.06
SPM (mg l^{-1})	24	27.62 ± 4.65	24.04 ± 1.99	38.21 ± 3.69	74.91 ± 11.75
Chl-*a* (μg l^{-1})	24	2.07 ± 0.53	2.07 ± 0.53	2.59 ± 0.98	5.91 ± 2.41
Chl-*b* (μg l^{-1})	24	0.36 ± 0.07	0.36 ± 0.07	0.42 ± 0.18	0.88 ± 0.43

*DIN, dissolved inorganic nitrogen (the sum of NH_4^+, NO_3^- and NO_2^-); SPM, suspended particulate matter. ^{R}Measurements taken in the water reservoirs, *Water temperature provided by multiprobe measurements, ‡ Water temperature provided by continuous measurement of Hobo loggers.*

not included in the analysis as they were below the quantification limit. However, even though nutrient concentration parameters in aquaria were low, other observable parameters suggested eutrophic conditions were occurring in the HN treatments. Algal blooms were observed in the HN treatments, showing different trophic conditions in the LN and HN treatments, especially in the HT + HN treatment. Algae were observable by naked eye on the glass of the aquaria and seagrasses. However the abundance of these microorganisms could not be quantified, as they formed fluffy layers that disintegrated when tried to sample them. SPM concentrations in the water column were also higher in the HN treatments (PERMANOVA, $P < 0.05$) with the highest concentrations in the HT + HN treatment (**Table 1**). Water column Chl-*a* concentrations were also higher in the HN treatments (2.6–5.9 μg l^{-1}) than in the LN treatments (2−2.1 μg l^{-1}) showing the highest mean value in the HT + HN treatment (5.91 μg l^{-1}). Even though Chl-*b* concentrations also increased in the HT + HN treatment, no significant differences were observed among treatments (PERMANOVA, $P > 0.05$). Therefore, eutrophic conditions were especially noticeable in the HT + HN treatment. The water column nutrient enrichment did not significantly change the sediment nutrient conditions, and sediment N concentrations were below the detection limits in all aquaria (data not shown). The other variables measured in the water column, including salinity, pH and Si concentrations, were constant throughout the experiment and did not show significant differences among treatments (**Table 1**).

Seagrass Biochemical Traits

Thalassia hemprichii showed the highest N content in all three tissues compared to that of the other species, followed by *C. serrulata* and *H. stipulacea* (**Figure 1**). Total C content was similar in all species, although allocation changed. While *T. hemprichii* showed higher C content in rhizome compared to leaves, *C. serrulata* showed similar content in these two tissues. On the other hand, *H. stipulacea* had slightly higher C content in AG than BG tissues (**Figure 1**).

Temperature treatments changed the biochemical contents and the nutrient allocation in the three species (**Table 2**). *C. serrulata* was the species most influenced by temperature in terms of its leaf biochemical traits, as leaf N and C content decreased, and C:N ratio significantly increased under HT treatments (**Figure 1**). Leaf C:N ratio also increased in *H. stipulacea*, although no significant effect was detected. *T. hemprichii* BG tissues were the most influenced by HT treatment, with significantly lower N content in rhizome and roots under HT treatments (**Table 2**). Root C content was also significantly lower under HT treatments in this species and *H. stipulacea*, while in *C. serrulata*, significantly higher C content was observed under HT treatments (**Figure 1**).

Nutrient treatments significantly influenced the nutrient contents of *T. hemprichii* and *C. serrulata* but not of *H. stipulacea* (**Table 2**). Both *T. hemprichii* and *C. serrulata* showed significantly higher leaf N content under HN treatments, and *T. hemprichii* also showed significantly lower C:N ratio ($P < 0.05$), while non-significant decreasing values were observed in *C. serrulata* ($P = 0.06$). Leaf FAAs content only positively responded to nutrient treatment in *C. serrulata* ($P = 0.06$). This species also showed the highest concentrations of NSC in the rhizome of the three species. In *C. serrulata* and *H. stipulacea*, the main NSC form was sucrose, while in *T. hemprichii*, starch concentrations were higher than sucrose under all treatments. The BG tissues of *C. serrulata* were the only ones that changed in response to nutrient enrichment, with lower starch and C content in rhizomes, and lower C content in roots in HN treatments (**Figure 1** and **Table 2**).

The effects of warming on *C. serrulata* and *H. stipulacea* biochemical traits, namely leaf C:N ratio in both species and root C content in *C. serrulata*, were significantly mediated by nutrient over-enrichment. This resulted in an antagonistic effect of both factors, as values were lower than expected for

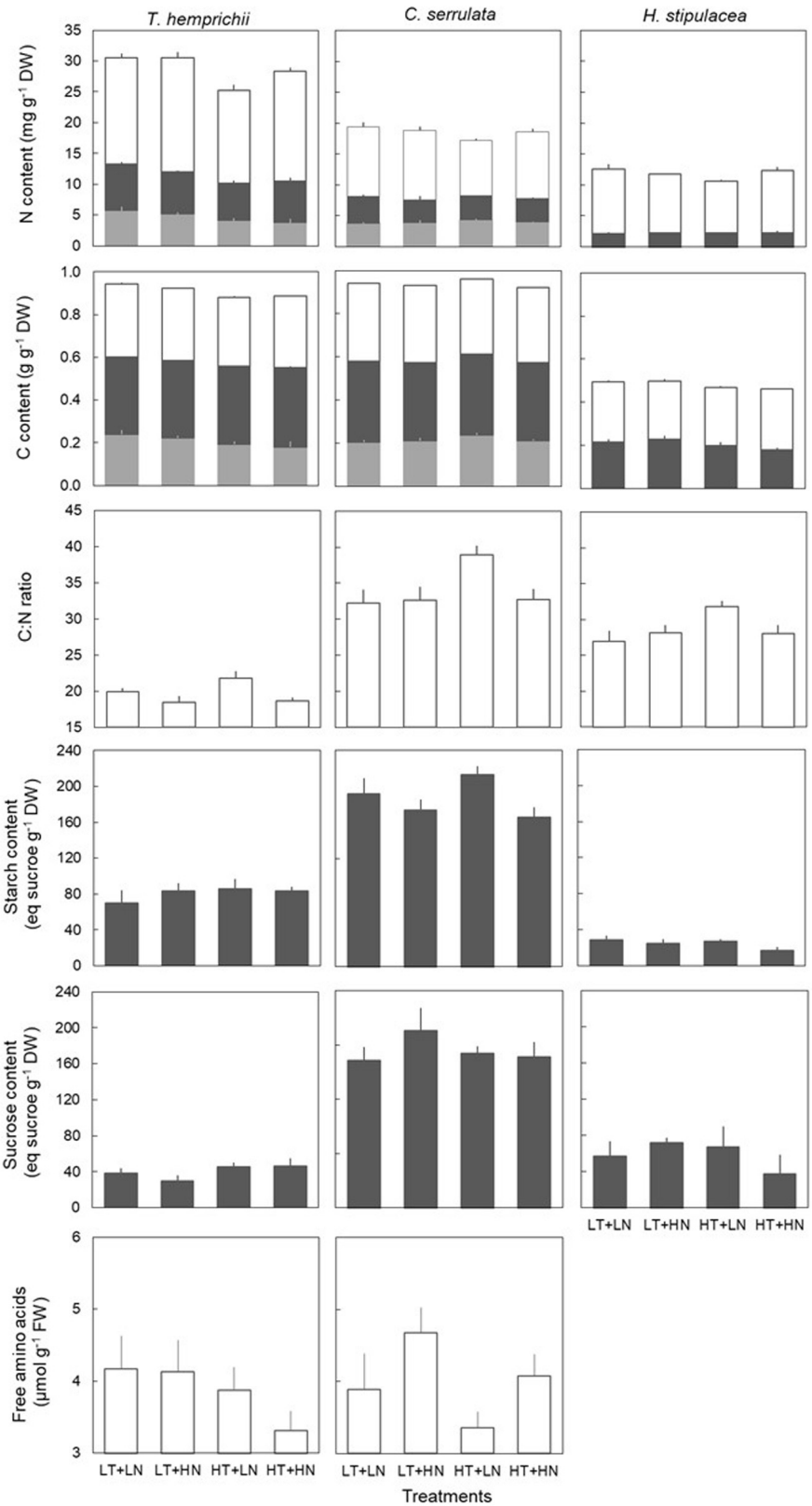

FIGURE 1 | Biochemical traits (mean ± SE, $n = 6$, except of *H. stipulacea*, $n = 3$) of *T. hemprichii*, *C. serrulata* and *H. stipulacea* in leaves (open bars), rhizome (dark gray bars) and roots (light gray bars) in the four treatments (LT, low temperature; LN, low nutrient; HT, high temperature; HN, high nutrient).

TABLE 2 | Permutational analysis of variance (PERMANOVA) of the effects of temperature (T) and nutrient (N) treatments on the biochemical traits of *T. hemprichii*, *C. serrulata*, and *H. stipulacea* in **Figure 1**.

		T. hemprichii					*C. serrulata*					*H. stipulacea*				
Trait	**Source**	**df**	**SS**	**R^2**	**Pseudo-F**	***P***	**df**	**SS**	**R^2**	**Pseudo-F**	***P***	**df**	**SS**	**R^2**	**Pseudo-F**	***P***
Leaf N	T	1	2.37	0.1	3.08	0.112	1	2.45	0.11	3.58	0.075	1	1.36	0.12	2.15	0.197
	N	1	4.86	0.21	6.32	**0.031***	1	2.78	0.12	4.07	**0.058***	1	0.39	0.04	0.61	0.437
	T:N	1	0.84	0.04	1.09	0.298	1	1.18	0.05	1.72	0.203	1	4.22	0.38	6.69	**0.045***
	T:ET	2	1.07	0.05	0.7	0.527	2	4.29	0.19	3.14	0.069	-	-	-	-	-
	Res	18	13.85	0.6			18	12.3	0.53			8	5.04	0.46		
Rhizome N ‡	T	1	4.42	0.19	4.75	**0.042***	1	0.07	0	0.07	0.781	1	0.09	0.01	0.07	0.789
	N	1	0.09	0.004	0.1	0.751	1	2.27	0.1	2.19	0.173	1	0.08	0.01	0.06	0.806
	T:N	1	1.44	0.06	1.55	0.223	1	1.53	0.07	1.48	0.259	1	0.04	0	0.03	0.852
	T:ET	2	0.29	0.01	0.15	0.847	2	0.51	0.02	0.25	0.814	-	-	-	-	-
	Res	18	16.76	0.73			17	17.61	0.8			8	10.78	0.98		
Roots N	T	1	8.12	0.35	16.86	**0.001*****	1	2.25	0.1	2.61	0.130	-	-	-	-	-
	N	1	0.83	0.04	1.72	0.223	1	0.15	0.01	0.17	0.704	-	-	-	-	-
	T:N	1	0.03	0	0.07	0.793	1	1.92	0.09	2.22	0.142	-	-	-	-	-
	T:ET	2	5.35	0.23	5.55	**0.017***	2	3.02	0.14	1.75	0.207	-	-	-	-	-
	Res	18	8.67	0.38			17	14.66	0.67			-	-	-		
Leaf C:N	T	1	1.41	0.06	2.05	0.184	1	3.07	0.13	4.78	**0.042***	1	2.58	0.23	4.18	0.084
	N	1	6.48	0.28	9.4	**0.010****	1	2.84	0.12	4.43	0.063	1	0.78	0.07	1.26	0.303
	T:N	1	0.87	0.04	1.27	0.276	1	2.81	0.12	4.39	**0.038***	1	2.72	0.25	4.42	**0.054***
	T:ET	2	1.83	0.08	1.33	0.309	2	2.74	0.12	2.13	0.158	-	-	-	-	-
	Res	18	12.4	0.54			18	11.54	0.5			8	4.93	0.45		
Leaf C	T	1	4.44	0.19	5.21	**0.029***	1	8.77	0.4	14.67	**0.002****	1	0.04	0	0.04	0.838
	N	1	0.34	0.01	0.4	0.521	1	0.02	0	0.04	0.855	1	0.03	0	0.02	0.864
	T:N	1	2.37	0.1	2.78	0.129	1	0.47	0.02	0.78	0.358	1	2.76	0.25	2.7	0.126
	T:ET	2	0.5	0.02	0.3	0.728	2	2.58	0.12	2.16	0.136	-	-	-	-	-
	Res	18	15.35	0.67			17	10.16	0.46			8	8.17	0.74		
Rhizome C‡	T	1	0.11	0.005	0.1	0.792	1	0.14	0.01	0.58	0.474	1	3.96	0.36	5.3	**0.045***
	N	1	0.03	0.001	0.02	0.902	1	15.01	0.68	60.44	**0.001*****	1	0.03	0	0.04	0.859
	T:N	1	0.22	0.01	0.2	0.690	1	0.36	0.02	1.44	0.258	1	1.04	0.09	1.39	0.265
	T:ET	2	2.7	0.12	1.22	0.339	2	2.26	0.1	4.55	**0.028***	-	-	-	-	-
	Res	18	19.95	0.87			17	4.22	0.19			8	5.97	0.54		
Roots C	T	1	5.04	0.22	8.97	**0.010****	1	3.12	0.14	4.7	**0.034***	-	-	-	-	-
	N	1	0.67	0.03	1.2	0.287	1	0.65	0.03	0.99	0.383	-	-	-	-	-
	T:N	1	0.01	0.0003	0.01	0.907	1	3.01	0.14	4.53	**0.041***	-	-	-	-	-
	T:ET	2	7.18	0.31	6.4	**0.011***	2	3.93	0.18	2.96	0.067	-	-	-	-	-
	Res	18	10.1	0.44			17	11.29	0.51			-	-	-		
Rhizome sucrose‡	T	1	2.21	0.1	2.01	0.165	1	0.32	0.01	0.28	0.606	1	0.34	0.03	0.31	0.578
	N	1	0.25	0.01	0.23	0.684	1	0.79	0.04	0.71	0.392	1	0.09	0.01	0.08	0.774
	T:N	1	0.34	0.02	0.31	0.606	1	1.46	0.07	1.3	0.230	1	1.78	0.18	1.6	0.263
	T:ET	2	0.54	0.02	0.25	0.794	2	0.46	0.02	0.21	0.801	-	-	-	-	-
	Res	17	18.65	0.85			17	18.98	0.86			7	7.79	0.78		
Rhizome starch‡	T	1	0.69	0.03	0.81	0.389	1	0.26	0.01	0.34	0.563	1	0.54	0.05	0.58	0.475
	N	1	0.31	0.01	0.36	0.567	1	5.23	0.23	6.81	**0.020***	1	2.39	0.24	2.58	0.145
	T:N	1	0.67	0.03	0.79	0.388	1	1.05	0.05	1.36	0.259	1	0.57	0.06	0.62	0.454
	T:ET	2	6.1	0.27	3.61	**0.052***	2	2.64	0.11	1.72	0.22	-	-	-	-	-
	Res	18	15.23	0.66			18	13.82	0.6			7	6.49	0.65		
Leaf FAA	T	1	2.13	0.09	2.24	0.169	1	2.15	0.09	2.3	0.162	-	-	-	-	-
	N	1	0.61	0.03	0.64	0.454	1	3.8	0.17	4.07	0.068	-	-	-	-	-
	T:N	1	0.48	0.02	0.5	0.475	1	0.01	0	0.01	0.917	-	-	-	-	-
	T:ET	2	2.65	0.12	1.39	0.283	2	0.22	0.01	0.12	0.891	-	-	-	-	-
	Res	18	17.13	0.74			18	16.82	0.73			-	-	-		

Significant differences are in boldface (: ≤ 0.05; **: ≤ 0.01; ***: ≤ 0.001). (ET: experimental tank, FAA: free amino acids). ‡Below-ground tissues (rhizome and roots) for H. stipulacea.*

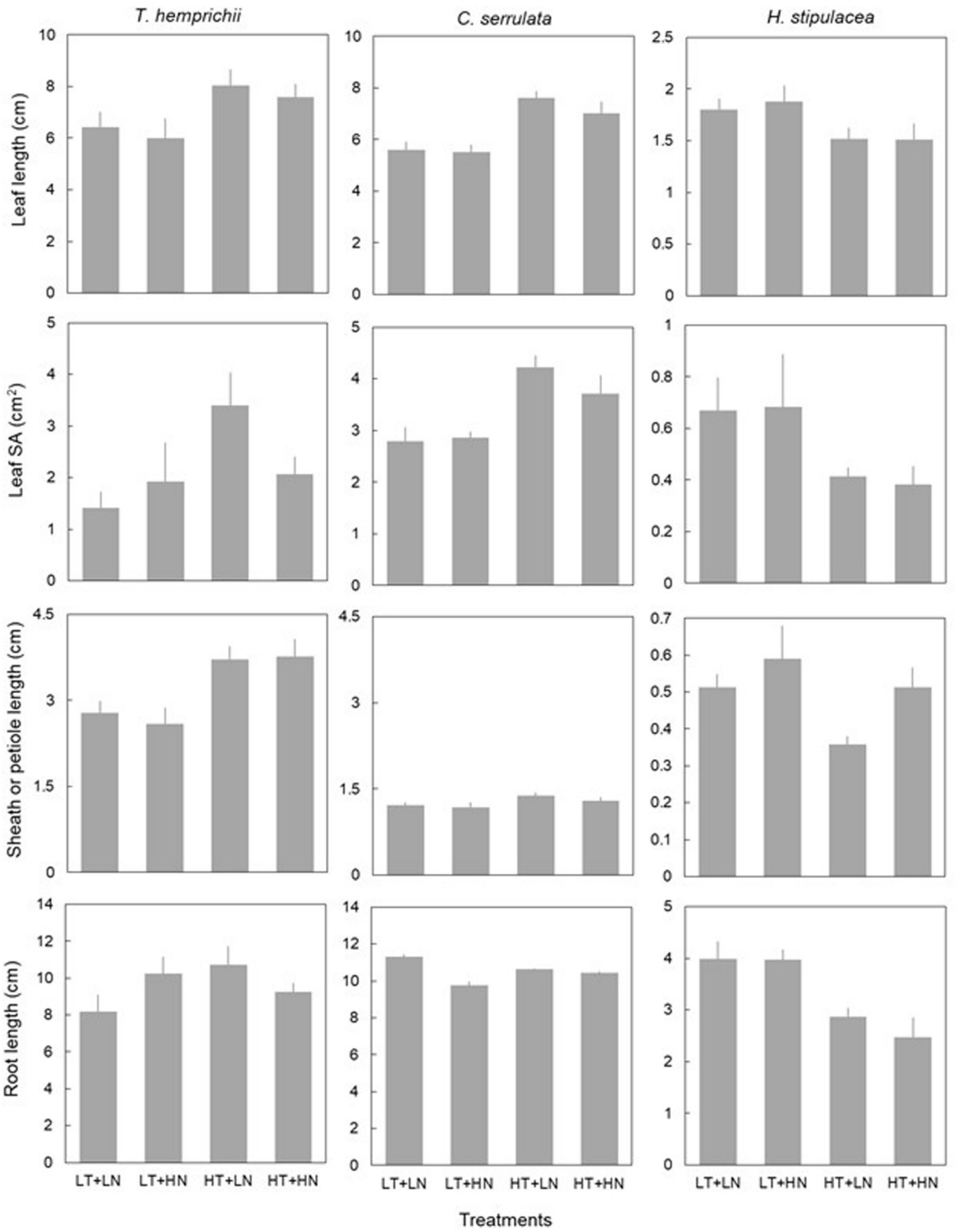

FIGURE 2 | Morphological traits (mean ± SE, $n = 6$) of *T. hemprichii*, *C. serrulata* and *H. stipulacea* in the four treatments (LT, low temperature; LN, low nutrient; HT, high temperature; HN, high nutrient). Note that *Y*-axis of *H. stipulacea* panels differ.

effects to be additive. Contrary, N content in AG tissues in *H. stipulacea* showed the only synergistic effect observed in this study. There were no interactive effects in the biochemical traits of *T. hemprichii* (**Table 2**).

Seagrass Morphological Traits

Temperature had an overall significant effect on seagrass morphological traits (**Figure 2** and **Table 3**). Leaf morphology was enhanced under HT treatments in *T. hemprichii* and

TABLE 3 | Permutational analysis of variance (PERMANOVA) of the effects of temperature (T) and nutrient (N) treatments on the morphological traits of *T. hemprichii*, *C. serrulata,* and *H. stipulacea* in **Figure 2** and **Supplementary Figure 1**.

		T. hemprichii					*C. serrulata*					*H. stipulacea*				
Trait	**Source**	**df**	**SS**	R^2	**Pseudo-F**	***P***	**df**	**SS**	R^2	**Pseudo-F**	***P***	**df**	**SS**	R^2	**Pseudo-F**	***P***
Leaf length	T	1	9.12	0.13	12.97	**0.002****	1	16.19	0.23	18.39	**0.001*****	1	7.97	0.13	13.06	**0.001*****
	N	1	0.63	0.01	0.9	0.349	1	0.6	0.01	0.68	0.404	1	0.92	0.01	1.51	0.246
	T:N	1	0	0	0	0.969	1	0.33	0	0.37	0.536	1	0.07	0	0.11	0.753
	T:ET:Aq	20	27.5	0.39	1.96	**0.028***	20	11.65	0.16	0.66	0.86	20	29.65	0.47	2.43	**0.008****
	Res	48	33.75	0.48			48	42.24	0.59			40	24.4	0.39		
Leaf SA	T	1	11.06	0.16	33.35	**0.001*****	1	12.19	0.17	13.11	**0.001*****	1	5.78	0.09	7.22	**0.008****
	N	1	1.54	0.02	4.66	**0.035***	1	0.46	0.01	0.49	0.478	1	0.08	0	0.1	0.757
	T:N	1	5.24	0.07	15.79	**0.001*****	1	0.81	0.01	0.87	0.364	1	0.07	0	0.09	0.795
	T:ET:Aq	20	37.23	0.52	5.61	**0.001*****	20	12.92	0.18	0.7	0.794	20	25.06	0.4	1.57	0.138
	Res	48	15.92	0.22			48	44.63	0.63			40	32.01	0.51		
Sheath length‡	T	1	17.93	0.25	27.47	**0.001*****	1	5.43	0.08	6.54	**0.016***	1	2.26	0.04	2.44	0.131
	N	1	0.08	0	0.12	0.711	1	1	0.01	1.2	0.266	1	1.4	0.02	1.51	0.253
	T:N	1	0.24	0	0.37	0.554	1	0.27	0	0.33	0.57	1	2.97	0.05	3.2	0.074
	T:ET:Aq	20	21.43	0.3	1.64	0.085	20	24.49	0.34	1.48	0.135	20	19.31	0.31	1.04	0.323
	Res	48	31.33	0.44			48	39.81	0.56			40	37.07	0.59		
Root length	T	1	1.49	0.02	2.39	0.138	1	0	0	0	0.994	1	23.51	0.37	46.39	**0.001*****
	N	1	0.48	0.01	0.77	0.37	1	1.21	0.02	1.3	0.254	1	0.59	0.01	1.16	0.316
	T:N	1	7.19	0.1	11.52	**0.002****	1	0.73	0.01	0.78	0.387	1	0.04	0	0.09	0.776
	T:ET:Aq	20	31.88	0.45	2.55	**0.006****	20	24.33	0.34	1.3	0.245	20	18.59	0.3	1.83	**0.058***
	Res	48	29.96	0.42			48	44.74	0.63			40	20.27	0.32		
IL	T	-	-	-	-	-	-	-	-	-	-	1	3.44	0.05	3.85	**0.054***
	N	-	-	-	-	-	-	-	-	-	-	1	3.29	0.05	3.69	0.069
	T:N	-	-	-	-	-	-	-	-	-	-	1	1.07	0.02	1.2	0.262
	T:ET:Aq	-	-	-	-	-	-	-	-	-	-	20	19.47	0.31	1.09	0.392
	Res	-	-	-			-	-	-			40	35.73	0.57		

Significant differences are in boldface (: ≤ 0.05; **: ≤ 0.01; ***: ≤ 0.001). (ET: experimental tank, Aq: aquarium, SA: surface area, IL: internode length). ‡Petiole length for H. stipulacea.*

C. serrulata, with longer leaves, bigger leaf SA, and longer sheaths. Root length did not respond to HT treatment in any of these two species. However, HT negatively influenced *H. stipulacea* leaf traits, with shorter leaves and smaller leaf SA, and also shorter roots and IL (**Figure 2** and **Supplementary Figure 1**).

Overall, nutrient addition did not show significant effects on morphological trait responses with the exception of lower leaf SA of *T. hemprichii* under HN treatments (**Figure 2**). *C. serrulata* morphological traits were unaffected by single nutrient treatments (**Table 3**).

Leaf SA and root length in *T. hemprichii* showed a significant interaction of temperature and nutrients, in which both traits showed an antagonistic effect, i.e., a cancelation of the enhanced effects of nutrients when also exposed to higher temperatures (**Figure 2** and **Table 3**). Petiole length in *H. stipulacea* showed some additive interaction of both factors, although the effect was not significant (**Table 3**). *C. serrulata* showed no interactive effects of both factors in the morphological traits considered (**Table 3**).

Morphological variables were also significantly affected by the nested blocking variables in *T. hemprichii* and *H. stipulacea,* but not in *C. serrulata* (**Table 3**). Therefore, the high variability between enclosures may have confounded some effects of the temperature and nutrient treatments.

Seagrass Physiological Traits

Growth rate responded both to temperature and nutrient treatments in *T. hemprichii* but showed no significant effects in *C. serrulata* (**Figure 3** and **Table 4**). On the contrary, the three species in this study significantly responded to both nutrient and temperature in terms of their photosynthetic efficiency (**Figure 3** and **Table 4**). Temperature was the main driver of these responses, and higher F_v/F_m and Alpha in *C. serrulata*, and Alpha in *H. stipulacea* were observed under HT treatments. In contrast, $rETR_{max}$ was negatively affected by HT treatments in the three species, and E_k in *C. serrulata* and *H. stipulacea* (**Figure 3**).

A single nutrient effect on photosynthetic performance was more limited than temperature alone. A significant effect of HN

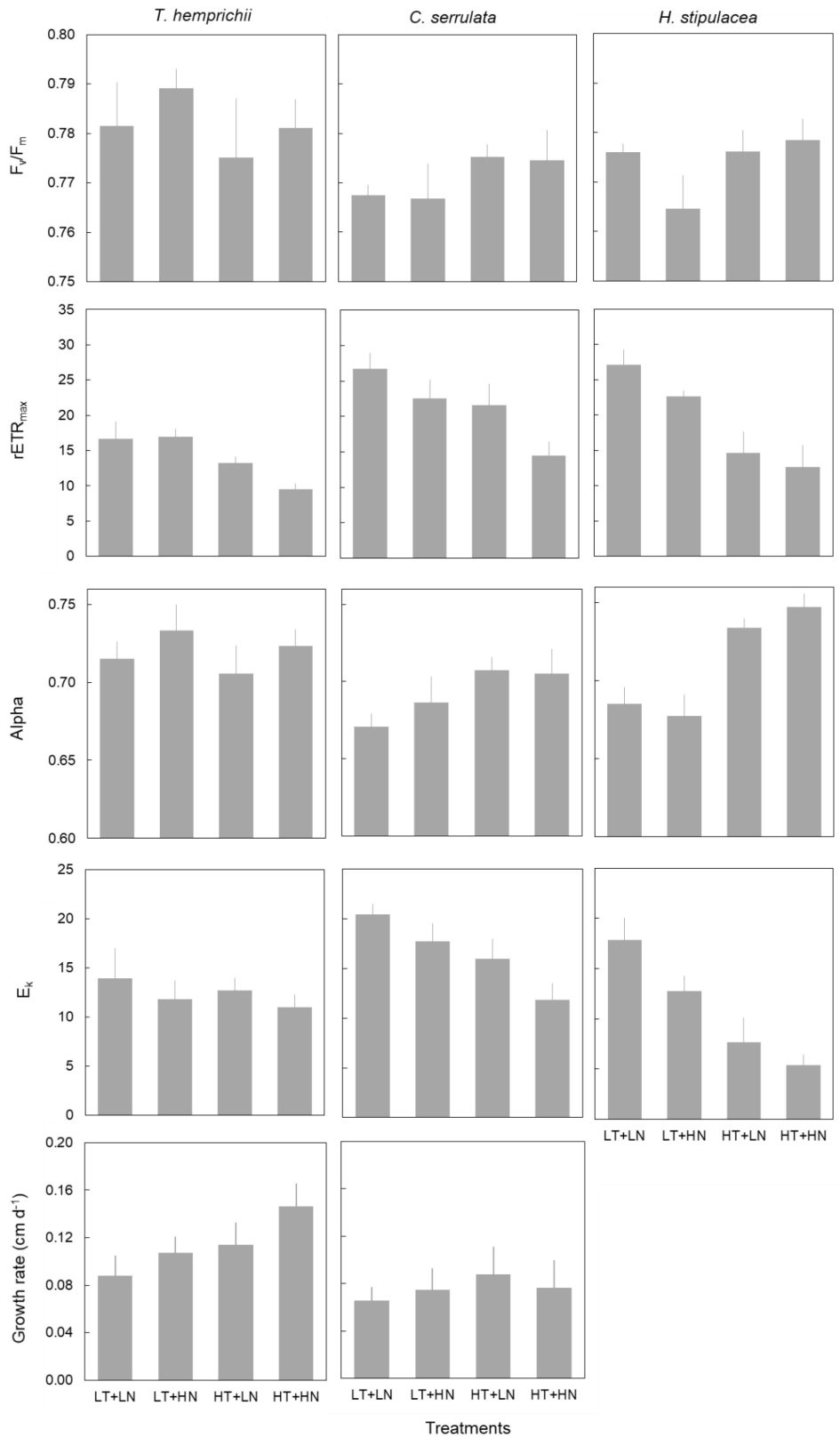

FIGURE 3 | Physiological traits (mean ± SE, *n* = 6, except of *H. stipulacea*, *n* = 3) of *T. hemprichii*, *C. serrulata* and *H. stipulacea* in the four treatments (LT, low temperature; LN, low nutrient; HT, high temperature; HN, high nutrient).

TABLE 4 | Permutational analysis of variance (PERMANOVA) of the effects of temperature (T) and nutrient (N) treatments on the physiological traits of *T. hemprichii*, *C. serrulata*, and *H. stipulacea* in **Figure 3**.

		T. hemprichii					*C. serrulata*					*H. stipulacea*				
Trait	**Source**	**df**	**SS**	R^2	**Pseudo-F**	***P***	**df**	**SS**	R^2	**Pseudo-F**	***P***	**df**	**SS**	R^2	**Pseudo-F**	***P***
F_v/F_m	T	1	1	0.01	1.42	0.254	1	3	0.04	3.68	**0.050***	1	1.12	0.03	1.08	0.319
	N	1	0.91	0.01	1.28	0.291	1	0.93	0.01	1.14	0.282	1	0.01	0	0.01	0.911
	T:N	1	0	0	0	0.997	1	0.05	0	0.06	0.796	1	1.48	0.04	1.43	0.234
	T:ET:Aq	20	34.59	0.5	2.45	**0.020***	20	27.55	0.4	1.69	0.068	7	6.45	0.19	0.89	0.55
	Res	46	32.51	0.47			46	37.47	0.54			24	24.94	0.73		
$rETR_{max}$	T	1	12.27	0.18	17.26	**0.002****	1	7.61	0.11	9.78	**0.002****	1	7.56	0.22	8.46	**0.006****
	N	1	1.93	0.03	2.72	0.100	1	0.58	0.01	0.74	0.415	1	0.19	0.01	0.21	0.649
	T:N	1	1.25	0.02	1.75	0.193	1	1.09	0.02	1.4	0.258	1	0.01	0	0.01	0.95
	T:ET:Aq	20	20.83	0.3	1.46	0.162	20	23.93	0.35	1.54	0.108	7	4.8	0.14	0.77	0.627
	Res	46	32.72	0.47			46	35.79	0.52			24	21.45	0.63		
Alpha	T	1	0.37	0.01	0.76	0.387	1	4.56	0.07	7.81	**0.004****	1	17.72	0.52	36.49	**0.001*****
	N	1	2.48	0.04	5.11	**0.034***	1	0.02	0	0.03	0.848	1	0.29	0.01	0.6	0.476
	T:N	1	0.21	0	0.44	0.502	1	1.62	0.02	2.77	0.099	1	0.59	0.02	1.21	0.304
	T:ET:Aq	20	43.65	0.63	4.5	**0.001*****	20	35.91	0.52	3.07	**0.001*****	7	3.74	0.11	1.1	0.389
	Res	46	22.3	0.32			46	26.89	0.39			24	11.66	0.34		
E_k	T	1	1.13	0.02	1.68	0.184	1	6.35	0.09	8.48	**0.007****	1	19.77	0.58	46.88	**0.001*****
	N	1	0.97	0.014	1.45	0.275	1	2.29	0.03	3.05	0.084	1	1.01	0.03	2.41	0.135
	T:N	1	0.05	0	0.07	0.806	1	1.37	0.02	1.83	0.181	1	0.21	0.01	0.51	0.472
	T:ET:Aq	20	35.95	0.52	2.68	**0.011***	20	24.55	0.36	1.64	0.091	7	2.89	0.08	0.98	0.469
	Res	46	30.9	0.45			46	34.45	0.5			24	10.12	0.3		
Growth	T	1	4.7	0.2	7.19	**0.014***	1	0.35	0.02	0.31	0.563	-	-	-	-	-
	N	1	3.19	0.14	4.87	**0.043***	1	0.02	0	0.02	0.902	-	-	-	-	-
	T:N	1	0.21	0.01	0.32	0.59	1	0.17	0.01	0.15	0.677	-	-	-	-	-
	T:ET	2	3.15	0.14	2.41	0.125	2	2.56	0.11	1.16	0.326	-	-	-	-	-
	Res	18	11.76	0.51			18	19.91	0.87			-	-	-		

Significant differences are in boldface (: ≤ 0.05; **: ≤ 0.01; ***: ≤ 0.001). (ET: experimental tank, Aq: aquarium).*

treatments was only detected by Alpha values in *T. hemprichii*, which was more evident in the combined HN + HT treatment (**Figure 3**). In *C. serrulata*, no trait showed a response to nutrients. The absence of responses of photosynthetic traits to nutrients was more evident when considering its interaction with temperature, as no significant effects were detected (**Table 4**). In the same way, no interaction between factors was observed in the other species in any of the physiological traits considered (**Table 4**).

Some photosynthetic variables, F_v/F_m, Alpha and E_k, were significantly affected by the nested blocking variables in *T. hemprichii* and *C. serrulata* (**Table 4**). Therefore, the high variability between enclosures may have confounded some effects of the temperature and nutrient treatments.

Relationships Between Biochemical, Morphological, and Physiological Seagrass Traits

We explored all correlations among biochemical, morphological and physiological traits within each of the three studied species (**Supplementary Tables 2–4**). After examining the data we recognize some correlations that were interesting to understand how trait responses differed among the three species (**Figure 4**).

Thalassia hemprichii showed no correlations between photosynthetic traits and growth, while leaf nutrient content was positively correlated with those traits (**Supplementary Table 2**). Specifically, leaf C content was positively correlated with $_rETR_{max}$ (**Figure 4A**). *T. hemprichii* was also the only species showing positive correlations between AG and BG traits, both through its nutrient content and photo-physiological traits (**Supplementary Table 2**), such as, for instance, between FAAs in leaves and the root N (**Figure 4E**).

Cymodocea serrulata showed positive correlations among physiological traits, while negative correlations between the AG and BG nutrient contents were observed (**Supplementary Table 3**). Particularly, leaf nutrient contents were negatively correlated with leaf morphological traits (**Figure 4C**), but in terms of photosynthesis or growth, no relations with leaf physiology were detected (**Figure 4A**). On the contrary, photosynthetic traits were positively correlated

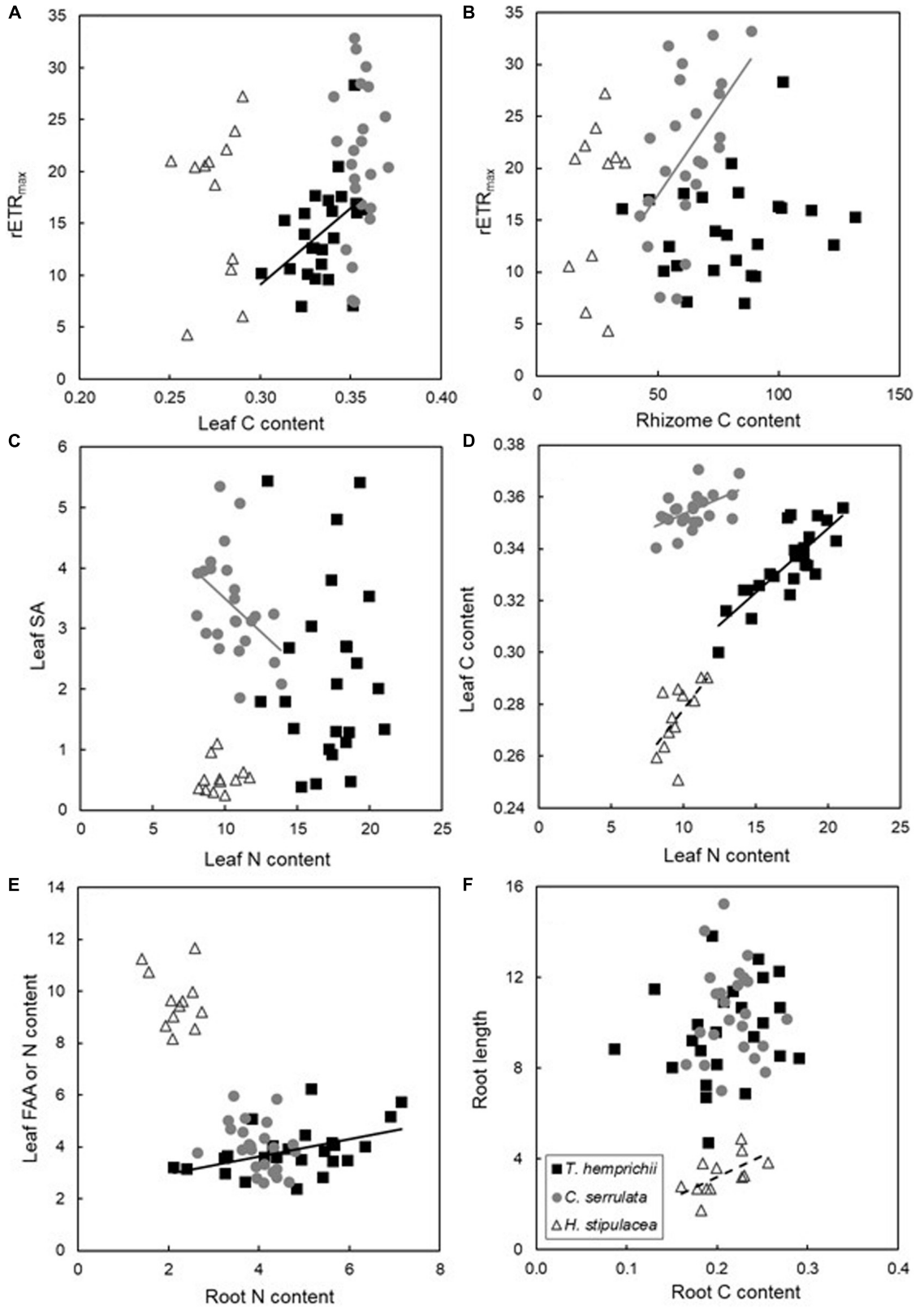

FIGURE 4 | Pearson ρ correlations among selected biochemical **(A–F)**, morphological **(C,F)** and physiological **(A,B)** traits (**Supplementary Tables 2–4**). Correlation lines are shown only when significant as black, gray and dashed lines for *T. hemprichii, C. serrulata* and *H. stipulacea* data respectively. Panel **(E)** show leaf FAA content excepting for *H. stipulacea* that shows leaf N content.

with leaf morphology and rhizome nutrient content. For example, $rETR_{max}$ and rhizome C content were positively correlated (**Figure 4B**).

Contrary to the other two species, all *H. stipulacea* correlations, when observed, were positive (**Supplementary Table 4**). Correlations between leaf length and BG C content, including sucrose, and E_k and root length were detected, linking AG and BG traits (**Supplementary Table 4**). Correlations between the same plant part were frequent, such as between leaf N and C contents (**Figure 4D**), or root C content and length (**Figure 4F**). But no correlations between photosynthetic performance and leaf morphometrics or nutrient content were detected (**Figures 4A,C**).

DISCUSSION

The present study aimed to examine the interactive and single effects on an extended exposure to the current ambient maximum temperature and nutrient enrichment in three tropical seagrass species. During the 5-week period, the two factors and their interaction differentially affected the biochemical, morphological and physiological traits considered, highlighting the varying strategies and tolerances among species to the treatments (**Figure 5**). Therefore, this study shows that *T. hemprichii*, *C. serrulata* and *H. stipulacea* have distinctly different responses to increasing temperature and nutrient enrichment despite their overlap in their distribution and the co-inhabitance of seagrass meadows. This suggests that ecosystem functioning and seagrass survivorship will be differently affected by changing environments.

Seagrass Responses to an Extended Exposure to the Maximum Ambient Temperature

Temperature was the main driver of seagrass responses during this study, as all trait categories showed some response to the single effect of this factor, and these responses showed overall bigger differences than responses to nutrients (**Figure 5**). In accordance with previous studies we did not observe any variation in F_v/F_m (maximum quantum yield) from reference values in any of the three species, showing that the highest target temperature (31°C) is lower than the maximum tolerance limits of the studied species (Campbell et al., 2006; Pedersen et al., 2016; Collier et al., 2017; Anton et al., 2020). However, the photosynthetic capacity ($rETR_{max}$), related with the investment in biochemical mechanisms for CO_2 fixation, is lower under HT treatments in the three species studied, showing some down-regulating mechanisms, and suggesting that all individuals were above their thermal optima.

In this study, *C. serrulata* and *H. stipulacea* showed photo-acclimation mechanisms to maximize C fixation and obtain extra energy, with lower saturating irradiance (E_k) and maximal photosynthetic efficiency (Alpha), as shown in congeneric species (Torquemada et al., 2005; Campbell et al., 2007). Contrary to this, and in accordance with previous studies, no physiological acclimation was observed in *T. hemprichii* under HT treatments (Campbell et al., 2006; Pollard and Greenway, 2013; George et al., 2018). Instead, this latter species showed a high morphological plasticity (longer leaves and sheaths and faster growth rate) that might maximize exposure of photosynthetic material to irradiance and minimize boundary layer thickness for gas, lowering its thermal tolerance. The ability of *T. hemprichii* to change its morphology without enhancing its photosynthetic performance, suggests this species my rely on its high N storage capacity (Viana et al., 2019a) shown by the high plant N content in comparison to the other two species (**Figure 1**). Similar to *T. hemprichii*, the opportunistic species *C. serrulata*, also enhanced its morphology during the study period. But contrary to the former species, *C. serrulata* was able to obtain greater energy from photosynthesis suggesting that the thermal limit of this species is higher than for *T. hemprichii*. While thermal optima values in the literature are highly variable even within species, our results are in accordance with previous findings in *T. hemprichii* from the Red Sea in which the optimum temperature for the metabolic rates was 30.4°C (Anton et al., 2020). Instead, experiments with *C. serrulata* showed large declines at 35°C or lower temperatures at an individual and community level (Collier et al., 2018; George et al., 2018; Burkholz et al., 2019).

The results in this study showed that the tolerant species *H. stipulacea* also acclimated to HT treatments similar to *C. serrulata*. This acclimation was performed both by increasing its Alpha values and, contrary to *C. serrulata*, with smaller-sized plants (lower leaf and root length). The decrease in BG growth is a common strategy in seagrasses plants to adjust their productivity to environmental resources (Alcoverro et al., 2001). Shorter IL, as a proxy of higher shoot and root density, is a common feature under stressful environmental factors, suggesting that *H. stipulacea* invests excess energy (increased Alpha values) in increasing number of shoots (Jensen and Bell, 2001; Kilminster et al., 2008) rather than on leaf length. In the literature, *H. stipulacea* thermal optima and limits showed differences between populations (Nguyen et al., 2020; Wesselmann et al., 2020) but thermal optima at the Red Sea was set at 30°C (Anton et al., 2020; Wesselmann et al., 2020). It is possible that these differences highlight the importance of the different population responses in coping with stressors (McMillan and Phillips, 1979; Winters et al., 2011; Nguyen et al., 2020; Wesselmann et al., 2020) where other environmental factors, such as light irradiance, or population genetics influence the responses (Collier et al., 2016).

Therefore, projections under future climate change scenarios might vary among co-habiting species, and within the same species, depending on the local adaptations and acclimation of seagrasses across geographical ranges. Morphological plasticity was a key acclimation response of the three species, but *H. stipulacea* and *C. serrulata* were the species that better acclimated to HT temperatures by primarily changing a combination of their biochemical, physiological and morphological traits. *T. hemprichii*, a climax species, which is expected to show slower acclimation times, surprisingly

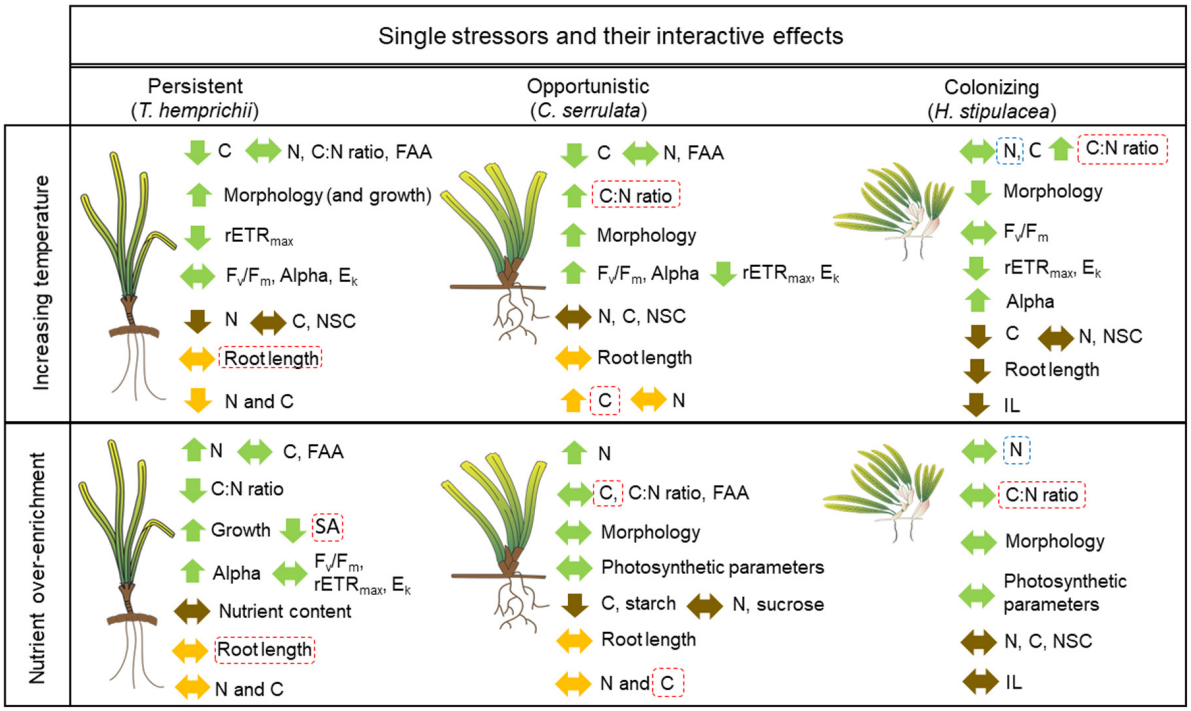

FIGURE 5 | Schematic representation of the significant responses ($P \leq 0.05$) to increasing temperature (31°C), nutrient over-enrichment and their antagonistic (dashed red lines) and synergistic (dashed blue line) interactive effects of leaves (green arrows), rhizome (brown arrows) and roots (orange arrows) in *T. hemprichii, C. serrulata* and *H. stipulacea*. Upward arrows show enhanced, downward arrows show depressed and horizontal arrows show no changes in the responses under single factor effects. (FAA, free amino acids; SA, surface area; NSC, non-structural carbohydrates; IL, internode length) Seagrass drawings are courtesy of the Integration and Application Network (www.ian.umces.edu/symbols/).

acclimatized to new environmental conditions by varying primarily its morphology, in the same time frame as *H. stipulacea*, a colonizing species. Further studies on *T. hemprichii* physiology are needed to understand its acclimation strategy and high plasticity.

Seagrass Responses to Water Column Nutrient Enrichment

In tropical environments, nutrients, as N, P or even Fe, are usually the limiting factor to growth (Pérez et al., 1991; Duarte et al., 1995; Agawin et al., 1996). Even though native species from these areas are adapted to survive in extremely low nutrient habitats, they usually respond to *in situ* or experimental nutrient additions by increasing their nutrient content, but also in terms of physiology and morphology, as shown in this study (**Figure 5**).

Although there were no changes in N content in the BG tissues, the enhanced leaf N content in *T. hemprichii* and *C. serrulata* suggests that they were nutrient limited. These differences between tissues might be explained because we enriched the water column but not the sediment, so it would take more time for the sediment to become nutrient enriched and to see changes in N storage in BG tissues. In contrast, the absence of biochemical and other trait responses both in AG and BG tissues of *H. stipulacea* suggests that this species was not N-limited. This interesting result might be related to the smaller size of *H. stipulacea*, which is related to the lower N demands, with lower N uptake and assimilation rates compared to the other two species, even under high N treatments (Alexandre et al., 2014; Viana et al., 2019a). But, surprisingly, *H. stipulacea* has been spotted in highly eutrophic areas where native species are not able to grow (Van Tussenbroek et al., 2016; Winters et al., 2020) showing significantly higher leaf N content and enhanced morphology compared to control sites (Mejia et al., 2016; Beca-Carretero et al., 2020). These differential trait responses between natural populations and this study suggests that trait's plasticity under nutrient-enrichment might be an adaptative response of the population to long-term changes rather than an acclimation response at an individual level. Although interspecific competition was not purposely studied, due to the experimental design in which all seagrasses were planted together, we cannot rule out competition among the primary producers in the aquaria for nutrient resources (Artika et al., 2020). *H. stipulacea* may be outcompeted for resources due to its smaller size when compared to the leaf and sheath length of *T. hemprichii* and *C. serrulata.* However, there are several occasions where the species forms mixed meadows with other seagrass species or macroalgae (Boudouresque et al., 2009; Sghaier et al., 2011; Gambi et al., 2018; Apostolaki et al., 2019).

As N and P are normally the limiting nutrients in the tropics, C could potentially become the limiting factor under HN scenarios (Buapet et al., 2013; Apostolaki et al., 2014). Therefore, species

with large C storage pools, such as *T. hemprichii*, might have their C demands already covered in all tissues (including leaves), and nutrient over-enrichment might first increase its photosynthetic efficiency (Alpha) and afterward enhance growth rate without C being limiting. Contrary to *T. hemprichii*, the opportunistic species *C. serrulata* followed a common strategy that mobilizes the rhizome carbohydrate reserves to perform photosynthesis (**Figure 4B**) and synthesize amino acids leading to lower levels of starch in BG tissues (Invers et al., 2004; Leoni et al., 2008). The correlation between $rETR_{max}$ and leaf C in *T. hemprichii* (**Figure 4A**) also supports the hypothesis that this species does not depend on storage tissues. Together with C storage depletion, *C. serrulata* showed no enhanced growth, and lower saturating irradiance (E_k), which are all typical responses to nutrient inputs in both tropical and temperate seagrasses (Martínez-Crego et al., 2008; Jiang et al., 2013).

Overall, *T. hemprichii* performed better than *C. serrulata* under short-term nutrient enrichment (> 5 weeks), while *H. stipulacea* showed a lack of response, which indicates a dormancy state typical for this tolerant species (Apostolaki et al., 2018; Hernández-Delgado et al., 2020).

Combined Effect of Different Drivers: Stressors Do Not Act Alone

Our results show that the interaction between both factors is limited to responses in morphological and biochemical traits, as no changes were observed in the physiological performance of the seagrasses, at least during the short-term experimental period (**Figure 5**). This study provides the first insights of the interactive effects of nutrient over-enrichment and increasing temperature in tropical species, therefore comparisons with other tropical species of other geographic areas is still not possible. While synergistic effects are frequent in coastal ecosystems, including seagrasses, in this study we mainly detected antagonistic interactive effects of the studied stressors (Gunderson et al., 2016; Stockbridge et al., 2020). Therefore, the results within this study show that the impact of increasing temperature average values will potentially be less detrimental to plant traits in tropical nutrient over-enrichment seagrass meadows in the short-term. However, this study does not take into account other biogeochemical processes taken place during the eutrophication process, as sediment anoxia or light deprivation (Burkholder et al., 2007).

Interactions between both factors were observed in both AG and BG tissues. The effects of warming on *C. serrulata* and *H. stipulacea* leaf C:N ratio were significantly mediated by nutrient over-enrichment, showing lower values than expected if effects would be additive (**Figure 1**). The enhanced productivity caused by higher temperatures in these species suggests that C limitation may occur in both AG and BG tissues when other nutrients, namely N and P, are not limiting in the water column. This would not be deleterious for these species, as C:N ratio values observed under the HT + HN treatment were, overall, closer to values in the control (LT + LN treatment) showing less unbalances among tissues than it would be expected under synergistic interactions. While leaf N content in *H. stipulacea* did not respond to any single-factor treatment, it showed the only synergistic interactive effect in this study. This shows that temperature might mediate responses to N over-enrichment through enhancing N metabolism, as shown for the temperate species *Z. marina* (Alexandre et al., 2020). In the literature, biochemical responses to the interactive effect of temperature and nutrients are variable, and have also been observed in leaves and BG nutrient content of *Z. marina* (Touchette and Burkholder, 2002; Moreno-Marín et al., 2018), but not in *C. nodosa*, or other *Zostera* species (Kaldy, 2014; Brodeur et al., 2015; Egea et al., 2018; Mvungi and Pillay, 2019; Ontoria et al., 2019b).

The interactive effects of both factors in *T. hemprichii*, contrary to the results of the other two species, were related to morphological traits. Leaf SA and root length values under the HT + HN treatment decreased drastically, showing an antagonistic effect of both factors. Morphological plasticity has been suggested to have adaptive advantages in heterogeneous environments, allowing organisms to maximize resource acquisition under unpredictable or changing levels of resource availability (Houston and McNamara, 1992). In this sense, the high plasticity shown by this species, both under single and cumulative stressors, is highly remarkable, especially when compared to *C. serrulata*, indicating that *T. hemprichii* can adopt a functional role closer to opportunistic or climax species when necessary. Interactive effects on morphology have been observed in leaf SA of *Z. marina*; leaf length of *Z. capensis* (Bintz et al., 2003; Mvungi and Pillay, 2019), and more frequently under natural conditions in *T. hemprichii* and other tropical species (Udy et al., 1999; Ali et al., 2018). Even though the mechanisms behind this interaction cannot be elucidated within the current study, high physiological activity in terms of photosynthesis and nutrient uptake and assimilation under enhanced temperature and nutrient over-enrichment (Touchette and Burkholder, 2007; Kaldy, 2014; Alexandre et al., 2020) may cause this limitation of energy to enhance growth, and therefore, morphology. Also, increasing organic matter in sediment has been related with lower BG growth (Ontoria et al., 2019b), although the low nutrient concentrations in sediment in this experiment does not sustain this hypothesis as the main reason. The interaction of both factors might be positive for the species survival, as no large differences in morphological traits might infer smaller differences between AG and BG biomass, one of the main reasons behind the increasing mortalities in seagrass meadows (Collier and Waycott, 2014).

In this study, physiological traits showed little variance under combined stressors in the three species studied. These results are in accordance with other studies with temperate species that showed no interactive effects on fluorescence parameters or growth (Bintz et al., 2003; Moreno-Marín et al., 2018; Ontoria et al., 2019b). However, in some studies significant interactions were found for yield, growth (Kaldy, 2014; Mvungi and Pillay, 2019; Ontoria et al., 2019a), or photosynthetic rate, as well as gross production rates (Egea et al., 2018; Moreno-Marín et al., 2018). Comparison among studies is difficult due to the variable traits measured; and care must be taken when

comparing results that differ in exposure period, the intensity of the stressors (e.g., nutrient concentration used), or even the different geographical areas of the species, as all these factors, among others, might affects the plasticity of seagrass traits.

It is generally accepted that plant acclimation follows a sequence of ordered changes that starts in the photosynthetic apparatus, followed by biochemical changes, as nutrient content, morphological changes and finally the population and community changes (McMahon et al., 2013; Roca et al., 2016). Such sequence will be also slower in foundation or persistent species, which are recognized to show higher physiological resistance than opportunistic or tolerant species. The simultaneous study of species with different life-history traits and trait categories have shown that different species might show different response and acclimation strategies. Therefore, despite the increasing number of studies on interactive effects of stressors, this study shows the need of further research to extract further conclusions on how seagrass meadows will respond to multiple stressors.

Ecological Implications

The varying responses of the three tropical species studied might imply that there are winners and losers under the changing scenarios studied. For instance, changes in biochemical traits have been observed under single-factor and cumulative treatments, especially in the large-blade species in this study. AG nutrient changes have been related with changes in palatability and herbivory preferences which might affect the food web structure and top-down pressure, affecting the resilience capacity of seagrass meadows (Jiménez-Ramos et al., 2017). Also, although C concentrations in AG tissues could be unaffected by changes in environmental nutrient concentrations (Brodeur et al., 2015), increases in C content with increasing dissolved N or P can occur (Lee and Dunton, 1999; Ali et al., 2018; Mvungi and Pillay, 2019) (**Figure 4D**). In fact, the ability of *T. hemprichii* to take advantage of the excess nutrients by increasing its photosynthetic efficiency suggests a dependence between nutrient limitation and photosynthetic C incorporation related to the synthesis of photosynthetically involved molecules such as chlorophyll, on nutrient supply (Agawin et al., 1996). This can result in a biochemical unbalance caused by the allocation of photosynthetically fixed C to leaf production at the expense of nutrient-deplete BG tissues.

The diminishing C concentrations in rhizome, as observed in *C. serrulata* under HN treatments, may ultimately affect C storage and nutrient retention, which are also important ecosystem services provided by seagrasses (Nielsen et al., 2004; Fourqurean et al., 2012), as C storage plays a key role in mitigating anthropogenic CO_2 emissions under the current climate change scenario. The biochemical disproportion caused by these stressors might eventually cause a biomass imbalance, increasing the AG:BG biomass ratios that will reduce the sediment stabilization or oxygenation capacity (Pedersen et al., 1998; Christianen et al., 2013), which are essential ecosystem services provided by seagrass meadows. Moreover, internal C reserves, as sugar and starch concentrations in the rhizome, are important energy suppliers under stressful events such as physical perturbations, shading or nutrient limitation (Soissons et al., 2018). Therefore, the diminishing C storage products would also be expected to exacerbate seagrass decline and to depress the ability of plants to survive dehiscence and dormancy periods (Burkholder et al., 2007). In fact, *T. hemprichii*, and not *C. serrulata,* was present in a highly eutrophic seagrass meadow in the Stone Town area (Zanzibar Archipelago, Tanzania), confirming that the former species have higher tolerance to these conditions even under long-term natural conditions (Teichberg et al., *in preparation*). Similarly, *T. hemprichii* was pointed as the most persistent species in other highly impacted seagrass meadows in China (Thomsen et al., 2020). Therefore, the unequal effect of eutrophication in different species might cause a biodiversity loss in tropical seagrass meadows.

Enhanced leaf morphological traits, primarily observed in *T. hemprichii*, may limit the biomechanical properties and the exposed surface area of the blades, which directly affect survival under high water dynamics during storms (La Nafie et al., 2012). *H. stipulacea*, contrary to the large blades species, did not appear to be negatively affected by the studied factors, showing potentially a dormancy strategy under the different treatments. Even though performance differences have been observed between individuals from its native and invasive ranges (Nguyen et al., 2020; Wesselmann et al., 2020) this species is generally recognized to be tolerant to a wide range of trophic conditions and temperatures (Winters et al., 2020). Although *H. stipulacea* is considered as invasive in the Mediterranean and Caribbean Seas, there is no evidence of competition and displacement of native species in these areas (Boudouresque et al., 2009; Al-Rousan et al., 2011; Gambi et al., 2018; Apostolaki et al., 2019). In addition, the provision of essential ecosystems services by this little seagrass is still under debate and needs further research (Apostolaki et al., 2019; Viana et al., 2019b; Muthukrishnan et al., 2020).

As the duration and frequency of heat waves has increased worldwide in the last century and is expected to continue increasing (Oliver et al., 2018), *C. serrulata* may show some advantages over *T. hemprichii* under these increasing temperature scenarios, at least under the target temperature of this study. On the other side, *T. hemprichii* may perform better under nutrient over-enrichment scenarios, even at higher temperature. If conditions are too detrimental for these species to grow, in terms of light deprivation or anoxic conditions in the sediment, *H. stipulacea* could potentially colonize the area, as it has been shown to be the most plastic and tolerant species in this study and, in general, among other seagrass species.

Overall, the tropical seagrasses in this study showed different tolerances and strategies to cope with stressors, but these responses could be critical in the long run for the seagrass survival and meadow persistence, compromising the maintenance of functions and services provided by the seagrass meadows. Seagrass individual trait responses to stressors are important as a first step to understand the upscaling of ecological consequences of climate change and eutrophication on ecosystem functioning and services. How the individual responses affect

their functioning and their abiotic and biotic interactions, however, is an issue that cannot be answered with this experiment and needs further study.

CONCLUSION

The holistic picture of seagrass responses highlights that the acclimation and resistance mechanisms behind photosynthesis are closely related with the whole-plant physiology, affecting the performance of the species in the long run. Seagrass species sharing the same original geographic area, or even the same meadow, may respond differently to temperature and nutrient conditions due to unique life history traits. *T. hemprichii* was positively influenced by nutrient over-enrichment conditions, while *H. stipulacea* was tolerant to these conditions, and *C. serrulata* was negatively affected. On the contrary, *C. serrulata* showed a better acclimation under HT scenarios than *H. stipulacea* and *T. hemprichii*. Interaction of both factors negatively affected some important traits of the three species.

Therefore, different scenarios might show winners and losers, but there is no trait that makes a winner under all circumstances, suggesting that if conditions change, some species survivorship might be endangered. Tropical seagrass meadows are characterized by high species diversity, but their functional roles are not always clearly interchangeable, as within the species in this study, and displacement of any of the three species may cause a functional or service loss of the seagrass community as a whole (Duarte, 2000).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

MT got the project funding. MT and IV designed the experiment. AM-S and IV carried out the experiment and processed the samples. AM-S and IV analyzed morphological, growth and nutrient data. AM-S analyzed water and PAM data. IV wrote a first version of the manuscript. MT and AM-S made significant contributions to the manuscript and critically revised the different versions of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Projected Rapid Habitat Expansion of Tropical Seagrass Species in the Mediterranean Sea as Climate Change Progresses

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During the last 150 years, the tropical seagrass species *Halophila stipulacea* has established itself in the southern and eastern parts of the Mediterranean Sea. More recently (2018), *Halophila decipiens* was observed for the first time in the eastern Mediterranean, and was described as the second non-native seagrass species in the Mediterranean Sea. We implemented a species distribution model (SDM) approach to (1) hindcast the habitat suitability of *H. stipulacea* over the last 100 years in the Mediterranean basin, and (2) to model the increase in the potential habitat suitability of *H. stipulacea* and *H. decipiens* during the current century under two very different climate scenarios, RCP 2.6 (lowest carbon emission scenario) and RCP 8.5 (highest carbon emission scenario). In addition, a principal component analysis (PCA) and *k*-means cluster based on temperature and salinity drivers were applied to visualize the distance and relatedness between the native and invasive *H. stipulacea* and *H. decipiens* populations. Results from this PCA suggest that the *H. stipulacea* populations of the Mediterranean and Red Sea are likely to be similar. In contrast, *H. decipiens* from the Mediterranean is more related to the Atlantic populations rather than to the Red Sea populations. The hindcast model suggests that the expansion of *H. stipulacea* was related to the increases in seawater temperatures in the Mediterranean over the last 100 years. The SDMs predict that more suitable habitat will become available for both tropical species during this century. The habitat suitability for *H. stipulacea* will keep expanding westward and northward as the Mediterranean continues to become saltier and warmer. In comparison, the SDMs built for *H. decipiens* forecast a restricted habitat suitability in the south-eastern Mediterranean Sea at the present environmental conditions and predicts a progressive expansion with a potential increase in habitat suitability along 85% of

the Mediterranean coastline. The predicted rapid expansion of non-native seagrass species could alter the Mediterranean's seagrass community and may entail massive impacts on associated ecosystem functions and services, impacts that have severe socio-economic consequences.

Keywords: biogeographical changes, global warming, *Halophila decipiens*, *Halophila stipulacea*, invasive spread, salinity, species distribution model

INTRODUCTION

Ocean warming, the increment of frequency and severity of extreme events such as marine heatwaves or storms, increases in salinity, and biological invasions are emerging as severe threats for marine ecosystems, resulting in a significant loss of biodiversity, functionality and associated ecosystem services (i.e., Rilov and Galil, 2009; Gattuso et al., 2015; Oliver et al., 2019). As a consequence, distributional shifts, new biotic interactions, and species extinctions are expected to alter local community compositions (Yang and Rudolf, 2010; Montero-Serra et al., 2015; Kleisner et al., 2017). In coastal ecosystems, seagrasses are key foundation species that create highly productive habitats and nursery grounds for fish and invertebrates, and provide essential functions including CO_2 sequestration, production of organic carbon and nutrient cycling (Duarte et al., 2010; Costanza et al., 2014; Nordlund et al., 2018). Seagrass habitats, however, are being lost worldwide at unprecedented rates in response to climate change and intensification of human pressures on nearshore coastal ecosystems (Waycott et al., 2009). Loss of seagrass ecosystems involves not only the loss of the associated biodiversity, but also leads to the loss of their associated goods and ecological services (Duarte, 2000; Jordà et al., 2012).

The Mediterranean basin is undergoing tropicalization where its waters are becoming warmer and saltier. Seawater warming rates in this enclosed sea are four times faster than the average of the warming rates of coastal waters of the world (Bianchi and Morri, 2003; Borghini et al., 2014), and these rates are predicted to increase as climate change progresses during this century (Somot et al., 2006; Rilov and Galil, 2009). During the last century, sea surface temperatures (SST) in the Mediterranean Sea have increased at an average rate of $+0.04 \pm 0.1$°C per decade, while in the eastern Mediterranean basin these rates were specially high with summer increases of $+0.12 \pm 0.07$°C year^{-1}; surface salinity has increased at an average rate of $+0.015 \pm 0.002$ per decade, and summer increases of $+0.008 \pm 0.006$ year^{-1} in the eastern Mediterranean basin (i.e., Axaopoulos and Sofianos, 2010; Borghini et al., 2014; Ozer et al., 2017).

The opening of the Suez Canal (1869) caused the vast spreading of tropical species from the Red Sea into the Mediterranean (Caspers, 1980). These Lessepsian migrations are expected to increase during this century not only due to the recent doubling of the shipping lane of the Suez Canal which will double the number of ships (Galil et al., 2017), but also, because the current and predicted environmental conditions within the Mediterranean Sea are becoming increasingly more suitable for warm-adapted and high salinity tolerant tropical species (i.e., Bianchi, 2007; Zenetos et al., 2010).

Autochthonous Mediterranean seagrass communities commonly include the large and slow-growing endemic seagrass species *Posidonia oceanica* (L.) Delile and the small and fast-growing species *Cymodocea nodosa* (Ucria) Ascherson, species with different ecological attributes, growth strategies and stress tolerances (e.g., Olesen et al., 2002; Procaccini et al., 2003; Ruiz et al., 2015). *Zostera noltii* and *Ruppia* spp. are also found in more confined areas within the Mediterranean, while *Zostera marina* has a smaller distribution range (Short et al., 2007). The two most abundant species (*P. oceanica* and *C. nodosa*) adopt different strategies in response to temperature increase (Marín-Guirao et al., 2018). *In situ* evidence alongside experimental and modeling studies further confirm that ocean warming will compromise *P. oceanica*'s distribution range as climate change progresses (i.e., Marbà and Duarte, 2010, Marba et al., 2014; Chefaoui et al., 2018; Traboni et al., 2018), while it seems that *C. nodosa* will be resilient to future climate changes in the Mediterranean Sea (Chefaoui et al., 2018; Ontoria et al., 2019).

Native to the Red Sea, Persian Gulf, and Indian Ocean, *Halophila stipulacea* arrived in the Mediterranean Sea shortly after the opening of the Suez Canal (one of the first ever Lessepsian migrants), and for the last 150 years has established itself in most of the eastern and southern Mediterranean Sea (Lipkin, 1975; Gambi et al., 2009). *H. stipulacea* is a small tropical seagrass species that can grow in a wide range of depths (1–70 m), light intensities, salinities (24–70 [partial salinity units]), temperatures (15–34°C), nutrients levels and substrates, making this species an exceptional competitor for resources and space (Steiner and Willette, 2015; Oscar et al., 2018; Beca-Carretero et al., 2020a; Winters et al., 2020). Currently, this species is distributed from the eastern to the central (Sicily coast, Italy) Mediterranean Sea, but it is expected to spread into the west Mediterranean basin as temperature and salinity increase (Gambi et al., 2009; Nguyen et al., 2020). In 2002, *H. stipulacea* was reported in the Caribbean Sea, and during the last 18 years, it has rapidly expanded to most of the Caribbean island nations and has even reached the South American continent (i.e., Vera et al., 2014; Willette et al., 2014; Winters et al., 2020). While its expansion has been so far slow in the Mediterranean Sea (Georgiou et al., 2016), its colonization has been rapid in the Caribbean Sea (i.e., Smulders et al., 2017; reviewed by Winters et al., 2020). Recent studies reported that growth rates from the Caribbean Sea are 8–30 times that of the native Red Sea populations (Azcárate-García et al., 2020; Willette et al., 2020). Recent studies from the Caribbean

Sea have additionally shown that *H. stipulacea* is physically displacing local Caribbean species, spreading its dominance within invaded habitats and changing the Caribbean's seagrass landscape (Willette and Ambrose, 2012; Steiner and Willette, 2015; reviewed by Winters et al., 2020).

A recent study reported the presence of the seagrass *Halophila decipiens* Ostenfeld in Salamina island for the first time (Saronikos Gulf, Aegean Sea, Greece) (38° N), making this species the second non-indigenous seagrass species in the Mediterranean Sea (Gerakaris et al., 2020). *H. decipiens* is considered the only real pan-tropical seagrass species, being broadly distributed in tropical, sub-tropical and warm-temperate nearshore systems in both the southern and northern hemispheres (i.e., Short et al., 2007). *H. decipiens* is generally an annual clonal species with a short life span (6 weeks), high turnover rates and an ephemeral nature present only in warmer months of the year and usually arising from seed banks (Fonseca, 1989; Hammerstrom et al., 2006). Gerakaris et al. (2020) suggested that the recently reported population could be only one year old due to the small size of the patchy meadows. The authors hypothesized that these plants were probably introduced through ballast waters of ships moving from the Red Sea to the Mediterranean Sea. Over the last 5 years, *H. decipiens* has also been expanding its distribution into subtropical Brazilian waters of the southern Atlantic (São Sebastião, 23°44S, 45°20W; Gorman et al., 2016, 2020), suggesting the potential invasive character of this species.

The aims of the present study were to (i) improve our understanding of the trait distance and relatedness between *H. stipulacea* and *H. decipiens* populations from the Mediterranean Sea and other worldwide populations, (ii) assess whether increases in SST over the last 100 years in the Mediterranean Sea could partially explain the colonization of *H. stipulacea* in this basin, and finally, (iii) examine the potential habitat expansion of *H. stipulacea* and *H. decipiens* in the Mediterranean basin during this century by implementing Species Distribution Models (SDMs) in relation to SSTs and salinities. SDMs have been widely applied to investigate the potential biogeographical shifts in response to the effects of global environmental changes of native and invasive terrestrial and aquatic species, including seagrass (i.e., Valle et al., 2014; Chefaoui et al., 2018; Beca-Carretero et al., 2020b; Gamliel et al., 2020). Here, we hypothesize that a wide potential expansions of the habitat suitability of *H. stipulacea* and to a lesser degree of *H. decipiens* are expected to occur during this century as the Mediterranean Sea becomes warmer and saltier. Understanding how invasive seagrass species will respond to different scenarios of global changes is particularly important since changes in the Mediterranean seagrass community will entail impacts on associated ecosystem functionality affecting all neighboring trophic levels and entailing severe socio-economic consequences to neighboring human populations. Despite their critical relevance, modeling studies, incorporating a set of traits to investigate the potential expansion of invasive tropical seagrass species in the Mediterranean Sea due to global change, are limited.

MATERIALS AND METHODS

Seagrass Species Occurrences and Environmental Variables

Data of distribution and presence of *H. stipulacea* and *H. decipiens* were obtained from different sources including published literature and online databases (e.g., the Global Biodiversity Information Facility [GBIF], 2020), detailed along with their date of access and DOI in the **Supplementary Material** (**Supplementary Table S1** and **Supplementary Figure S1**).

Temperature and salinity have been identified as some of the most important environmental descriptors controlling seagrass growth, survival and distribution; they control physiological processes such as photosynthesis and respiration, but also affect reproduction, flowering and seed germination (i.e., Lee et al., 2007; Oscar et al., 2018; Beca-Carretero et al., 2020a; Nguyen et al., 2020; Winters et al., 2020).

To hindcast the habitat suitability of *H. stipulacea* over the last 100 years (1920, 1950, 1970, and 2019) in the Mediterranean basin, we used SST data derived from the Extended Reconstructed Sea Surface Temperature (ERSST)[1] (i.e., Meng et al., 2018). This is a monthly global dataset of SSTs, where missing data were filled by applying statistical methods. Collinearity of the environmental variables at a regional scale (presence of *H. stipulacea* in its native areas) were assessed using the Pearson correlation coefficient ($r > 0.85$) (Werneck et al., 2011). We used a final set of 6 environmental and continuous variables of SST including the winter (the months of January–March) and summer (July–September) months. Data were produced with a 2° × 2° resolution (2° is equal to 222 km). The rest of the monthly SST data were excluded due their high collinearity ($r > 0.85$).

To perform the population analysis and the SDMs of both *H. stipulacea* and *H. decipiens*, we used environmental predictors of temperature and salinity available for present (2020) and future climate scenarios (2050 and 2100) of two contrasting greenhouse gas concentration projections including RCP 2.6 (representative concentration pathway; lowest carbon emission) and the RCP 8.5 (highest carbon emission) (IPCC, 2014). Collinearity of the environmental variables at the regional scale (presence of the species) were assessed using the Pearson correlation coefficient ($r > 0.85$). We used a final set of five environmental variables including the mean, maximum and minimum values of SST and maximum and minimum surface salinity, which were continuous variables with a 30 arc-seconds (500 × 500 m) resolution (Tyberghein et al., 2012)[2]. Mean salinity was excluded from the analysis due its high collinearity ($r > 0.85$).

Population Analysis

To visualize distance and relatedness between the native and invasive populations of *H. stipulacea* and *H. decipiens*, we applied a principal component analysis (PCA) based on a *k*-means cluster analysis for each species (Ding and He, 2004). *K*-means

[1] https://www.ncdc.noaa.gov/

[2] www.bio-oracle.org

clustering works by assigning a number of centroids based on the number of clusters given. The combination of CA and *k*-means cluster analysis allowed to reduce the dimensions of the dataset and group the different seagrass populations based on the similarity of their environmental conditions (sea surface temperature and surface salinity). Both statistical techniques, PCA and *k*-means cluster, were based on a Euclidean similarity matrix. Two resemblance levels (ellipses) for the PCA and k-means cluster analysis were applied for each seagrass species (*H. stipulacea* and *H. decipiens*). Green ellipses represent levels of 0.035 and red ellipses levels of 0.07. For *H. stipulacea*, we used populations from the Red Sea, Mediterranean Sea, Caribbean Sea, Indian coast, Eastern Africa and Persian Gulf, which correspond with the known populations to date. For *H. decipiens*, we selected populations from the Red Sea, Mediterranean, Arabian Sea, Canary Islands, Bermuda, Caribbean Sea and South American coast, Australian coast, Fiji, French Polynesia, Gulf of California, Andaman Sea, South Chinese Sea and Java Sea (**Table 1** and **Supplementary Figure S1**).

Species Distribution Models (SDMs)

To determine potential habitat suitability of the two seagrass species in the Mediterranean Sea, we used the MAXENT model, which is based on a maximum-entropy algorithm and has a consistent predictive performance using only occurrence records (Elith and Leathwick, 2009). As there is a potentially wider distribution of *H. stipulacea* and *H. decipiens* in the Mediterranean Sea because these species may be continually expanding into new areas, and also because there are no specific monitoring program that follow-up the spreading of either species in this basin, we consider that models using only occurrence records are more appropriate than models using presence and absence records (Franklin, 2010). MAXENT (Phillips et al., 2006) was run with the default response settings. Firstly, a SDM was preformed to investigate the potential effect of SST rise over the last 100 years (1920, 1950, 1970, and 2019) in the Mediterranean Sea on the habitat expansion of *H. stipulacea* in this basin. To hindcast the habitat suitability of *H. stipulacea* we only used data of the target species in its native area, excluding the records from the Mediterranean Sea (**Supplementary Figure S1**). Secondly, additional SDMs were applied to (i) investigate the potential variation in their biogeographical range experienced during this century, (ii) to determine the main environmental drivers determining the habitat preferences of the target species, and (iii) to assess the annual linear coastline potentially suitable as new habitats for colonization. The model was built with the following assumptions: (i) the distribution of the seagrass (*H. stipulacea* and *H. decipiens*) has remained constant since first records of its distribution, (ii) the presence of the target species is in equilibrium with the current environmental descriptors,

TABLE 1 | Summary of the environmental descriptors, temperature (°C), and salinity (PSU), of the biogeographical regions of *Halophila stipulacea* and *H. decipiens*.

Halophila stipulacea	**Min. Temp.**	**Mean Temp.**	**Max. Temp.**	**Min. Salinity**	**Max. Salinity**
Mediterranean Sea	15.2 ± 1.1	21.4 ± 1.1	28.3 ± 1.2	37.3 ± 1.7	39.2 ± 0.5
Red Sea	21.4 ± 1.7	25.3 ± 1.5	29.4 ± 1.3	37.9 ± 0.9	39.6 ± 0.4
Persian Gulf	17.9 ± 0.9	27.2 ± 0.7	34.4 ± 0.3	35.5 ± 1.6	39.2 ± 0.5
Caribbean Sea	25.9 ± 0.4	27.8 ± 0.2	29.8 ± 0.1	32.4 ± 0.8	36.4 ± 0.2
Eastern Africa	24.9 ± 1.1	27.1 ± 0.8	29.5 ± 0.5	32.7 ± 0.9	35.4 ± 0.1
Indian Ocean	26.3 ± 0.3	28.6 ± 0.4	30.9 ± 0.3	29.7 ± 1.3	35.3 ± 0.1
Average values	21.9 ± 4.2	26.2 ± 2.4	30.4 ± 2.0	34.3 ± 2.9	37.5 ± 1.9
Halophila decipiens					
Mediterranean Sea	13.9 ± 0.0	20.4 ± 0.0	27.7 ± 0.0	32.4 ± 0.0	38.9 ± 0.0
Red Sea	23.5 ± 2.0	27.6 ± 1.4	31.3 ± 1.2	36.2 ± 0.9	39.1 ± 0.7
Canary Islands	18.3 ± 0.2	21.3 ± 0.2	25.0 ± 0.3	36.4 ± 0.1	37.1 ± 0.1
Arabic Sea	23.9 ± 0.3	26.5 ± 0.5	29.8 ± 0.5	35.4 ± 0.1	36.9 ± 0.1
Caribbean Sea	22.1 ± 2.1	26.8 ± 0.7	30.4 ± 0.5	34.9 ± 1.3	36.5 ± 0.3
South American Coast	23.9 ± 0.3	26.1 ± 0.5	28.6 ± 0.5	35.2 ± 0.1	37.0 ± 0.1
Bermuda Islands	18.6 ± 0.0	23.3 ± 0.0	28.5 ± 0.0	36.1 ± 0.0	36.8 ± 0.0
Gulf of California	18.2 ± 0.0	24.8 ± 0.0	30.3 ± 0.0	33.7 ± 0.0	35.2 ± 0.0
French Polynesia	25.8 ± 0.0	27.7 ± 0.0	29.3 ± 0.0	35.3 ± 0.0	36.6 ± 0.0
Andaman Sea	27.8 ± 0.0	29.4 ± 0.0	31.3 ± 0.0	30.3 ± 0.0	33.6 ± 0.0
South Chinese Sea	23.4 ± 0.0	27.3 ± 0.0	30.1 ± 0.0	29.9 ± 0.0	34.3 ± 0.0
Java Sea	27.4 ± 0.0	28.9 ± 0.0	30.5 ± 0.0	30.6 ± 0.0	34.5 ± 0.0
Fiji	24.7 ± 0.01	27.0 ± 0.0	29.5 ± 0.0	33.8 ± 0.0	35.7 ± 0.0
Coral Sea	21.1 ± 1.5	25.4 ± 1.1	29.1 ± 0.7	34.0 ± 0.7	35.9 ± 0.2
Tasmanian Sea	15.3 ± 1.6	19.6 ± 1.6	23.4 ± 0.9	35.3 ± 0.1	35.7 ± 0.1
Southwest Australia	22.6 ± 1.1	27.4 ± 1.1	31.2 ± 0.3	31.6 ± 1.0	35.6 ± 0.1
Northwest Australia	16.8 ± 0.0	19.6 ± 0.0	22.5 ± 0.0	35.2 ± 0.0	35.9 ± 0.0
Average values	21.6 ± 4.1	25.1 ± 3.2	28.6 ± 2.6	33.8 ± 2.1	36.2 ± 1.4

Max, maximum; Min, minimum. Data are presented as means ± SD.

and (iii) there is no exclusion through competition with other species. MAXENT generated a continuous raster file with a pixel value ranging from 0 to 1, with 0 representing the absence of the target species, and 1 representing the highest probability for potential habitat suitability. The predicted probabilities derived from the SDMs were transformed into classified maps with two possible categories: suitable habitat or unsuitable habitat. To assume the potential habitat suitability of the seagrass, we used two different logistic thresholds. To hindcast the habitat suitability of *H. stipulacea* over the last 100 years (1920–2019) based on SST, we used the "10 percentile training presence" threshold which corresponded to 0.13. To model the present and future habitat expansion of *H. stipulacea* and *H. decipiens* we used the logistic threshold "equal training sensitivity and specificity" which corresponded to 0.152 for *H. stipulacea* and 0.278 for *H. decipiens* (**Supplementary Table S2**).

Data of the species records were divided five times into a calibration group (80%) and a validation group (20%). Final maps of distribution (**Figures 1**–**3**) were obtained based on the average of the five independent predictions. The SDMs were evaluated using two different evaluation measures chosen according to the nature of the data and the SDMs. The first evaluation approach was based on the threshold-independent metric "area under the curve (AUC)" "receiver operating characteristic (ROC)"; ROC values between 0.5 and 0.7 indicated that the model is performing poorly; values from 0.7 to 0.9 were considered to have a moderate discriminatory ability, and models with values higher than 0.9 were considered to perform excellently (Manel et al., 2001). The significance of the AUC was tested using a cross-validation procedure covering 100 interactions. The second evaluation approach was the sensitivity parameter, which is the proportion of the presence of the target species correctly predicted by the SDMs (Allouche et al., 2008).

Finally, we assessed the potential annual linear coastline that could become potentially suitable as new habitat for colonization based on SST and surface salinity (km year^{-1}). The annual linear coastline was calculated as the number of new kilometers of coastline which are suitable to be colonized by *H. stipulacea* and/or *H. decipiens*, excluding those kilometers where the SDMs built for the present scenario predicts the distribution of the species. The modeled Mediterranean basin accounts for a total of 36,600 km of coastline.

RESULTS

This study encompassed an updated review of the distribution of *H. stipulacea* and *H. decipiens* in the studied area (**Supplementary Table S1** and **Supplementary Figure S1**). The SDM built to hindcast the habitat suitability of *H. stipulacea* over the last 100 years (1920–2019) (**Figure 1**) had an AUC of 0.97 and a sensitivity of 92% (**Supplementary Table S2**). The SDMs built to model the present and future habitat suitability of *H. stipulacea* and *H. decipiens* (**Figures 2**, **3**) showed a marked discriminatory ability, with AUC values of 0.94–0.96, and a high sensitivity, with MAXENT models accurately predicting 94 and 96% of the records of *H. stipulacea* and *H. decipiens*, respectively (**Supplementary Table S2**). Results of the relative contribution of the environmental descriptors indicated that the most important variables of the SDMs built for *H. stipulacea* were maximum temperature with a contribution of 70.2% and maximum salinity (18.4%) (**Table 2**). While the most relevant environmental descriptors for *H. decipiens* were minimum and

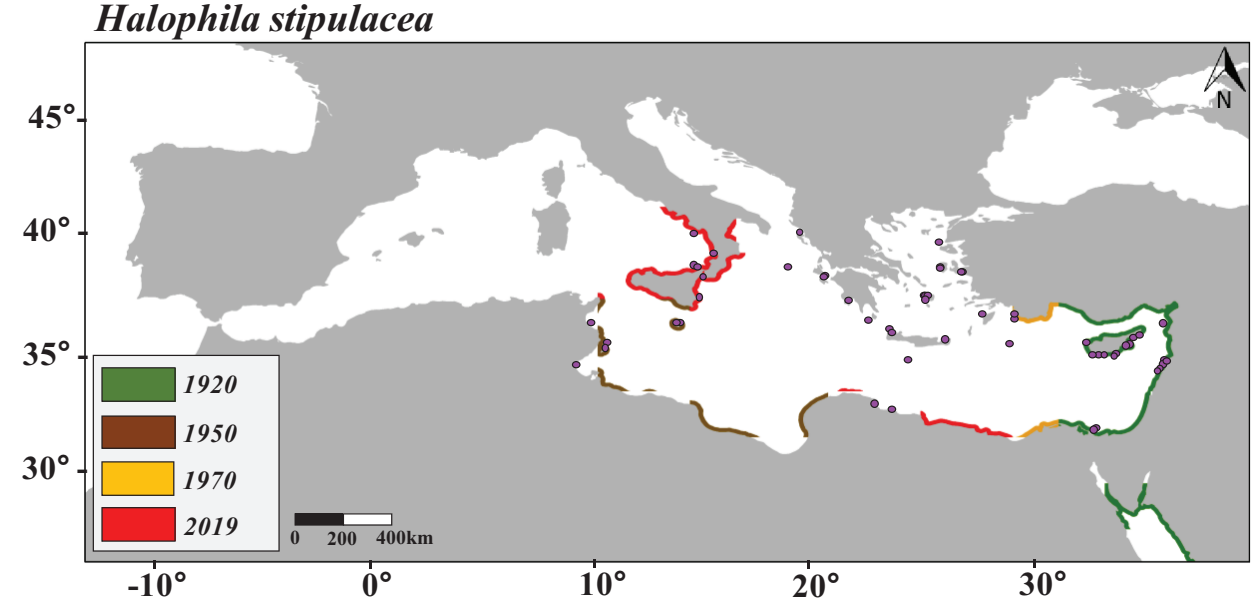

FIGURE 1 | Predicted maps of habitat suitability and distribution of *Halophila stipulacea* over the last 100 years (1920, 1950, 1970, and 2019) based on sea surface temperature (SST). Purple points represent the current distribution of *H. stipulacea* in the Mediterranean Sea.

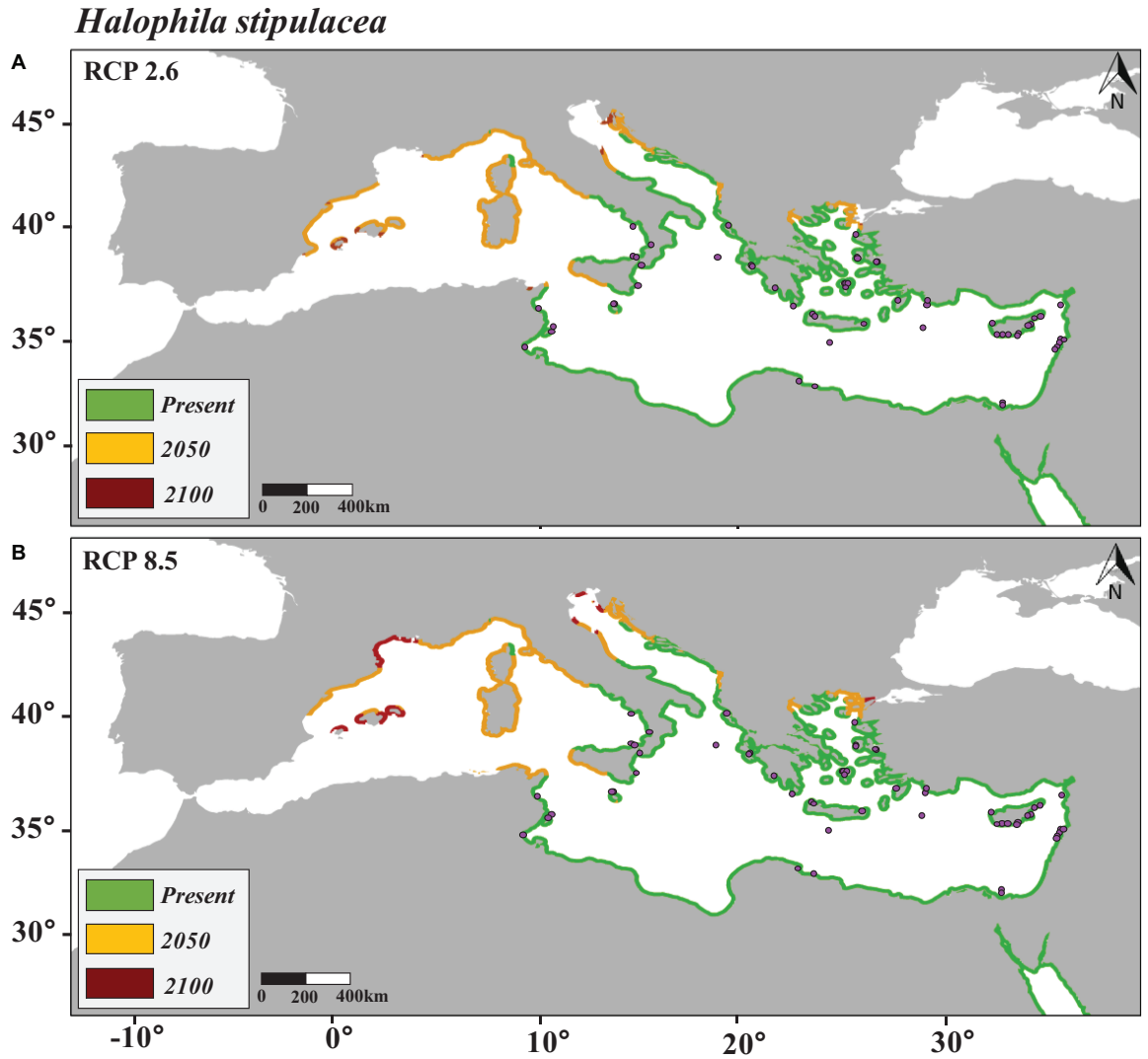

FIGURE 2 | Maps of the distribution of predicted habitat (salinity and temperature) suitability for *Halophila stipulacea* in the Mediterranean Sea under present conditions and under future scenarios of climate change of two contrasting carbon emission projections (RCP 2.6 **[Panel A]** and RCP 8.5 **[Panel B]**) by 2050 and 2100. Note that yellow is additional habitat to the present and red additional to 2050. Purple points represent the current distribution of *H. stipulacea* in the Mediterranean Sea.

maximum temperatures with a contribution of 31.9 and 29.9%, respectively, followed by maximum salinity (25.2%).

The SDM built to hindcast the habitat suitability of *H. stipulacea* over the last 100 years (1920–2019) showed a progressive habitat expansion as the Mediterranean Sea became warmer. Compared with the 2,947.6 km of the Mediterranean's coastline that was predicted by the SDM to be a suitable habitat for *H. stipulacea* in 1920, our SDMs predicted 3,924.2 km in 1950, 4,467.9 km in 1970, and 7,686.6 km in 2019 (**Supplementary Figure S2**). By 2019, the SDM built for *H. stipulacea* adequately predicted the presence of 62% of the Mediterranean records that occur today (**Figure 1**).

The PCA and *k*-means cluster grouped Mediterranean and some of the Red Sea *H. stipulacea* records in the same group, indicating that these populations are exposed to similar environmental conditions (**Figure 4A**). Compared with Red Sea *H. stipulacea* populations, the Mediterranean Sea *H. stipulacea* populations were distributed in significantly colder average temperatures (*t*-test, $p < 0.05$), while there were no significant differences of maximum temperatures (Mediterranean Sea = 29.4 ± 1.3°C; Red Sea = 28.3 ± 1.2°C) (**Table 1**). Mean, minimum and maximum salinities of the Mediterranean and Red Sea populations did not show significant differences. In addition, PCA clustered native *H. stipulacea* populations from the Indian Sea and East Africa coast in the same group with the invasive Caribbean Sea population. In contrast, the PCA of the *H. decipiens* populations reported that the environmental conditions from the Mediterranean Sea

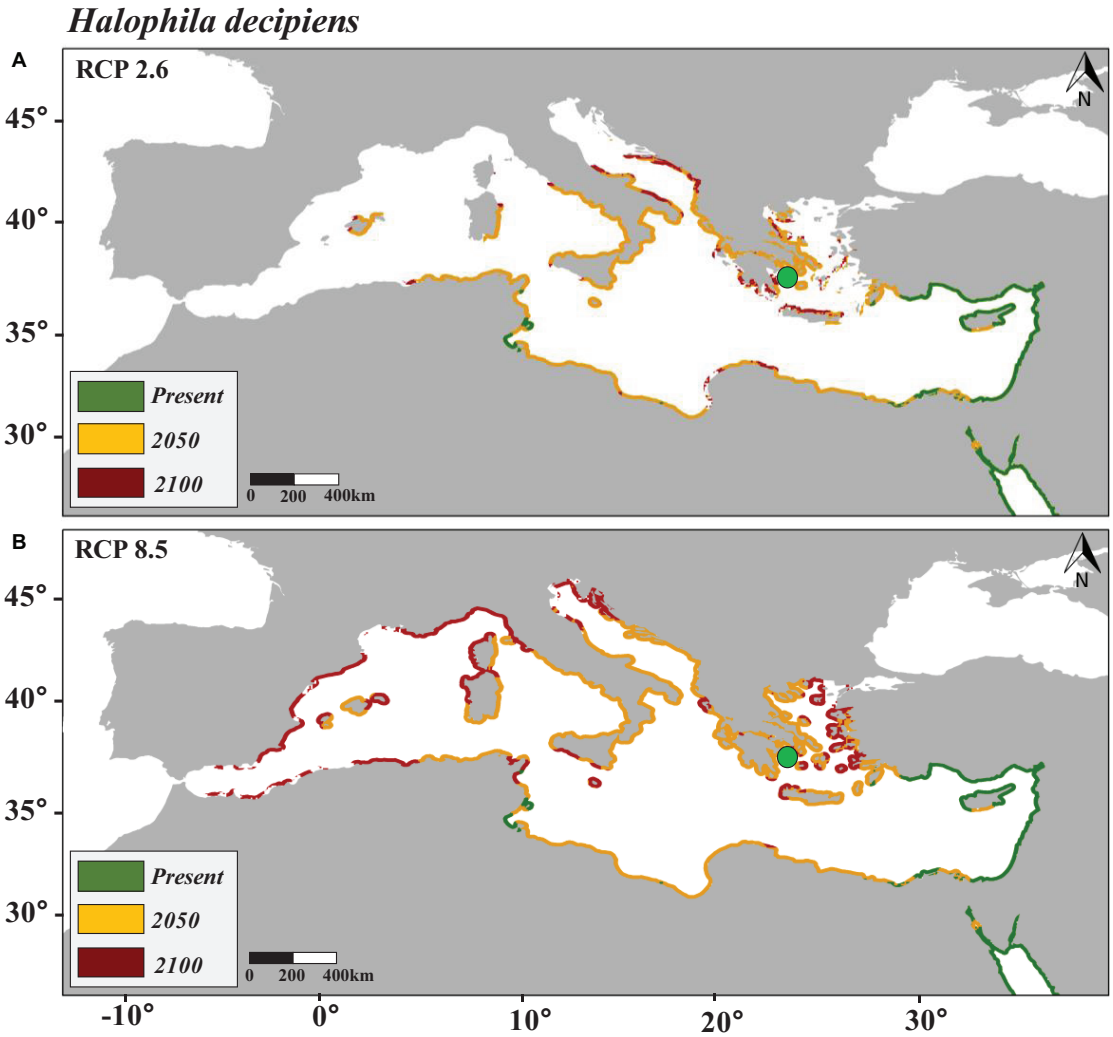

FIGURE 3 | Maps of the distribution of predicted habitat (salinity and temperature) suitability for *Halophila decipiens* in the Mediterranean Sea under present conditions and under future scenarios of climate change of two contrasting carbon emission projections (RCP 2.6 **[Panel A]** and RCP 8.5 **[Panel B]**) by 2050 and 2100. Note that yellow is additional habitat to the present and red additional to 2050. Purple points represent the current distribution of *H. decipiens* in the Mediterranean Sea.

(only one record) were more related to warm-temperate adapted populations, such as populations from Tasmania Sea, southwest Australia or Canary Islands populations than to the tropical Red Sea populations (**Figure 4B**). For instance, the Mediterranean (20.4 ± 0.0°C) and Canary Island (21.3 ± 0.2°C) populations were distributed in significantly (*t*-test, $p < 0.05$) colder habitats than the Red Sea populations (27.6 ± 1.4°C). Similar trends were found for salinity values, whereby the Mediterranean and Canary Islands plants were distributed in significantly (*t*-test, $p < 0.05$) less salty waters than the Red Sea populations (**Table 1**).

The current westernmost distribution of *H. stipulacea* in the Mediterranean Sea was observed in the Palinuro harbour (Italy, 40.03°N, 15.27 E°) (Gambi et al., 2009, 2018; Di Genio et al., 2020) in the central part of the Mediterranean basin, and the coast of Sousse (Tunisia, 35,9° N, 10.6 E) (Sghaier et al., 2014) in the south (**Supplementary Figure S1**). However, the SDM built for *H. stipulacea* for the present climate scenario predicted a habitat suitability in further western areas, such as the Island of Corsica (France, 42.7° N, 8.5° E) (**Figure 2A**). The SDMs built for

TABLE 2 | Scores (%) of the relative contributions of the environmental variables (sea surface temperature and salinity) based on their permutation importance to the Maxent models built for *Halophila stipulacea* and *H. decipiens*.

Relative contribution (%)	*H. stipulacea*	*H. decipiens*
Minimum temperature	4.4	31.9
Mean temperature	1.6	10.4
Maximum temperature	70.2	29.9
Minimum salinity	5.4	2.6
Maximum salinity	18.4	25.2

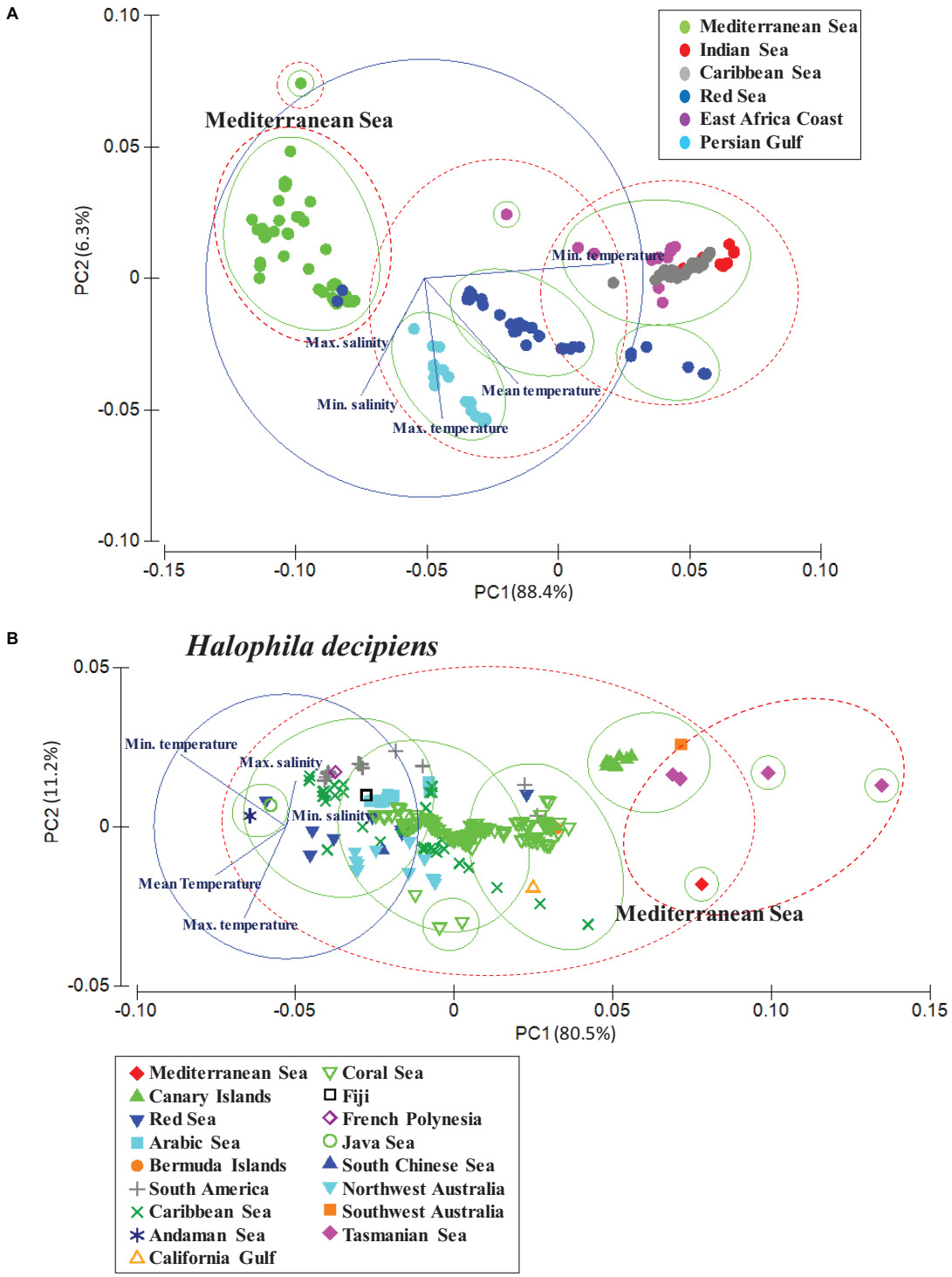

FIGURE 4 | Results of PCA based on the cluster analysis (**Supplementary Figure S3**) revealing the relatedness and dissimilarity of *Halophila stipulacea* **(A)** and *H. decipiens* populations **(B)** in relation to the mean, maximum and minimum values of sea surface temperature (SST) and maximum and minimum of surface salinity (Tyberghein et al., 2012, www.bio-oracle.org).

H. stipulacea under future climate projections showed a marked range expansion into northern and western areas as seawater temperatures and salinity levels increase in the Mediterranean Sea. By 2050, the SDMs predicted an average annual increase of the habitat suitability of 221 km year^{-1} under the scenario RCP 2.6 and 239 km year^{-1} under the scenario RCP 8.5, although

these habitat increase rates significantly decreased after 2050 (87 km year^{-1}) (**Figure 5**). By 2100, the SDM predicted a continued westward expansion reaching areas of western Spain (40.5° N, 1.0° E) and the Balearic Islands. By 2100 under the worst scenario of carbon emission (RCP 8.5), the SDM predicted a habitat suitability of 30,271 km, which represents 83% of the total Mediterranean modeled coastline (**Table 3** and **Figure 2B**).

In comparison, the SDM built for *H. decipiens* for the current climate scenario predicted a habitat suitability of 13,413 km of coastline which represents t 37% of the entire Mediterranean coast (**Table 3**). The fundamental niche (defined as the range of environments where a species is found) of *H. decipiens* in the Mediterranean Sea is divided into two main areas: (i) the south-eastern coast including Cyprus (34.7° N, 33.1° E), and (ii) to a lesser degree, the Strait of Sicily (Tunisia; 33.85° N, 10.37° E) (**Figure 3**). By 2050, under the RCP 2.6 and 8.5 scenarios, the SDMs predicted similar projections; however, by 2100 the SDMs predicted two contrasting scenarios. Between the present distribution and 2050, the models predicted an average increase in habitat suitability of 323 km year^{-1} under both RCP 2.6 and 8.5, primarily connecting areas of habitat suitability predicted by the present climate scenario and expanding into the central and western part of the Mediterranean basin, reaching the Balearic Islands (40.17° N, 3.88° E) (**Figure 5**). In contrast, between 2050 and 2100, the SDMs showed two different predictions for RCP 2.6 and 8.5 carbon emission scenarios. Under the RCP 2.6 scenario, the SDM built for *H. decipiens* predicted a habitat suitability of 19,459 km with a suitable habitat expansion of 198 km year^{-1}, potentially covering 53% of the Mediterranean coastline. Habitat suitability under the scenario of higher carbon emissions of RCP 8.5 is predicted to expand westward and northward into the Mediterranean basin potentially reaching 31,002.7 km of coastline, representing 85% of the basin, with a habitat expansion of 342 km year^{-1} (**Table 3** and **Figure 5**). Interestingly, for both *H. stipulacea* and *H. decipiens* the SDMs of 2050 and 2100 were not predicting the loss of suitable habitat distribution under both scenarios of carbon emission RCP 2.6 and RCP 8.5.

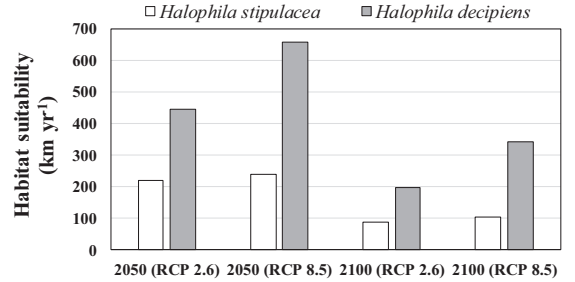

FIGURE 5 | Average length of coastline (km year^{-1}) of suitable new habitat (compatible in terms of salinity and temperature) predicted by the species distribution models built for *Halophila stipulacea* and *H. decipiens* over two periods (2020–2050 and 2050–2100) under future scenarios of climate change of two contrasting carbon emission projections (RCP 2.6 and RCP 8.5).

TABLE 3 | Kilometers of coastline of suitable sea surface temperature and salinity habitat predicted by the species distribution models (SDMs) built for *Halophila stipulacea* and *H. decipiens* under present and future scenarios (2050 and 2100) of climate change of two contrasting carbon emission projections (RCP 2.6 and RCP 8.5).

		2050		2100	
	Present	**RCP 2.5**	**RCP 8.2**	**RCP 2.5**	**RCP 8.2**
H. stipulacea	21,795	28,436	28,956	28,752	30,271
H. decipiens	3,594	17,007	23,299	19,459	31,003

The Mediterranean basin account for a total of 36.600 km of coastline.

DISCUSSION

Future climate projections estimate that marine Mediterranean ecosystems will experience the most substantial worldwide change in biodiversity (i.e., Rilov and Galil, 2009; Huntley, 2017; Corrales et al., 2018). Predicted increasing temperatures of ∼3–4°C and salinity levels of ∼1.0–1.2 PSU by the end of this century in the Mediterranean Sea (IPCC, 2014) could exceed the adaptive capacity of some native species and cause the migration or disappearance of their ecological niche. This will result in local distribution losses and the emergence of new habitats potentially available for colonization by better-adapted species (Jordà et al., 2012). Climate change is expected to compromise the distribution of the large and slow-growing endemic Mediterranean seagrass species *P. oceanica* and, to a lesser degree, the fast-growing *C. nodosa* (i.e., Olesen et al., 2002; Chefaoui et al., 2018; Ruocco et al., 2018; Ontoria et al., 2019). In contrast, increases in temperature and salinity are expected to promote the performance and distribution of the invasive species *H. stipulacea* (Oscar et al., 2018; Nguyen et al., 2020; Wesselmann et al., 2020). Our results clearly show a critical expansion of the biogeographical range of *H. stipulacea* and *H. decipiens* as climate change progresses during this century, with 80% of the Mediterranean coastline predicted to be suitable for colonizing based on temperature and salinity conditions alone. However, there is less evidence of the invasive capacity of *H. decipiens*, as this species was just recently found for the first time in 2018 along the coast of Greece (38°N) (Gerakaris et al., 2020).

Our hindcast model was implemented using only SST, while our SDMs were implemented with only one additional environmental parameter (SST and surface salinity) (i.e., Chefaoui et al., 2018; Wilson et al., 2019; Gamliel et al., 2020). Temperature and salinity are some of the main factors defining the physiological thresholds and distribution of seagrasses (Lee et al., 2007; Marbà and Duarte, 2010), although, other factors (biochemical, physical and biological) may also contribute to determining seagrass habitat ranges. A well-known limitation of SDMs applied to past and future assessments in marine ecosystems is the scarcity of accessible environmental descriptors over large temporal and spatial scales, as well as depicting the multitude of interactions intrinsic for complex natural systems. On local scales biological interactions such as grazing or competition, physical settings (light availability, wave exposure) or chemical water composition (most importantly nutrient

concentration) are decisive if a species can settle and if a population can survive and persist. Therefore, our study should be taken as a proxy for the potential change of the fundamental niche of *H. stipulacea* and *H. decipiens* in relation to SST and salinity. Our SDMs were built on a regional scale; therefore, fine-scale factors were not incorporated into these models, such as biological interactions or dispersion mechanisms alongside some environmental factors including current velocity, wave exposure or sediment type. These, could potentially limit or even enhance the presence and expansion of *H. stipulacea* and *H. decipiens* into new predicted habitats (Greve and Binzer, 2004; Kraan et al., 2020). Previous modeling studies reported that the most important variables defining the distribution of seagrass species for present climate conditions at a local scale were salinity, irradiance, depth and nutrient concentrations (Fourqurean et al., 2003; Kraan et al., 2020). Conclusively, with future large-scale availability of other parameters which have been proven to affect seagrass performance such as pH, irradiance or nutrients (i.e., Lee et al., 2007; Winters et al., 2020) the model complexity will increase and will result in a more accurate spatial prediction of suitable habitats.

While *H. stipulacea* in the Mediterranean Sea is considered a Lessepsian migrant, it is native to the Red Sea, Persian Gulf, and the Indian Ocean (Winters et al., 2020). Results from the PCA revealed that the environmental conditions, at least at the surface, from the Mediterranean populations are actually more similar to Red Sea conditions than to Indian or Persian Gulf waters. Interestingly, PCA results also showed that environmental conditions from the Caribbean *H. stipulacea* populations match more closely to Eastern Africa and Indian Ocean populations than to the Mediterranean populations. Over the last 150 years, the expansion of *H. stipulacea* in the Mediterranean has been related with two main factors, the opening of the Suez canal which allowed the entrance of native species from the Red Sea to the Mediterranean, and the increase of seawater temperatures experienced over the last decades in this region (reviewed by Winters et al., 2020).

Our SDM built to hindcast the colonization of *H. stipulacea* in the Mediterranean basin over the last 100 years suggested that the habitat expansion of *H. stipulacea* was correlated with the recorded increases in SST. The hindcast model predicted the presence of *H. stipulacea* in 62% of the current records in the Mediterranean Sea, while the remaining 38% of the points were not predicted. This may suggest that other environmental factors which are not incorporated in the model may more adequately explain the expansion of *H. stipulacea* into northern latitudes during the last century. Other factor that could constrain the habitat suitability of the target species in northern regions of the Mediterranean Sea is the fact that we only used records of *H. stipulacea* from its native regions to perform the hindcast model. Nevertheless, the model was conducted at a regional scale, and variations of temperature at a local scale due to the local topography, presence of currents or up-welling events may constrain or expand the habitat suitability range of the seagrasses. Yet, the colonization of *H. stipulacea* in the Mediterranean basin has been moderate within an expansion rate of 12 km year^{-1} (Georgiou et al., 2016). Based on the increases in SSTs and surface salinities predicted in the region (discussed above), our SDMs predicted that more suitable coastline habitats will become available to be colonized by *H. stipulacea* with a potential habitat suitability expansion of 239 km year^{-1} by 2050 under the worst carbon emission scenario (RCP 8.5). By the end of 2050, in both scenarios of carbon emission (RCP 2.6 and 8.5), the habitat suitability of *H. stipulacea* is predicted to reach the Iberian Peninsula and the Balearic Islands in the westernmost coast of the Mediterranean basin. The SDMs pointed out that maximum annual temperatures, and to a lesser degree maximum annual salinity, are essential factors determining the fundamental niche of *H. stipulacea* in the Mediterranean Sea. Our results are consistent with recent experimental studies demonstrating the first evidence of optimal acclimatization of native and invasive Mediterranean populations of *H. stipulacea* to projected scenarios of Mediterranean climate change, including temperatures of 30–32°C and salinities higher than 40 PSU (Georgiou et al., 2016; Oscar et al., 2018; Nguyen et al., 2020; Wesselmann et al., 2020). Similarly, native populations from the Persian Gulf are adapted to even more extreme conditions, with *in situ* summer temperatures of 33–34°C and summer salinities of 39.0–39.5 PSU (Tyberghein et al., 2012), defining *H. stipulacea* as an exceptional euryhaline and thermal-tolerant species. Adaptability to such high salinities and temperatures suggests the potential capacity of this species to successfully thrive and compete for space and resources under future climate conditions in the Mediterranean Sea.

From the present scenario to 2100, the increase of suitable areas available to be colonized by *H. stipulacea* is predicted to be moderate, with an annual increase of 97 km year^{-1}. Although this rate is considered slow in comparison with other marine macrophyte invaders (∼300–400 km year^{-1}), such as the macroalgae *Caulerpa* and *Sargassum* spp. (i.e., Lyons and Scheibling, 2009; Mineur et al., 2015), it will provide the opportunity for this species to expand into 83% of the Mediterranean coastal area, with only the north of the Adriatic Sea and the southwest of the basin as environmentally unsuitable areas for its distribution. This habitat restriction is potentially explained by the relatively cold winter conditions and upwelling events that maintain SST lower than 13–14°C, which might represent a physiological minimal threshold for the survival of *H. stipulacea* (Gambi et al., 2009). While the presence of cold waters may represent a barrier to the expansion of *H. stipulacea* into westernmost habitats in the Mediterranean Sea, a recent study identified this region as a habitat refuge for the foundation species *P. oceanica* and *C. nodosa* under future climate scenarios (Chefaoui et al., 2018). Similarly, an even newer study reported that *H. stipulacea* in the Mediterranean Sea can cope with significantly lower temperatures (∼14°C) than what observed in its native habitats (∼23°C), suggesting a thermal niche shift to the lower temperatures in the Mediterranean Sea and potentially allowing this species to colonize temperate regions (Nguyen et al., 2020; Wesselmann et al., 2020). This physiological capability may be explained by the capacity to produce and accumulate high levels of unsaturation in the lipidic structures of the leaves of *H. stipulacea* (Beca-Carretero et al., 2019). In the northern Red Sea, *H. stipulacea*'s lipid composition, which

partially determines the thermal tolerance of primary producers (Murakami et al., 2000), is characterized by high levels of n-3 polyunsaturated fatty acids (PUFAs), more related to species from the Mediterranean Sea (*P. oceanica* and *C. nodosa*) and the temperate species *Z. noltii* (Portugal, 37°N) than to tropical species. Finally, SDMs did not predict any loss in the *H. stipulacea* habitat suitability range under any climate scenario, indicating a potential stable habitat distribution in the Mediterranean basin.

Until recently, the spread of *H. stipulacea* in the Mediterranean basin was considered to be from clonal propagation as these plants exclusively developed male flowers (Procaccini et al., 1999; Gambi et al., 2009). However, the recent documentation of both female and male flowers (Nguyen et al., 2018) and mature seed capsules in the Mediterranean (Gerakaris and Tsiamis, 2015) demonstrates its capacity for sexual reproduction in this invasive habitat, suggesting a potential increase in its genetic diversity. Genotypic diversity, the production of seed banks and seed recruitment have been documented to have positive effects on seagrass production, enhancing resilience and recovery to different stresses, including storms, diseases, or warming events (i.e., Ehlers et al., 2008; Reynolds et al., 2012; Rasheed et al., 2014). The recent enlargement of the Suez Canal (July 2015; Galil et al., 2017) may also favor the arrival of new populations of *H. stipulacea*, further contributing to the interaction between different genotypes. Another factor that can favor the expansion of *H. stipulacea* in the Mediterranean basin is its proved capacity to acclimate and survive in disturbed areas under high nutrient concentrations and human pressures, such as harbors or polluted bays in both native (Red Sea; Beca-Carretero et al., 2020a) and non-native regions (Mediterranean and Caribbean Sea) (Gambi et al., 2009; Willette and Ambrose, 2012; Van Tussenbroek et al., 2016; Beca-Carretero et al., 2020a). However, in an ongoing study we observed that *H. stipulacea* populations from Eastern Africa were highly vulnerable to experimental warming and nutrient increase conditions, suggesting different populations-specific stress-tolerance and resilience capacity (Viana et al. in revision).

While Gerakaris et al. (2020) suggested that *H. decipiens* was probably introduced from the Red Sea to the Mediterranean, our PCA analysis indicated that the environmental conditions of the Mediterranean were more related to the conditions of warm-temperate waters from the south hemisphere or North Atlantic, such as, Canary Islands, Bermuda or Caribbean Sea, and indicated that these populations were more likely to be closely related than that of the Red Sea population. Nevertheless, a genetic study will be necessary to confirm the origin of the Mediterranean population. Our models indicated that *H. decipiens* could become a future invader in the Mediterranean Sea and potentially establish itself in 40–80% of the shallow waters of this basin during this century; however, some factors can compromise its further colonization and stabilization. The habitat expansion of *H. decipiens* was previously reported in warm-temperate Brazilian waters of the southern Atlantic, where this species has been expanding and firmly developing extended monospecific meadows as ocean warming progresses (Gorman et al., 2016, 2020). Similarly, in the Canary Islands, a consistent expansion of *H. decipiens*' distribution range has been shown since 1980 (Pavón-Salas et al., 2000; Ruiz et al., 2015); this was suggested to potentially be associated with the reported increase in SSTs at a rate of 0.2°C/decade since 1970 (Luque et al., 2013).

In the Mediterranean Sea, *H. decipiens* was only reported in Salamina Island (Greece), however for the present climate scenario, it was predicted to grow in one-third of this basin. Our SDMs suggested that based on the environmental conditions required for this species, it could already inhabit other Mediterranean regions. However, the fact that this species was not reported in more Mediterranean coastal areas could indicate that some factors may restrict its entrance and spread in the basin. For instance, the low abundance of *H. decipiens* populations in the Red Sea may restrict its successful colonization into the Mediterranean Sea. Furthermore, two main processes have been related with the success of species invasions: death and reproduction (Williamson and Fitter, 1996; Sakai et al., 2001). In its native habitat, this species can adapt to a wide range of temperatures, including anomalous winter temperatures reaching 13–14°C in Bermuda in the Atlantic Sea (23.3°N, −64.8°O) (Manuel et al., 2013). These observations suggest that Mediterranean winter temperatures will not represent a critical physiological threshold for this species, thus not preventing its survival from year to year. In its native habitat, *H. decipiens* colonizes new areas by propagating via seed transport or vegetative fragments (Hammerstrom et al., 2006). Gerakaris et al. (2020) observed flowers in an early stage of maturity in Greece, suggesting potential expansion via sexual reproduction and seed production, although this hypothesis has not been confirmed. However, if the species does not produce seeds, its settlement and dispersal into the Mediterranean basin may be limited. Remarkably, its recently reported occurrence provides a unique case study to examine whether non-native seagrass species can acclimate in a new habitat and evolve within their new environmental conditions.

The coexistence of native Mediterranean seagrass species (e.g., *P. oceanica*, *C. nodosa*, and *Z. noltii*) with invasive species (such as *H. stipulacea* and *H. decipiens*) enables potential biological interactions between them, and the possibility of generating diverse response mechanisms, such as competition, exclusion, or species facilitation. Indeed, similar questions arise with *H. stipulacea* and *H. decipiens* as the SDMs predicted large areas where their fundamental niche can overlap. To date, both species were observed forming mixed assemblages in the Caribbean Sea (Willette et al., 2014; Winters et al., 2020); however, there is no clear evidence of the consequence of their potential interactions over time. Recent reports from the Mediterranean Sea (eastern Tunisian coast) have demonstrated first signs of species interactions showing a potential physical replacement or displacement of the local seagrass *C. nodosa* by the invasive *H. stipulacea* (Sghaier et al., 2014). Studies from the Caribbean have shown that *H. stipulacea* is physically displacing local Caribbean species (Willette and Ambrose, 2012; reviewed by Winters et al., 2020). However, to date, there are no mechanistic explanations of competition between invasive and native seagrass species. In the context of climate change, the adaptability

of *H. stipulacea* and *H. decipiens* to predicted Mediterranean conditions suggests their potential to successfully thrive and compete for space and resources with native Mediterranean Sea seagrass species. In this basin, studies predicted a potential loss of habitat suitability of *P. oceanica* as climate change advances during this century in the Mediterranean Sea (Marbà and Duarte, 2010; Marba et al., 2014), and to a lesser extent of *C. nodosa* (Chefaoui et al., 2018). The increase in frequency of extreme weather events such as heatwaves, storms and "hurricanes" (Cavicchia et al., 2014) and other local factors such as nutrient pollution, which have been found to be detrimental to native seagrass populations (Danovaro, 2003) in the Mediterranean Sea, will lead to more frequent disturbance of seagrass habitats. Although *P. oceanica* has been shown to be much more resilient to temperature increases than previously thought and may have escape mechanisms such as flowering (Marín-Guirao et al., 2018, 2019), its loss by human-related impacts may provide an opportunity for fast-growing and rapid colonizing seagrass species to spread into these unoccupied areas. Moreover, both *H. stipulacea* and *H decipiens* are some of the deepest-adapted seagrass species worldwide, often occurring at more than 30 m but also growing at a depth of 60–70 m, (i.e., Duarte, 1991; Coles et al., 2009; Winters et al., 2020). This exceptional capacity may allow these invaders to occupy the potential habitat loss of deep-adapted Mediterranean seagrass meadows which were reported to be highly vulnerable to anomalous increments in water temperature (Marbà and Duarte, 2010; Marba et al., 2014). Therefore, as a consequence of climate change, we may predict a possible transition from Mediterranean seagrass communities with relatively large seagrass species, diverse ecological attributes and growth strategies, to a community dominated by small and fast-growing species. The potential re-structuring of the native Mediterranean seagrass communities may lead to impacts on associated goods, ecological services and ecosystem functionality affecting all the trophic levels (i.e., Fourqurean et al., 2012; Jordà et al., 2012; Viana et al., 2019).

With Mediterranean marine ecosystems facing unprecedented anthropogenic threats, it is critical to understand the potential role that invasive species may play in the configuration of new benthic communities. To better monitor the potential spread of invasive seagrass species in the Mediterranean sea and understand their ecological role, we suggest to (i) implement SDMs to develop habitat conservation and invasive species management plans, including environmental risk assessments to potentially anticipate the arrival of invasive species and effectively implement contingency actions, (ii) create specific areas to continually monitor the vegetative development of invasive species and their potential interaction with native species, and finally (iii) increase public awareness of the impacts of invasive species on native ecosystems and gain support from local divers, fisherman and coastal communities.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

MT, GW, GP, and HR developed the theoretical idea of the manuscript and collaborated in the final version of the manuscript. PB-C developed the models and statistics and wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.555376/full#supplementary-material

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Efficient Heat Dissipation and Cyclic Electron Flow Confer Daily Air Exposure Tolerance in the Intertidal Seagrass *Halophila beccarii* Asch

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Seagrasses inhabiting the intertidal zone experience periodically repeated cycles of air exposure and rehydration. However, little is known about the photoprotective mechanisms in photosystem (PS)II and PSI, as well as changes in carbon utilization upon air exposure. The photoprotective processes upon air exposure in *Halophila beccarii* Asch., an endangered seagrass species, were examined using the Dual-PAM-100 and non-invasive micro-test technology. The results showed that air exposure enhanced non-photochemical quenching (NPQ) in both PSII and PSI, with a maximum increase in NPQ and Y(ND) (which represents the fraction of overall P700 that is oxidized in a given state) of 23 and 57%, respectively, resulting in intensive thermal energy dissipation of excess optical energy. Moreover, cyclic electron transport driven by PSI (CEF) was upregulated, reflected by a 50 and 22% increase in CEF and maximum electron transport rate in PSI to compensate for the abolished linear electron transport with significant decreases in pmf_{LEF} (the proton motive force [pmf]) attributable solely to proton translocation by linear electron flow [LEF]). Additionally, H^+ fluxes in mesophyll cells decreased steadily with increased air exposure time, exhibiting a maximum decrease of six-fold, indicating air exposure modified carbon utilization by decreasing the proton pump influxes. These findings indicate that efficient heat dissipation and CEF confer daily air exposure tolerance to the intertidal seagrass *H. beccarii* and provide new insights into the photoprotective mechanisms of intertidal seagrasses. This study also helps explain the extensive distribution of *H. beccarii* in intertidal zones.

Keywords: air exposure, intertidal seagrass, photoprotective mechanisms, non-photochemical quenching, cyclic electron flow

INTRODUCTION

Seagrass ecosystems have a global distribution, offering indispensable ecosystem services (Hemminga and Duarte, 2000; Barbier et al., 2011), including carbon sequestration (Duarte and Krause-Jensen, 2017), sediment stabilization (Bos et al., 2007), and provision of food and habitats, as well as the reduction of pathogenic microorganisms (Lamb et al., 2017). Seagrasses inhabit both inter- and subtidal coastal zones. Intertidal seagrasses, the focus of this study, are periodically exposed to air during low tide. This frequent tidal exposure could result in desiccation, excessive light, and high-temperature stress in such intertidal seagrasses (Kohlmeier et al., 2017; Manassa et al., 2017; Suykerbuyk et al., 2018). Therefore, the ability of seagrasses to tolerate air exposure and regain competence upon rehydration probably determines their distribution in the intertidal zone.

A diversity of photoprotective mechanisms during air exposure has been described in other intertidal plants. In most cases, non-photochemical quenching (NPQ), photosystem (PS)I-driven cyclic electron flow (CEF), and energy redistribution between PSI and PSII play critical roles in photosynthetic plasticity, allowing the plants to survive environmental stress during air exposure (Gao et al., 2011; Zia et al., 2016; Tan et al., 2017). NPQ is a method for heat dissipation associated with quenching of excitation energy similar to overloading optical energy (Müller et al., 2001; De Carvalho et al., 2011; Yamakawa et al., 2012; Yamakawa and Itoh, 2013). It mainly involves a low thylakoid lumen pH- and zeaxanthin-dependent quenching mechanism (Xu et al., 2015). It has been reported that carotenoids and zeaxanthin that accumulate upon desiccation are crucial to the activation of NPQ, as well as protection of the thylakoid membranes from peroxidation (Havaux et al., 2007; Du et al., 2010; Beckett et al., 2012). Additionally, another protection mechanism mentioned above is involved in the compensation for abolished linear electron flow (LEF) (Gao et al., 2011). CEF promotes electron transfer from the acceptor side of PSI back to plastoquinone, thus maintaining ATP production and supplying the proton gradient (Fan et al., 2016; Yamori and Shikanai, 2016). Therefore, it has been proposed that both NPQ and CEF contribute largely to the protective and adaptive mechanisms of intertidal plants (Gao et al., 2011; Zia et al., 2016; Tan et al., 2017).

In previous studies, considerable attention has been paid to the desiccation-tolerant mechanism in seagrasses (Björk et al., 1999; Shafer et al., 2007; Jiang et al., 2014). However, very few studies have attempted to assess the role of cyclic electron transport driven by PSI in protecting the photosystem (Kohlmeier et al., 2017; Manassa et al., 2017; Suykerbuyk et al., 2018). Additionally, it is not yet known whether there is a tradeoff between PSI and PSII photoprotection for seagrasses. Furthermore, elevated gaseous CO_2 concentration with less water resistance during air exposure may greatly affect the carbon utilization in seagrass because of the substantial changes from those of the submerged environment, with a lack of instantaneous parameters to confirm it (Silva et al., 2005; Clavier et al., 2011).

The seagrass *Halophila beccarii* Asch, one of the two species in the oldest lineage of seagrasses and listed as a vulnerable species on the IUCN Red List of threatened species (Short et al., 2011), has a relatively large range, with a patchy distribution in the intertidal areas of the tropical Indo-Pacific region (Aye et al., 2014). Recently, the broader distribution of *H. beccarii* was found in the intertidal zone along the coastline of South China (Jiang et al., 2017, 2020). *H. beccarii* in the high intertidal area has even been associated with land plants (**Supplementary Figure 1**) and endures long-term air exposures. *H beccarii* may show unique photoprotective mechanisms during air exposure. It has been reported that NPQ and cyclic electron transport play critical roles for photoprotection in intertidal plants (Gao et al., 2011; Zia et al., 2016; Tan et al., 2017). However, it is not yet known whether NPQ and cyclic electron transport confer daily air exposure tolerance in the intertidal seagrass *H. beccarii*. Furthermore, it is not known whether HCO_3^- utilization is lowered due to the higher CO_2 concentration with less transport resistance.

Aiming to close some of these gaps of knowledge about intertidal seagrasses, we conducted an indoor experiment to culture the seagrass *H. beccarii* with different periods of daily air exposure to investigate the photosynthetic, morphological, and biochemical responses to air exposure. Furthermore, we compared instant air exposure with long-term daily air exposure to determine whether long-term adaptation is necessary for photoprotective mechanisms. *In vivo* chlorophyll fluorescence and the P700 redox state of leaves were measured using the Dual-PAM-100. Additionally, measurements of the dual-beam 550- to 515-nm signals simultaneously with the Dual-PAM-100 P515/535 served to identify module proton motive force (pmf) and ATP-synthase activity. Moreover, the net H^+ flux at mesophyll cells of *H. beccarii* was detected using non-invasive micro-test technology to determine the changes in carbon utilization (Beer et al., 2014). Our hypotheses include the following: (1) photoprotective processes in *H. beccarii* require long-term adaptation to air exposure; (2) enhanced NPQ together with elevated CEF could contribute substantially to photoprotection during air exposure; (3) upon air exposure, leaves would downregulate H^+ fluxes at mesophyll cells to lower HCO_3^- utilization, based on a tradeoff between elevated gaseous CO_2 concentration and the reduced demand for carbon sources.

The results obtained in this study will undoubtedly strengthen our understanding of the photosynthetic plasticity and adaptive mechanisms of intertidal seagrasses.

MATERIALS AND METHODS

Plant Materials and Experiment Design

Halophila beccarii was collected with its natural sediment using a PVC corer at the intertidal zone of the monospecific seagrass bed in Yifengxi, along the South China coast (Jiang et al., 2020). Following collection, seagrasses were placed into 16 glass tanks (290 × 160 × 190 mm) with natural seawater in a closed system. For the laboratory acclimation, seagrasses were cultured at 80 μmol photos m^{-2} s^{-1} to simulate natural light intensity for 1 week. After initial laboratory acclimation, *H. beccarii* was cultured with different periods of daily air exposure, including a CT (control group without any air exposure), ST (short time, 1 h of daily air exposure), MT (middle time, 2 h of daily air exposure),

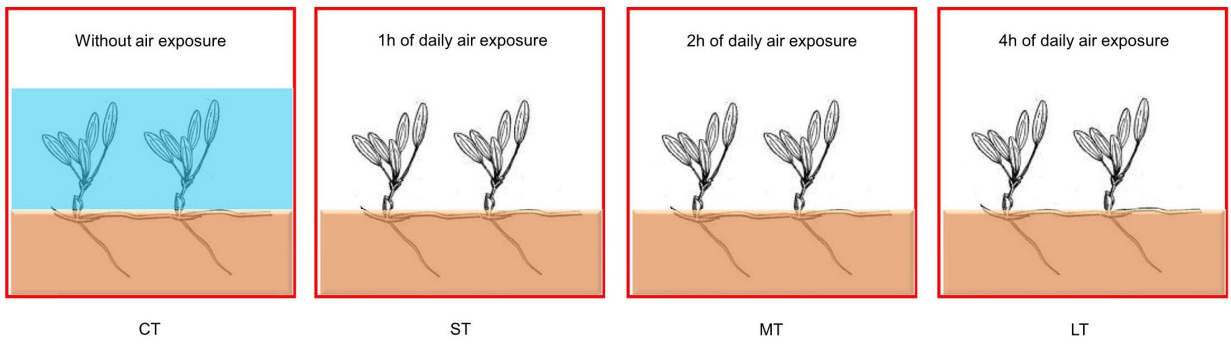

FIGURE 1 | Experimental set-up of the laboratory treatment. CT: Control group without any air exposure; ST: 1 h of daily air exposure; MT: 2 h of daily air exposure; LT: 4 h of daily air exposure.

and LT (long time, 4 h of daily air exposure) (**Figure 1**) to imitate the tidal exposure from the high tide area to the low tide area. The average air temperature and humidity were maintained at 20°C and 60%, respectively. In this study, air exposure treatment did not simulate changing temperatures or light intensity. The experimental design emphasized the effect of desiccation, which was in agreement with natural observations. According to the statistical analysis of low tide when seagrasses emerged during 2018–2019 (**Supplementary Figure 2**), the exact timing at low tide in the daytime primarily occurred between 5:00 and 8:00 a.m. or between 7:00 and 9:00 p.m.; at these times, intertidal exposure to irradiance and the air temperature was expected to be relatively low. After 4 weeks of daily tidal simulation treatment, photosynthetic and other physiological parameters of *H. beccarii* were detected. In particular, we also measured the photosynthetic parameters of seagrass in the CT with instant air exposure of 2 h (Ins-2 h) and 4 h (Ins-4 h), to compare the response mechanism with that of the long-term daily air exposure.

Chlorophyll Fluorescence and P700$^+$ Signal Measurement

In vivo chlorophyll fluorescence was measured using the Dual-PAM-100 (Heinz Walz, Effeltrich, Germany). Plants were dark-adapted for 30 min before measurement, and three randomly chosen leaves were detached. The actinic light for measurements of Chl fluorescence was 131 μmol photons m^{-2} s^{-1} (635 nm). The chlorophyll fluorescence parameters were calculated as follows: $F_v/F_m = (F_m–F_o)/F_m$, Y(II) = ΦPS II = $(F_m'–F_s)/F_m'$ = Fq'/Fm', NPQ = $(F_m–F_m')/F_m'$ = F_m/F_m'–1, Y(NPQ) = $F/F_m'–F/F_m$, Y(NO) = F/F_m (Lu et al., 2020). F_o and F_m are the minimum and maximum fluorescence after dark-adaptation, respectively. F_o' and F_m' represent the minimum and maximum fluorescence after light adaptation, respectively. F_s is the light-adapted, steady-state fluorescence. For PSI: Y(I) = $(P_m'–P)/P_m$, Y(NA) = $(P_m–P_m')/P_m$, Y(ND) = P/P_m (Baker, 2008; Sun et al., 2020). The CEF value was estimated as the electron transfer rate ETRI-ETRII (Lu et al., 2020).

The redox state of P700 was determined *in vivo* using automated routines provided by the Dual-PAM software (Schreiber, 2008; Lu et al., 2020). The P700 signal was measured during a single turnover flash (200 000 μmol photons $m^{-2}s^{-1}$, 10 μs, PQ pools being oxidized) followed by multiple turnover flashes (20 000 μmol photons $m^{-2}s^{-1}$, 2 ms, PQ pools were fully reduced) in the presence of a far-red (FR) background light. The change in the P700 signal reflected the dynamic of the P700 oxidation state after FR light exposure and P700 reduction after removal of the FR light. Balancing and calibration of the P700 signal using the automated routine of the Dual-PAM-100 software were performed before each measurement. Kinetic measurements of the dark re-reduction of P700$^+$ after turning off the FR light were used for estimation of the half-time ($t_{1/2}$) of dark decay of the P700$^+$ signal.

Measurement of Fast Chlorophyll Fluorescence Induction Kinetics Curves

The fast induction kinetics of chlorophyll fluorescence were monitored using the Dual-PAM-100 (Heinz Walz, Effeltrich, Germany). All formulae and glossary of terms used in the JIP-test in the analysis of the O-J-I-P fluorescence transient are presented in **Supplementary Table 1**. They were used to analyze the changes in the donor side, receptor side, and reaction center of PSII (Straaer, 1995; Stirbet, 2011).

Measurement of Rapid Light Curves

Rapid light curves (RLCs) of rETR (relative electron transport rate through PSII and PSI) versus irradiance were obtained by exposing leaves to a range of light intensities (13 steps) from 0 to 610 μmol m^{-2} s^{-1}. The ETR_{max} (maximum electron transport rate), photosynthetic light-harvesting efficiency (the initial slope of the RLC, α), and l_k (half-saturation light intensity) were derived by fitting the RLCs to the equation (Eilers and Peeters, 1988), which was available in the Dual-PAM software.

Proton Motive Force Measurement

The dual-beam 550- to 515-nm signals were monitored simultaneously using the Dual-PAM-100 P515/535 module. All samples were dark-adapted overnight. Dark interval relaxation kinetics were analyzed to calculate the proton gradient (ΔpH), membrane potential (ΔΨ), and the proton

conductivity of the thylakoid membrane (gH^+) (Sun et al., 2020; Tan et al., 2020). Additionally, pmf_{LEF} (the pmf attributable solely to proton translocation by LEF) was calculated using the following equation: $pmf_{LEF} = LEF/gH^+$, in which was used in the estimation of the light-driven proton flux through the ATP synthase based on the extent of LEF and the kinetic properties of the ATP-synthase turnover (Avenson et al., 2005).

Measurement of H^+ Fluxes at Mesophyll Cells

In vivo net H^+ fluxes at mesophyll cells were measured with a non-invasive micro-test technology (NMT, Younger, Amherst, MA, United States; Xuyue Science and Technology Co., Ltd., Beijing, China). Washed leaves were cut in half and incubated in the standard medium buffer (100 mM NaCl) for 5 min at room temperature. Microelectrodes were positioned close to the mesophyll cells. Finally, we entered the data Origin (1) millivolts (mV) and AvgOrigin-X microvolts (μV) as measured by the ASET 2.0 software (The imFlux® Software). H^+ flux was then calculated directly by using the JCal v3.2.1 software. Each sample was monitored for the kinetics of net H^+ fluxes for 10 min. Three biological replicates were performed per group.

Chemical and Morphological Analysis

The content of chlorophyll and carotenoids in leaves was determined by a simplified spectrophotometry method (Shu et al., 2010). A leaf with known weight was extracted overnight in 5 mL of 80% acetone solution, and the volume of the extracted solution was determined in 5 mL. The optical density was read at 663, 645, and 470 nm, respectively, by spectrophotometry, and the content of photosynthetic pigments was calculated by the following formulae:

Chl a ($\mu g\ mL^{-1}$) = 12.7 OD_{663}–2.69 OD_{645}

Chl b ($\mu g\ mL^{-1}$) = 22.9 OD_{645}–4.86 OD_{663}

Carotenoid ($\mu g\ mL^{-1}$) = (OD_{470}–3.27 Chl a–104 Chl b)/229

Leaf length, leaf width, and root length were measured for five randomly selected leaves from each group. Seagrasses were carefully retrieved, separated into aboveground and belowground tissues, and subsequently dried at 60°C for 72h until a constant weight was achieved. Seagrasses were then homogeneously powdered. The total carbon and nitrogen of seagrasses were analyzed using an Elementary Analyzer (Flash EA 3000, Thermo Scientific, Milan, Italy).

Statistical Analysis

The measurements were conducted on randomly selected samples, and the values presented are the mean ± SD of a minimum of three replicates. The data were analyzed using SPSS 23 Software (IBM SPSS Statistics, Armonk, NY, United States) using ANOVA, and a statistically significant difference was set at a probability level of 0.05. LSD (least significant difference)-*post hoc* tests were performed to evaluate *post hoc* pairwise comparisons. The figures were drawn with Origin 9 Software (Origin Lab, Northampton, MA, United States).

RESULTS

Responses of ETR and CEF

Our results demonstrated that the increase in transient post-illumination did not affect chlorophyll fluorescence when subjected to air exposure treatment (**Figure 2A**). Daily air exposure did not affect CEF, although instant air exposure decreased significantly (Fa,b = 16.03, n = 18, $P < 0.01$) (**Figure 2B**). Similar trends were observed in both ETR(I) (Fa,b = 27.07, n = 18, $P < 0.01$) (**Figure 2C**) and ETR(II) (Fa,b = 43.941, n = 18, $P < 0.01$) (**Figure 2D**), strongly indicating attenuated electron transfer in instant air-exposed leaves. In contrast, daily air-exposed leaves showed considerably higher CEF and ETR than did the instant air-exposed leaves.

Daily air exposure also enhanced the maximum $P700^+$ signals (P700 fully oxidized) of leaves (**Figure 2E**). The half-time ($t_{1/2}$) of dark decay kinetics to the steady-state (re-reduction of $P700^+$) after turning off the FR light declined with the increase in daily air-exposed hours (Fa,b = 7.615, n = 18, $P < 0.05$) (**Figure 2F**), suggesting exacerbated CEF driven by PSI.

ETR_{max} from the curve fitting for RLCs showed distinctly changing trends in PSII and PSI. Daily air exposure did not affect ETR_{max} of PSII, whereas instant air exposure considerably decreased it (Fa,b = 3.347, n = 18, $P < 0.05$) (**Figure 3A**). Similarly, only instant air exposure significantly enhanced ETR_{max} in PSI (Fa,b = 4.918, n = 18, $P < 0.05$) (**Figure 3A**).

Concomitantly, daily air exposure markedly promoted I_k (half-saturation light intensity) in PSII, whereas instant air exposure showed no effect (Fa,b = 5.824, n = 18, $P < 0.01$). However, daily and instant air exposure both enhanced I_k in PSI (Fa,b = 14.403, n = 18, $P < 0.01$), with greater enhancement by instant air exposure (**Figure 3B**).

Similarly, daily and instant air exposure both significantly decreased the alpha (initial slope of the fitting curve) of PSII (Fa,b = 3.876, n = 18, $P < 0.05$) (**Figure 3C**), although a non-significant trend was observed in PSI with a given decrease in daily air-exposed leaves (Fa,b = 2.674, n = 18, $P > 0.05$).

Response of PSII Activity

No significant difference was observed in maximal quantum yield of PSII (F_v/F_m) among treatments (**Table 1**) (Fa,b = 0.572, n = 18, $P > 0.05$). Only instant air exposure markedly stimulated Y(II) (Fa,b = 90.228, n = 18, $P < 0.01$), Y(NO) (Fa,b = 9.059, n = 18, $P < 0.01$) and relative Q_A reduction (Fa,b = 9.248, n = 18, $P < 0.01$), but reduced Y(NPQ) (Fa,b = 180.521, n = 18, $P < 0.01$) and NPQ (Fa,b = 51.722, n = 18, $P < 0.01$).

Although non-significant, Y(NPQ) and NPQ had higher values in the daily air-exposed leaves than in the control and instant air-exposed leaves, suggesting a higher energy allocation in NPQ. Additionally, daily air-exposed leaves showed lower levels of Y(NO) and relative Q_A reduction, indicating a greater extent of PSII photoinhibition in instant air-exposed leaves.

Rapid chlorophyll fluorescence induction kinetics curve (OJIP curve) was only detected in daily air-exposed leaves (**Figure 4**). The changes in the donor side of PSII, the maximum fluorescence

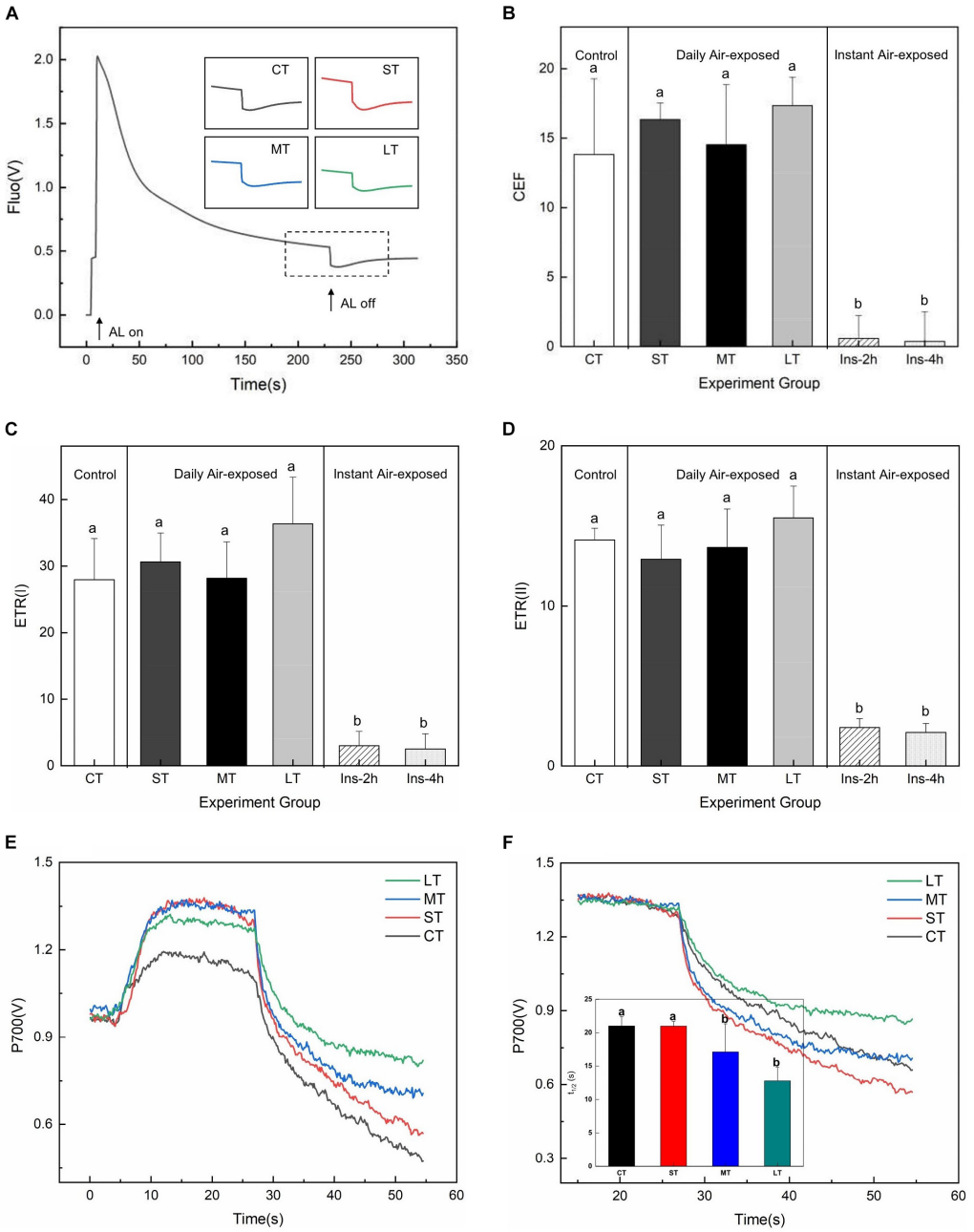

FIGURE 2 | Effects of air exposure treatments on ETR and cyclic electron flow. **(A)** Post-illumination increases of chlorophyll fluorescence in the treatments. **(B)** Changes in CEF in the treatments. **(C)** Changes in ETR(II) in the treatments. **(D)** Changes in ETR(I) in the treatments. **(E)** The P700 signal in the treatments. **(F)** Enlarged display of P700 signal after FR light was removed and the half ($t_{1/2}$) of dark decay kinetics to the steady state estimated from the P700 signal. All data are expressed as means ± SD based on experiments in triplicate. Different letters over columns in panels **(B–D,F)** indicate significant difference ($P < 0.05$) among means. CT: Control group without any air exposure; ST: 1 h of daily air exposure; MT: 2 h of daily air exposure; LT: 4 h of daily air exposure; Ins-2 h: instant air exposure of 2 h; Ins-4 h: instant air exposure of 4 h.

intensity (P point), decreased as the air-exposed time increased (**Figure 4A**). Changes in the receptor side of PSII, the maximal photochemical efficiency (φ_{Po}), decreased slightly in air-exposed leaves. The PQ pool decreased, which was reflected by lower S_m, and further led to a slight decline in φ_{Eo}. Restore times of Q_A (N) decreased, which was consistent with the decreased ability to transfer electrons. No significant change was observed in the opening degree of the active reaction center (ψ_O) (**Figure 4B**). The changes in the reaction center of PSII, the performance index (PIcs), indicated the effect of air exposure on PSII, which was significantly decreased with increased exposure time. There were no significant changes in V_J, in accordance

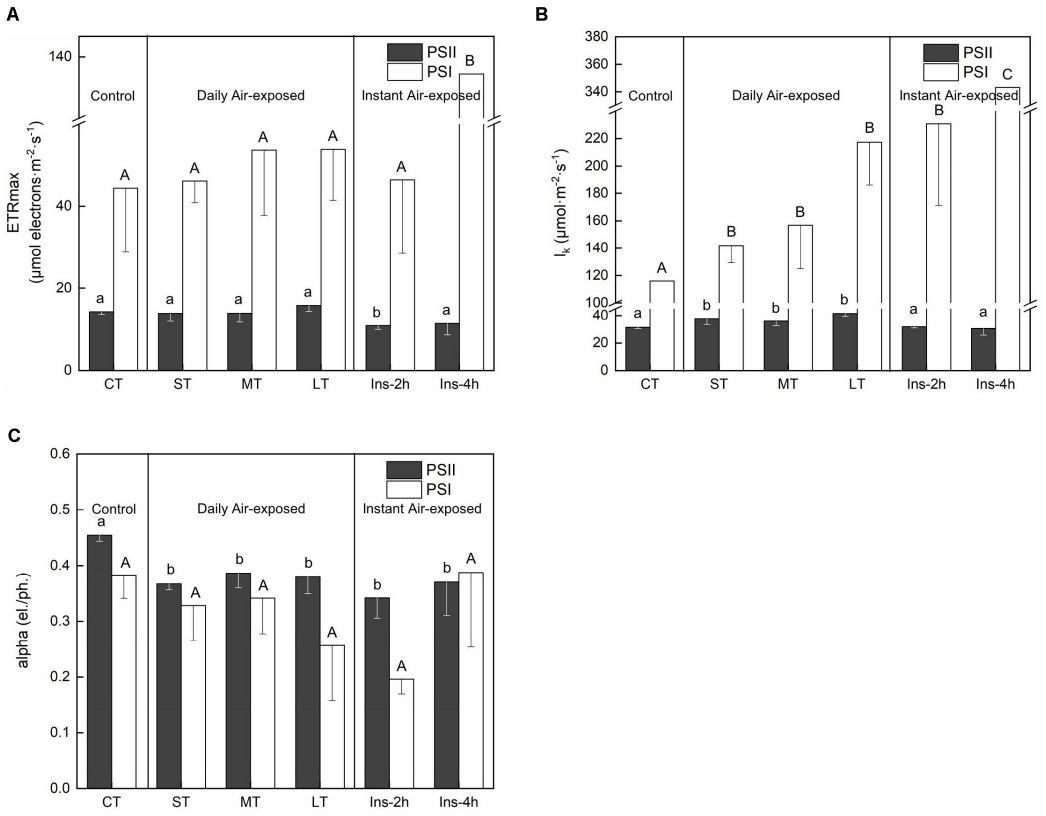

FIGURE 3 | Rapid light curves fitting parameters under different air exposure treatments. **(A)** ETR_{max} estimated in PSII and PSI. **(B)** I_k (half-saturation light intensity) estimated in PSII and PSI. **(C)** Alpha (initial slope of fitting curve) estimated in PSII and PSI. All data are expressed as means ± SD based on experiments in triplicate. Different letters over the columns indicate significant difference ($P < 0.05$) among means. Upper- and lower-case letters are used to distinguish different parameters within each figure part. CT: Control group without any air exposure; ST: 1 h of daily air exposure; MT: 2 h of daily air exposure; LT: 4 h of daily air exposure; Ins-2 h: instant air exposure of 2 h; Ins-4 h: instant air exposure of 4 h.

TABLE 1 | Variation in PSII activity during daily and instant air exposure treatment (values are means of three replicates ± SD).

	F_v/F_m	Y(II)	Y(NPQ)	Y(NO)	NPQ	Relative Q_A Reduction
CT	0.79 ± 0.01^{ns}	0.26 ± 0.01^{a}	0.45 ± 0.01^{a}	0.29 ± 0.01^{a}	1.55 ± 0.02^{a}	0.29 ± 0.01^{a}
ST	0.78 ± 0.02^{ns}	0.24 ± 0.04^{a}	0.50 ± 0.05^{a}	0.27 ± 0.03^{a}	1.88 ± 0.37^{a}	0.27 ± 0.03^{a}
MT	0.79 ± 0.01^{ns}	0.25 ± 0.04^{a}	0.46 ± 0.01^{a}	0.29 ± 0.03^{a}	1.63 ± 0.16^{a}	0.29 ± 0.03^{a}
LT	0.78 ± 0.00^{ns}	0.27 ± 0.02^{a}	0.48 ± 0.04^{a}	0.25 ± 0.01^{a}	1.90 ± 0.24^{a}	0.27 ± 0.02^{a}
Ins-2 h	0.78 ± 0.01^{NS}	0.58 ± 0.01^{b}	0.08 ± 0.01^{b}	0.33 ± 0.01^{b}	0.25 ± 0.02^{b}	0.34 ± 0.01^{b}
Ins-4 h	0.79 ± 0.01^{NS}	0.58 ± 0.04^{b}	0.07 ± 0.01^{b}	0.35 ± 0.03^{b}	0.20 ± 0.03^{b}	0.35 ± 0.03^{b}

CT: control group without any air exposure; ST: 1 h of daily air exposure; MT: 2 h of daily air exposure; LT: 4 h of daily air exposure; Ins-2 h: instant air exposure of 2 h; Ins-4 h: instant air exposure of 4 h.

with the unchanged proportion of inactive reaction centers (**Figures 4B**, **2B**).

Response of PSI Activity

Air exposure did not affect Y(I) ($Fa,b = 0.233$, $n = 18$, $P > 0.05$) or Y(NA) ($Fa,b = 0.449$, $n = 18$, $P > 0.05$) (**Figures 5A,B**). Daily air-exposed leaves showed lower levels of both Y(I) and Y(NA), in contrast to instant air-exposed leaves. Additionally, daily air exposure did not affect Y(ND) (which represents the fraction of overall P700 that is oxidized in a given state), whereas instant air exposure significantly decreased it ($Fa,b = 8.220$, $n = 18$, $P < 0.01$), with Y(ND) in Ins-4 h reaching zero and suggesting serious photoinhibition. In comparison, daily air-exposed leaves showed much higher levels of Y(ND), indicating an increased quantum yield of PSI non-photochemical energy dissipation (**Figure 5C**).

Response of Electrochromic Shift (ECS) and H^+ Fluxes

The membrane potential ($\Delta\psi$) and proton gradient (ΔpH) of the pmf in daily air-exposed leaves were estimated by analyzing the light-off responses of the P515 signal (**Figure 6A**). Significant

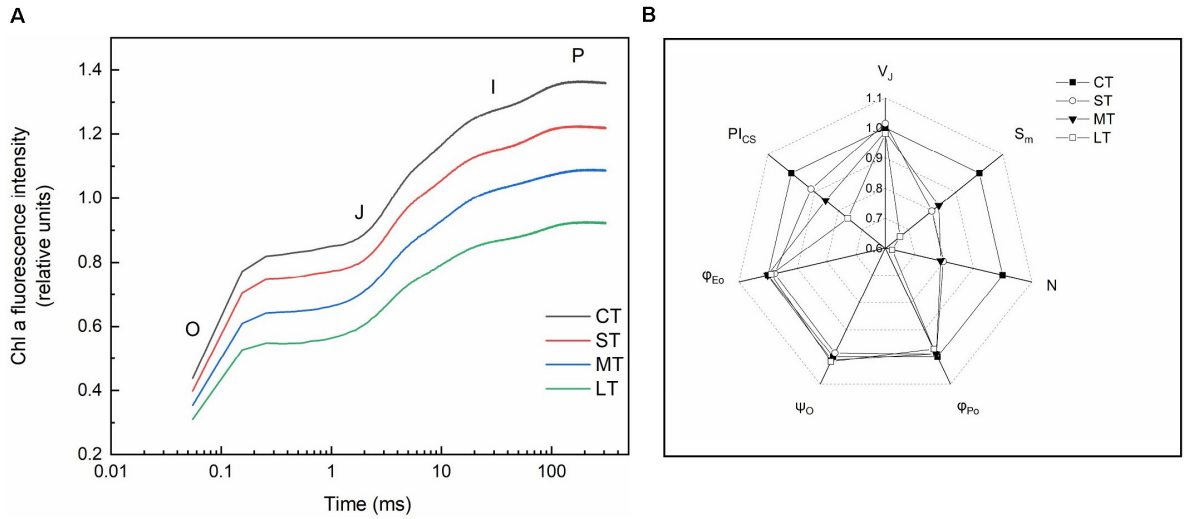

FIGURE 4 | Rapid chlorophyll fluorescence induction kinetics. **(A)** OJIP transient (values are means of three replicates). **(B)** The spider plots of the chlorophyll fluorescence parameters in different treatments. V_J: Relative variable fluorescence intensity at J point. S_m: The complementary area between the O-J-I-P induction curve, F = FM and the *Y*-axis. N: The number of times that Q_A was restored during the period from the start of illumination to FM. φ_{Po}: Maximal photochemical efficiency. ψ_O: The ratio of the excitons captured by the reaction center to the other electron acceptors used to promote electron transfer to the electron transfer chain, which exceeds Q_A, to the excitons used to promote Q_A reduction. φ_{Eo}: Quantum yield for electron transfer. PI_{CS}: Performance index based on unit area. All data are expressed as means ± SD based on experiments in triplicate. CT: Control group without any air exposure; ST: 1 h of daily air exposure; MT: 2 h of daily air exposure; LT: 4 h of daily air exposure.

changes in $\Delta\psi$ were detected (Fa,b = 7.195, n = 12, $P < 0.01$). A transient increase in $\Delta\psi$ was observed in ST, followed by a decline in $\Delta\psi$ in MT and LT (**Figure 5B**). Furthermore, though not significant (Fa,b = 2.308, n = 12, $P > 0.05$), a fluctuating decline in ΔpH is shown in **Figure 6B**. The pmf was lowest in LT, probably because it had the longest air exposure. Additionally, air exposure did not significantly affect gH^+ (Fa,b = 0.903, n = 12, $P > 0.05$) or pmf_{LEF} (Fa,b = 0.560, n = 12, $P > 0.05$), but a trend was observed. Though not significant, air-exposed leaves showed higher levels of gH^+ (proton conductivity of the thylakoid membrane). Additionally, the pmf attributable solely to proton translocation by LEF decreased slightly in air-exposed leaves as the exposure time increased (**Figure 6C**), in accordance with exacerbated CEF (**Figure 2B**).

The net H^+ fluxes at mesophyll cells of *H. beccarii* in different air exposure treatments are depicted in **Figure 7**. The net H^+ fluxes under CT, ST, MT, and LT were 31.00 ± 1.24, 25.40 ± 0.70, 21.19 ± 0.85, and 5.18 ± 1.56 pmol cm^{-2} s^{-1}, respectively. Air exposure significantly decreased the net H^+ fluxes (Fa,b = 3.936, n = 12, $P < 0.05$).

Changes in Chemical and Morphological Properties

There were no significant differences in carbon content in both the aboveground (Fa,b = 1.247, n = 8, $P > 0.05$) and belowground (Fa,b = 0.138, n = 8, $P > 0.05$) tissues among air exposure treatments (**Figure 8A**). However, air-exposed leaves showed higher levels of nitrogen content in aboveground and belowground tissues (aboveground: Fa,b = 19.258, n = 8, $P < 0.05$; belowground: Fa,b = 0.997, n = 8, $P > 0.05$), resulting in a lower C/N ratio (aboveground: Fa,b = 9.008, n = 8, $P < 0.05$; belowground: Fa,b = 3.728, n = 8, $P > 0.05$) (**Figure 8A**). Furthermore, although non-significant, photosynthetic pigments, including Chl a (Fa,b = 2.221, n = 12, $P > 0.05$), Chl b (Fa,b = 2.740, n = 12, $P > 0.05$) and carotenoids (Fa,b = 0.430, n = 12, $P > 0.05$), increased in all air exposure treatments, whereas the Chl a/Chl b ratio remained stable (Fa,b = 0.577, n = 12, $P > 0.05$) (**Figure 8B**).

No statistically significant difference was observed in leaf length (Fa,b = 3.002, n = 20, $P > 0.05$), leaf width (Fa,b = 2.532, n = 20, $P > 0.05$), or root length (Fa,b = 1.234, n = 20, $P > 0.05$). The changing trend in leaf length and width was similar, with an increase in ST and a decrease in MT and LT (**Figure 8C**). Root length in the air exposure groups was lower compared to that of CT (**Figure 8C**).

DISCUSSION

Efficient Heat Dissipation and Cyclic Electron Flow (CEF) Confer Daily Air Exposure Tolerance

The seagrass *H. beccarii* inhabits the intertidal zones and periodically experiences repeated cycles of air exposure and rehydration. To our knowledge, very few studies have attempted to assess the difference in photosynthetic responses of seagrasses exposed to instant and long-term daily air exposure. In this study, protective processes were found only in daily air-exposed leaves. In contrast, the NPQ of PSII was absent in instant air-exposed leaves, reflected by higher levels of Y(NO)

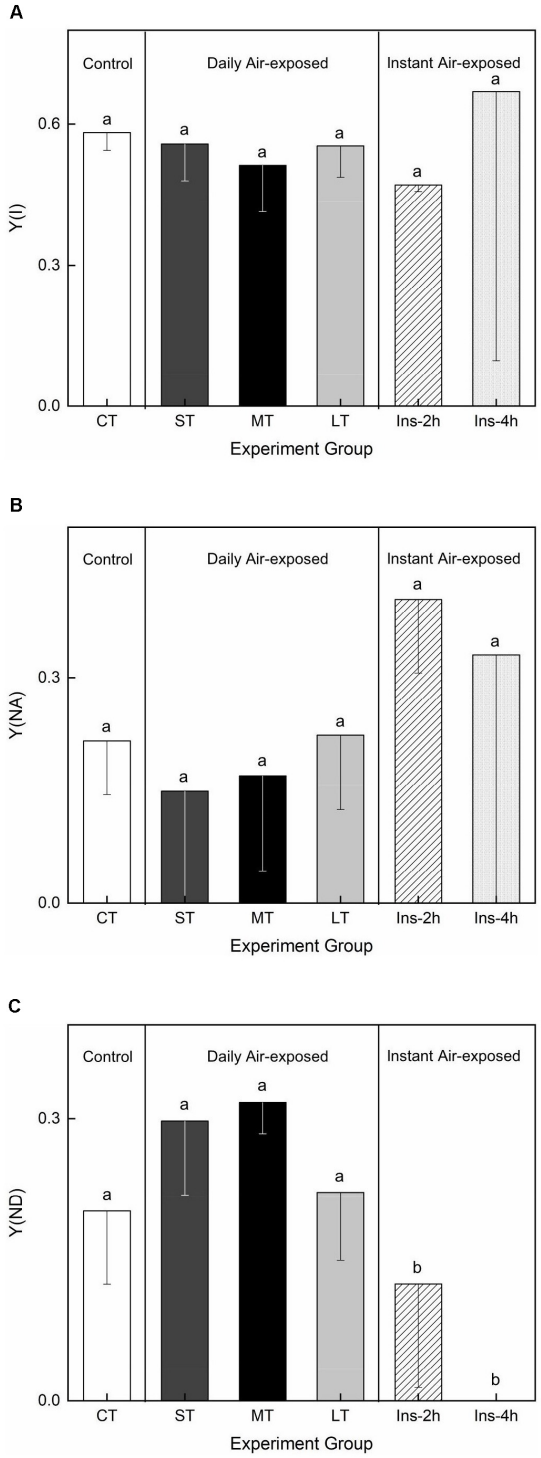

FIGURE 5 | Effects of air exposure treatment on PSI activity. **(A)** Changes in Y(I) in the treatments. **(B)** Changes in Y(NA) in the treatments. **(C)** Changes in Y(ND) in the treatments. All data are expressed as means ± SD based on experiments in triplicate. Different letters over the columns indicate significant difference ($P < 0.05$) among means. CT: Control group without any air exposure; ST: 1 h of daily air exposure; MT: 2 h of daily air exposure; LT: 4 h of daily air exposure; Ins-2 h: instant air exposure of 2 h; Ins-4 h: instant air exposure of 4 h.

and relative Q_A reduction. This indicated that instant air exposure caused photodamage and a higher turn-off proportion of the PSII reaction center. Previous studies have indicated that the extent of PSII photoinhibition was dependent on the reduction in Q_A (Kramer et al., 2004), which could reduce the D1 protein content by recombination with $P680^+$ (Krieger-Liszkay et al., 2008; Vass, 2011). It has also been reported that carotenoids, which accumulated upon desiccation, were crucial to activating NPQ in plants (Demmig-Adams, 2003; Du et al., 2010; Beckett et al., 2012). Thus, the deactivated NPQ leading to photoinhibition in instant air-exposed leaves might be caused by low levels of carotenoids. Moreover, CEF, essential for efficient photoprotection (Pinnola and Bassi, 2018; Lu et al., 2020), was also absent in instant air-exposed leaves, which was also reflected in significantly lower CEF and ETR in both PSII and PSI. It should be noted that the distinct difference in photosynthetic physiology between instant and daily air exposure was that daily air exposure allowed the repeated rehydration period to relax. Similar research on the effect of fluctuating light on rice and *Arabidopsis thaliana* suggested that PSI is more sensitive to fluctuating light than to constant high light, and CEF is required to alleviate photodamage of both photosystems by fluctuating light (Kono et al., 2014; Yamori and Shikanai, 2016; Yamori et al., 2016). Therefore, based on our results and published studies, we deduced that only daily air exposure enhanced CEF because of the sensibility of PSI to repeated cycles of air exposure and dehydration.

In comparison, a protective and adaptive mechanism for mediating daily air exposure stress was observed. It consisted of two main processes (**Figure 9**). First, NPQ in both PSII and PSI was observed to be exacerbated with higher levels of Y(NPQ) and Y(ND), which resulted in intensive thermal energy dissipation of excess optical energy. Consequently, the activity of PSII and PSI was maintained, as reflected by the unchanged F_v/F_m and Y(I). The unchanged F_v/F_m and Y(I) suggested that photosynthetic efficiency was stable at 0 to 4 h of air exposure, which could be explained by the photosynthetic plasticity, as well as flexible leaves that easily make contact on the moist sediment surface. Similar trends were observed in terrestrial plants and aquatic plants under moderate levels of drought or desiccation stress (Bian and Jiang, 2009; Kang et al., 2013; Li et al., 2019). Similar to the previous study, elevated NPQ allowed energy to bypass reaction centers at the beginning of the absorbed excitation energy partition, and it was rapidly converted to thermal energy (Sharkey and Zhang, 2010; Yamakawa et al., 2012; Hernández-Fuentes et al., 2019). Despite the protected photosynthetic active centers, photochemical efficiency was affected under air exposure. Notably, $P700^+$ signal and S_m were observed to decrease, suggesting lower levels of the PQ pool as a consequence of water deficit. Additionally, LEF was inhibited because of limited electron production from water photolysis (Gao et al., 2011), further resulting in lower pmf_{LEF}. Second, to compensate for the linear electron transport, the CEF transport driven by PSI was upregulated. The results were higher levels of CEF and ETR_{max} in PSI, as well as lower $t_{1/2}$. This was interpreted as elevated CEF, in accordance with decreased pmf_{LEF}. A similar study on the seagrasses *Enhalus acoroides* and *Thalassia hemprichii* indicated

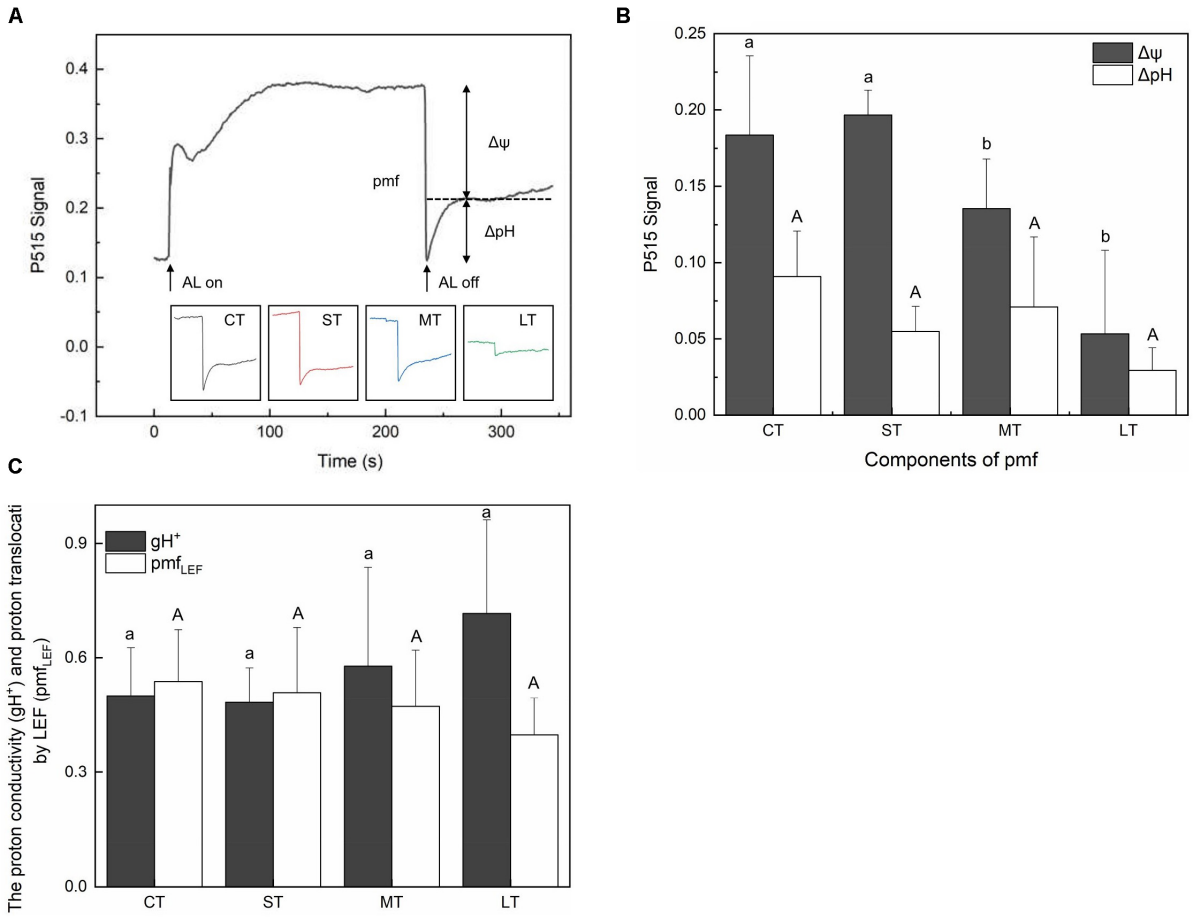

FIGURE 6 | Effects of air exposure treatment on Electrochromic shift (ECS). **(A)** Changes in the P515 signal of slow dark–light–dark induction transients. **(B)** Two components of the proton motive force (membrane potential and proton gradient) derived from the slow dark–light–dark induction transients of the 550 to 515 nm signals. **(C)** Proton conductivity of the thylakoid membrane and the pmf attributable solely to proton translocation by LEF estimated from P515 signal. All data are expressed as means ± SD based on experiments in triplicate. Different letters over columns in panels **(B,C)** indicate significant difference ($P < 0.05$) among means. Upper- and lower-case letters are used to distinguish different parameters in panels **(B,C)**. CT: control group without any air exposure; ST: 1 h of daily air exposure; MT: 2 h of daily air exposure; LT: 4 h of daily air exposure.

that enhanced ETR_{max} upon desiccation was consistent with relative water content being higher than the critical threshold, which caused no irreversible damage (Jiang et al., 2014). It is also noteworthy that this experimental design emphasized the effect of desiccation with suitable temperature and light intensity, in accordance with natural observations. Thus, the degree of stress might not yet induce significantly upregulated CEF but induced certain increased trends, which further confirmed a high extent of photosynthetic plasticity. In the following processes, CEF could promote electron transfer from the acceptor side of PSI back to plastoquinone, thus maintaining ATP production and supplying the proton gradient (Fan et al., 2016; Yamori and Shikanai, 2016), consistent with a slightly increased ΔpH of the ST group in this study. The formation of ΔpH was further suppressed with increasing air exposure hours because of the increased thylakoid H^+ efflux activity from the luminal to the stromal side, reflected by elevated gH^+. Based on these results, we tentatively propose that cyclic electron transport contributed substantially to air exposure tolerance for intertidal *H. beccarii* (**Figure 9**), which helped explain the broad distribution of *H. beccarii* in intertidal zones.

Air Exposure Decreased the Proton Pump Influxes

Seagrass can use CO_2 and HCO_3^- as inorganic carbon sources, depending on the species (Beer et al., 2014). The pathways of inorganic carbon source utilization in seagrass leaves are quite different from those of other plants because of the absence of stoma on the leaf epidermis. For example, the seagrass *Halophila ovalis*, the same genus as *H. beccarii* in this study, was previously studied using HCO_3^- as a carbon source (Uku et al., 2005). More specifically, protons are extruded by an active H^+ pump somewhere along the leaf, then transported back into the cells, accompanied by HCO_3^- (Beer et al., 2014). Under high light or drought, plants usually close stoma to reduce water loss by transpiration and decrease the internal leaf CO_2

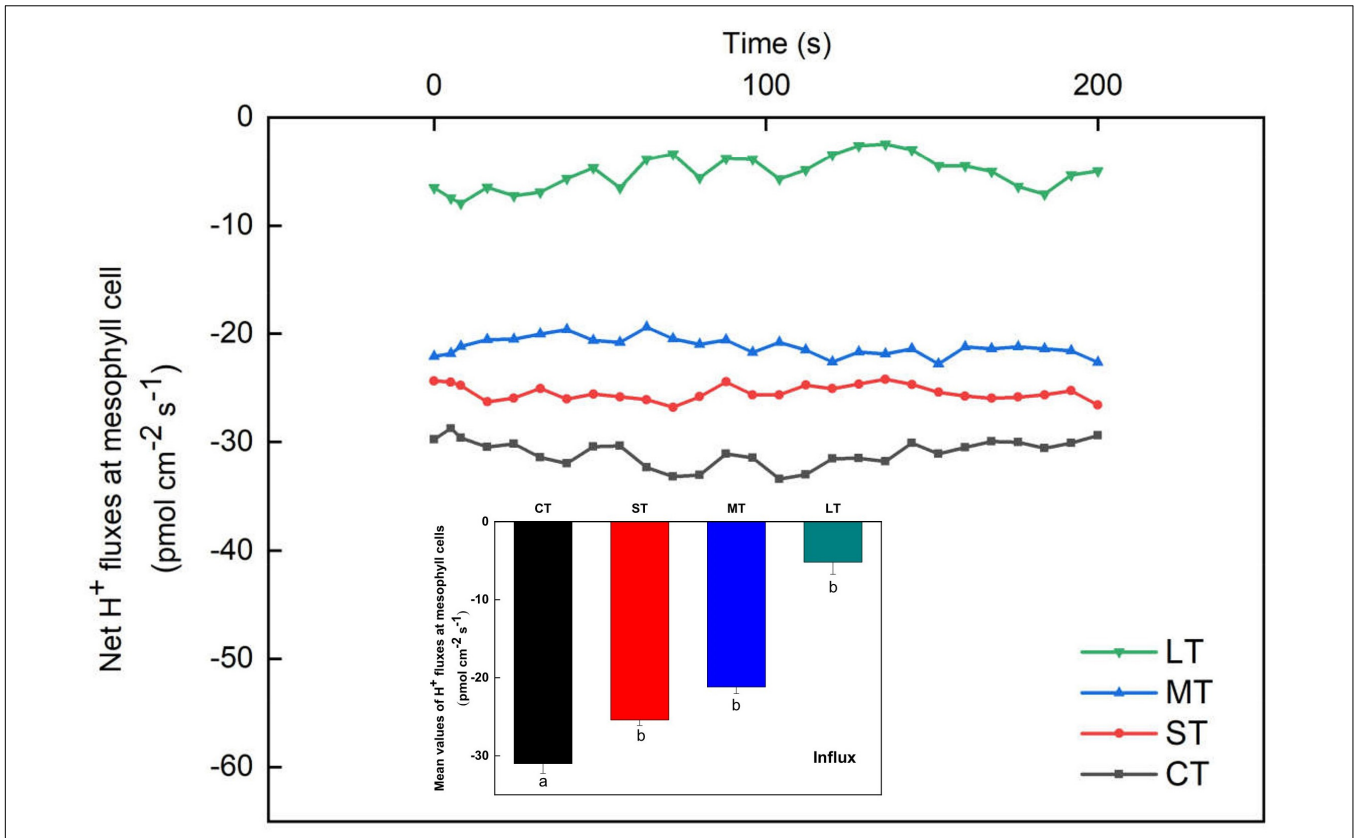

FIGURE 7 | Net H^+ fluxes at mesophyll cells of *H. beccarii* in different air exposure treatments. All data are expressed as means ± SD based on experiments in triplicate. Different lowercase letters under the four columns indicate significant difference ($P < 0.05$) among means. CT: Control group without any air exposure; ST: 1 h of daily air exposure; MT: 2 h of daily air exposure; LT: 4 h of daily air exposure.

concentration (Dubey, 1996; Beer et al., 2014; Zhang et al., 2017). However, for seagrass without the stomatal regulation mechanism, air exposure may increase the photosynthetic rate in seagrass because of the higher ambient CO_2 concentration (Jiang et al., 2014). However, as exposure time increased, the carbon intake decreased along the gradually rising water loss. In this study, air-exposed leaves made a tradeoff between elevated gaseous CO_2 concentrations and the reduced demand for carbon sources by downregulating the HCO_3^- intake. More specifically, air-exposed leaves exhibited lower levels of H^+ fluxes as the exposure time increased. It should be noted that this study has examined only changes in H^+ fluxes to confirm HCO_3^- utilization. Detection of carbonic anhydrase activity and changes in HCO_3^- concentration in water could further characterize carbon utilization under air exposure (Beer et al., 2014). Notwithstanding its limitation, this study does suggest that proton pump and carbon intake were downregulated.

Air Exposure Enhanced Photosynthetic Pigments but Reduced Root Length

The present study indicated that photosynthetic pigments, including Chl a, Chl b, and carotenoids, increased in all air exposure treatments (**Figure 9**). Previous studies proposed that photosynthetic pigments, such as carotenoids and zeaxanthin, were necessary for inducing NPQ, quenching of singlet oxygen, and protecting thylakoid membranes from peroxidation (Havaux et al., 2007; Du et al., 2010; Beckett et al., 2012), in accordance with strengthened NPQ in both PSII and PSI, as mentioned above. Additionally, it was reported that the content of carotenoids in *Boea hygrometrica* was partially increased upon dehydration (Tan et al., 2017), which was similar to the findings in this study. Therefore, based on our results and published studies, we suggest that accumulated photosynthetic pigments in air-exposed leaves contribute to the photoprotective mechanism (**Figure 9**). It should be noted that this study has only detected the total carotenoid content without quantifying zeaxanthin or violaxanthin, which merits further study. In addition to the photosynthetic pigment discussed in this study, it would also be worthwhile to study the role of secondary metabolites in plants, including flavonoids and phenols that could play a role in the protection of photoinhibition against high irradiance and increased temperature during air exposure (Close and Beadle, 2003; Abdala-Díaz et al., 2006). This requires further investigation.

Although morphological changes in this study were not statistically significant, there were some interesting findings. The changing trend in leaf length and width was similar, with a rise in 1 h long-term exposure and a drop in 2 h and 4 h

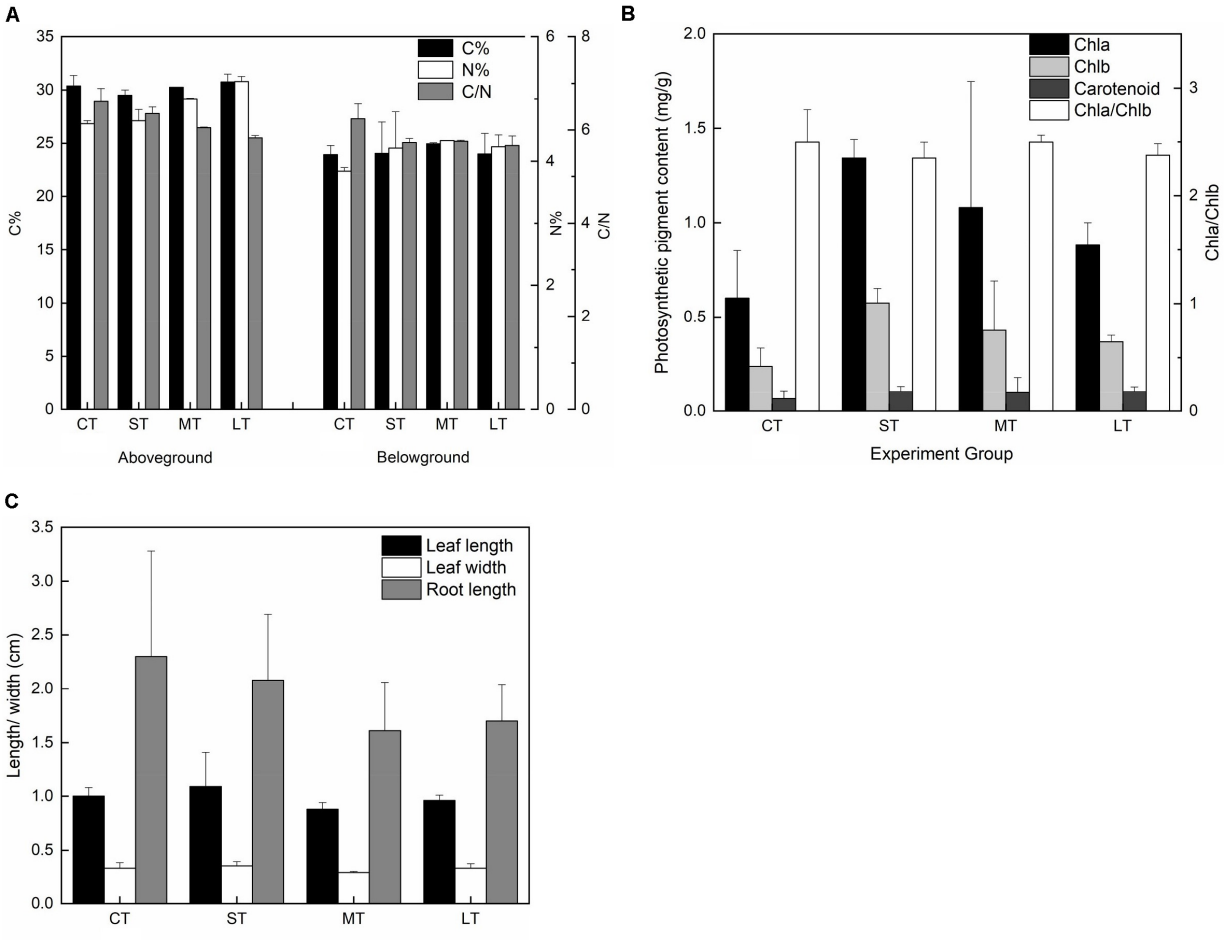

FIGURE 8 | Effects of air exposure on the chemical and morphological changes. **(A)** The changes in carbon and nitrogen content in air exposure treatments (values are means of two replicates). **(B)** The changes in photosynthetic pigments including of Chl a, Chl b, and carotenoids in air exposure treatments (values are means of three replicates). **(C)** The changes in morphology including of leaf length, leaf width, and root length in air exposure treatments. All data are expressed as means ± SD based on experiments in triplicate. CT: Control group without any air exposure; ST: 1 h of daily air exposure; MT: 2 h of daily air exposure; LT: 4 h of daily air exposure.

long-term exposure. The leaf change trend of being smaller was similar in other seagrass species (*Zostera marina*; *Zostera noltii*; *Zostera japonica*) inhabiting high tide zones (Harrison, 1982; Short and Burdick, 1996; Invers et al., 2004; Heck and Valentine, 2006). It seems possible that smaller leaves are more flexible to maintain contact with the moist sediment surface to reduce water loss. Additionally, the energy cost for both shedding damaged leaves upon desiccation and growing new leaves under suitable conditions would be much lower with smaller leaves, which are utilized by *Ruppia cirrhosa* and *Zostera capensis* to rapidly recover from desiccation stress (Pérez-Lloréns and Niell, 1993). Similar changes were also found in the response mechanism of leaf abscission in land and floating plants under water deficit (McMichael et al., 1973; Adams and Bate, 1994; Beer and Bjork, 2000; Saltmarsh et al., 2006). Furthermore, stress-mediated reduction in cell turgor of the meristem could lead to suppressed cell division and elongation (Nelissen et al., 2018; Todaka et al., 2019). Regarding the observed changes in root length in this study, it was surprising that root length in air exposure groups decreased because longer roots might benefit from drawing water from the deeper sediment. It has been reported that increased rooting depth is often observed as a primary response to drought in terrestrial plants (Kashiwagi et al., 2005; Comas et al., 2013). While considering the different aquatic habitat of seagrass, the water content of surface sediment is higher; thus, root elongation for drawing water may be unnecessary. Therefore, decreased root length was probably caused by attenuated carbon assimilation during the air exposure period. However, higher efficiency of carbon assimilation for submerged seagrass, in accordance with the higher pmf, might lead to longer roots by providing a greater carbon source. Further long-term research on root growth and turnover is needed to investigate seagrass plasticity under air exposure. Moreover, no differences in total carbon content and slightly higher levels of total nitrogen in exposed leaves were observed, resulting in a lower C/N ratio (**Figure 9**). This is seen as a consequence of the

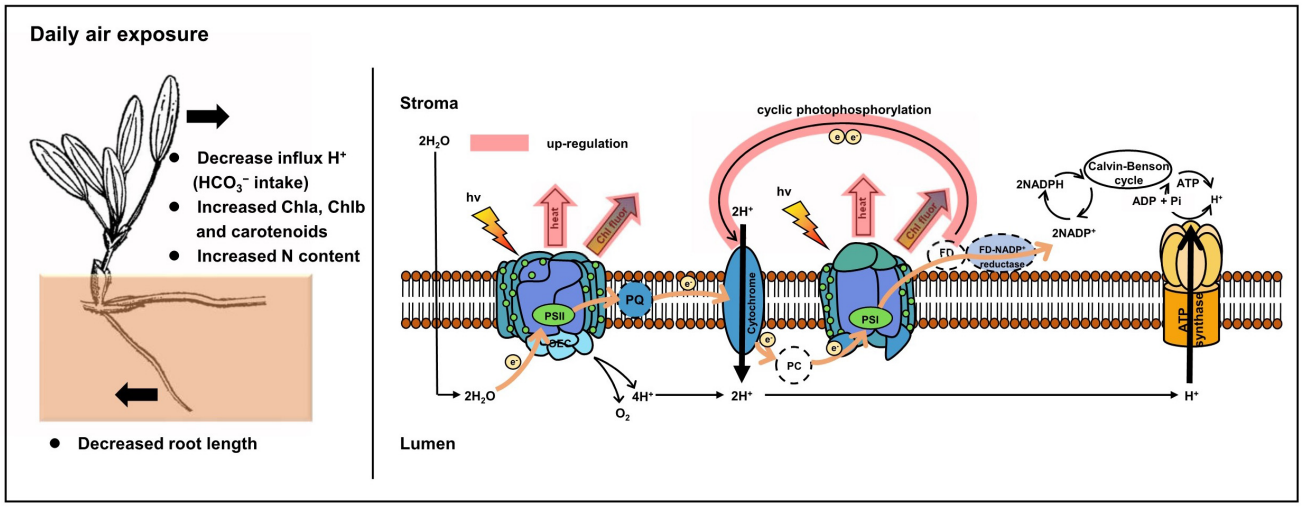

FIGURE 9 | Photoprotective mechanisms in intertidal *H. beccarii* upon daily air exposure. NPQ and CEF indicated in red were upregulated in tolerance with air exposure stress.

gradual dilution of stored nutrient resources in the control group because of a high growth rate (Peralta et al., 2002; Jiang et al., 2010). Lower carbon assimilation upon air exposure with smaller seagrass leaves might also cause non-change of total carbon content. Furthermore, free amino acid accumulation, such as proline, is needed to adjust osmotic pressure to enhance tolerance to desiccation (Pandey et al., 2010; Pizarro et al., 2019), which might explain the increased nitrogen accumulation.

CONCLUSION

In summary, our results indicated that two main photoprotective mechanisms found in the intertidal seagrass *H. beccarii* required long-term adaptation to daily air exposure. Efficient NPQ in both PSII and PSI resulted in intensive thermal energy dissipation of excess optical energy upon air exposure. CEF driven by PSI was upregulated to compensate for the abolished linear electron transport, although the role of water–water cycles in tolerating daily air exposure warrants further research (Huang et al., 2019). More detailed quantification of zeaxanthin and violaxanthin could further determine the role of photosynthetic pigments in photoprotective processes. Additionally, H^+ fluxes at mesophyll cells were downregulated for lower inorganic carbon uptake. Furthermore, modern approaches based on transcriptomics, proteomics, and metabolomics will hopefully be involved in investigations on molecular mechanisms of desiccation-tolerant seagrass in intertidal zones in future research.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

XH and ZJ designed the study. YF, CZ, LL, CR, SL, and YW performed the experiments. YF, ZJ, and XH analyzed the data and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.571627/full#supplementary-material

Supplementary Figure 1 | *H. beccarii* associated with the land plant in the high intertidal area.

Supplementary Figure 2 | Statistical analysis of the exact timing and tidal height of low tide when seagrasses can emerge in Yifengxi, along the South China coast, during 2018–2019.

Supplementary Table 1 | Formulae and glossary of terms used in the JIP test in the analysis of the O-J-I-P fluorescence transient. CT: control group without any air exposure; ST: 1 h of daily air exposure; MT: 2 h of daily air exposure; LT: 4 h of daily air exposure.

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Historical Analysis Exposes Catastrophic Seagrass Loss for the United Kingdom

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The spatial extent of seagrass is poorly mapped, and knowledge of historical loss is limited. Here, we collated empirical and qualitative data using systematic review methods to provide unique analysis on seagrass occurrence and loss in the United Kingdom. We document 8,493 ha of recently mapped seagrass in the United Kingdom since 1998. This equates to an estimated 0.9 Mt of carbon, which, in the current carbon market represents about £22 million. Using simple models to estimate seagrass declines triangulated against habitat suitability models, we provide evidence of catastrophic seagrass loss; at least 44% of United Kingdom's seagrasses have been lost since 1936, 39% since the 1980's. However, losses over longer time spans may be as high as 92%. Based on these estimates, historical seagrass meadows could have stored 11.5 Mt of carbon and supported approximately 400 million fish. Our results demonstrate the vast scale of losses and highlight the opportunities to restore seagrass to support a range of ecosystems services.

Keywords: blue carbon, ecosystem change, habitat loss, intertidal, historic change, marine, shifting baseline syndrome, Zostera spp.

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INTRODUCTION

Increased interest in the Blue Carbon capacity of seagrass means knowledge of the location, extent, and condition of seagrasses has become increasingly important (Fourqurean et al., 2012; Greiner et al., 2013; Röhr et al., 2018; Green et al., 2018). Seagrasses are highly productive, and represent one of the largest global carbon sinks despite occupying only 0.1% of the ocean floor (Hemminga and Duarte, 2000; Orth et al., 2006; Fourqurean et al., 2012; Duarte et al., 2013). An estimated 19.9 Pt carbon is stored in the top 1 m of seagrass worldwide, the equivalent to the CO_2 emissions from fossil fuel and cement production in 2014 (Kerr, 2017). Seagrasses also support biodiversity, as well as contributing to the productivity of 20% of the world's biggest fisheries (Unsworth et al., 2019), supporting coastal livelihoods, increasing shoreline stability, cycling nutrients, and making our coastlines make more affable places to live (Cullen-Unsworth et al., 2014; Nordlund et al., 2016).

Under the Paris agreement countries pledged to outline National Determined Contributions (NDC's) to reduce their emissions (Martin et al., 2016), and nature-based solutions are increasingly being adopted within these strategies. To date, six countries name seagrass directly in their NDCs (Martin et al., 2016). Although these inclusions are encouraging, to the best of our

knowledge, there has been no attempt by any country to document the total areal extent and historic loss of seagrass in their coastal waters. One of the main global challenges of seagrass conservation is that the status of many seagrass meadows is unknown (Unsworth et al., 2019). Knowing how much seagrass a country has is clearly an important step to knowing how to protect it; but knowing where seagrass was, or where seagrass could thrive, gives countries an opportunity to re-plant and restore seagrass in favourable areas (Cunha et al., 2012; Greiner et al., 2013; Paulo et al., 2019).

It is increasingly accepted that restoration of natural habitats must play a crucial role in global efforts to mitigate climate change (European Commission, 2009). That seagrasses can absorb more carbon up to 40 faster than terrestrial forests (Mcleod et al., 2011) should make them a significant component of these attempts. Global loss of seagrasses since the 1980's is thought to be at least 29% (Waycott et al., 2009; Short et al., 2010), and seagrasses continues to be lost at a rate of 1.4% a year (Short et al., 2010). These losses must be stemmed if seagrasses are to play a role in climate mitigation and understanding where losses have occurred is an important first step towards appropriate conservation planning (Cullen-Unsworth and Unsworth, 2016).

Seagrasses are highly sensitive to degrade water quality and conditions which impose light limitations to photosynthesis (Orth et al., 2006). Coastal development and nutrient enrichment have historically been responsible for worldwide declines, which threaten the substantial ecological services seagrass meadows provide (Fraser and Kendrick, 2017). Global seagrass declines only account for mapped populations, and in many countries, data on extent are limited. Even in developed countries, such as those of the United Kingdom, spatial data on seagrass extent are largely incomplete. Given the paucity of seagrass mapping to date, the baseline from which global seagrass declines are calculated are almost certainly significant underestimations. The most up-to-date estimate of seagrass coverage indicate that a minimum of 325,178 km^2, occurs globally, but these values include data from the United Kingdom that is largely out of date (Short, 2018; McKenzie et al., 2020). Recent efforts have been made to demonstrate the substantial services afforded by United Kingdom seagrass habitats through sediment stabilisation (Wilkie, 2011), fisheries support (Bertelli and Unsworth, 2014), and carbon sequestration (Green et al., 2018). Understanding the significance of these services is challenging without robust estimates of the current and historic areal extent of United Kingdom seagrass meadows.

As with global seagrass observations, monitoring and mapping of United Kingdom seagrasses occurs with limited consistency (McKenzie et al., 2020). Where studies have occurred, the resulting data are largely in the grey literature or held disparately by local councils, national and devolved governments, and non-government organisations (NGO's). This has resulted in a lack of current and robust estimates on spatial coverage of seagrasses. The needs for these estimates are multiple but recent studies highlight the poor status of seagrasses in the United Kingdom (Jones and Unsworth, 2016).

Once considered a significant component of the natural heritage of United Kingdom waters (Davidson and Hughes, 1998), seagrass is now accepted to be nationally scarce and sparsely distributed (Hiscock et al., 2005; Jones and Unsworth, 2016). Conceptions of environmental degradation tend to shift depending on our temporal reference point. In the United Kingdom, this "shifting baseline syndrome" (SBS; Pauly, 1995) occurs when the earliest known data of areal extent are assumed as an unaffected baseline condition. This is further exacerbated by data being supported by qualitative accounts that refer to healthier conditions within a scientist's lifetime (Butcher, 1941; Pauly, 1995). With each generation, the concept of a healthy ecosystem shifts, depending on their perceived baseline.

The earliest attempts to document seagrass extent already pointed to declines and the need for more data (Butcher, 1934, 1941). It is likely that Butcher's 1930's reports were already subject to SBS. Two periods of decline are emphasised throughout the literature: one immediately after WWI, and another during the northern Atlantic outbreak of wasting disease in the early 1930's (Butcher, 1934; Cottam, 1934). The wasting disease "epidemic" has been perpetually attributed as the main cause of declines (Den Hartog, 1983; Garrard and Beaumont, 2014) without consideration for the pervasive environmental degradation that occurred in the centuries before. Regardless of the cause of these declines, more efforts are needed to evaluate the status and trends of these valuable marine habitats. To fully appreciate the extent of declines, we must find a way to look beyond these earliest evaluations, which are almost certainly underplayed due to SBS. The objectives of this study were accordingly to estimate for the United Kingdom: (1) the current areal extent of seagrass; and (2) the recent (since 1998) and historic (since before 1998) percentage loss of seagrass. The paper places our results in the context of conservation and provision of ecosystem services.

MATERIALS AND METHODS

The United Kingdom contains two species of seagrass; *Zostera marina* and *Zostera noltii*. The former is predominately sublittoral, and the latter occurs intertidally (Wilkinson and Wood, 2003). Both species are protected under the EU Habitats Directive (92/43/EEC) as features included in annex 1 (habitats), are indicators of Good Ecological Status under the EU Water Framework Directive (WFD; Foden and Brazier, 2007), and gain protection from a number of other EU Directives due to their need for good water quality [i.e., EU Nitrates Directive (91/676/EEC), Urban Wastewater Treatment Directive (91/271/EEC)], and their importance as habitat for wildfowl [Birds Directive (79/409/EEC); Jackson et al., 2016]. In addition, seagrass habitats across a range of United Kingdom waters are offered protection associated with amend and devolved legislation stemming from the Wildlife and Countryside Act 1981.

For the purpose of this work, we have categorised any data collected since 1998 as "contemporary" (similar to current conditions), and any data older as "historical" (not reflecting current conditions), since we cannot reliably confirm the presence of something that has not been mapped for over 20 years. To fulfill the first objective, multiple datasets were collated

with other isolated data, to determine the current mapped areal extent of seagrasses in the United Kingdom. Due to the paucity of available data, we have used three methods to assess seagrass loss with high, medium, and low certainty. High certainty loss estimates were generated collating data older than 1998 and cross-checking them against contemporary data to confirm loss of areal extent. Medium certainty loss includes data on sites where no contemporary data are available, i.e., sites that have not been revisited since 1998. All these methods were supplemented by a systematic review to provide qualitative and quantitative data on seagrass loss. Low certainty loss estimates, not subject to SBS and data limitations, were derived using best available data on historic seagrass extent and additional data regarding sub- and intertidal, mud- and sandflat area of England, Scotland, and Wales (mainland Britain) to estimate maximum seagrass extent and percentage loss in mainland Britain. These estimates excluded Ireland and Northern Ireland because accurate data on mud- and sandflat area were not available.

Contemporary and Historical Areal Estimates of Seagrass Habitats

Two datasets were identified as containing records of *Zostera* from multiple sites. The OSPAR Commission (2017) dataset represents the current known areal extent of seagrasses in the United Kingdom and includes records on *Zostera* between 1986 and 2015. Under the WFD, a range of government agencies (e.g., English & Northern Ireland Environment Agency, Scottish Environment Protection Agency, and Natural Resources Wales) are required to assess the condition of seagrass to help determine the biological condition of United Kingdom water bodies (Foden and Brazier, 2007). The outcome of this is another dataset that includes areal extent of *Zostera* meadows monitored under the WFD between 2007 and 2017. These data were analysed by region and date on QGIS (version 3.2.1) and were shown to contain substantial gaps. To supplement these, we contacted stakeholders from a multitude of organisations targeting local councils, national and devolved governments, government advisory organisations, fisheries authorities, private environmental consultants, and scientists who work on seagrasses in the United Kingdom. From these searches, 14 additional contributors supplemented the OSPAR and WFD datasets, the collective of which makes up all the known available data, based on the searches we undertook (see Supporting information). Since species identification was not provided across all data sets, they have not been included here. However, as an intertidal species, there are far less technological constraints associated with surveying *Z. noltii*. Because of this we expect it to be in the majority, and further expect many *Z. marina* meadows to have gone unreported. Because of these inconsistencies, we have decided not to discriminate between species, since greater abundance of one species is likely due to mapping inconsistencies rather than variances in the conditions that allow one species to proliferate over another. It should also be noted that *Zostera angustifolia* was once considered its own species, although is now recognised as a phenotype of *Z. marina* (Becheler et al., 2010). As we do not distinguish between species, *Z. angustifolia* is treated in the same way as *Z. marina* and *Z. noltii*.

Data were provided in the form of individual observations (point data) and area estimates (polygon data). Polygon data were used to provide the area estimates contained herein. Spatial assessments were made using QGIS (version 3.2.1) and all data were analyzed for duplicates or overlaps. Where they occurred the most recent data was used, unless differences between years occurred that represented data collection restraints rather than area changes. For example, when data were present in the same location from 2016 to 2018 but the 2018 data held substantially reduced area, it was assumed that this was not representative of habitat degradation but restrictions to accessing the full extent of the meadow. This was supported by several data fluctuating between 3 years – e.g., 2014 and 2016 showed the same area cover, but 2015 showed far reduced cover. The area of each polygon was calculated in m^2 using a cylindrical WGS-84 projection and converted to hectares (ha) for reporting purposes.

The contemporary data represent the minimum area of seagrasses in the United Kingdom since some meadows have certainly gone unreported. OSPAR data were used to provide high and medium certainty estimates of historic mapped areal extent. The maximum seagrass extent for each record within the dataset was checked against contemporary records and where contemporary records were found the difference between largest (oldest) and current meadow size was used to provide high certainty loss estimates. Where no contemporary record of the meadow was found, these were considered as spatial loss and included in medium certainty loss estimates. We acknowledge our approach, and the subsequent estimates of changes in coverage through time, is constrained by sampling efforts and data reporting of past research efforts, but this represents the best use of the best available data.

Systematic Review of Qualitative and Quantitative Data on Seagrass Declines

Systematic reviews are used to encapsulate a broad range of literature on a discrete subject by aggregating large data and rigorously extracting relevant information (Minx et al., 2017). We followed the distinct protocols required to achieve a systematic review by: (1) defining the discrete subject parameters and the timeframe of interest; (2) creating a search term to encapsulate all data that might be relevant to the subject; (3) inputting this into Web of Science (Thompson Reuters) to extract a literature database; (4) justifying and making a transparent selection of the literature; and (5) providing a synthesis of the relevant literature. Full details of these methods are provided in the supporting information (SI).

Web of Science includes published, peer-reviewed articles as far back as 1990. Because of the distinct lack of published data on seagrass area cover in the United Kingdom, and a need to capture data as far back in time as possible, it was necessary to broaden the search to include published, unpublished, and grey literature. These data were collected by extensive internet searches, through contacting stakeholders from government, private organisations, and NGO's, and from

scientists and the public who work in seagrass science, conservation, and management throughout the United Kingdom. Papers were qualified and data extracted based on the same criteria as the systematic search (SI). Both search unearthed 179 papers that were considered relevant to this work.

Modelling the of Seagrass Throughout England, Scotland, and Wales

Because of the scarcity of historic empirical data, and the observation that many of the early qualitative reports are almost certainly subject to SBS, we used available data to model the maximum historic extent and low certainty loss estimates of seagrasses in mainland Britain.

In 1991, the Nature Conservancy Council (NCC) undertook a report on the 155 estuaries that exist in mainland Britain (Davidson et al., 1991). In 1932, Butcher reported on the distribution of *Zostera* in the United Kingdom, including a spatial distribution map of mainland Britain that corresponds very closely with the estuaries map presented in the NCC report (**Figure 1**; Butcher, 1934). Further, qualitative data suggest that before WWI seagrass would once have been found across a large proportion of subtidal mud- and sandflats and on the lower ranges of most intertidal flats throughout the United Kingdom, especially prevalent in estuaries (Cotton, 1933; Butcher, 1934, 1941; Cottam, 1934). Sub and intertidal mud- and sand-flats, in particular, estuarine ones are, therefore, a good proxy for modelling historic seagrass distribution.

We identified locations with best available data on seagrass area cover from either historic or contemporary estimates, where contemporary estimates represent meadows that are in reasonably good condition, and where total mud- /sandflat area for each site was available. This restricted the inclusion of sites to those designated as Special Protected Areas (SPA's) and Special Areas of Conservation (SAC's; JNCC, 2005, 2015, 2018) since such sites have been accurately mapped by the Joint Nature Conservation Committee (JNCC; **Table 1**).

The chosen sites represent typical environmental variation for United Kingdom seagrasses including intertidal and subtidal habitats within estuaries, rivers, lochs, and spits. Seagrass area coverage per hectare of mud- / sandflat was calculated for each site by dividing total seagrass area by total mud- /sandflat area (**Table 1**; **Figure 2i**). Seagrass coverage was estimated using bootstrapping techniques (Manly, 2006). Our 10 sites were randomly selected with replacement 1,000 times and multiplied by total mud-/sandflat area estimates from (a) the OSPAR dataset (2017), which includes data in and outside of estuaries, and (b) the NCC report (Davidson et al., 1991), which includes data from estuaries only (**Figures 2a**,**b**). The average of these 1,000 estimates were used to estimate maximum extent of seagrasses around mainland Britain (**Figures 2ai**,**bi**). We used this simple bootstrapping procedure (Manly, 2006), rather than more typical parametric statistical methods, due to the paucity of available data. The data provide low certainty maximum seagrass extent around mainland Britain. In addition to the simple modelling approach, datasets were also obtained from previous studies using habitat suitability modelling to estimate potential seagrass distribution (Brown, 2015; Defra, 2020).

RESULTS

Contemporary and Pre-1998 Areal Estimates of Seagrass Habitats

The total mapped areal extent of contemporary seagrass records (post-1997) from the OSPAR dataset, the WFD dataset, and all other contributors includes 47 surveys spanning 20 years, 79% of which are from the last 10 years (see Supporting Information). In total, the data confirm the presence of 8,493 ha of seagrasses in the United Kingdom (**Table 2**). Occurrence of seagrasses is not uniform. Half of all mapped seagrass occurs in the Scottish Highlands (20%), Devon (16.2%), and Northern Ireland (14.3%; **Table 2**). Seagrass occurrence ranges from patches less than $1m^2$ to meadows up to 1,200 ha (i.e., Cromarty Firth, East Scotland). The average size of seagrass record is 2.64 ± 32.22 ha.

The OSPAR dataset, which represents the currently used known areal extent of seagrasses in the United Kingdom, includes 13,753 ha of seagrass. Of this, 8,835 ha (64.2%) was recorded pre-1998 and 4,919 ha (35.76%) was contemporary (post-1997). Within the OSPAR dataset, there is an inverse relationship between average meadow size relative to age of record, i.e., the average area of historic seagrass meadows is 71 ± 218 ha whilst the average area of contemporary seagrass meadows is 2 ± 238 ha. The average for all the meadows included in the OSPAR dataset between 1986 and 2015 is 4 ± 50 ha. GIS point data exist beyond the extent of these mapped areas but the extent of any seagrasses associated to such an observation is unknown and therefore not included within this analysis.

The total mapped historic extent of seagrasses in the United Kingdom is 16,524 ha. The total documented loss of seagrasses since 1936 is 6,697 ha. A further 1,364 ha of seagrass habitat has not been revisited since 1998 (**Table 3**), a disproportionate amount of which is from Scotland (**Table 4**). With high certainty the UK has, therefore, lost 44% of its seagrass since 1936, 34% since the 1980s. With medium certainty, including historic data with no recent observations, 50% has been lost since 1936, and 42% since the 1980s.

Systematic Review of Qualitative and Quantitative Data on Seagrass Declines

The first published account of seagrass in the United Kingdom that we are currently aware of was in 1831 (Winch, 1831), where it was included in a publication on the "*Flora of Northumberland and Durham*." This observation is from a location on the Tyne River long since reclaimed and now an industrial estate.[1] A peak in publications occurred around the time of the 1930's wasting disease when naturalists became concerned with the substantial degradation of sites throughout the United Kingdom (**Figure 3**). Publications were sporadic until 1990 ($n = 20$) and since then have occurred more frequently as a series of peaks and troughs ($n = 66$).

Attempts to describe seagrasses across the United Kingdom were made by Butcher in 1932, but without any defined methodology (Butcher, 1934). He considered the occurrence of

[1]https://seagrassspotter.org/sighting/1816

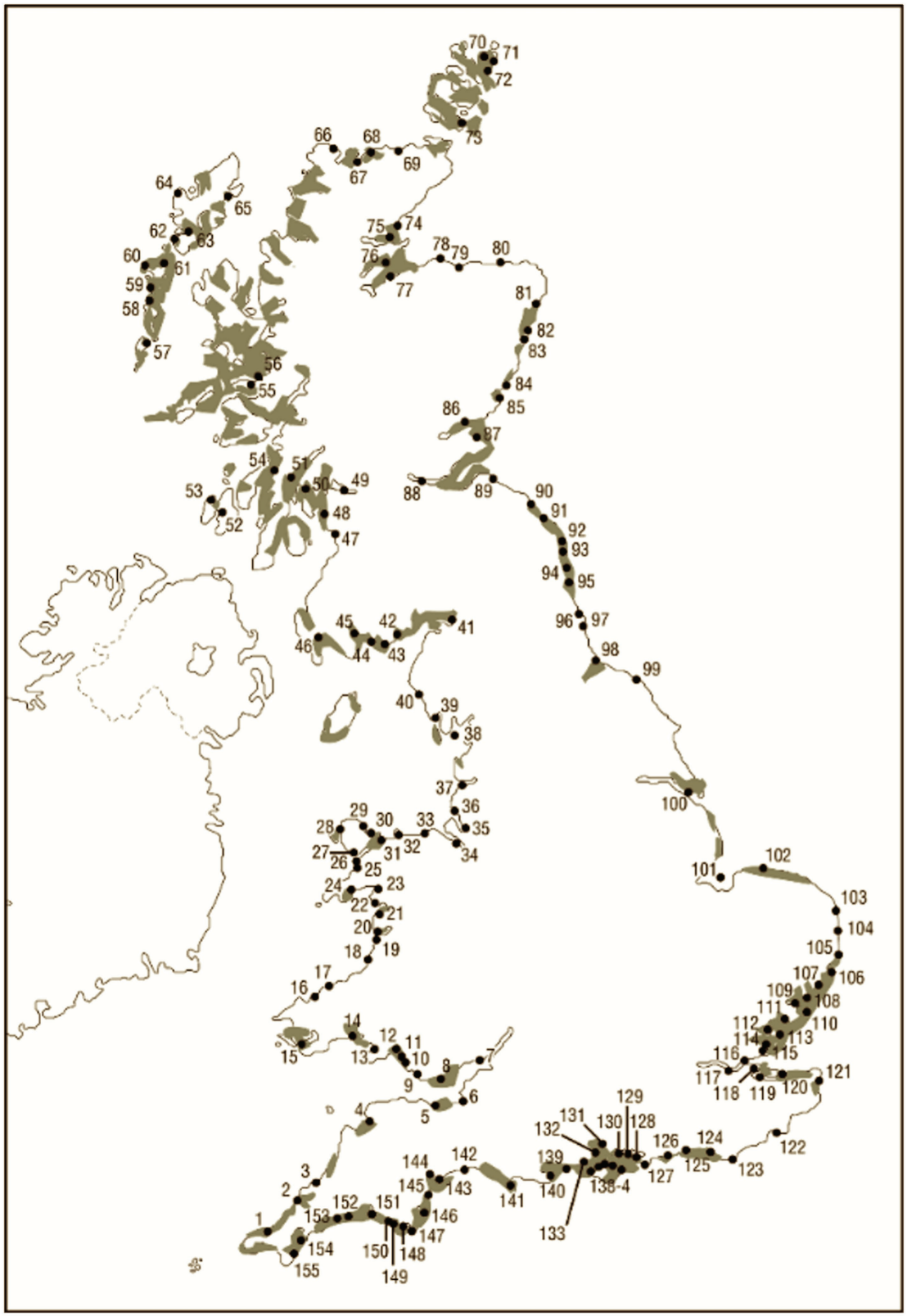

FIGURE 1 | Butcher's 1930s estimate of seagrass area cover (shaded; Butcher, 1934) and numbers referring to estuaries identified by Nature Conservancy Council (Davidson et al., 1991; image created by UCL drawing office).

TABLE 1 | Seagrass meadow area data used to calculate historic seagrass loss in the United Kingdom.

Site name	Site location	Current extent ha	Historic extent ha	m/s-flat area ha	Seagrass area/ha m/s-flat	Reference for m/s-flat area
Spurn bight	Humber estuary	0.59	550	4,842	0.11	JNCC, 2018
Lindisfarne	NE England	679	1,046	1,571	0.67	JNCC, 2015
Foulness/maplin sands	Thames estuary	40.	320	8,746	0.04	JNCC, 2005
Fal and helford	Cornwall	104	208	645	0.32	JNCC, 2018
River stour and orwell	Thames estuary	1	380	2,620	0.15	JNCC, 2018
Exe estuary	Cornwall	146	**N.D.**	900	0.16	JNCC, 2018
Dornoch firth	East Scotland	117	2,546	6,787	0.38	JNCC, 2018
Cromarty firth	East Scotland	1,200	3,241	3,766	0.86	JNCC, 2018
Moray firth	East Scotland	**N.D.**	1,098	2,339	0.47	JNCC, 2018
Plymouth sound	Devon	92	**N.D.**	2,555	0.04	JNCC, 2018

Current and historic extent (where available) and mud-/sandflat (m/s-flat) area were used to determine average seagrass area per hectare (ha) of mud-/sandflat. N.D. = no available data

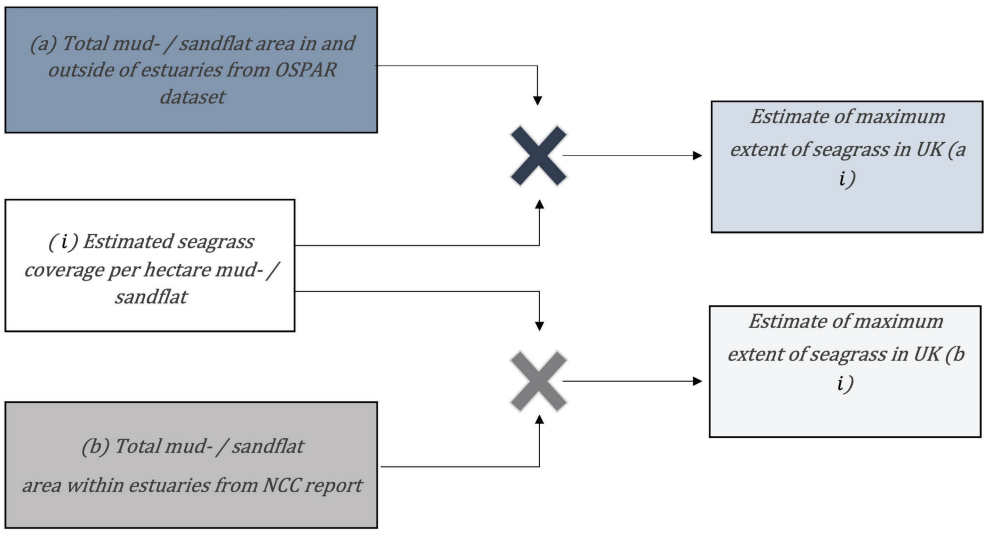

FIGURE 2 | Calculations used to estimate maximum areal extent of seagrass in mainland Britain: $a \times i = ai; b \times i = bi$. i is the average seagrass area from 11 sites with good historic or contemporary estimates divided by known mud- and sand flat area.

seagrasses regionally but did not provide area estimates. Seagrasses seemingly occurred ubiquitously, "apart from wave-swept, shingly and rocky shores to the west of the country" (Butcher, 1934). The abundance of seagrasses in sheltered and protected areas on the east coast was noted, as were plentiful populations in the lochs of Ireland and the west of Scotland (Butcher, 1934). Aside from this early attempt, efforts were made to document the status of seagrasses in Scotland in 1933 (Cleator, 1993), in Wales (Kay, 1998), and in Cornwall and the Isles of Scilly in 2002 (Hocking and Tompsett, 2002a,b). These reports provide presence and absence data and indicate widespread declines, but do not provide usable spatial estimates of area.

A full analysis and synthesis of the systematic search is provided in Supplementary Information. The literature analysed through the systematic review show ubiquitous declines across almost every region of the United Kingdom. Areas where good historic data are available show declines of between 40% (Cornwall) and 100% (Suffolk; **Table 3**). Although historic quantitative data are rare, the ubiquitous declines in seagrass areal extent are evident. Further, these historic declines are matched by pervasive recent declines, which suggest we are yet to stem this trend.

Modelling the Loss of Seagrass Throughout England, Scotland, and Wales

The proportion of seagrass area per hectare of sub- and intertidal mud- /sandflat ranged from 4 to 86% with an average of 32 ± 27%. The 1991 NCC report (Davidson et al., 1991) established that estuaries comprised a total of 530,000 ha of coastal waters in mainland Britain. Of these, half are in England and almost one third are found within Scottish waters (Davidson et al., 1991). Within these, mud- /sandflats make up about 43%, totaling 254,400 ha (Davidson et al., 1991). The OSPAR dataset does not include any data from Ireland and is lacking in Scottish datapoints. It reports 143,571 ha of sub- and intertidal mud- /sandflats in mainland Britain, including those outside of estuaries. Considering one third of estuaries is found in Scotland it is unsurprising that these figures do not align. The total current mapped areal extent of seagrasses in mainland Britain (from this papers data) is 6,760 ha.

Using the NCC data on total mud- /sandflats area, the estimated maximum seagrass extent for mainland Britain is 81,953 ha, with an upper 95% CI ranging from 126,430 to 40,964 ha (**Table 5**; **Figure 4**). Using the OSPAR data on total mud- /sandflats area, the maximum seagrass extent for mainland Britain is 43,559 ha, with 95% CI ranging between 72,647 and 24,267 ha (**Table 5**; **Figure 4**). Statistical comparisons between the two seagrass coverage values were made by comparing 95% CIs. Overlapping CIs indicated no significant difference between area estimates. The modelled data suggests that, with low certainty, between 36,799 and 75,193 ha of seagrasses has been lost from mainland Britain, this would represent an 84 and 92% decline, respectively (**Table 3**).

DISCUSSION

This study, to the best of our knowledge, is one of the first to systematically estimate the current and historic extent of seagrasses in any country and place it in the context of associated ecosystem services (see also, Ruiz et al., 2015; Harcourt et al., 2018).

TABLE 2 | Distribution of contemporary mapped seagrass area from the OSPAR and Water Framework Directive datasets, and other collected data sources since 1998.

Location	Area ha	% of total
Scottish highlands	2,056	24.21
Devon	1,392	16.39
Northern Ireland	1,810	14.44
Hampshire and Isle of wight	714	8.41
Northumbria	680	8.01
South Wales	460	5.42
Dorset	372	4.38
Scilly Isles	196	2.31
North Wales	172	2.03
Suffolk, Essex, and Kent	170	2.00
Cornwall	166	1.95
East Scotland	108	1.27
West Wales	90	1.06
Cumbria	65	0.77
Norfolk	42	0.49
Total	**8,493**	

Data present total known areal extent of seagrass in the United Kingdom by region, including relative contribution to the total mapped area.

With high certainty, at least 44% of United Kingdom's seagrasses has been lost since 1936. Of this, 39% has been lost since the 1980's, which is substantially more than the suspected global decline of 29% in the same period (Waycott et al., 2009; Short et al., 2010). It also provides a different narrative to that emerging from the recent European review of seagrass status and trends that focused exclusively around monitoring data mostly from the previous 10 to 15 years (de los Santos et al., 2019). With medium certainty, 48% of seagrasses have been lost since 1936, 44% since the 1980s. The modelled potential distribution of seagrasses suggests with low certainty that up to 92% of seagrass has been lost from mainland United Kingdom waters.

We provide estimated loss ranges because the paucity of survey data means it is impossible to know exactly how much seagrass has been lost from these waters. Our high certainty estimates are almost certainly under-representative of the true scale of occurred losses. They only represent those meadows that have been documented, and almost all of these would have undergone some level of degradation prior to their documentation. Our model has limitations, but without data, it is an important step to understanding the wide-scale losses that have occurred. In consultation with ABPmer (Defra, 2020) the EA undertook a suitability model to assess where seagrasses could occur in United Kingdom waters. They documented 43,346 ha of suitable habitat in England alone. Based on the current areal extent of seagrasses in England (3,873 ha), this would represent a 91% loss. A similar suitability model was conducted for Wales, which indicated 4,541 ha of suitable habitat (Brown, 2015). Based on the current areal extent of seagrasses in Wales (551 ha), this would represent an 88% loss. Together, these models suggest a total of 47,888 ha of suitable seagrass habitat in England and Wales. This total is comparable to our lower estimate for the

TABLE 4 | Estimated seagrass loss from high (known) and medium (unmapped) estimates across all regions and Scotland, calculated by analysing data older than 1998 from the OSPAR dataset.

	High certainty, known seagrass loss	Total unmapped seagrass	Medium certainty seagrass loss
All regions	6,697	1,364	8,061
Scotland	4,790	1,358	6,148

Known loss is from sites which have been revisited and data captured since 1998, unmapped is from sites that have not been revisited.

TABLE 3 | High certainty seagrass loss (by area and percent reduction) in regions where good historic data are available based on the systematic review.

	Max extent (pre-1998)	Contemporary area	High certainty loss since 1936	
	ha	*ha*	*ha*	*%*
UK	16,524	8,335	6,826	41
Cornwall	271	166	167	62
Essex	450	170	280	62
Northumbria	1,595	679	916	57
NW England	224	65	159	71
Scilly Isles	325	196	129	40
Scotland	8,312	2,164	4,790	58
Suffolk	380	1	379	100

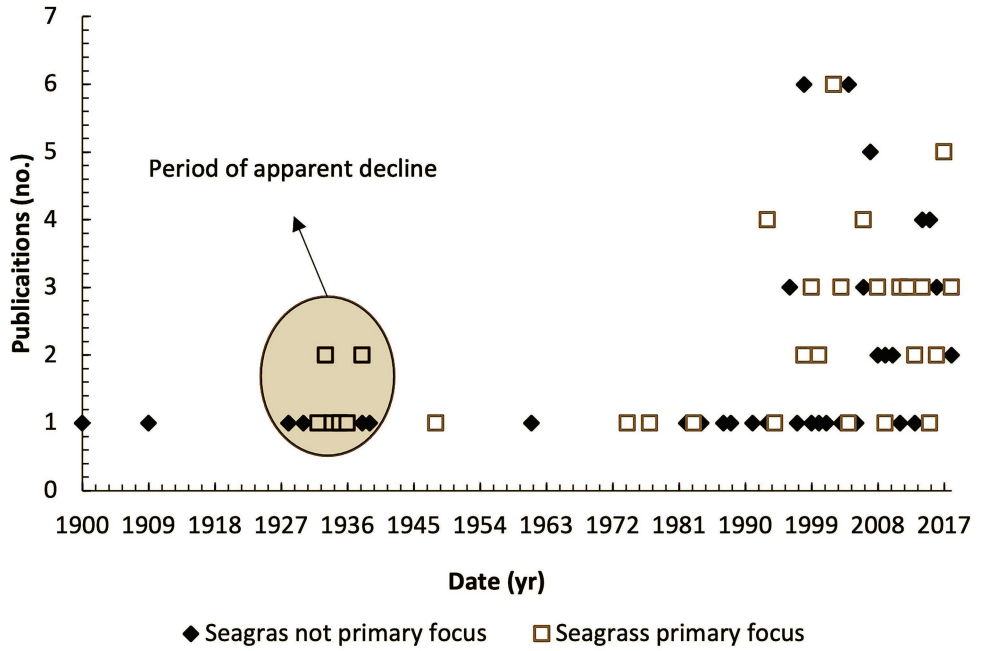

FIGURE 3 | Number of publications (grey and peer reviewed) relating to United Kingdom seagrass habitats by date where seagrass is primary focus (orange square) and seagrass is significant secondary focus (blue diamond).

TABLE 5 | Modelled maximum seagrass area extent, area and percentage loss in mainland Britain from the Nature Conservancy Council (NCC) report (Davidson et al., 1991) and the OSPAR dataset.

	NCC total mud- /sandflat area ha			OSPAR total mudflat area ha		
	254,400			143,571		
	Maximum seagrass extent ha	Seagrass loss ha	% decline	Maximum seagrass extent ha	Seagrass loss ha	% decline
Average	81,953	75,193	92	43,559	36,799	84
Upper 95%	126,430	119,670	95	72,647	65,887	91
Lower 5%	40,965	34,205	83	24,267	17,507	72

Average and 95% confidence interval displayed.

whole of mainland Britain (43,559 ha). Considering the suitability models and our lower estimate do not include any or many data points from Scotland, it would be reasonable to assume that the actual number is much closer to our higher estimate (81,953 ha). The EA also undertook a modelling project to map the historic areal extent and loss of saltmarsh habitats in England. Digitally overlaying ordinance survey maps from 1860, they combined these with historic maps of saltmarsh extent, and estimated coastline flooding capacity using LIDAR data to calculate an historic areal extent estimate of 215,624 ha (Mike Best EA, 2019, *personal communication*). This represents an 85% reduction on current saltmarsh extent in England. Although our modelled estimates may seem high, they are seemingly not out of character with other estimates of coastal degradation in the United Kingdom. Oyster reefs too are thought to have suffered similar extensive declines (Thurstan et al., 2013). If 85% of saltmarsh habitat has been lost in the United Kingdom, then the likelihood is that the environment which fringes it has also experienced widespread declines. The reef function of Oysters and their capacity to filter vast quantities of water rapidly means their loss would have had a significant negative impact upon the environmental conditions (light and shelter) of many areas that would have historically contained seagrass.

This study brings records together from disparate sources and provides the most up to date and accurate estimates of seagrass cover possible. The large-scale loss of seagrasses described here redefines the severity and spatial extent of what is known about biodiversity loss in our coastal seas, setting a new baseline upon which future management and restoration (Valdez et al., 2020) can aspire to build. Given the need to restore and improve management of these ecosystems, in light of work highlighting their importance to United Kingdom fisheries (Bertelli and Unsworth, 2014) and carbon sequestration (Green et al., 2018), and work highlighting the declining state of United Kingdom seagrass meadows (Jones and Unsworth, 2016), this work is much needed and timely in its arrival. Not all United Kingdom seagrass meadows has been lost and degraded, our research finds seagrasses persisting at many sites across the United Kingdom, to varying degrees of extent, with occurrences of seagrass recovery at some sites.

The rare accounts of documented areal extent of seagrasses in the early 1900's provide an example of the changes that

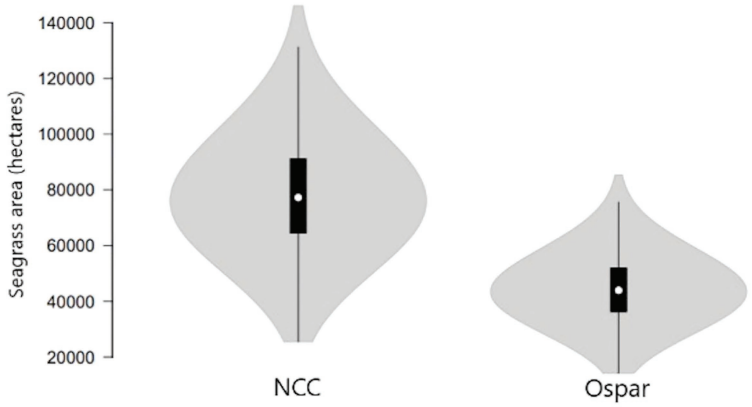

FIGURE 4 | Modelled maximum seagrass area extent in mainland Britain from the NCC report (69) and the OSPAR dataset. Error bars indicate 95% CI.

have likely occurred throughout the United Kingdom. Although these data are in isolation, the consistent declines noted in the literature, along with the documented 96% decline in average meadow size, confirms the trend of degradation pointed to by earlier studies (Butcher, 1934, 1941). Historic declines since the 1900's are mirrored by more recent declines noted in the last decade (Jackson et al., 2016) and numerous incidences of small-scale disturbances in recent years (Unsworth et al., 2017).

There is strong evidence to suggest that seagrass loss can lead to a state of negative feedback preventing ecosystem recovery (Maxwell et al., 2017). However, this has not been the case for all United Kingdom sites. Intertidal (but not subtidal) recoveries observed in Milford Haven, Wales, where historic pollution encroachment and oil spills had previously reduced seagrasses (Bertelli et al., 2018), indicate removing or reducing stressors can, in some locations, lead to habitat recovery. The recovery of other intertidal seagrasses in the Leigh Marshes in the Greater Thames Estuary further supports this observation. History suggests meadows are capable of fluctuating and can recover from dramatic losses. The ability of seagrass meadows to regain abundance is encouraging and should help spur conservation initiatives globally, especially current attempts to promote seagrass restoration (Valdez et al., 2020).

Understanding the Trends of Seagrass Decline in the United Kingdom

Rationalising the probable causes of such vast losses of seagrass in Britain is at best difficult, mostly because robust estimates regarding historic spatial extent of *Zostera* are limited. Typically, the seagrass wasting disease *Labyrinthula* has been described as the primary cause of virtually all seagrass loss in the United Kingdom (Butcher, 1934; Cottam, 1934; Den Hartog, 1983; Garrard and Beaumont, 2014). We argue that this assumption is itself a result of SBS. This assumption stems from the discussion within Butcher's report, where undoubtedly seagrasses were lost due to disease (Butcher, 1934). Butcher reported changes to habitat based on his own intertidal experiences of seagrass abundance, supported by those of individuals whose baselines only go as far back as their own inherited knowledge (Butcher, 1934; Pauly, 1995). This case of SBS means the basis on which Butcher referred to healthy populations of seagrasses is likely a gross underrepresentation of what once occurred in these waters and is an excellent example of how SBS impacts contemporary environmental knowledge (Butcher, 1934). There has been no enquiry as to whether the seagrass habitats Butcher was assessing were already heavily degraded, with almost all the literature pointing to this period for the cause of seagrass degradation (Butcher, 1934). Considering the early industrialisation of the United Kingdom and a long history of mining activity, it is almost certain that the systems Butcher assessed in 1930's would have already undergone dramatic declines. His assumptions were also largely based on visits to sites on the eastern shores of England. It is likely that persistent gradual declines had been occurring for centuries before Butchers report and these have continued to the present day (Jackson et al., 2016; Jones and Unsworth, 2016; Unsworth et al., 2017; Jones et al., 2018).

As the first country to industrialise in the 17 and 18th centuries, Britain had been undergoing dramatic land-use transformation long before Butcher assessed the status of seagrasses (Butcher, 1934). Industrialisation is intrinsically linked to environmental degradation. By the time, Butcher had been writing, dramatic physical alterations to the United Kingdom landscape had been occurring for at least 300 years (Butcher, 1934, 1941). A reference to seagrasses in the Tyne estuary in the early 1800's (Winch, 1831) referred to a site that has since been reclaimed, now containing an industrial estate. Coastal reclamation, dredging and building of sea walls were prevalent in the 17th century, 200 years prior to this account (Batty, 1997), and are highly likely to have been as in conflict with seagrass as they still are today (Erftemeijer and Lewis, 2006). Importantly, the UK was at the forefront of the global metal industry, with metal mining prevalent throughout many parts of the United Kingdom during the 1700s and 1800s, with many of these mines (e.g., Wales) still producing extensive metal contaminated discharge into coastal and estuarine waters today. The negative impacts of a suite of heavy metals, causing toxic conditions for seagrasses, are well documented

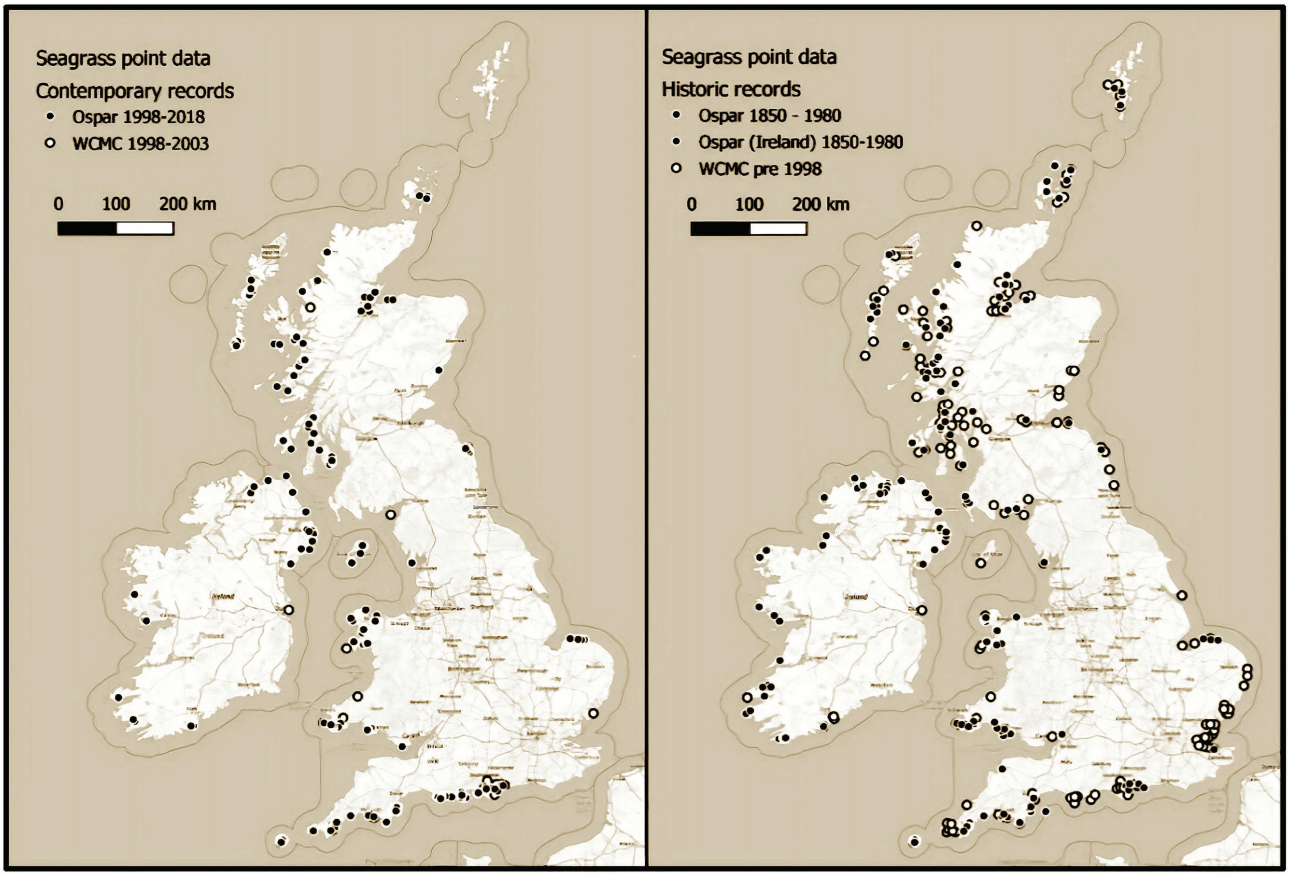

FIGURE 5 | Seagrass point data from the OSPAR and UNEP-World Conservation Monitoring Centre datasets showing pre-1998 surveys (left) and post-1998 surveys (right).

(Prange and Dennison, 2000; Macinnis-Ng and Ralph, 2002). Many areas thought to be viable seagrass sites within Welsh habitat suitability models are areas of historic heavy metal mining contamination, such as NE Anglesey (Whiteley and Pearce, 2003).

In addition to industrial development, the scale of overfishing of oysters around the United Kingdom cannot be ignored as a fundamental change and major disturbance to the environmental conditions. There is growing evidence of the close positive interactions that occur between seagrasses and many bivalves (Perkins, 1988; Gagnon et al., 2020). Locations such as the Firth of Forth have entirely lost up to 5,000 ha of oyster beds (Thurstan et al., 2013). These oyster beds would have fundamentally influenced the volume of suspended particles in the water column and hence the water clarity, creating conditions suitable for photosynthetic production by seagrasses. Similar estimates are available for areas, such as the Solent, the Thames, The Clyde, the Humber, and the Severn (Thurstan et al., 2013). The extraction activities of these Oyster fisheries were also likely to have disturbed and remobilised sediments at a high frequency over major areas for prolonged periods, potentially negatively impacting seagrasses. It is not only Oyster fisheries that would have likely had a historic negative impact upon seagrass. As early as the 1700s, bottom trawling was already widespread around the coastal waters of the Great Britain (Jones, 2018) and such activity is well known to directly damage seagrasses (Blaber et al., 2000). Seagrasses remain threatened in the United Kingdom today by activities which reduce water quality, and direct physical disturbance (Jones and Unsworth, 2016; Green et al., 2018).

The Need for Improved Seagrass Assessment

The present analysis highlights a lack of coherent and systematic monitoring and mapping programmes of seagrass meadows in the United Kingdom. That 64% of records in the OSPAR dataset are older than 20 years highlights the prolific lack of constant effort in seagrass mapping. Despite this fact, the OSPAR dataset is the baseline for estimates of United Kingdom seagrass extent included by the UNEP World Conservation Monitoring Centre (WCMC). Since this is the first attempt to provide an accurate map and up-to-date map of seagrass occurrence and declines for an entire country it is likely that these are not the only data included by WCMC that are inaccurate (McKenzie et al., 2020). The paucity of current data means that estimates, even coarse ones, are a necessary step to evaluating the pressures imposed on seagrasses, in keeping with the accepted approach of "*use available data*" (Hiscock, 1997). However, data

inconsistencies can make it challenging to talk meaningfully about global seagrass trends and arguably managers should only be working with temporal and spatial data that we are reasonably confident is accurate. Regional and local mapping of sites around the world is important in ensuring that current attempts to increase seagrass abundance through restoration, rehabilitation, and conservation have any hope to succeed.

Data inconsistencies found within this work are most obvious in Scotland, where most data were collected over 20 years ago. Although the isolation from human population likely means there is potential for these meadows to remain intact, many have likely been impacted by the extensive and continually expanding salmon aquaculture industry, with fish farming a known cause of seagrass loss in other parts of the world (Berry and Davison, 2001). The meadows where historic and contemporary data are available show mass declines. The once huge meadows in the Cromarty and Dornoch Firths have been reduced from 3,241 to 1,200 ha and 2,546 to 117 ha, respectively. Regardless, huge swathes of Scottish waters have not been surveyed for over 2 decades and could represent a vital stronghold of this once ubiquitous United Kingdom habitat. Their condition and extent should be assessed with urgency. Scotland is not the only region where survey efforts since 1998 have been insubstantial. Pre- and post-1998 maps show a reduced survey effort across all regions (**Figure 5**).

Impact of Declines on the Ecosystem Services Afforded by United Kingdom's Seagrass

Recent efforts have been made to estimate the amount of carbon stored within the United Kingdom seagrass meadows (Röhr et al., 2018; Green et al., 2018; Lima et al., 2020). These papers analysed sediment from 13 meadows along the southwest coast of the United Kingdom for organic carbon content. For areas around the Solent and adjacent harbours, Lima et al. (2020) found 33.8 ± 18.5 Mg C ha^{-1} in the top 30 cm of sediment. Whilst Green et al. (2018) found meadows contained a total of 141 ± 73 Mg C ha^{-1}, within the top 1 m of seagrass sediment. We have used mean values across both United Kingdom seagrass species as current carbon values are limited and explicit assessment of the impact of species and environmental variation upon this are largely absent. Based on these average figures, the estimated total carbon stored in the top 100 cm of recently mapped seagrasses of the United Kingdom is ~1.2 Mt carbon. In mainland Britain, this figure is 0.9 Mt of carbon (**Table 5**). Based on the upper (81,952 ha) and lower (40,965 ha) estimates of historic seagrass distribution, mainland United Kingdom seagrass meadows could once have contained between 5.7 and 11.5 Mt of carbon (**Table 5**). The upper value of this is equivalent to 3% of the United Kingdoms CO_2 emissions in 2017 (Eaton, 2019).

There exists only one value currently of United Kingdom seagrass carbon sequestration, but this value itself is an estimate (Garrard and Beaumont, 2014), hence there are no reliable estimates of seagrass sequestration rates in the United Kingdom. Reasonable and frequently used rates in the literature give low (0.044 cm $yr.^{-1}$), medium (0.202 cm $yr.^{-1}$), and high (0.42 cm $yr.^{-1}$) bounds to frame carbon sequestration estimates (Duarte et al., 2013; Lavery et al., 2013; Macreadie et al., 2013; Miyajima et al., 2015; Röhr et al., 2018). Here, sequestration rates were estimated by dividing the total carbon estimates by the amount of time it takes to accumulate this stock using the sedimentation rates above, to provide estimates on average annual carbon accumulation of United Kingdom seagrass meadows (Lavery et al., 2013; Röhr et al., 2018; **Table 6**.). Assuming a medium sedimentation rate, the seagrass meadows of the United Kingdom are accumulating roughly 0.024 Mt C yr^{-1} (**Table 6**). Assuming this medium sedimentation rate, historic undisturbed seagrass meadows of the United Kingdom could have been absorbing 0.232 Mt C yr^{-1} (**Table 6**).

Considering the need to include natural ecosystems in climate mitigation strategies, there is increasing interest in placing monetary valuations on carbon stock and sequestration estimates. The United Kingdom government has recently implemented a legal commitment to achieve Net-Zero greenhouse gas emissions by the year 2050. To reach this target will require major economic reforms and substantial increases in natural carbon sequestration capacity. The current United Kingdom carbon value is £24/t C (DECC, 2011; Green et al., 2018). However, according to the Grantham Institute, a price that is consistent with the Net-Zero targets needs to begin at £50/t C, rise to £75/t C in 2030, and to £160/t C in 2050 (Burke et al., 2019). Based on today's market price, the value of the carbon stored in the top 100 cm of recently mapped seagrass stands at a value of £29 million with yearly (medium) sequestration value (**Table 6**) of £0.58 million, rising to £3.9 million over the next 30 years, if the projections hold true (**Table 7**). Taking the upper (**Table 6**) range of the historic estimates of seagrasses in mainland Britain, at today's market value, these would once have contained

TABLE 6 | Estimates of total carbon (Mt C) of modeled historic and contemporary seagrass distribution of mainland Britain, and of contemporary seagrass distribution of the United Kingdom, with low (0.044 cm yr^{-1}), medium (0.202 cm yr^{-1}), and high (0.42 cm yr^{-1}) estimates for carbon sedimentation per year (Mg C $yr.^{-1}$).

	Carbon stock		Sedimentation rates		
	Area ha	Total carbon Mt	Low Mt. C yr^{-1}	Medium Mg C yr^{-1}	High Mg C yr^{-1}
Upper historic estimate	81,953	11.5	0.050	0.232	0.483
Lower historic estimate	40,965	5.7	0.025	0.115	0.239
Contemporary area United Kingdom	8,493	1.2	0.005	0.024	0.050
Contemporary area mainland Britain	6,760	0.9	0.004	0.018	0.038

TABLE 7 | Estimates of total carbon (Mt C) and current and projected increases in carbon economic value (£million) of modelled historic and contemporary seagrass distribution of mainland Britain, and of contemporary seagrass distribution of the United Kingdom, medium (0.202 cm yr^{-1}) estimates for carbon sedimentation per year (Mg C $yr.^{-1}$), and associated current and projected increases in carbon economic value (£million).

	Area	Total carbon	Total market value £million			Sequest-ration	Total market value £million		
			Today	NZR Today	30 years		Today	NZR Today	NZR 30 years.
	ha	*Mt*	£24/t C	£50/t C	£160/t C	*Mg C yr–1*	£24/t C	£50/t C	£160/t C
Upper historic estimate	81,953	11.5	276	575	1840	0.23	5.6	11.6	37.2
Lower historic estimate	40,965	5.7	136.8	285	912	0.12	2.8	5.8	18.4
Contemporary area United Kingdom	8,493	1.2	28.8	60	192	0.02	0.6	1.2	3.9
Contemporary area mainland Britain	6,760	0.9	21.6	45	144	0.02	0.4	0.9	2.9

NZR, net zero requirement.

£276 million worth of carbon in their sediments. In this undisturbed state, these seagrass meadows could have been responsible for sequestering £5.6 million worth of carbon every year, rising, if the projections hold true, to £37.2 million over the next 30 years (**Table 7**). These figures, although crude, offer a powerful indicative snapshot of what has been lost through long-term environmental degradation, and support the need to offer protection to those seagrass meadows that remain. Seagrasses use to be ubiquitous along the shores of the United Kingdom, and if restored to even part its former extent; this ecosystem will provide valuable support to reaching a carbon neutral future.

The value that seagrasses provide for other ecosystem services cannot be readily quantified financially with current data; however, the functions they play are extensive in the United Kingdom, particularly with respect to fisheries support, biodiversity, nutrient cycling, and sediment stabilisation (Nordlund et al., 2016). United Kingdom studies have revealed that seagrass harbours 4.6 times the abundance of fish of unvegetated habitat at a density of 6,000 fish per hectare (Bertelli and Unsworth, 2014), resulting in United Kingdom seagrasses currently supporting approximately 50 million fish, many of commercial importance (e.g., juvenile whiting, cod, plaice, and pollack). Based on the upper (81,952 ha) and lower (40,965 ha) estimates of historic seagrass distribution, mainland United Kingdom seagrass meadows could once have contained between 200 and 400 million fish, a potential loss of 274 million fish based on the comparison to unvegetated seabed.

Given the eutrophication issues faced by many coastal waters around the United Kingdom, the loss of seagrasses will have had a detrimental impact upon water quality. For example, *Z. marina* meadows may cycle approximately 49 kg of nitrogen per hectare year (Watson et al., 2020). This equates to the current annual cycling of 416 tonnes per year that could have historically been as high as 4,015 tonnes per year. Seagrasses also help reduce coastal erosion through improved stability of sediments. The extent of this role is often context dependent (Ondiviela et al., 2014) but available evidence indicates that at high density *Z. marina* may increase by up to 10 fold the sheer strength of the sediment (Widdows et al., 2008). Given the vast loss of seagrasses in eastern parts of the United Kingdom in areas know to be subject to coastal erosion and predicted to suffer from increasing impacts of rising sea levels, we hypothesise that at its historic extent seagrasses would have played a pivotal role in reducing coastal erosion.

CONCLUSION

Although the United Kingdom has arguably been altering its natural habitats for longer than almost any other country, the trends and impacts of declines exposed in this paper are likely occurring in many other developing and developed counties. This analysis shows the devastating ecosystem services losses that this decline has caused. It is hoped that this paper will not only generate a better understanding of seagrass losses in the United Kingdom, but also spur efforts to protect remaining seagrasses and restore historical losses and drive other countries to take stock of this vital coastal habitat to the same goal.

DATA AVAILABILITY STATEMENT

The seagrass distribution data used in this study have been deposited in the Dryad Digital Repository (Green et al., 2021).

AUTHOR CONTRIBUTIONS

All authors conceived the ideas and designed the methodology. AG collected the data and led the writing of the manuscript. AG and MC analysed the data. All authors contributed critically to the drafts and gave final approval for publication. All authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.629962/full#supplementary-material

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frontiers in Marine Science

REVIEW
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Impediments to Understanding Seagrasses' Response to Global Change

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Uncertainties from sampling biases present challenges to ecologists and evolutionary biologists in understanding species sensitivity to anthropogenic climate change. Here, we synthesize possible impediments that can constrain research to assess present and future seagrass response from climate change. First, our knowledge of seagrass occurrence information is prevalent with biases, gaps and uncertainties that can influence inferences on species response to global change. Second, research on seagrass diversity has been focused on species-level metrics that can be measured with data from the present – but rarely accounting for the shared phylogenetic relationships and evolutionary distinctiveness of species despite species evolved and diversified from shared ancestors. Third, compared to the mass production of species occurrence records, computational tools that can analyze these datasets in a reasonable amount of time are almost non-existent or do not scale well in terms of computer time and memory. These impediments mean that scientists must work with incomplete information and often unrepresentative data to predict how seagrass diversity might change in the future. We discuss these shortfalls and provide a framework for overcoming the impediments and diminishing the knowledge gaps they generate.

Keywords: marine macrophytes, sampling biases, computational infrastructure, Alismatales, biodiversity shortfalls, climate change

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INTRODUCTION

Human activities, through fossil fuel emissions and widespread deforestation, have contributed to increased global temperature above pre-industrial levels (IPCC, 2018). As a consequence, global increases in temperature and atmospheric carbon dioxide can influence species by altering their growth rates, physiological functions, sexual reproduction, distribution, community composition, and primary productivity (Short and Neckles, 1999; Campbell et al., 2006). Such changes in environmental climate outside species' tolerable thresholds will cause some species to relocate in order to stay within their tolerance zones (Bradshaw and Holzapfel, 2001; Parmesan, 2006; Miller-Rushing and Primack, 2008; Anderson et al., 2012; MacLean et al., 2018). For instance, species on land generally ascend to higher elevations or latitudes as temperatures warm, but may run out of room, which can lead to local extirpation (Parmesan et al., 1999; Freeman et al., 2018). The sensitivity and responsivity of seagrasses or other marine species, whose distributional ranges lie at the land-sea margin and with very different evolutionary histories may show different responses to climate change.

Seagrasses are a major vascular plant clade of about 70 species belonging to the Alismatales, an order that includes ∼4,000 other non-marine species (Berry, 2019). They are widely distributed across marine coastlines or estuarine environments, often growing submerged in marine water (Hemminga and Duarte, 2000). Seagrasses display a wide variety of morphological diversity including turtlegrass (*Thalassia testudinum*) which forms long and jointed rhizomes, rhizome matts in *Posidonia*, ribbonlike leaves in eelgrass (*Zostera marina*), and paddle-shaped leaves in paddle grass (*Halophila decipiens*) (**Figure 1**). They play key ecosystem roles including primary productivity, nutrient cycling, and carbon sequestration (Hemminga and Duarte, 2000; Duarte, 2002; Les et al., 2002; Orth et al., 2006a,b; McGlathery et al., 2007; Nordlund et al., 2018). Seagrass meadows are an important nursery ground for many invertebrates and fishes (Beck et al., 2001), and directly provide food for marine herbivores including manatees, dugongs, and green sea turtles (Green and Short, 2003; Larkum et al., 2006). As threats from global climate change intensify, the impacts across seagrass communities are mixed. Some studies have found a decline in seagrass habitats especially in Australasia with decline rates of about 110 km^2 per year (Waycott et al., 2009). This pattern is not true in North America and Europe where seagrass communities are no longer in decline, but in fact show positive trajectories in some cases (de los Santos et al., 2019), perhaps as a result of the proliferation of seagrass monitoring and conservation programs such as Seagrass-Watch[1] and SeagrassSpotter.[2] Indeed, the vulnerability to the impacts of climate change on seagrass communities may be scale or context dependent (Day et al., 2008).

A number of studies indicate that global climate change can impact seagrass communities in a variety of ways. Short and Neckles (1999) reviewed the potential effects of climate change on seagrass growth rates, reproduction and spatial distributions; Duarte et al. (2018) explored relationships between climate change and phenotypic variation in seagrasses (including physiological variation, propagation success, and herbivore resistance); whereas Erry et al. (2019) used a mesocosm experiment to assess response of a multi-trophic seagrass ecosystem to several global change factors. The findings overwhelmingly demonstrated that these factors in unison could lead to deleterious effects on seagrass ecosystems if they are unable to rapidly adapt to changes in climate. Similar trends have been observed for specific seagrass locations e.g., Great Barrier Reef (Waycott et al., 2007), Mediterranean (Pergent et al., 2014), tropical Pacific Ocean (Waycott et al., 2011), and Western Australia (Arias-Ortiz et al., 2018; Strydom et al., 2020); or in selected species (e.g., Chefaoui et al., 2018). Other threats to seagrass populations can be attributed to overexploitation, physical modification, nutrient and sediment pollution, and introduction and spread of invasive species (Zieman, 1976; Ralph et al., 2006; Moksnes et al., 2008; Bryars et al., 2011; Dewsbury et al., 2016). By contrast, research to elucidate effects of global climate change on seagrass meadows and how to improve the prediction of future risks under varying scenarios of climate change have received less attention (Pernetta et al., 1994; Bijlsma et al., 1995; Short and Neckles, 1999).

Here, we argue that the extension of research agenda to assess seagrasses' response to climate change may be constrained by at least three factors. First, our knowledge of seagrass occurrence information is widespread with biases, gaps and uncertainties that can influence downstream inferences. Second, most of the research on seagrass diversity has been focused on species-level metrics (e.g., species richness, endemism or threat) that can be measured with data from the present – but rarely accounting for the shared phylogenetic relationships and evolutionary distinctiveness of species. Species are not independent units but

[1]https://www.seagrasswatch.org/
[2]https://seagrassspotter.org/

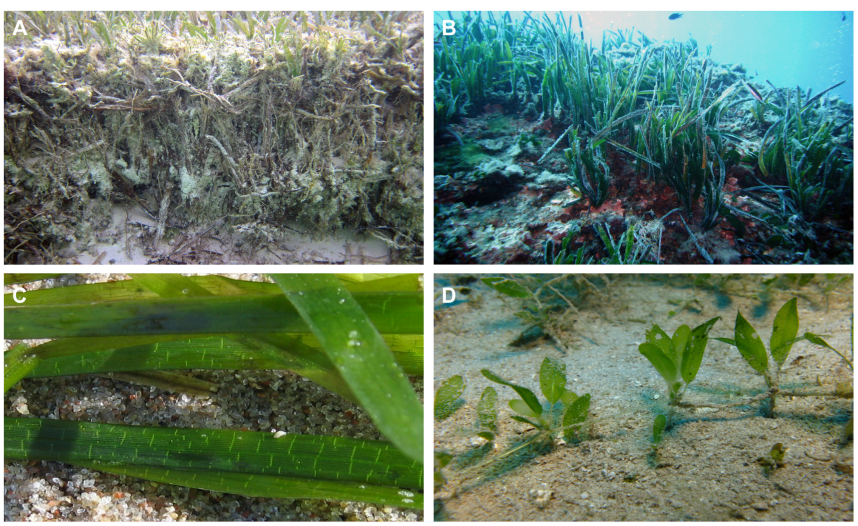

FIGURE 1 | Morphological diversity of selected species of seagrasses. **(A)** *Thalassia testudinum* (turtle grass) bed with view of jointed rhizomes, San Salvador Island, Bahamas. **(B)** *Posidonia oceanica* (Neptune grass) meadow with view of rhizome matts, Portofino, Italy. **(C)** *Zostera marina* (eelgrass) with ribbon-like blades. **(D)** *Halophila decipiens* (paddle grass) with paddle-shaped blades. (https://commons.wikimedia.org and https://calphotos.berkeley.edu/).

are lineages that evolve and diversify from shared ancestors (Diniz-Filho et al., 2013). Third, compared to the mass production of species occurrence records, computational tools that can analyze these datasets in a reasonable amount of time are almost non-existent or do not scale well in terms of computer time and memory. These impediments mean that scientists must work with incomplete information and often unrepresentative data to predict how seagrass diversity might change in the future. These shortfalls need be carefully recognized and remedied. The objectives of this review are therefore to first identify the knowledge gaps to understanding seagrasses' response to climate change, and secondly propose strategies and tools to overcome these impediments.

KNOWLEDGE GAPS IN SEAGRASS SAMPLING PRACTICES

Global change has become a central focus of modern ecology. Yet, our knowledge of how anthropogenic drivers affect seagrass evolutionary diversity is limited by a lack of biological data spanning the Anthropocene that equally represents all seagrass species. We define the Anthropocene as a period of profound human impact on biodiversity, characterized by widespread migration by humans as initiated by the Columbian Exchange circa 1492 (Nunn and Qian, 2010). The vast amounts of specimens of seagrasses deposited in herbaria can serve as a historical lens into the ecological processes by which present-day seagrass diversity arose, are maintained, and may evolve in the future. However, occurrence records archived in herbaria and museums are non-randomly collected over space and time, and thus present biases and uncertainties that can complicate ecological inferences (e.g., Boakes et al., 2010; Meyer et al., 2016; Daru et al., 2018; Dias Tarli et al., 2018). As a consequence, the use of occurrence records has not fully permeated the field of global change biology. The gap between specimen availability and use is widening as hundreds of thousands of specimens are being mobilized through massive digitization efforts worldwide. We argue that sampling biases in seagrass occurrence records can manifest in at least three ways: geographic, taxonomic, and temporal biases (**Figure 2**). We distinguish between the biases and describe how these limitations can inhibit progress in understanding seagrass response to global change.

Biases in Geographic Sampling

Geographic bias is the disproportionate sampling of a species in some regions of its range relative to others (Meyer et al., 2016; Stropp et al., 2016; Daru et al., 2018; Menegotto et al., 2019). Seagrass geographic data is commonly available as point records or polygons. Point records are commonly derived from major data hubs such as the Global Biodiversity Information Facility (Edwards et al., 2000; GBIF.org, 2020), United Nations Environment World Conservation Monitoring Centre (UNEP-WCMC, and Short, 2020) or Ocean Biodiversity Information Facility (OBIS) whereas polygons are derived from the International Union for the Conservation of Nature's (IUCN) spatial database and United Nations Environment World Conservation Monitoring Centre (Green and Short, 2003; UNEP-WCMC, and Short, 2020). Despite the fundamental importance of occurrence data for species distribution modeling, the sampling of seagrasses across most of their ranges are underrepresented in collections (Green and Short, 2003). For instance, extensive spatial gaps exist across regions that harbor high concentrations of seagrass diversity, especially in Western and Central Indo-Pacific, whereas Europe and North America are well sampled (**Figure 3**) (see Methods and Source Data file in **Supplementary Material** for details). This pattern is consistent with previous studies. For example, Waycott et al. (2009) found wide sampling gaps in West Africa, northeast South America, and the northwest Pacific area of the United States, most of which correspond to seagrass areas of endemism. Moreover, since biogeographic patterns are scale dependent, varying along spatial grains, geographic extents and taxonomic treatments

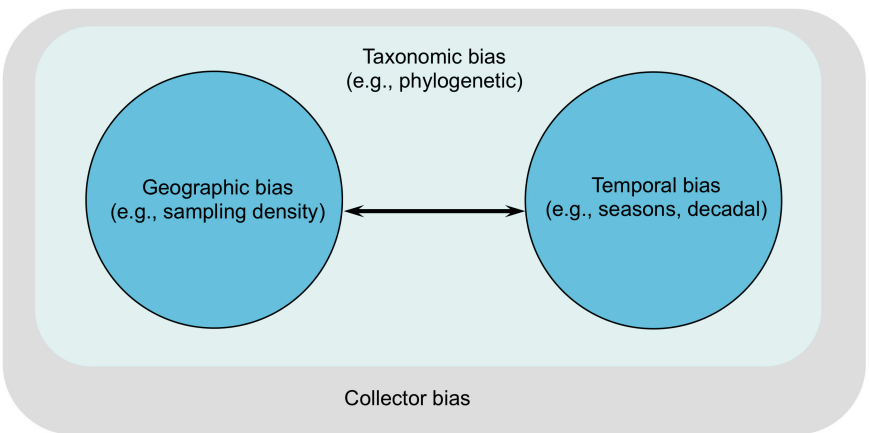

FIGURE 2 | Interactions between sampling biases indicating the extent of influence of each bias on the others. Taxonomic bias affects all other biases whereas arrows indicate direction of influence between the other two. However, all three types of biases ultimately reflect the personal preferences, biases, and proclivities of collectors.

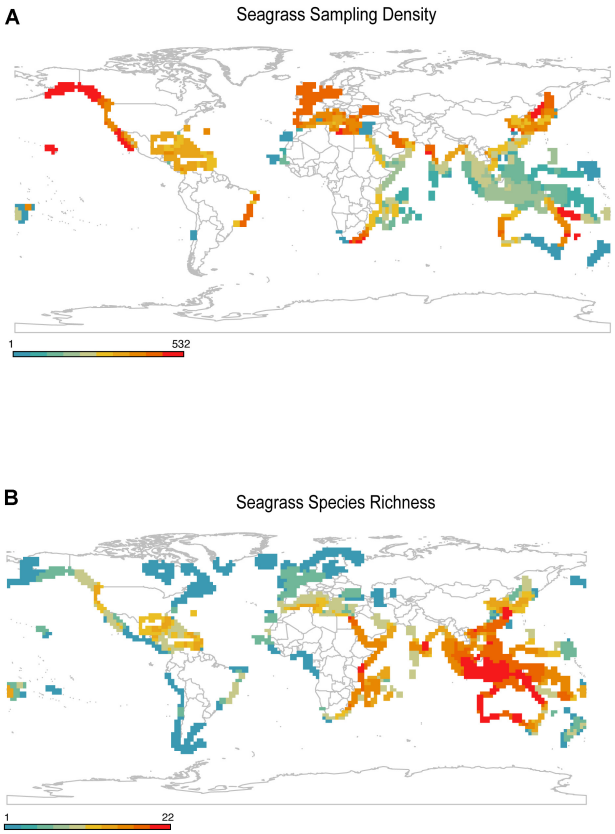

FIGURE 3 | Gaps in geographic sampling of seagrasses. **(A)** Seagrass occurrence records showed strong density of sampling in temperate regions, while sampling within the tropics was generally low. **(B)** Geographic distribution of seagrass known species richness based on expert delineated polygons. Source data are provided as a Source Data file.

(Jarzyna and Jetz, 2018; Daru et al., 2020a; GBIF.org, 2020), the extent to which geographic biases in seagrass sampling vary with spatial extent, grain size and taxonomic treatment remains poorly explored. However, it has been predicted that as grain size decreases, the knowledge gap in geographic sampling correspondingly increases (Hortal et al., 2015).

The mismatch between observed seagrass diversity and maps of survey efforts can be attributed to several factors: (1) knowing data exists in the first place and where it is, (2) harvesting data collected in native languages not common to science, (3) getting permission to access data collected under commercial license or from uncooperative governments, (4) validating data both spatially and taxonomically, (5) the difficulty in sampling specimens especially species in remote and inaccessible waters e.g., *Halophila decipiens* occurring >70 m deep in the Central Indo-Pacific (Short et al., 2007) or large parts of Northern Australia that are only accessible by helicopter, (6) lack of reliable research infrastructure e.g., West Papua and Papua New Guinea, (7) un-inhabited reef lagoons in large parts of the tropics and Western Pacific, (8) the cost of gathering long-term data (Wolfe et al., 1987), (9) perhaps a reversing trend of seagrass loss in Europe, North America, and subtropical Atlantic, e.g., increasing population trends in *Cymodocea nodosa* (Schäfer et al., 2021), *Zostera marina* and *Zostera noltei* (de los Santos et al., 2019; Guerrero-Meseguer et al., 2021), and (10) budget constraints for seagrass research. If seagrass species observations are made near accessible areas e.g., seaports, harbors or marine research stations, their application in analysis of species distribution modeling can compromise model performance (Kadmon et al., 2004; Lobo and Tognelli, 2011; Bystriakova et al., 2012; Kramer-Schadt et al., 2013; Varela et al., 2014). In practice, this means that most observations only reflect the climate space of accessible areas (e.g., Daru, 2021), and correspondingly areas of human activities where surface temperatures are higher than in surrounding natural areas (Kalnay and Cai, 2003). Additionally, regions known to contain seagrass meadows (e.g., Canada, Indonesia, and Russia) have inadequately mapped distributions, while other currently mapped regions most likely only represent a small portion of seagrass diversity (McKenzie et al., 2020). Targeting the places that are underrepresented in future collecting expeditions could remedy these limitations and aid in evaluating how species are responding to recent and future environmental change across biomes.

Biases in Temporal Sampling

The sampling of seagrasses can manifest as temporal bias – the unbalanced collecting of specimens in some years or parts of a given year. This can influence conclusions drawn from analyses of such non-randomly sampled collections records (Syfert et al., 2013). Temporal data is increasingly used in a wide range of applications in ecology and evolutionary studies including tracking changes in phenology – the timing of seasonal events such as flowering, leafing, and fruiting – and monitoring the spread of invasive species (Iler et al., 2013; Veeneklaas et al., 2013; Daru et al., 2019; Meerdink et al., 2019). Yet, while there is general agreement that climate change can influence phenological patterns by disrupting the timing of life cycle events and consequently drive changes in fitness and population demography (Ovaskainen et al., 2013; CaraDonna et al., 2014; Thackeray et al., 2016; Kharouba and Wolkovich, 2020), most have been observed in terrestrial species and to a lesser extent in marine flowering plants. In a meta-analysis of GBIF occurrence records over the course of 250 years (1770–2020) to understand the nature and evolution of seagrass sampling (GBIF.org, 2020), sparser records were observed in earlier years and high collection densities between the 1900s and present-day (**Figure 4**). Although over the 250-year time span, occurrence data was absent for a total of 131 years. Seasonally, seagrass specimens were overwhelmingly biased toward spring and summer months (regardless of hemisphere location) for most marine ecoregions including Temperate Southern Africa, Temperate Australasia, Temperate Northern Pacific, and Temperate Northern Atlantic (**Figure 5**; see Methods and Source Data file in **Supplementary Material**). Interestingly, these periods are spanned by comprehensive time series data of ocean climate including sea temperature and salinity (Benway et al., 2019). This means that the time series of changes in seagrass communities across years or seasons are fewer than the available climate records (cf. Duarte, 1992).

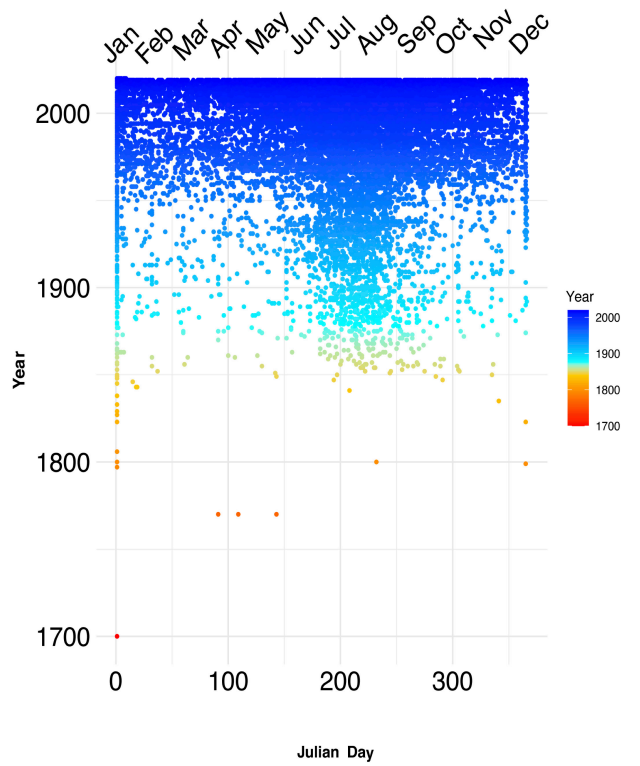

FIGURE 4 | Temporal sampling of seagrasses reveal drastic increases midway throughout the 18th century. Temporal data from seagrass records over the course of three centuries (roughly 1700–2000) display dense amount of sampling records accumulating after 1850. Each dot represents an occurrence record of a seagrass in Julian day of year format, with the color gradient representing recent years with colder color tones, and older years represented by warmer color tones. These data also support the previously identified global trend of increased sampling occurring predominantly within the summer months (early June through early October). Source data are provided as a Source Data file.

As a consequence, the non-random sampling of seagrasses in some years or parts of a year could mean that occurrence records are not reliable sources of phenological change driven by climate or population demography. If seagrasses are collected only when it is climatically convenient coupled with lack of reproductive structures on most specimens (Pearson et al., 2020), botanists may miss important phenological events such as winter bud formation, which protects the embryonic shoot of species during development and elongation (van der Schoot et al., 2013). Similarly, climate change can influence population demography through range change (Hunter et al., 2010; Dalgleish et al., 2011; Hugo, 2011; Gaillard et al., 2013; Selwood et al., 2015) or facilitate the spread of invasive species (Hellmann et al., 2008; Clements and Ditommaso, 2011; Vicente et al., 2013; Hou et al., 2014; Thapa et al., 2018). However, the skewed sampling of seagrass occurrence data suggests that the data is insufficient to track demographic changes or monitor spread of invasive species. We recognize that several aspects can influence seagrass sampling across years or seasons. For instance, some seagrass species are annuals, completing their life cycle within one growing season (e.g., *Halophila decipiens*). Other reasons include inaccessibility to most sites in the West Indo-Pacific during monsoon times, resulting in overrepresentation of specimens during maximum growing season/flowering season.

Biases in Taxonomic Sampling

The sampling and collection of seagrass data may be disproportionately higher in some taxa over others (Hortal, 2008). Taxonomic bias can manifest as phylogenetic bias and be assessed by testing for phylogenetic signal in collection frequency. A strong phylogenetic signal – closely related species share similar collecting frequency – would suggest phylogenetic bias in collections (Daru et al., 2018). Phylogenetic bias can hamper prospects of identifying species that are climate change indicators and those most likely to be affected by future climate change, especially given that species' response to climate change tends to be phylogenetically non-random (Willis et al., 2008; Davis et al., 2010; Davies et al., 2013). A phylogenetic analysis of long-term monitoring data in Concord Massachusetts, for instance, revealed a strong association between change in abundance with flowering time response such that the response traits are shared among closely related plant species (Willis et al., 2008). However, taxonomically non-random collection may mask such patterns and therefore bias conclusions of seagrass response to climate change. These data limitations may result from a research focus on specific seagrass lineages over other groups or simply lack of data on some species. For example, Coyer et al. (2013) estimated divergence times in 20 species in the family Zosteraceae at 14.4 Ma, whereas Dilipan et al. (2018) assessed phylogenetic relationships by focusing on only family Hydrocharitaceae. Not only do these clade-based approaches point to different divergence times, but the phylogenetic reconstructions also used different gene regions with likely different rates of evolution. Seagrass occurrence data on GBIF tends to display a weak phylogenetic signal in the tendency of closely related species to be sampled similarly; with an average of ∼9 specimens per species representing most *Halophila*, and ∼6–9 specimens per species representing most *Zostera*, whereas *Halodule* and *Posidonia* had far fewer records (**Figure 6**; see Methods and Source Data file in **Supplementary Material** for details).

Another factor that can induce taxonomic bias is the lack of comprehensive phylogeny for seagrass species. Inferring evolutionary patterns based only on phylogeny of the taxa within the community of interest without fully accounting for the overall phylogenetic diversity of the entire lineage can potentially lead to spurious results (Park et al., 2018). The available DNA sequences of seagrasses in GenBank/EBI are sufficient to construct a molecular phylogenetic tree for only 55 (of 72) species (Daru and le Roux, 2016). The 17 species without available DNA sequences are often manually grafted to the molecular tree in a multichotomy to the node of their close relatives using a Bayesian framework (Thomas et al., 2013). Such incomplete sampling or misplaced taxa on the phylogeny can influence the final tree topology and compromise rates of evolution (Nee et al., 1994; FitzJohn

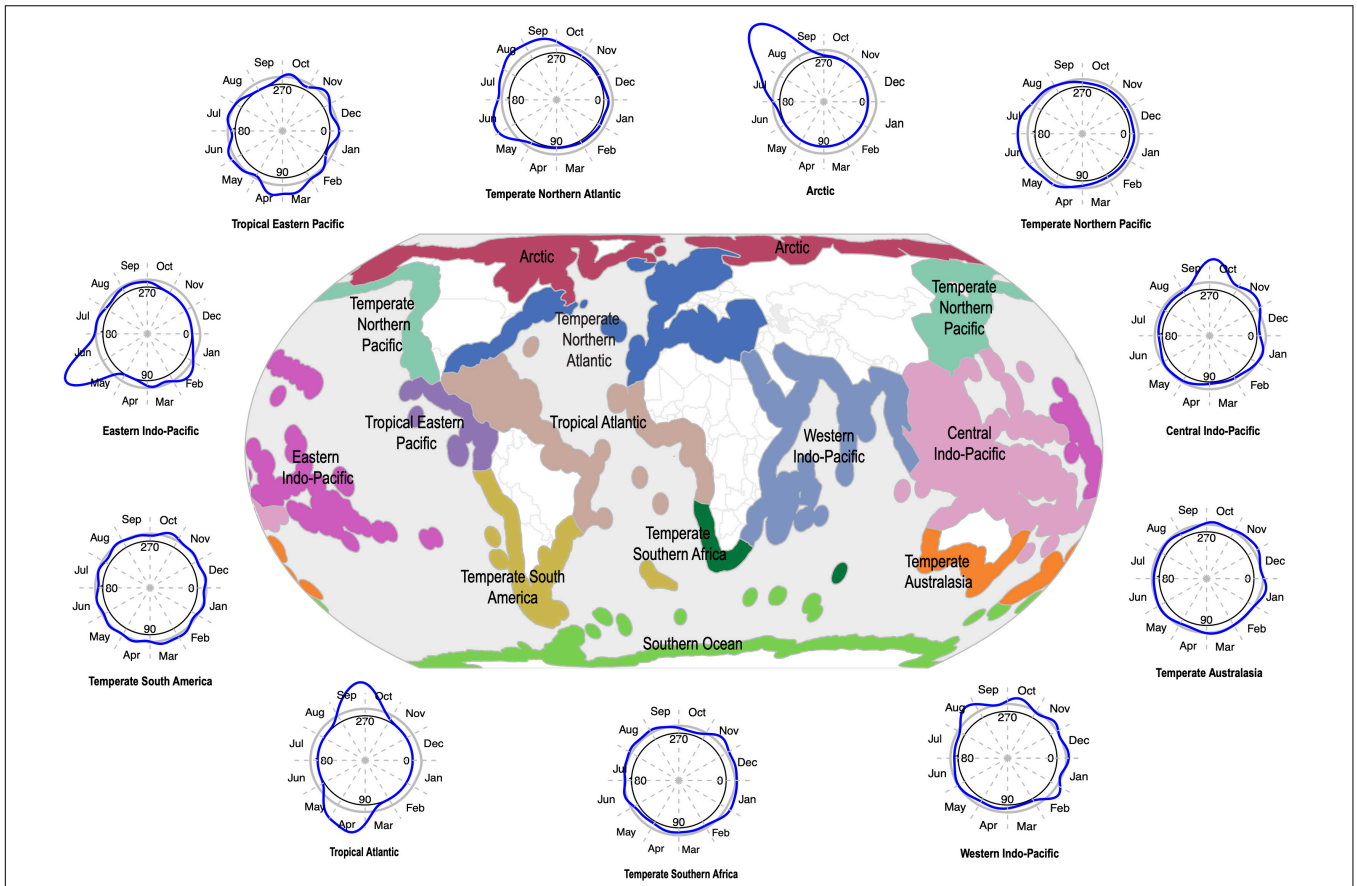

FIGURE 5 | Temporal trends in seagrass sampling are not consistent across seasons within marine ecoregions of the world (MEOWs). Temporal data from seagrass occurrence records were converted into Julian day of year format in order to analyze trends in the monthly sampling of seagrasses for all MEOWs. The blue line around each temporal sampling plot represents seagrass sampling density in monthly intervals over an extensive time period (1770–2019), with corresponding temporal sampling plot for each MEOW. Seagrass sampling rates increase during summer seasons associated with northern and southern hemispheres. The central plot provides a reference for the geographic location of each MEOW included in the analysis. Source data are provided as a Source Data file.

et al., 2009), especially when biases are also geographically non-random (Daru et al., 2018). Even with complete DNA sequences for all seagrass species, there are large uncertainties in the estimation of divergence times, and unknown evolutionary models linking phylogenies to underlying ecological traits and life history variation (Diniz-Filho et al., 2013). Moreover, the polyphyletic nature of seagrasses, drawing from several lineages within the Alismatales, might also compound our understanding of phylogenetic sampling biases.

The aforementioned sampling biases can combine with each other in several ways (**Figure 2**). Taxonomic bias can influence all other biases because it reflects knowledge gaps on the fundamental unit of ecology and evolutionary biology. Geographic bias is strongly influenced by temporal bias as limited accumulation of data over time can alter accurate estimations of species' range size or population demographic history (Pybus et al., 2000; Drummond et al., 2005). Similarly, geographic bias can compromise estimates of species' phenological response to climate change or demographic change, owing to lack of geographical coverage in many regions (Poelen et al., 2014). Ultimately, these sampling biases are human artifacts such that any personal preferences, biases, and proclivities of collectors can greatly skew our understanding of seagrass diversity.

GAPS IN KNOWLEDGE OF SEAGRASS EVOLUTIONARY DIVERSITY

Understanding what drives variation in the distribution of biodiversity can provide insights into the ecological and historical processes underlying community assembly (Cavender-Bares et al., 2009) and for prioritizing conservation (Kreft and Jetz, 2010; Holt et al., 2013; Daru and le Roux, 2016). However, data gaps in the sampling of seagrasses (as outlined above) can influence estimates of broad-scale patterns and underlying processes (e.g., extinction, speciation and niche conservatism). Traditionally, identifying broad-scale patterns in seagrasses has been based on species-level metrics (e.g., species richness, and endemism) (Short et al., 2007; Mtwana Nordlund et al., 2016; Duffy et al., 2019). Although indispensable in providing baseline biodiversity knowledge, these metrics alone fail to detect the substantial evolutionary and conservation implications captured

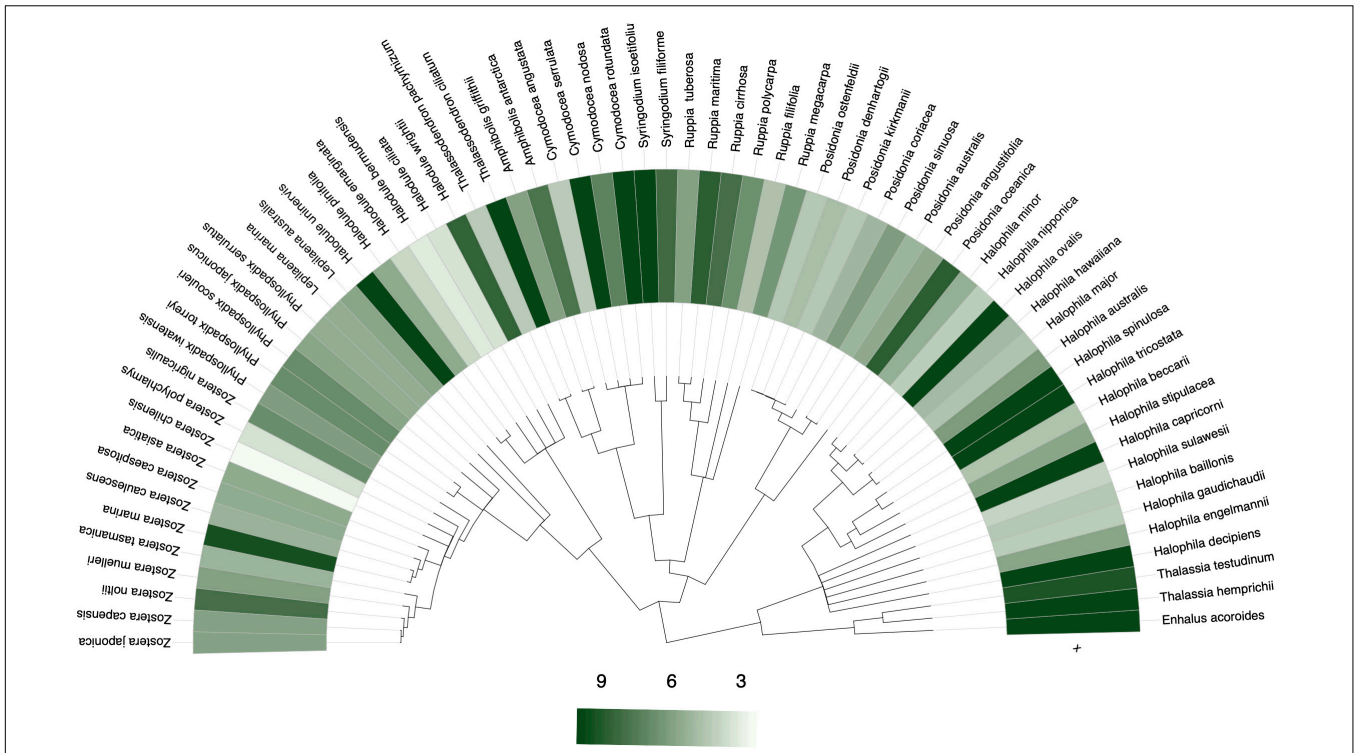

FIGURE 6 | Phylogenetic bias in seagrass sampling. Phylogenetic distribution of the number of specimens sampled per seagrass species to assess the tendency of closely related species to be similarly collected. No statistically significant phylogenetic signals were detected, although there was slight favoring for sampling of the *Thalassia, Enhalus,* and *Halophila* genera over other seagrass genera. Source data are provided as a Source Data file.

by the shared phylogenetic relationships and evolutionary distinctiveness of species (Mace et al., 2003; Redding and Mooers, 2006; Cadotte, 2013). Recent approaches harmonized metrics that consider evolutionary components, for example, phylogenetic diversity (Faith, 1992), evolutionary distinctiveness (Redding and Mooers, 2006), phylogenetic endemism (Rosauer et al., 2009), or a combination of these metrics. As pressures from climate change induced by anthropogenic activity mount, we will eventually observe range shifts and losses that can erase unique evolutionary history (Waycott et al., 2009). There is some evidence that evolutionarily distinct temperate seagrass assemblages might be disproportionately at risk of extinction (Daru et al., 2017a), which could elevate losses of phylogenetic diversity (Redding et al., 2008). However, the associated directionality of species' responses to climate change and impact on phylogenetic diversity under a scenario of non-random extinction is unclear (Purvis et al., 2000). This means that as global temperatures increase, tropical seagrass species might be capable of expanding their distributions (Beca-Carretero et al., 2020) into regions traditionally utilized only by temperate seagrass species. This can induce selection pressures on temperate species that can result in the loss of distinct evolutionary diversity of seagrasses as the available climate space for temperate species is reduced by warming temperatures. Such pressures would inhibit our ability to understand the evolutionary history of seagrasses, as evolutionarily distinct species are lost or greatly reduced.

The global decline of seagrasses along a latitudinal gradient is imbalanced, with greater declines documented in temperate than tropical regions, requiring urgent conservation action (Hauxwell et al., 2001; Orth et al., 2006a,b; Moksnes et al., 2008; Bryars et al., 2011; Erry et al., 2019). The recent finding that temperate seagrass assemblages tend to be those that are most evolutionarily unique also warrants concern given that their extinction would result in a greater loss of phylogenetic diversity (Daru et al., 2017a). In this regard, the familial membership of threatened seagrass species across marine ecoregions (see Methods and Source Data file in **Supplementary Material**) showed a tendency of threatened species in the Temperate Northern Pacific and Tropical Eastern Pacific clustering within similar families (**Figure 7**). This phylogenetic and taxonomic structuring suggests that evolutionary history is an important predictor of species decline, possibly reflecting a non-random pattern of extinction risk (Purvis et al., 2000). Van Allen et al. (2012) demonstrated the importance of life-history traits for predicting how natural assemblages are likely to be impacted by anthropogenic and climatic disturbances using modeled declines in population growth rates under simulated stochastic disturbance. With regard to species extinctions and extinction risk, an important link has been identified between the loss of species and the loss of unique evolutionary history (National Research Council [NRC-US], 2008). Furthermore, the extinction of evolutionarily distinct or paleoendemic species can elevate losses of evolutionary history (Veron et al., 2015; Daru et al.,

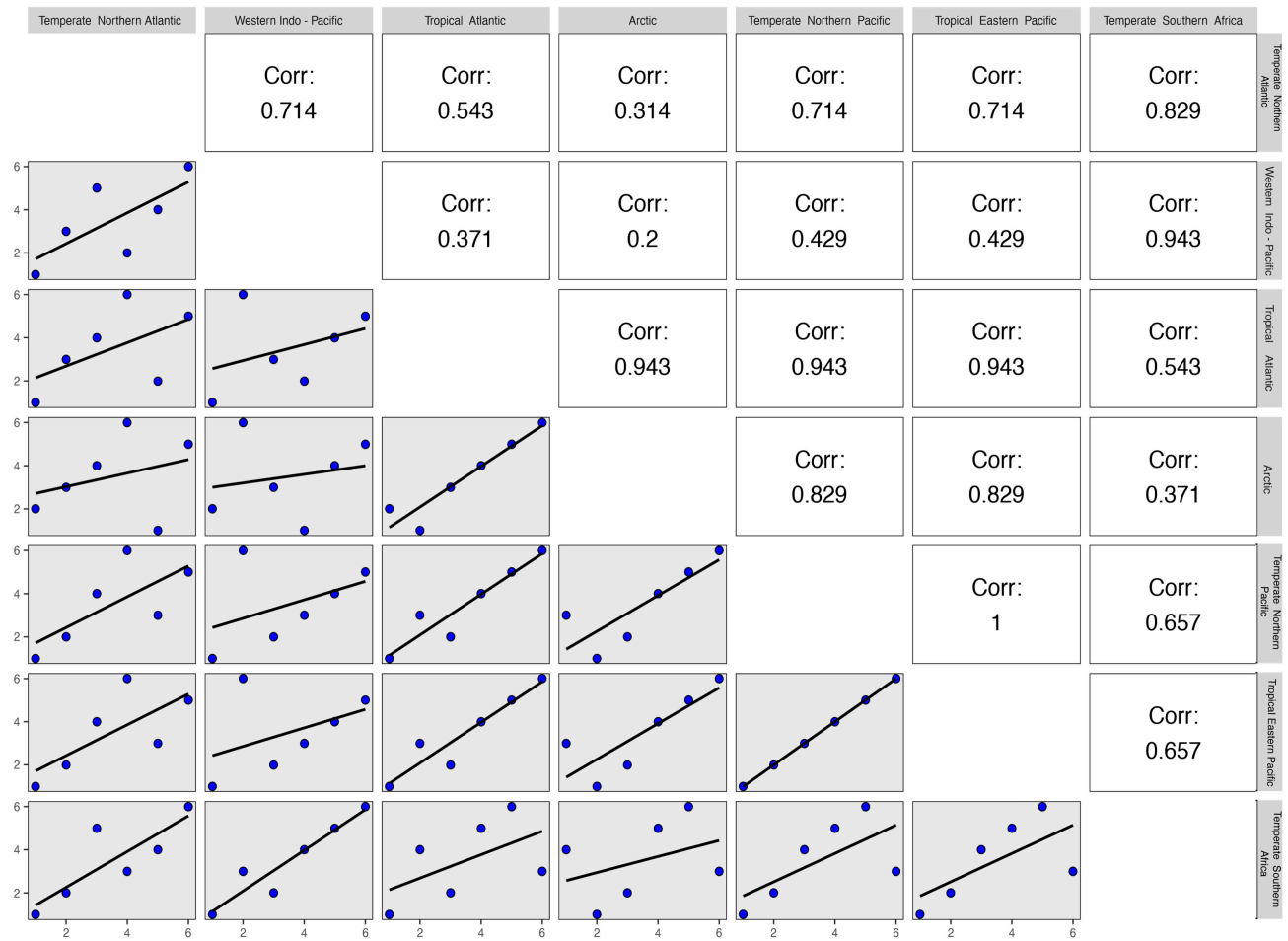

FIGURE 7 | Correlations of family ranks possessing threatened seagrass species across marine ecoregions of the world (MEOWs). The pairwise correlational analysis assigned values based on the level of overlap of seagrass families across MEOWs that possessed seagrass species classified as threatened by the International Union for Conservation of Nature. Low correlation values were generally reported between temperate and tropical MEOWs, indicating that the threatened seagrass species in these regions are unique to those areas. Source data are provided as a Source Data file.

2017b). These patterns might be indicative that seagrasses are characterized by species that subtends longer phylogenetic branches perhaps representing once diverse clades that have been lost through historical extinctions.

As seagrasses are increasingly threatened along their taxonomic structure spanning several marine ecoregions, we argue that seagrass extinctions are unlikely to be random. Previously, Short et al. (2011) determined that roughly 14% of seagrass species were at an elevated risk of extinction based on the IUCN's Red List of Threatened Species criteria. Currently, the IUCN indicates that 31% (22 out of 72) of seagrass species are in global decline, and 22% lack information for proper assessment of conservation status (IUCN, 2020). Therefore, the question of why some species persist while others decline across regions will require an understanding of the shared evolutionary history underlying changes in species richness and composition (Waycott, 1999; Arnaud-Haond et al., 2010; Massa et al., 2013). With many species' ranges greatly reduced or unknown, it is even more challenging to track patterns in seagrass population successes or failures that could be indicative of their resilience to climate change. In the absence of these key insights for the adaptive potential of seagrass species, we are unable to fully predict how individual species of seagrasses will respond to drastic, widespread environmental changes.

In order to facilitate effective conservation action, it is important to accurately determine which species are currently at the greatest risk for extinction, and which species will be at risk in the future. One successful approach has been to collect expert opinion data to prioritize seagrass management actions at regional scales (Grech et al., 2012) for species that may be unequally impacted. To this end, phylogenetic information can be very useful for predicting vulnerabilities at individual or familial levels (Gallagher et al., 2015). For example, families with a high proportion of species in global decline include Zosteraceae, Hydrocharitaceae, Posidoniaceae, and Cymodoceaceae; with Zosteraceae contributing about half of the total number of species in decline (**Figure 8**). Therefore, Zosteraceae and other evolutionarily similar families may

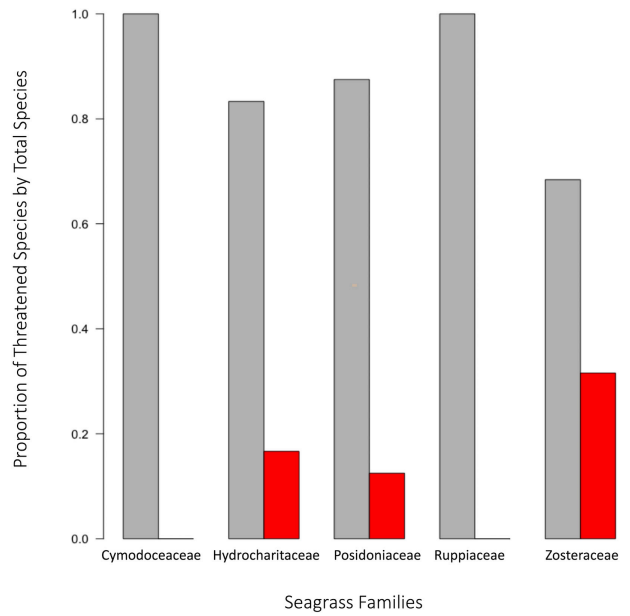

FIGURE 8 | Taxonomic distribution of extinction risk in seagrass. Population status of seagrasses were assessed using the classifications set forth by the International Union for Conservation of Nature. Proportion of threatened species was assessed as number of threatened species in a family divided by the total number of species assessed within that family. When comparing the proportions of threatened species per family to the calculated 95% confidence interval, three families were significant: Zosteraceae, Posidoniaceae, and Hydrocharitaceae. Source data are provided as a Source Data file.

possess a phylogenetic signal for extinction pressures. Families with seagrasses having unknown population trends include Hydrocharitaceae, Cymodoceaceae, Ruppiaceae, Posidoniaceae, and Zosteraceae according to the IUCN (see Methods and Source Data file in **Supplementary Material** for details). These groups are of high conservation concern given that species associated with these families may be currently threatened or already in decline without notice. Such population trends, or lack thereof, imply that certain species of seagrasses may be too heavily impacted in the future to prevent complete losses or extinctions given the rapid pace of climatic change.

SHORTFALLS IN COMPUTATIONAL TOOLS FOR ASSESSING SPECIES RESPONSE TO CLIMATE CHANGE

It is possible that the aforementioned impediments can be solved by increasing biological knowledge and computational capacity. However, compared to the mass production of occurrence records and climate data, tools that can analyze these datasets in a reasonable amount of time are almost non-existent or do not scale well in terms of computer time, memory, or other resources. This is particularly true for seagrasses that have wide geographic ranges, colonizing every coastline. As a consequence, ecologists and conservationists wishing to address questions related to seagrass response to climate change may be deterred by lack of analytical tools.

The occurrence data typically used for species distribution modeling is generated from massive digitization of museum records and citizen science campaigns (e.g., Seagrass-Watch, see text footnote 1) and are often available as point records; whereas global oceanographic variables are measured by instruments on satellites daily (NOAA Climate.gov, 2020), which increase the size of the dataset many-folds. This exponential increase in species occurrences and oceanographic information inflate the size of running time for modeling algorithms (Farley et al., 2018; Allen et al., 2019), and consequently increases the challenges for visualizing downstream patterns. In **Figure 9**, the number of seagrass occurrence records in GBIF has increased over time. Where there used to be access to only a few dozen records, the rapid expansion of biodiversity occurrence data has now made it common for there to be a few thousand records per species (see Source Data file in **Supplementary Material**). This poses computational challenges for researchers. For analysis of species distribution modeling under different representative concentration pathway scenarios, for instance, researchers rapidly run into a spatial scale exponentiation problem. At a spatial resolution of 0.5 degrees (equivalent to ∼50 km at the equator) covering the geographic ranges of seagrasses, there are 201,600 possible pixels for the algorithm to evaluate from. Computing probabilities across a 201,600-possibility data frame is a challenge. Such large-scale analysis can easily reach thousands of bytes and analysis using current tools would be prohibitively expensive computationally.

Presently, the software that can facilitate analysis of species distribution modeling of seagrasses includes *maxent* (Phillips et al., 2017), *dismo* (Hijmans et al., 2011), *biomod2* (Thuiller et al., 2014), *esdm* (Woodman et al., 2019), ModEco (Guo and Liu, 2010), SDMtoolbox 2.0 (Brown et al., 2017), ArcGIS, and ARCMap. Several of these packages contain some statistical capabilities by integrating occurrence information and climate data. For instance, *biomod2* facilitates species distribution modeling by averaging across different methods including generalized additive models, generalized linear models, generalized boosting trees, maximum entropy, and random forest (Thuiller et al., 2014). However, these packages differ in their inferences, and analytical and computational capacity to process the massively mobilized occurrence records spanning tens of thousands of pixels across the globe (depending on the measurement scale). Some of these packages are developed for use in command-line while others are graphical user-interface (GUI). Most packages are developed to address a specific biological question and may have restricted analytical options that can limit computational flexibility. Ultimately, scientists wishing to address more complex hypotheses will have to use a compilation of multiple computational workflows.

More recent approaches to scale existing software to handle the exponential growth of biodiversity datasets include developing parallel algorithms (McCallum and Weston, 2011) and using modern computational architectures, such as multicore systems, graphics processing units, and supercomputers (Maruyama et al., 2011). The advantages of these methods are

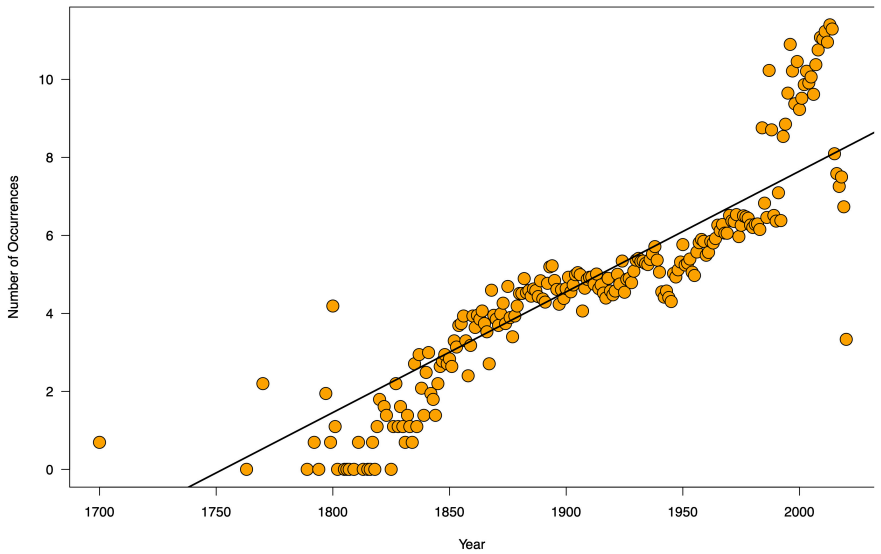

FIGURE 9 | Temporal change in the amount of seagrass occurrence records over time. Seagrass point records downloaded from GBIF were mapped over time based on the chronological date listed in the occurrence data for each record to demonstrate that seagrass occurrences have greatly increased within recent decades. This indicates that analyses with these data will be computationally expensive. Source data are provided as a Source Data file.

that they provide reproducible source codes. However, they might require the user to have a good background in high performance computing. These limitations should not detract from exploring other outstanding questions that remained to be addressed with the available tools: (1) What are the effects of reduced area and increased isolation of marine habitats? (2) Where will seagrass species disperse to under alternative scenarios of climate change? and (3) How have anthropogenic activities e.g., marine pollution, sedimentation, and coastal urbanization changed the geography of seagrasses?

OVERCOMING THE IMPEDIMENTS

Seagrass occurrence records are increasingly being utilized in biogeographical investigations and prioritizing conservation (Valle et al., 2014; Chefaoui et al., 2018; Jayathilake and Costello, 2018; Beca-Carretero et al., 2020; Heck et al., 2021). As possible solutions for the geographic uncertainty, we suggest enhanced funding for local, regional, and global inventories such as SeagrassNet for seagrass habitats in the Western Pacific (Short et al., 2006), Seagrass-Watch in Australasia (McKenzie et al., 2000, 2009), ResilienSEA[3] in West Africa, Texas Seagrass Monitoring program,[4] SeagrassSpotter (see text footnote 2) a global tool for locating seagrasses, or Zostera Experimental Network[5] for eelgrass (*Zostera marina*). Overcoming gaps in geographic sampling can also include collectors using best practices for collecting and vouchering specimens such as capturing accurate geolocations. It could also require the digitization and mobilization of vouchered seagrass specimens stored in herbaria and museums across the world. The iNaturalist project is a platform for sharing species observations along with geographic coordinates for terrestrial organisms and can be leveraged for filling in the data gaps in seagrass sampling. Kew's Plants of the World Online portal (POWO) provides distribution information on the seed-bearing plants of the world based on level 3 of the Taxonomic Diversity Working Group distribution scheme which corresponds to country borders (POWO, 2019) and can be extended to cover seagrasses as well. High resolution cameras attached to unmanned aerial vehicles can be deployed to survey seagrasses in remote and inaccessible waters; however, special permits can often be required to access some sites (Johnston, 2019).

Species distribution models – the statistical estimation of species geographic distributions based on only some known occurrences and environmental conditions (Peterson et al., 2011) – can also provide an unbiased and easily interpretable estimate of improving representativeness and coverage of seagrass distributions. For example, a recent species distribution model predicts more than two-fold increase in the potential global distribution of seagrasses (Jayathilake and Costello, 2018). However, the accuracy of this prediction has attracted particular scrutiny because of inconsistent measures and widespread sampling gaps in seagrass occurrence records (McKenzie et al., 2020). Additionally, modeling approaches can contribute other useful measurements of seagrass meadows such as assessing ecosystem services as well as estimating broad-scale seagrass resources as was exemplified by Collier et al. (2021) who used historical data to accurately predict the below-ground biomass of five seagrass species. Because geographic scale is an important consideration in ecological analyses (Jarzyna and Jetz, 2018; Daru et al., 2020a), a multi-scale approach varying

[3]http://resiliensea.org/3

[4]http://www.texasseagrass.org/

[5]http://zenscience.org

along spatial extents (local, regional and global) and grain resolutions should be considered in assessing seagrass response to global change and model testing. Temporal bias can be diminished by carrying out new field surveys that are more consistent and evenly distributed across seasons and years. Collectors should use best practices such as capturing and documenting accurate dates of collection. For the taxonomic bias: increased support for marine plant taxonomy and advances in taxonomic publications could minimize biases. Next-generation DNA sequencing combined with bioinformatics (Taberlet et al., 2012) will help diminish taxonomic bias such as sequencing old herbarium specimens of very rare species such as *Halodule bermudensis*. The rapid growth of large databases such as GenBank,[6] SeagrassDB (Sablok et al., 2018), and Treebase,[7] allows researchers to download available phylogenies or DNA sequences to build their own (Morell, 1996; Piel et al., 2000; Page, 2007). Taxonomic bias can also be reduced by targeting future collecting in poorly sampled clades.

Improvement of analytical and computational tools is an important priority for handling the analyses for large-scale comparative analyses of seagrass species. For instance, the US National Science Foundation-funded software *BiotaPhy* facilitates integration, data collection and analysis by connecting to existing data repositories such as the Open Tree of Life, iDigBio, and Lifemapper (BiotaPhy, 2020), whereas the open-source package *sampbias* allows quantification of geographic sampling biases in species distribution data (Zizka et al., 2020). The R software package *phyloregion* – designed for biogeographic regionalization and macroecology – can overcome some computational challenges (Daru et al., 2020b). It contains tools for biogeographical regionalization, macroecology, conservation, and visualizing biodiversity patterns, and has potential application in diverse fields including evolution, microbial diversity, systematics, ecology, phylogenetics, and many others (Daru et al., 2020b). We expect that the proliferation of more open-source analytical tools to greatly facilitate comprehensive understanding of seagrass sensitivity to ecological change driven by anthropogenic causes.

CONCLUDING REMARKS

Here, we outlined impediments that limit progress in understanding seagrass sensitivity to global change induced by human activities. These knowledge gaps are interconnected and represent only few of the possible issues related to research in seagrass diversity and evolution. Taxonomic bias can influence all other types of biases as it reflects knowledge gaps on the fundamental unit of ecology and evolutionary biology. The geographic and temporal biases are strongly related and capture knowledge gaps about species distributions in space and time, respectively. Even when the aforementioned impediments are resolved, many of the critical questions about seagrass sensitivity to global change, can be out of reach for scientists without the right analytical tools. The recent development of efficient and replicable computational tools, massive mobilization of natural history collections, and increased funding for seagrass research could remedy these shortcomings. Most of the management tools designed for use in developed countries can be extended to remote areas in developing countries where most seagrass diversity resides e.g., the Central Indo-Pacific. Although research on a single taxon or selected taxa is useful to a certain extent, species are lineages that evolve and diversify from shared ancestors, suggesting an integrative approach that accounts for their shared phylogenetic relationships.

[6] http://www.ncbi.nlm.nih.gov/genbank

[7] http://www.treebase.org

AUTHOR CONTRIBUTIONS

BHD conceived and designed the study. BMR ran the analyses with help from BHD. BMR wrote the manuscript with substantial contributions from BHD. Both authors approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.608867/full#supplementary-material

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Relationships Between Annual and Perennial Seagrass (*Ruppia sinensis*) Populations and Their Sediment Geochemical Characteristics in the Yellow River Delta

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Annual and perennial populations commonly occur for the same submerged aquatic angiosperm species, yet relationships between population types and sediment characteristics are poorly understood. In the current study two *Ruppia sinensis* habitats with annual and perennial populations were surveyed in the Yellow River Delta (YRD). Biomass and seasonal seed bank size were used to evaluate population status and potential recruitment capacity. Sediment geochemical parameters including moisture, sulfide, Chl *a*, carbohydrate, OM, TOC, TN, and TP were measured to compare sediment nutrient composition and variability. The results revealed a higher biomass and larger seed bank in the annual *R. sinensis* population compared with the perennial population. The P levels in sediments between the two *R. sinensis* populations were similar; while the N level in the sediment of the annual population was significantly higher than the perennial population, which might support the recruitment of vegetative shoots when a large amount of seeds germinated during wet periods. The annual population exhibited greater resilience after habitat desiccation, with the population recovering rapidly once water appeared. The results of this study add to the knowledge of *R. sinensis* populations and their sediment geochemical characteristics, and can be used as a reference for *Ruppia* population conservation and management.

Keywords: submerged aquatic vegetation, *Ruppia*, population traits, sediment geochemical characteristics, sediment carbon cycling, sediment nutrients

Abbreviations: ANOVA, analysis of variance; OM, organic matter; PCA, principal component analysis; TN, total nitrogen; TOC, total organic carbon; TP, total phosphorus; YRD, Yellow River Delta.

INTRODUCTION

The Yellow River Delta (YRD) is the broadest, and best conserved wetland ecosystem in temperate China (Han et al., 2006). Rich salt-tolerant plants are found in this area, with *Phragmites australis*, *Suaeda salsa*, and *Tamarix chinensis* being the dominant species (Zhang et al., 2013; Yu et al., 2016). Increasing anthropogenic activities, have resulted in the coastal wetlands of the YRD changing from natural wetlands to farmlands, and salt-culture ponds (Yu et al., 2012). These changes result in diverse sediment characteristics. For instance, the topsoil of *Deyeuxia angustifolia* wetlands contains more labile fraction organic carbon than an upland forest and two farmlands in the Sanjiang Plain of northeast China (Zhang et al., 2006). Meanwhile, the redistribution of local plants, including aquatic plants, have also caused shifts in the nutrient composition and chemical processes in the sediment, due to the close relationship between plants and sediment composition. For instance, *Thalassia testudinum* leaves are capable of inducing $CaCO_3$ precipitation and increasing habitat sediment carbon storage (Enríquez and Schubert, 2014).

Ruppia, a genus of submerged marine angiosperms, commonly inhabit shallow systems, such as coastal lagoons and saltmarshes (Verhoeven, 1979; Mannino and Sara, 2006; Strazisar et al., 2015). Similar to other seagrass species, *Ruppia* species act as nursery areas for a variety of fishes and birds (Congdon and Mccomb, 1979; Rodriguez-Perez and Green, 2006; Mannino et al., 2015), provide food, increase water clarity by enhancing sedimentation (Barbier et al., 2011), and are key sites for global carbon storage (Fourqurean et al., 2012; Jiang et al., 2018). The high environmental adaptability of *Ruppia* not only leads to it being widely distributed around the world (Aedo and Casado, 1988; den Hartog et al., 2016; Martinez-Garrido et al., 2017), but also to high phenotypic plasticity, which characterizes the taxonomic confusion of this genus (Aedo and Casado, 1988; Yu and den Hartog, 2014). Yu and den Hartog (2014) recently updated the distribution and taxonomy of *Ruppia* in China and named two new species, *R. brevipedunculata* and *R. sinensis*, based on genetics and morphological characteristics. *R. sinensis* is widely distributed in abandoned salt pans and salt-culture ponds of the YRD (Gu et al., 2019) and forms dense, monospecific beds like other *Ruppia* species (Verhoeven, 1979; Mannino and Sara, 2006). Even though *R. sinensis* has the potential to be used as a phytoremediation species, little is known about the population characteristics of this species (Gu et al., 2020), and it is often ignored when researchers investigate plant distributions and calculate carbon storage (Zhang et al., 2013; Yu et al., 2016).

Annual or perennial life cycle strategies are commonly found in seagrass populations (Moore and Short, 2006). Sexual and asexual reproduction commonly occur in all seagrass populations, including annual and perennial populations (Sato et al., 2016; Entrambasaguas et al., 2017; Xu et al., 2018). Moreover, the life cycle strategy for seagrass individuals is considered to be genetically fixed, and annual and perennial seagrasses can be mixed within populations. Environmental factors, such as underwater photon flux density and the presence of water, have been recognized as major factors controlling seagrass survival, and may result in a seagrass population being perennial or annual (Kim et al., 2014; Mannino and Graziano, 2014). In perennial populations, the vegetative shoots of seagrass appear throughout the year and succession primarily relies on clonal reproduction (Verhoeven, 1979; Malea et al., 2004; Xu et al., 2018). In contrast, annual seagrass populations are absent during unfavorable conditions (e.g., freezing or desiccation) and re-establishment is completely dependent on seed germination (Verhoeven, 1979; Strazisar et al., 2013a,b). Many studies have suggested that these two kinds of shoots, perennial and annual, could be found in both perennial and temporary *Ruppia* populations. *Ruppia* populations with vegetative shoots present throughout the year are considered to be perennial populations. Populations exhibiting an annual life history complete their life cycle within a few months, and survive as seeds before producing the next generation of plants (Verhoeven, 1979; Brock, 1982; Malea et al., 2004; Mannino and Graziano, 2014). Many studies have compared the morphological variations in different *Ruppia* population types. Longer roots and bigger leaves are found in *R. maritima* and *R. cirrhosa* perennial populations, while more flowers are found in annual populations (Malea et al., 2004; Mannino and Graziano, 2014).

Sediment nutrition is vital for seagrasses and population strategy may affect sediment nutrition status. Phosphorus, nitrogen, sulfur, and carbon can be measured in the sediment to reveal the relationships between vegetation and sediment nutrition. Nitrogen and phosphorus are considered to be the two most important elements related to vegetative growth (Liao et al., 2008; Ailstock et al., 2010; Qu et al., 2018), and their concentrations in the sediment are strongly linked to seagrass biomass development and rapid recruitment (Menendez, 2009; Strazisar et al., 2013a,b). The reproductive organs of vegetative plants are often phosphorus-rich (Kerkhoff et al., 2006), while there are higher nitrogen requirements in photosynthesizing leaf tissue (Feller et al., 2008). Sulfide is considered toxic to seagrass. For instance, a direct link between high sediment sulfide levels and mortality of *T. testudinum* has been noted (Carlson et al., 1994). Total organic carbon (TOC), organic matter (OM), and carbohydrates are the three parameters used to describe the carbon sink in the sediment. TOC represents the quantity of buried organic carbon, whereas the compositional features of OM identify the source of organic matter (Geraldi et al., 2019; Kaal et al., 2020a,b). Carbohydrates are an important source of organic matter in the aquatic environment (Burdige et al., 2000), which mainly come from the microbial degradation of organic matter such as photosynthetic organisms and provides an index to assess recent sediment conditions (Bianchelli et al., 2016; Artifon et al., 2019).

In the current study, we selected two *R. sinensis* populations considered to be annual and perennial in the YRD. We recorded the seasonal changes of these two populations, and investigated their sediment characters, including inorganic nutrient concentrations and physical parameters. This study aimed to (1) elucidate the main factors resulting in *R. sinensis* populations being differentiated as annual or perennial and (2) assess the effect of *R. sinensis* absence on habitat sediments. The results of this study could serve as simple records for *Ruppia*

populations in YRD, further our understanding of annual and perennial *Ruppia* characteristics and provide more information to inform *Ruppia* management.

MATERIALS AND METHODS

Study Site

The Yellow River Delta (YRD) is a wetland ecosystem in the warm temperate zone of China covering an area of approximately 5400 km^2 (Yu et al., 2016). The YRD is considered a carbon sink hotspot due to the large amounts of particulate carbon that are transferred here (Zhao et al., 2020). Yu and den Hartog (2014) identified a new species, *R. sinensis*, which is widely distributed in the YRD (36°55′–38°16′, 117°31′–119°18′, Li et al., 2009). In this study, we selected two *R. sinensis* populations located 45 km apart (**Figure 1**), comprising an annual (Site 1) and perennial population (Site 2), respectively. Site 1 was a 1,200 m^2 ditch, which was near the YRD Nature Reserve (37° 45′ 55.83″ N; 118° 58′ 13.03″ E). The water level at Site 1 fluctuates seasonally, becoming dry in winter. Site 1 has an annual *R. sinensis* population. Site 2 occurred around a brackish water pond (37° 59′ 52 N; 118° 36′ 33″ E) and was approximately 5,000 m^2 in area. The salinity at the two sites ranged from 7.2–11.6 to 9.3–16.7 psu, respectively, and there was little anthropogenic influence at either site.

Environmental Parameters and *R. sinensis* Populations

We investigated the two *R. sinensis* populations over a period of 2 years. This observation period included an unusual extreme desiccation event from March 2018 to May 2018 (**Figures 1b,d**), which resulted in both survey sites drying up. Environmental data including temperature and precipitation, were downloaded as daily records from Tianqi.2345.com[1]. Precipitation and evaporation are two of the main factors impacting the water levels of these two closed, natural *R. sinensis* habitats. Temperature is an indirect parameter that indicates water evaporation at the survey sites. The quantity of precipitation was used as an indirect parameter to indicate water input. Water temperature is closely related to air temperature in these shallow water systems. Thus, air temperature was used to represent the seasonal temperature in *R. sinensis* habitats, and weather records indirectly indicated the precipitation at the study sites. The mean monthly temperatures of the two sites were calculated to represent seasonal temperature changes. The weather records were divided into four categories: light rain or snow (precipitation < 10 mm/24 h, value = 1), showers or thunderstorms (value = 2), moderate rain or snow (10 mm/24 h < precipitation < 28 mm/24 h, value = 3), and heavy rain or rainstorm (precipitation > 25 mm/24 h, value = 4). We then quantified the monthly precipitation for each of these four precipitation categories.

The vegetative status of both *R. sinensis* populations were observed from October 2016 to July 2018 (observations were recorded in October and December 2016; March, May, June, August, October, and December 2017; and March, May, and July 2018). We randomly sampled four *R. sinensis* cores (diameter = 10.5 cm, depth = 10 cm, sample interval = 5 m) from May to August 2017 to compare the biomass of the two populations. The recruitment capacity of the two populations was assessed using seed bank size. To do this, we surveyed dynamic seed bank changes at the two sites using four replicate core samples (diameter = 6 cm, depth = 20 cm).

[1]http://tianqi.2345.com/wea_history/70617.htm

Sediment Sample Collection

Sediment cores at two different depths (deep and shallow) were collected from the two sites. Four deep sediment cores (diameter = 4 cm, depth = 60 cm, sample interval = 2 m) were collected at each site in May 2017 and 2018, respectively, to investigate the vertical sediment characteristics in the two *R. sinensis* populations. For each deep sediment core, the upper 40 cm was analyzed, which was cut into 2 cm segments (a total of 20 subsamples) using a cutting ring and packaged into ziplock bags. The seasonal differences in the sediments at the two sites were investigated through four shallow sediment cores (diameter = 6 cm, depth = 35 cm, sample interval = 2 m) collected at five sampling periods, May, October, and December 2017, and May and July 2018. Each shallow core (upper 20 cm) was divided into 5 cm segments (a total of 4 subsample) and packaged into ziplock bags. All of the sediment samples were transported to the laboratory within 2 days (stored on ice to avoid the water in the samples evaporating during transportation), where they were stored at −20°C prior to sample analysis.

Deep Sediment Core Analysis

Sulfide content, Chlorophyll *a* (Chl *a*), moisture content, carbohydrate, OM, TOC, total nitrogen (TN), and total phosphorus (TP) were measured in every second segment of the deep sediment cores (10 segments from each deep sediment core). Sediment grain size was determined by pooling five segments, i.e.,10 cm/sample, with three replicates. The sulfide content was determined using the iodometric method (National Marine Environmental Monitoring Center (NMEMC), 2007), and Chl *a* was analyzed fluorometrically following the Welschmeyer method (Welschmeyer, 1994). Pigments were extracted with 90% acetone over a period of 36 hours in the dark at 4 °C. The samples were centrifuged at 3,000 rpm for 15 min and the supernatant was used to determine the Chl *a* content. The rest of the sediment samples were freeze-dried. The moisture content (MC; %) of the sediment samples was determined using the following equation:

$$MC = \frac{M_W - M_D}{M_W} \times 100\%$$

where M_W represents the fresh weight (g) of the initial sediment, and M_D represents the weight (g) of the dried sediment. The dried sediment samples were sieved through a 250-μm sieve, after being ground by hand with a mortar, to remove coarse debris and stones. A number of analyses were then conducted to determine the physical and chemical properties of the sediments. Carbohydrates were analyzed using the phenol-sulfuric acid method (Gerchako and Hatcher, 1972)

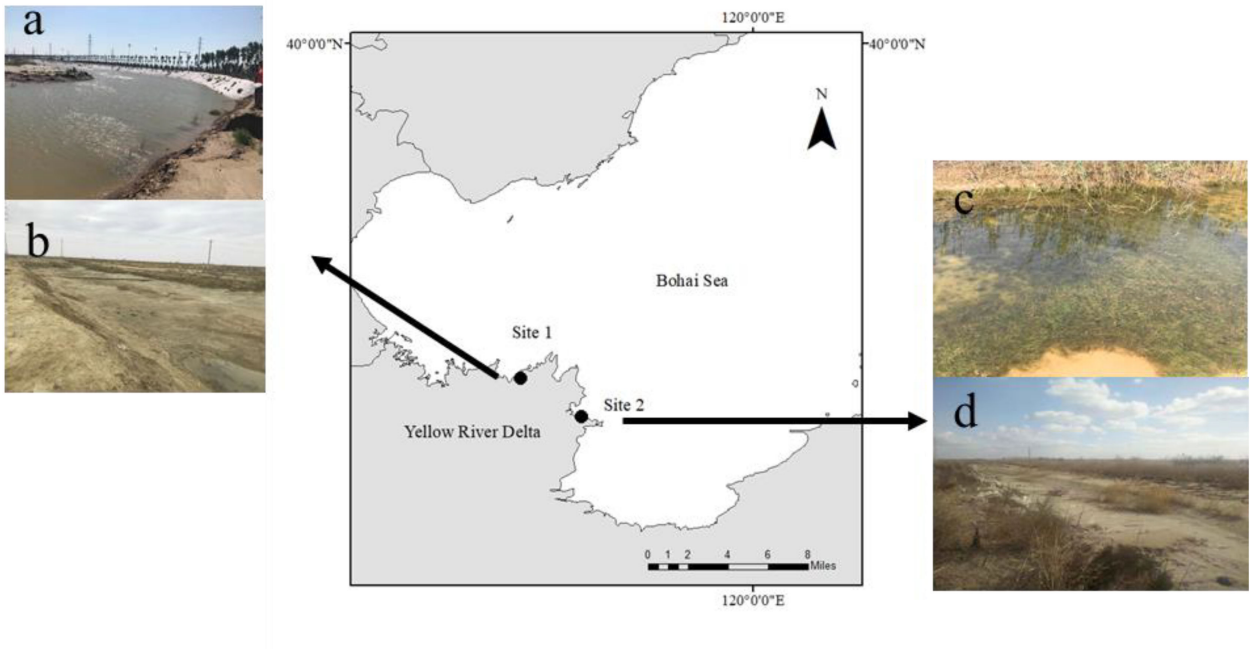

FIGURE 1 | Study sites in the Yellow River Delta. **(a)** high water levels at Site 2 in August 2017; **(b)** extreme desiccation period at Site 2 in March 2018; **(c)** high water level at Site 1 in May 2017; and **(d)** dry season at Site 1 in December 2017.

and expressed as glucose equivalents. OM was determined as the difference between the dry weight of the sediment and the residue left after combustion at 450°C for 4 h (Parker, 1983). Before TOC analysis, the sediment samples were treated with an excess of 10% HCl to remove carbonates that could interfere with TOC measurement (Hedges and Stern, 1984). TOC and TN content was then measured using a VARIO EL III elemental analyzer. TP was measured using the method modified for particulate TP determination (Zhou et al., 2003). The grain sizes were measured using the 10 cm sections of sediment examined through a Laser Particle Size Analyzer. The sediment type was determined by considering the proportion of clay (C) in the silt (S) as follows:

$$C/S = \frac{Cr}{Sr}$$

where, Cr represents the clay accumulation rate in the sediment sub-sample, and Sr represents the silt accumulation rate in the sediment sub-sample. The sediment is considered to be clay when $C/S > 2$, mud when $0.5 < C/S < 2$, and silt when $C/S < 0.5$ (Folk et al., 1970).

Shallow Sediment Core Analysis

The shallow sediment cores were each divided into four 5 cm segments. Each of these sub-samples was analyzed for moisture content, carbohydrate, OM, TOC, TN, and TP using the same analysis as described for the deep cores.

Statistical Analysis

A three-way analysis of variance (three-way ANOVA) was employed to compare the general effects of population type (sample site), sediment vertical distribution (sample depth), and season (sample time) on the sediment indexes including moisture content, TP, TN, OM, carbohydrate, TOC, Chl *a*, and sulfide. When the interaction between sample time, sediment depth, and site was significant, a one-way ANOVA with Tukey's multiple comparison was conducted to compare their effects ($p < 0.05$). The general differences between the two sites and the monthly differences of each site in terms of biomass (2017: May to August) and seed density (2017: March, May; October, December; 2018: May) were compared using a one-way ANOVA. The grain sizes including medium diameter (D_{50}) and the clay/silt ratio at every sediment depth of the two populations were compared with one-way ANOVA. Principal component analysis (PCA) was used to assess the relationship between the sediment characteristics and the variables investigated including sample time, sediment depth, and sample site. PCA was performed using the "prcomp" function in the R software program to determine the multivariate ordination of the 11 sediment parameters for seasonal and vertical sediment assessment, and PCA plots were constructed using the FAC-TOEXTRA package (Kassambara and Mundt, 2017) in the R software program.

RESULTS

Ruppia sinensis at the Two Study Sites

The *R. sinensis* at both study sites differed in terms of population type (**Table 1**). The *R. sinensis* at Site 1 was an annual population, with vegetative shoots dying off each winter when the habitat became dry. Increasing precipitation and an accumulation of

TABLE 1 | Seasonal changes in *R. sinensis* populations and water levels at two study sites.

	Survey time	Water level		Biomass (g/m^2)		Seed density (seeds /m^2)	
		Site1	Site 2	Site1	Site 2	Site1	Site 2
2016	October	High	High	reproductive shoots	reproductive shoots	—	—
	December	None	Low	0	adult shoots	—	—
2017	March	High	High	—	—	109814 ± 15659*	4836 ± 1924
	May	High	High	367.06 ± 54.84*	206.21 ± 61.66	59684 ± 19669*	5662 ± 433
	June	High	High	439.51 ± 107.62	282.75 ± 138.40	—	—
	August	High	High	220.93 ± 65.49	151.25 ± 7.19	—	—
	October	Low	Low	short shoots	flowering and immature reproductive shoots	113470 ± 19810*	18519 ± 5819
	December	None	Low	0	adult shoots	112703 ± 22736*	26008 ± 9247
2018	March	None	None	0	0	—	—
	May	Low	Low	29.22 ± 9.56	0	39278 ± 3148*	26362 ± 3134
	July	None	None	0	0	—	—

**Indicates significant differences between the two sites, $p < 0.05$; "—" represents no investigative data for the corresponding month; Water level "High" indicates water level > 20 cm; "Low" represents ≤20 cm; "None" represents no flooding water.*

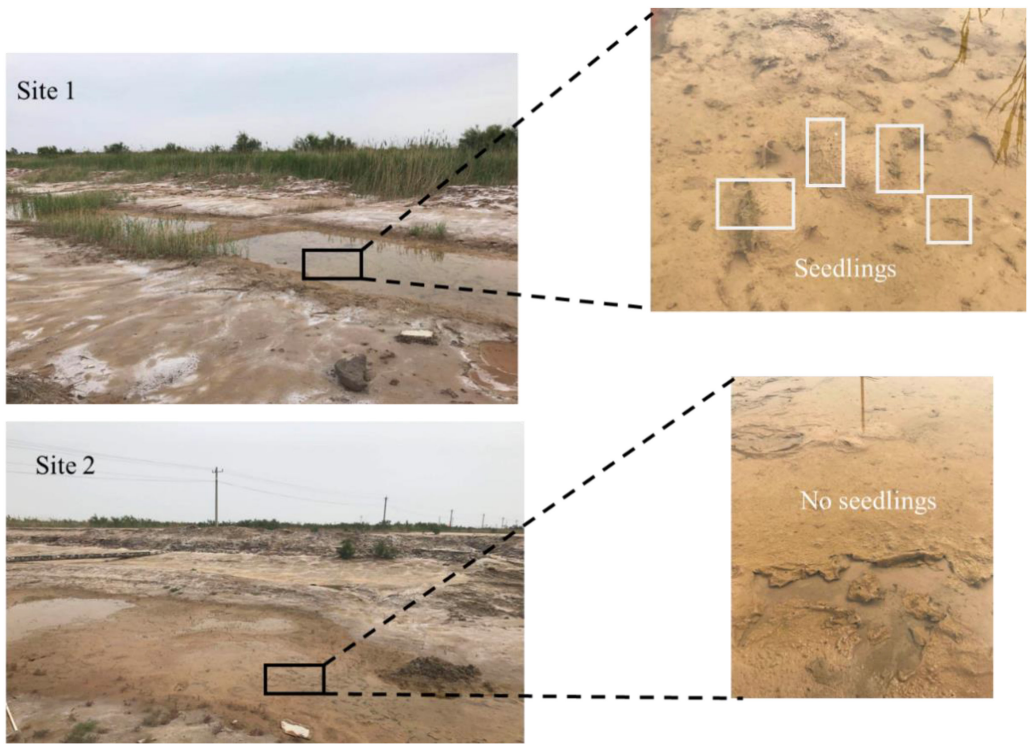

FIGURE 2 | *Ruppia sinensis* growth status in May 2018.

TABLE 2 | Grain sizes at different sediment depths at the two study sites in the Yellow River Delta.

Depth	D_{50}		Clay / Silt Ratio		Type of sediment	
	Site 1	Site 2	Site 1	Site 2	Site 1	Site 2
1-10 cm	24.07 ± 1.53	27.36 ± 4.57	3.61 ± 0.28	2.45 ± 0.51	Clay	Clay
11-20 cm	31.09 ± 5.00	33.58 ± 0.82	2.42 ± 0.88	1.80 ± 0.08	Clay	Mud
21-30 cm	26.43 ± 1.40	37.85 ± 2.36	3.41 ± 0.52	1.39 ± 0.23	Clay	Mud
31-40 cm	27.62 ± 1.86	25.20 ± 4.28	3.88 ± 0.32	1.59 ± 0.81	Clay	Mud

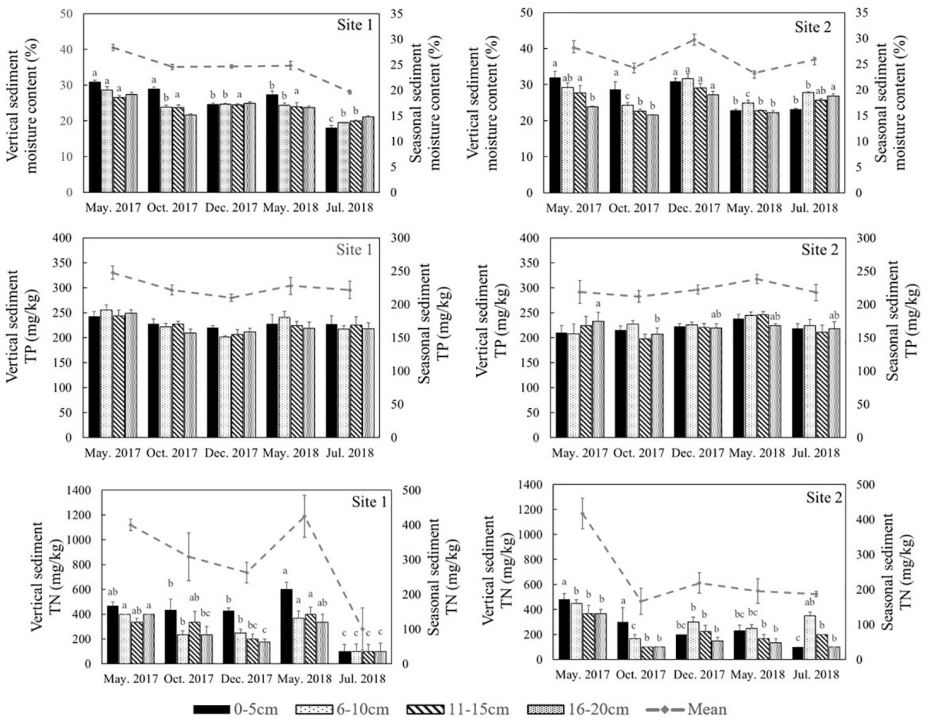

FIGURE 3 | Moisture content, total phosphorus (TP) and total nitrogen (TN) in different sediment layers in different seasons at the two sites in the Yellow River Delta. The dotted lines represent the seasonal mean values of all parameters. Different letters represent significant differences between different seasons in the sediment ($p < 0.05$).

water in the habitat facilitated seed germination and the start of a new growing cycle. Although the surface water at Site 2 froze over in December 2017, the vegetative shoots survived, and green *R. sinensis* shoots could be observed under the ice layer. There was no significant difference in monthly population biomass between the two *R. sinensis* populations from May to August 2017 ($p_{site\ 1} = 0.174$, $p_{site2} = 0.346$). Although the monthly biomass at Site 1 was higher than at Site 2, these differences were not statistically significant, with the exception of May 2017 ($p_{May2017} = 0.03$, **Table 1**). The seed densities at Site 1 were significantly higher than at Site 2 ($p < 0.05$, **Table 1**). An unexpected desiccation event occurred in December 2017, which caused the *R. sinensis* habitats at both sites to dry up until April 2018, with a small amount of water recorded in May 2018. Once water was present, the *R. sinensis* seeds at Site 1 germinated quickly, and several seedlings were observed. In contrast, no seedlings were observed at Site 2 when the water level increased (**Figure 2**). Both sites dried up again in July 2018 and all the vegetation died.

Relationship Between Sediment Nutrients and *R. sinensis* Growth

The environmental conditions of the two study sites, including temperature (evaporation) and rainy value (precipitation), were similar (**Supplementary Figure 1**). The highest mean temperature in this area during the study period was 28.5°C in July, while the lowest mean temperature was −2°C in January (**Supplementary Figure 1A**). The two sites had similar mean annual rainfall levels of 192 and 193 mm, respectively. The monthly precipitation at the two sites showed significant seasonal differences (**Supplementary Figure 1B**), with May to July being the three wettest months. In general, the sediment grain sizes at both sites were similar, indicating similar water storage capacities at the two sites (**Table 2**), though there was a slight difference in their vertical composition. The moisture content of the top 5 cm of the sediments was closely correlated with the appearance of water (**Figure 3**) and there was significant seasonal variation at both sites (**Supplementary Table 1**, $p < 0.001$). In 2017, the surface sediment contained more water than the deeper layers. In contrast, the deeper layers of sediment were wetter than the surface sediment in 2018, which was the dry year (**Figure 3**).

Similar levels of TP were found at both sites ($p = 0.466$, **Supplementary Table 1**), although they showed slight seasonal variations. In general, TN in the sediment at Site 1 was higher than the sediment at Site 2 (**Figure 3**, $p < 0.001$). In 2018 TN was lower than 2017 levels at both sites (**Figure 4**, $p < 0.001$). TN in the surface sediment of Site 1 was higher than that of the other sediment layers; however, this pattern was not observed at Site 2 (**Figure 3**). Higher levels of TN were recorded during the vigorous growth period of *R. sinensis*, which occurred at both sites in May 2017 and at Site 1 in May 2018 (**Figure 3**). This changing trend was closely correlated with the appearance of *R. sinensis* (**Table 1**). Highest levels of sulfides were found at a

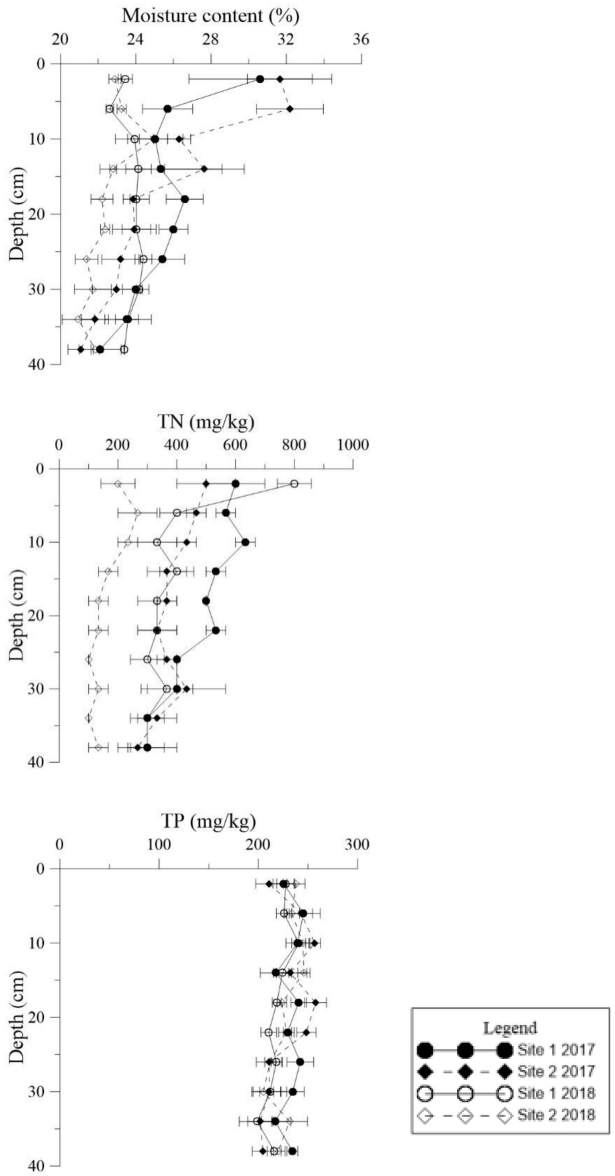

FIGURE 4 | Vertical distributions of moisture content, total phosphorus (TP), and total nitrogen (TN) in the deep sediment cores from the two study sites in May 2017 and May 2018.

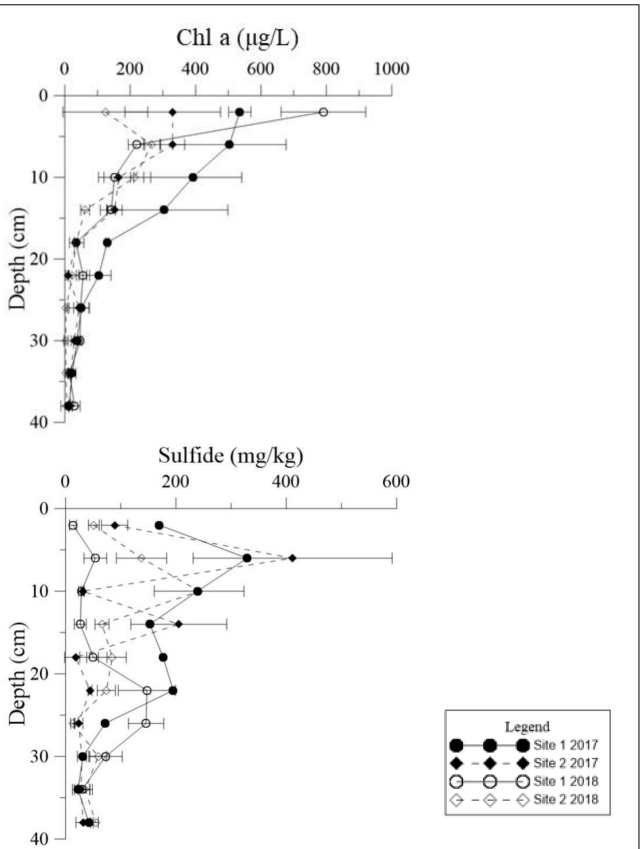

FIGURE 5 | Vertical distributions of Chl *a* and sulfide in the deep sediment cores from the two study sites in May 2017 and May 2018.

depth of 6–10 cm at both sites, and sulfide levels in sediments deeper than 14 cm were not statistically different to the top 2 cm (**Figure 5**, $p < 0.001$), indicating that 6–14 cm was the most suitable sediment layer for sulfide accumulation.

The Relationship Between *R. sinensis* Shoot Decomposition and Sediment Composition

Both Chl *a* and sulfide were measured in the deep sediment cores from both sites. Sediment layers deeper than 18 cm had low Chl *a* values at both sites (**Figure 5**, $p < 0.001$). During the regular year (2017), the Chl *a* level in the sediment at Site 1 was higher than at Site 2, which was correlated with the biomass differences between the two sites (**Table 1**). The Chl *a* levels in the sediment at Site 2 in 2017 were significantly higher than 2018; however there was no significant annual variation at Site 1. This may have been due to the disappearance of *R. sinensis* at Site 2 in 2018, while the *R. sinensis* population at Site 1 recovered rapidly. (**Figure 5**, $p_{site1} = 0.139$, $p_{site2} = 0.012$). There were seasonal changes in the three sediment carbon parameters (carbohydrates, TOC, and OM), and these changes differed between the two sites (**Figure 6**). While seasonal changes in OM content were observed at both sites, Site 1 had higher OM values than Site 2, corresponding with the presence of the *R. sinensis* population in May 2017 and 2018. No particular sediment layer exhibited a distinctive OM content, which indicated that its vertical distribution in these two sites was relatively balanced (**Figure 7**). The TOC at Site 1 was generally higher than Site 2. In contrast to OM, seasonal changes in TOC at both sites showed no significant seasonal differences, except for the sharp decrease in TOC when the water dried up in July 2018 at Site 1 (**Figure 6**). Moreover, the significant annual differences in TOC were only observed in the surface sediment, above 6 cm (**Figure 7**). In most seasons, there were higher levels of carbohydrates in the surface sediment (0-5 cm) of Site 1 than the deeper sediment layers (5-20 cm)

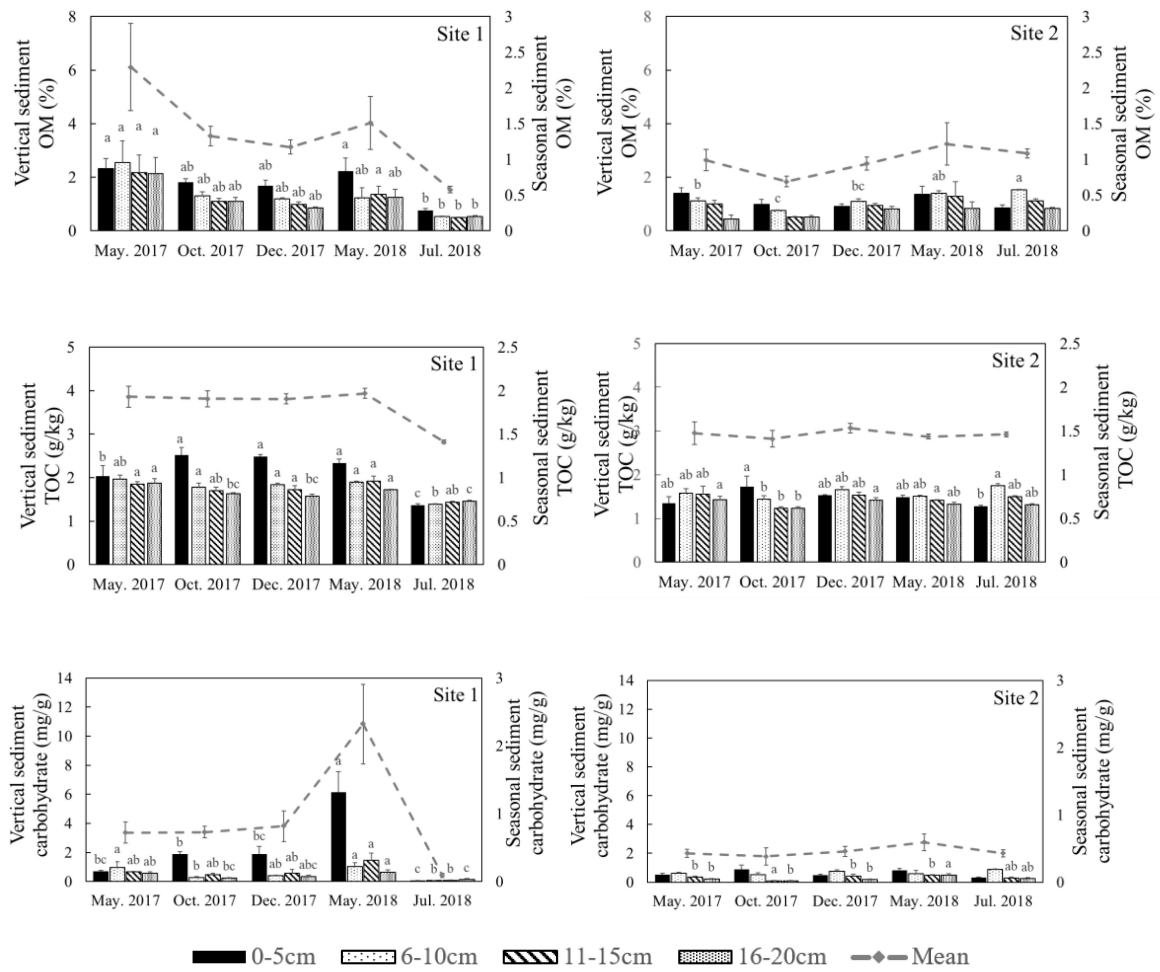

FIGURE 6 | Organic matter (OM), total organic carbon (TOC), and carbohydrate content in different sediment layers in different seasons at the two study sites. The dotted lines represent the seasonal mean values of all parameters. Different letters represent significant differences between different seasons ($p < 0.05$).

(**Figure 6**, $p < 0.001$). Carbohydrate levels at Site 1 showed significant seasonal differences, which were twice as high as Site 2 (**Supplementary Table 1**, $p < 0.001$). The highest carbohydrate level at Site 1 was observed in May 2018, when water was present again and new *R. sinensis* seedlings were growing. No significant changes in carbohydrate values were found at Site 2 at this point.

Sediment Assessment Index

The analysis results of the deep sediment cores with eight sediment assessment parameters (TOC, OM, carbohydrate, TN, Chl a, sulfide, moisture content, and TP) and three classifying parameters (time, depth, and site) explained 64.4% of the variation in the first two components (**Figure 8A**). The first component (PC1) represented 47.3% of the variance, while the second component (PC2) represented 17.1% of the variability and was dominated by different sample sites. Sulfide levels were closely related to the moisture content of the sediment (**Figure 8A**). Sampling site, which represented the different population types, was the highest contributing factor for variation in sediment characteristics in the deep cores with an annual sampling interval (**Supplementary Table 2**). In addition, in both shallow and deep cores, TOC contributed more than OM and carbohydrates when explaining the variation (**Supplementary Table 3**).

DISCUSSION

Ruppia sinensis is a commonly distributed seagrass in the YRD. Information relating to population types and the relationships between sediment characteristics and vegetative status are essential for population management. Similar to *R. cirrhosa* and *R. maritima* (Malea et al., 2004; Mannino and Graziano, 2014), annual and perennial traits occurred in the two *R. sinensis* populations in the YRD in the current study. Temperature and light availability are two key environmental parameters impacting the phenology of seagrass (Qin et al., 2020; von Staats et al., 2021). The presence of water in the habitat is another important factor that impacts the life cycle of this aquatic plant (Malea et al., 2004).

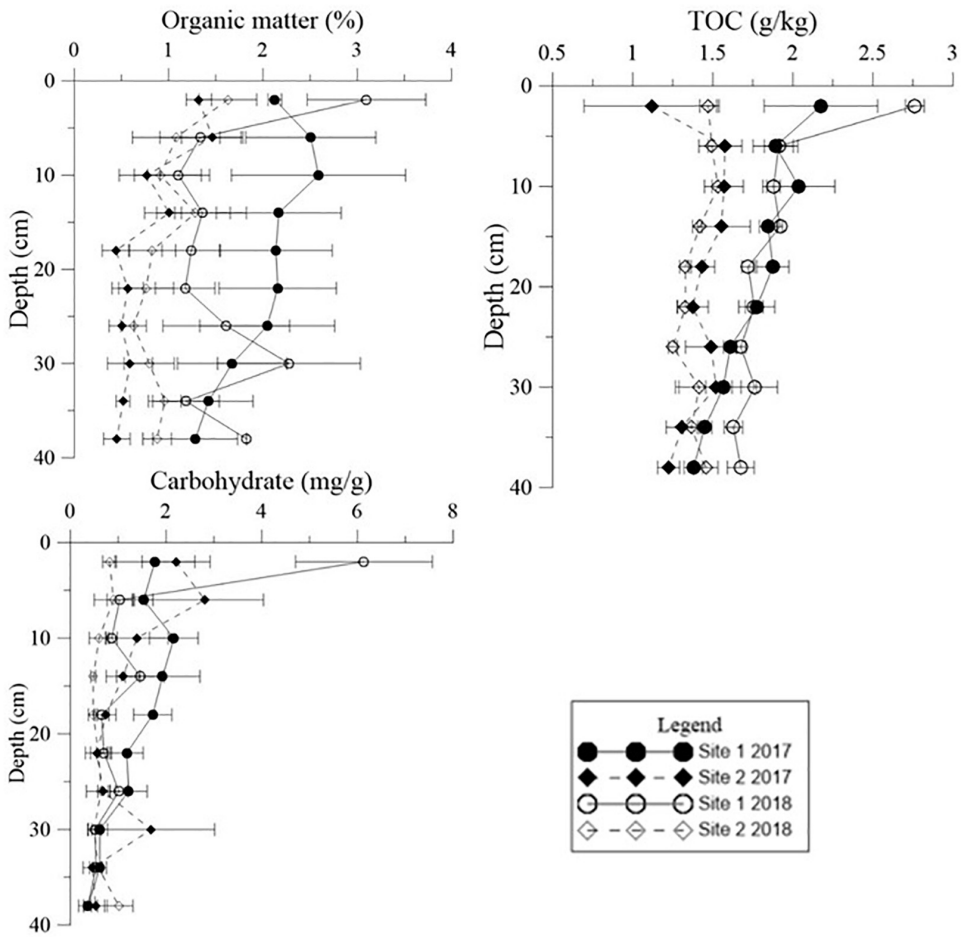

FIGURE 7 | Vertical distribution of organic matter (OM), carbohydrate levels, and total organic carbon (TOC) in the deep sediment cores from the two study sites in May 2017 and May 2018.

In our previous study, around 70% of *R. sinensis* seeds collected from Site 2 germinated immediately under optimal germination conditions, and the dormancy of the remaining seeds could be broken by low temperatures (Gu et al., 2018). However, after an unusual desiccation event in 2018, *R. sinensis* seeds at Site 1 germinated immediately when surface water appeared, while no seedlings appeared at Site 2 (**Figure 2**). It appears that annual populations have higher stress resilience and a quick re-establishment capacity compared with perennial populations.

Although perennial populations of other *Ruppia* species are more reliant on clonal growth for population regeneration, both annual and perennial populations flower and produce seeds in the reproduction season (Mannino and Graziano, 2014). The larger seed densities of the *R. sinensis* annual population in the current study suggested that this population placed more reproductive energy into producing seeds than the perennial population (**Table 1**). However, similar to *Ruppia* habitats in the Everglades-Florida Bay ecotone (Strazisar et al., 2016), TP in the YRD was limited, and was not the main factor resulting in the two different *R. sinensis* life cycles. The TN levels in the sediment at Site 1 were higher than Site 2, which likely benefited the growth of shallow shoots, increasing the capacity for clonal growth within the population (Feller et al., 2008). In addition, the higher biomass of *R. sinensis* at Site 1 might also result in more TN input after decomposition of plant material at the end of the life cycle. The higher nutrient levels in the sediment of the annual *R. sinensis* population might support the recruitment of vegetative shoots when a large amount of seeds germinated during wet periods. In contrast, the nutrient conditions in the sediment of the perennial population were relatively stable.

Detritus from aquatic macrophytes is one of the most important endogenous sources of nutrients in wetlands (Wu, 2009). The Chl *a* content of sediments is a good representation of the abundance of primary producers such as living algae and undegraded macrophyte tissues (Mannino and Sara, 2006; Pusceddu et al., 2009). Low Chl *a* levels below depths of 18 cm in the sediments could imply limited presence of algal and *R. sinensis* detritus at this depth. However, in the shallow sediment layers, higher Chl *a* values were found when more *R. sinensis* was growing, which may be a result of more *R. sinensis* detritus settling in sediment and more suitable conditions for algae growth at this time. Moreover, a previous study indicated

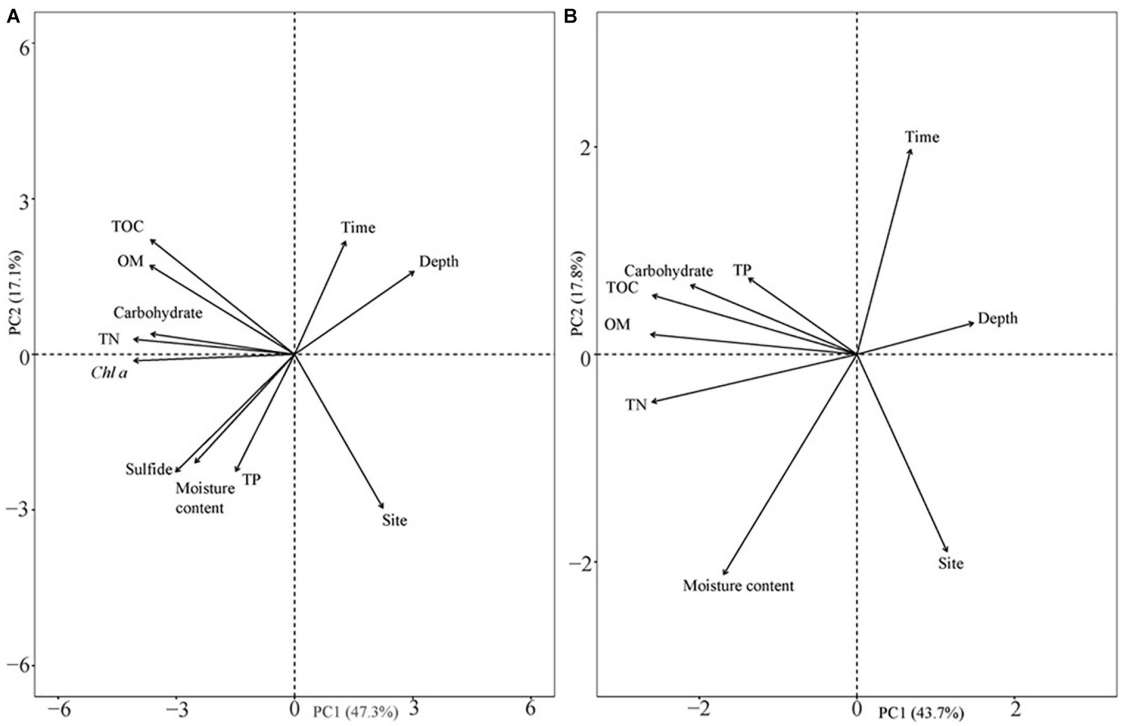

FIGURE 8 | Principal component analysis of sediment parameters in different seasons and their vertical distribution. **(A)** Eight sediment parameters in deep sediment cores with annual differences and different vertical distributions in two different years; and **(B)** six sediment parameters in shallow sediment cores with seasonal changes and vertical distribution at the two sites.

that *Ruppia* not only provided organic carbon directly to the biogeochemical cycle but also provided physical support for the attachment of other macrophytes, which also amplified the production and diversity of the system (Mannino and Sara, 2006). Sediment sulfide content was also closely related to organic deposition. A sharp decrease in *R. sinensis* biomass occurred at both sites after the dry spell, which may have resulted in a decrease in sediment sulfide levels. The results of the current study also showed a peak in the vertical sulfide gradient at a depth of 6 cm, indicating that the sediment conditions at this depth were suitable for sulfide accumulation (**Figure 5**).

The organic carbon from *Ruppia* detritus supports a complex food web through bacterial decomposition (Mannino and Sara, 2006). Organic carbon also indicates the quantity of leaf decomposition which increases nutrient supply for the survival of vegetation (Fan et al., 2015). The three different carbon parameters used in the current study showed slightly different trends. OM decreased with the deposition of *R. sinensis* shoots, and then increased significantly when surface water appeared in May 2018, when *R. sinensis* seedlings germinated at Site 1. Although there was a similar trend in OM at Site 2, there was no germination of *R. sinensis* seeds. This variation indicated the presence of algae, which emerged quickly when surface water appeared and is also an important resource of sediment OM (**Figures 2**, **7**). TOC content has previously been used to represent OM (Sardans et al., 2017). However, even though the correlations of these two parameters were similar (**Figures 6**, **8**), the seasonal changes in these two parameters were slightly different. A recent study noted that the main components of available OM to biota in aquatic ecosystems are carbohydrates, lipids, and proteins (Dias et al., 2017). Meanwhile, carbohydrates are more closely related to phytoplankton origin and vegetal detritus (Cotano and Villate, 2006). The carbohydrate levels in the sediments of the two survey sites differed more than OM and TOC (**Figure 6**). This variation might be related to the appearance of vegetation, as the highest carbohydrate level at Site 1 was recorded in May 2018, when both algae and *R. sinensis* were rapidly growing. However, the carbohydrate levels in the sediment at Site 2 were always low (**Figure 6**).

In summary, the two different *R. sinensis* population types in the YRD exhibited different resilience strategies under extreme desiccation conditions. An annual *R. sinensis* population was present at Site 1. Higher levels of TN were observed at this site which could benefit *R. sinensis* seedling growth, and promote population recovery when the water re-accumulated after drying up in winter. The appearance of *R. sinensis* was also accompanied by more algal growth. This not only increased primary productivity, but also increased carbon deposition and enriched the sediment. The appearance of water was the key factor resulting in the two different *R. sinensis* population types, which could be represented through sediment characteristics such as water moisture content. Of the different carbon parameters used to evaluate sediment carbon deposition in the current study, TOC was the most indicative in explaining the

differences between the different *R. sinensis* populations. There were higher TOC levels recorded in the annual *R. sinensis* population compared with the perennial population. The results of this study provided a useful reference for the conservation and management of both annual and perennial *Ruppia* populations. This study also provided an example of the sensitivity of different carbon parameters in assessing the relationships between vegetation and sediment carbon.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

RG did the conceptualization, investigated the data, and composited the original manuscript. XS performed the methodology. YZ carried out the funding acquisition, supervised the data, and reviewed and edited the manuscript. ScX, SX, SY, YuZ, and XZ investigated the data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.634199/full#supplementary-material

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Unravelling the Spatial and Temporal Plasticity of Eelgrass Meadows

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The phenotypic plasticity of seagrasses enables them to adapt to changes in environmental conditions and withstand or recover from disturbance. This plasticity was demonstrated in the large variation recorded throughout a suite of bioindicators measured within *Zostera marina* meadows around Wales and SW England, United Kingdom. Short-term spatial data were analysed alongside long-term monitoring data to determine which bioindicators best described the status of eelgrass meadows subjected to a range of environmental and anthropogenic drivers. Shoot density, leaf length, leaf nutrients (C:N ratio, %N, %P) including stable isotope of $\delta^{13}C$ and $\delta^{15}N$ provided insight into the longer-term status of the meadows studied and a good indication of the causes of long-term decline. Meadows ranged from those in the Isles of Scilly with little evidence of impact to those in Littlewick in Milford Haven, Wales that showed the highest levels of impacts of all sites. Bioindicators at Littlewick showed clear warning signs of nutrient loading reflected in the long-term decline in shoot density, and prevalence of wasting disease. This study highlights the need for continuous consistent monitoring and the benefits of using extra tools in the form of shoot nutrient analysis to determine causes of decline.

Keywords: ***Zostera marina*, plasticity, bioindicator, water quality, nutrient, resilience**

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INTRODUCTION

Seagrass is protected under International, European, and United Kingdom legislation and monitoring of meadows has been integrated into management and Water Framework Directives as an indicator of good ecological status of coastal waters (Krause-jensen et al., 2005; Foden and Brazier, 2007; Marbà et al., 2013; de los Santos et al., 2019). This has led to an increase in monitoring of seagrass meadows around Europe in recent decades (de los Santos et al., 2019). However, the diverse range of seagrass indicators used (Marbà et al., 2013) and the difference in frequency of monitoring surveys make it difficult to make assumptions on the true status of these vital habitats. Baselines for monitoring have major implications for how the interpretation of the status of seagrass meadows is or has altered over time. Monitoring enables the management and protection of seagrass meadows from direct existing or potential impacts, such as reductions in water quality. This ultimately improves the overall health and resilience of the seagrass to increasing threats from climate change (Björk et al., 2008). As an important carbon store in the marine environment, it is even more pertinent that seagrass meadows are protected and, where viable, restored so that they can continue to absorb CO_2 from the atmosphere (Röhr et al., 2018).

Zostera marina meadows around the British Isles are degraded in status, with estimations of 25–49% decline in the last 35 years (Hiscock et al., 2005; Jackson et al., 2013) although recent evidence has this value at 92%, a loss of approximately 75,000 ha (Green et al., 2021). To be able to set criteria for monitoring and mitigation strategies within management plans, it is important to understand environmental drivers of seagrass meadows. Environmental conditions such as light, temperature and depth will affect many physiological, morphological, and structural parameters of seagrass meadows (Martínez-Crego et al., 2008). The plasticity of seagrasses enables them to adapt to changes in environmental conditions and in turn to withstand certain levels of disturbances (Short and Wyllie-Echeverria, 1996). These changes can be used as bioindicators of reduced light levels, nutrient input and other impacts that can be attributed to anthropogenic disturbance or other causes for decline in water quality. Detailed studies of seagrass responses to light reduction have revealed a number of consistent and robust bioindicators such as reductions in shoot density, biomass, growth and production, and shorter narrower leaves (McMahon et al., 2013). Above ground biomass is reduced in this way in order to reduce the respiratory and energetic costs that come from the production and maintenance of new leaves (Fourqurean and Zieman, 1991; Collier et al., 2012). Chlorophyll content of leaves can increase under low light, with the chlorophyll *a:b* ratio lowering to increase photosynthetic efficiency (Silva et al., 2013). However, if light stress is prolonged, the production of more chloroplasts may prove too costly and resulting in the rapid decline in photosynthetic performance within a relatively short time-frame (Ralph and Gademann, 2005; Bité et al., 2007). Based on such evidence it can be assumed that the morphology and physiology of *Z. marina* can provide an insight into the overall light environments *in situ* and hence the status of coastal waters (Dennison et al., 1993).

Leaf biochemistry of seagrass can also be used to signify changes in ecological health of coastal waters from eutrophication (Fourqurean et al., 1997; Jones and Unsworth, 2016). Such studies in the United Kingdom found most seagrass to be in a poor condition, with nutrient values in excess of global averages (Jones and Unsworth, 2016). Additionally, shoot C:N ratio and the stable isotope of carbon, $\delta^{13}C$ have both been identified as a robust and early indicator of light stress (McMahon et al., 2013), with C:N shown to have a positive relationship with seagrass cover (McKenzie et al., 2011). Also, the stable isotope of nitrogen $\delta^{15}N$ in seagrass can be used to identify anthropogenic sources of nutrient inputs from agricultural or urban effluents (Lepoint et al., 2004; Jones et al., 2018), providing indications of the source of eutrophication threat to the ecosystem (Short et al., 1995; Lee et al., 2004).

In order to understand the status of seagrass, monitoring of abiotic factors such as temperature, turbidity and light are also important (Jackson et al., 2013; Burton et al., 2015; McDonald et al., 2016) as natural environmental processes also effect seagrass growth. Temperature has been found to effect the morphology of *Z. marina* with wider leaved plants being found in areas where the annual temperature fluctuation is small such as the Scilly Isles (Den Hartog, 1970). Also, *Z. marina* growing in higher wave exposure will have significant morphological differences to plants growing where relative wave exposure is lower (Krause-Jensen et al., 2003). Changes in depth limits of seagrass growth is one of the bioindicators used to inform the WFD of changes to water quality as deeper maximum depth limits suggest clearer waters (Dennison and Alberte, 1985; Dennison, 1987; Krause-jensen et al., 2005). Density will also be lower at increased depths as a response to lower light in order to reduce self-shading and reduce respiratory demand (Collier et al., 2007). This supports the need for monitoring a number of robust bioindicators alongside abiotic parameters within seagrass meadows when assessing status. When bioindicators at the meadow or plant-scale change, hypothesising the potential drivers is compromised by gaps in explanatory environmental and seagrass data. Specifically, it is important to determine if changes are natural processes such as yearly fluctuations in sunlight hours and sea surface temperature, or are being caused by anthropogenic sources such as light limitation caused by nutrient loading (Rasheed and Unsworth, 2011). The need for detailed reference conditions need to be taken into account for such changes to be properly assessed (Krause-jensen et al., 2005).

The aims of this study were to investigate the plasticity of *Z. marina* using a suite of morphological and physiological indicators over a range of environmental conditions and hypothesise that these responses can be used to explain changes occurring in these meadows over time using available long-term monitoring survey data. These sites include one in the Isles of Scilly used as a low impact "reference site" thought to have reduced anthropogenic pressures.

MATERIALS AND METHODS

Seagrass Condition

Six *Z. marina* meadows around the coast of Wales and the Isles of Scilly (United Kingdom) were assessed for morphological and physiological factors. The sites were as follows: Littlewick bay 51.706°N, 5.067°W (Milford Haven), North Haven 51.738°N, 5.280°W (Skomer), Pen-y-chain 52.899°N, 4.322°W, Criccieth 52.917°N, 4.227°W and Porthdinllaen 52.943°N, 4.565°W (Llyn Peninsula) and Little Arthur 49.948°N, 6.265°W within the Isles of Scilly (**Figure 1**). All sites were surveyed in August and September 2016.

At each site a PAR logger (Odyssey, Dataflow systems Ltd) and a temperature logger (Tinytag aquatic 2) were deployed and left *in situ* for a month to record light availability and temperature in the middle of the seagrass meadows. The light logger was placed vertically attached on the mooring block at 50 cm above the seabed so it would be recording at the top of the canopy, and to avoid shading. A Secchi disk was used to measure turbidity, and depth was recorded using a dive computer (Suunto zoop) on the survey days and corrected to Chart Datum using tidal prediction software (POLTIPS v3, Bell, 2016). Wave energy index for each site was calculated using data taken from EMODnet seabed habitats portal which provides data on variables that influence habitat type taken from various survey sources. For each site the three grid squares (0.3 km resolution) closest to the

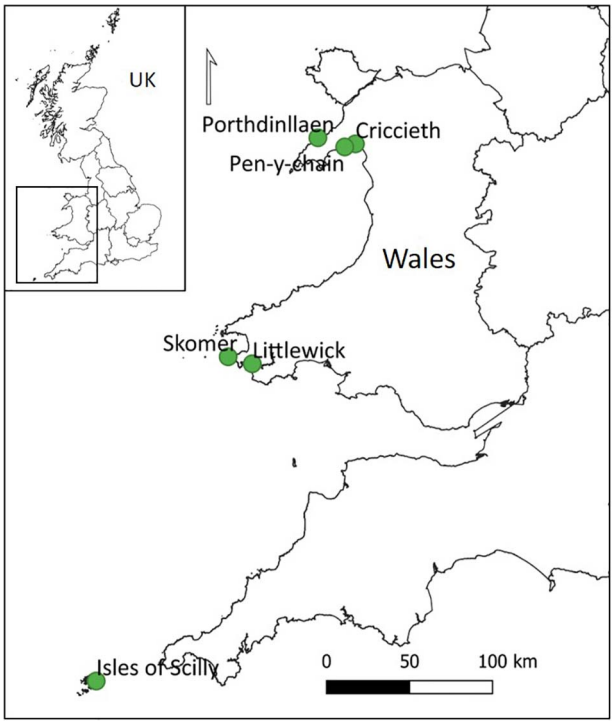

FIGURE 1 | Locations of seagrass sites surveyed around Wales and on the Isles of Scilly, United Kingdom.

survey position that contained wave energy data were averaged to give an overall value.

At each Welsh site, the mid-meadow and meadow edges were identified from previous site data collection and drop-down camera work (Nagle, 2013; Brown, 2015; Burton et al., 2015). Ten 50 cm × 50 cm quadrat were placed haphazardly through the middle of the meadow, perpendicular to the shore. Within each quadrat, 25 cm × 25 cm area of seagrass was removed, with shoots being cut just at the level of the substrate and cut shoots placed in separate zip lock bags. Where visibility was good enough, a Go-Pro®Hero 4 camera attached to the top of the quadrat frame was used to video the quadrats. This allowed extra data to be collected including percentage coverage of seagrass and algae which were analysed from video footage. This was repeated at the edge of the meadow in order to get a good representation overall. At Pen-y-chain and Criccieth, the seagrass was found to be relatively patchy and a distinct edge was not found owing to poor visibility, so only measurements through the middle of the meadow were possible.

All shoots collected were counted and each leaf measured. Shoot measurements included leaf length (taken from top of sheath to tip of leaf), leaf width, epiphyte and wasting disease cover. Leaf length was measured with a measuring tape to the nearest mm, and leaf width was measured using callipers to the nearest 0.1 mm. Canopy height was estimated by taking the maximum leaf length of each shoot. Epiphyte and wasting disease cover was scored between 0 and 5 for each leaf (whereby "0" = 0%, 0% < "1" ≤ 2%, 2% < "2" ≤ 25%, 25% < "3" ≤ 50%, 50% < "4" ≤ 75%, and 75% < "5" ≤ 100%) based on the index developed for wasting disease (Burdick et al., 1993).

Shoot data for the Isles of Scilly site, Little Arthur, was obtained from Natural England annual surveys which follow a comparable method outlined in Lobelle et al. (2013) and Potouroglou et al. (2014). This allowed for the inclusion of metric data from the 2016 annual survey to be included into this study.

Leaf Nutrient Analysis

Samples of seagrass were taken from each of the sites and leaves were separated, scraped free of epiphytes, and dried. The dried seagrass was ground up with a pestle and mortar to a fine homogenous powder. Samples were sent to OEA laboratories Limited for analysis of the % composition of Carbon, Nitrogen and Phosphorus by weight using a continuous flow isotope ratio mass spectrometer (Sercon 20–20 IRMS coupled to Thermo EA1110 elemental analyser). The ratios of stable isotopes 13C to 12C ($\delta^{13}C$) and 15N to 14N ($\delta^{15}N$) were also determined to give values which can indicate light availability, nutrient availability and anthropogenic sources of nutrients (Jennings et al., 1997; Lepoint et al., 2004). Leaf nutrient data for the Isles of Scilly was obtained from a previous study by Jones et al. (2018).

Long-Term Data Analysis

Four long-term monitoring datasets for Skomer (Burton et al., 2019), Littlewick (Hiscock, 1987; Irving and Worley, 2000; Nagle, 2013; Unsworth et al., 2017a), Porthdinllaen (Project Seagrass, 2019), and Isles of Scilly (Lobelle et al., 2013; Alotaibi et al., 2019) were collated and standardised. All comparable data were extracted for analysis for temporal changes and trends.

Statistical Analysis

All averages are reported ± Standard Deviation. GLM is a flexible method of analysis that can be used on different types of data including count data (shoot density) and continuous data (leaf lengths) without being limited by the assumptions of normally distributed data (Crawley, 2005). For leaf lengths and widths, GLMs with Gamma errors were used which is most appropriate for continuous data such as measurements (Crawley, 2005; Zuur et al., 2009). For epiphyte, wasting disease, seagrass cover and algae cover, GLM with binomial errors which is appropriate for proportion data. All scores and percentages were converted to proportions (0–1). For over or underdispersed data whereby the residual deviance was higher or lower than the degree of freedom, quasi-binomial GLM was used instead to correct for this, making the models more conservative with lower chance of type 1 error (Crawley, 2005). For count data, shoot density and number of leaves, Poisson (or quasi-poisson for overdispersion) GLM with log link was used which ensures all fitted values are positive (Crawley, 2005). All GLM were carried out using R Studio (R version 4.0.2). Model comparisons were made using a likelihood ratios test with and without site as a factor to assess significance of site on the parameter. Where appropriate, Tukey pairwise comparisons between sites were undertaken using the "glht" function in the "multcomp" package in R studio. This analysis was also carried on long-term datasets using year as a factor.

Principal Components Analysis (PCA) was carried out using shoot level data for maximum leaf length, leaf width, epiphytes and wasting disease. All data were scaled before analysis. As not all data were collected at the same resolution separate PCA were conducted including shoot metric data, quadrat level data (to include shoot density), and meadow-scale data (to compare nutrient data). PCA conducted on quadrat level data to include shoot density and leaves per shoot. Leaf nutrients and stable isotopes (C:N, %N, %P, δ^{15}N, δ^{13}C) were analysed using PCA separately alongside average shoot density to see if they were having an effect on shoot count as has been found in other studies. Owing to cost of nutrient analysis, sample number for nutrients was limited therefore a separate PCA was conducted to visualise similarities between meadows. Principal components with eigenvalues > 1.0 were considered, and eigenfactors or variable coefficients ≤ -0.3, or ≥ 0.3 were selected. All PCA was carried out using Primer-e (version 6).

RESULTS

Seagrass Condition

The morphological plasticity of seagrass throughout our six survey sites from 2016 was highly variable and likelihood ratios tests showed that site as a factor had a significant effect on all metrics (**Supplementary Table A.1**). Leaf length was significantly longer in the Isles of Scilly (630.68 ± 162.71 mm) than any other site (**Figure 2** and **Supplementary Table A.2**). Littlewick had the widest leaves (3.41 ± 0.78 mm) although width data was not available for Isles of Scilly. Density was highest in Porthdinllaen (189.18 ± 109.43 shoots per m^2) along with Skomer and Isles of Scilly, all of which were found to have significantly higher shoot densities than other sites (**Figure 2** and **Supplementary Table A.2**). Criccieth and Pen-y-chain were found to have similar shoot densities to Littlewick, albeit with shorter and narrower leaves (**Figure 2** and **Supplementary Table A.2**). Wasting disease was significantly higher in Littlewick (1.29 ± 0.51) than Porthdinllaen, Skomer, and Isles of Scilly (**Figure 2** and **Supplementary Table A.2**) with the lowest scores in Porthdinllaen (0.47 ± 0.47). Pen-y-chain had the highest epiphyte score (2.12 ± 0.59) and the lowest scores were in the Isles of Scilly (0.67 ± 0.39, **Figure 2** and **Supplementary Table A.2**) although most sites were not different from each other. Numbers of leaves per shoot were highest on the Isles of Scilly (4.38 ± 0.86) and significantly higher than all sites except for Porthdinllaen (**Figure 2** and **Supplementary Table A.2**).

Seagrass cover and algae percentage cover from the drop-down camera varied significantly between the sites surveyed (no data for Isles of Scilly). Model comparisons found that site as a factor was found to having a significant effect on seagrass and algae cover (**Supplementary Table A.1**). Seagrass cover was significantly higher in Porthdinllaen (54.2 ± 37.69%) than all other sites (**Figure 2** and **Supplementary Tables A.1, A.2**). Algae cover was highest in Littlewick (44.8 ± 28.51%, **Figure 2** and **Supplementary Table A.2**), significantly higher cover than Skomer and Pen-y-chain (**Figure 2** and **Supplementary Table A.2**).

Nutrient Analysis

Seagrass nutrient results show high levels of variability between sites (**Table 1**). Isles of Scilly had the lowest %P and δ^{15}N content showing little, if any, evidence of nutrient enrichment from anthropogenic sources at this site. These nutrient parameters were found to be highest in seagrass from Littlewick indicating nutrient enrichment. Skomer, however, had the lowest C:N, δ^{13}C and the highest %N suggesting light limitation and nutrient enrichment.

Principal Components Analysis

Principal components analysis (PCA) was carried out on all available parameters measured at both shoot and quadrat levels for all Welsh sites. All of the shoot level metrics (width, length, epiphyte, and wasting score) were shown by PCA to be strongly contributing to the variability between the seagrass meadows (**Supplementary Figure A.1A** and **Supplementary Table A.4**) with all factors found to be significant across the first two components explaining over 80% of the variation. PC1 and shows a significant correlation between leaf length, width and wasting disease with all eigen factors over 0.3 (**Supplementary Table A.4**). The second PCA (**Supplementary Figure A.1B**) shows leaf length and width contribute strongly to explaining the variation between meadows in Wales at the quadrat level with PC1 and PC2 explaining nearly 65% of the variation. PC2 shows a strong positive correlation with epiphyte and wasting disease cover and a negative correlation with leaves per shoot. The third PCA was used to compare shoot nutrient data for each of the sites in Wales and plotted with shoot density and shows a higher level of clustering of sites. PC1 shows a strong positive correlation of C:N and isotope δ^{13}C with decreasing %N (**Supplementary Figure A.2C**) demonstrating higher light availability (↑C:N, δ^{13}C) with decreasing nutrient inputs (↓%N). PC2 (33.2% variation) shows a positive correlation with δ^{15}N and %P, both of which would increase in seagrass meadows with nutrient loading. PC3 (18.6% variation) shows a positive correlation with δ^{15}N but negative correlation with shoot density (**Supplementary Table A.4**) suggesting an increase in anthropogenic sourced nutrients having a negative effect on shoot density.

Nutrient data available for the Isles of Scilly included all nutrient parameters (except for δ^{13}C) and relevant shoot metrics and was therefore included in a fourth PCA (**Figure 3** and **Table 2**). Epiphytes, δ^{15}N and %P showed significant negative correlation with leaf length, width and leaves per shoot in PC1 (47% variation). Clustering of sites shown in **Figure 3** shows the Isles of Scilly sharing no overlap with other sites particularly on PC1 axis, whereas Skomer, Pen-y-chain, and Criccieth show more similarity.

Environmental Variables

Environmental variables are shown in **Table 3**. No data was available for the Isles of Scilly site. Pen-y-chain and Porthdinllaen were found to have the highest light availability based on PAR logger data, whereas light Criccieth had the lowest (**Table 3**). Temperature results showed little difference between sites so is likely having limited effect on the meadows that can be discerned from this short-term data (**Table 3**) Wave energy data shows

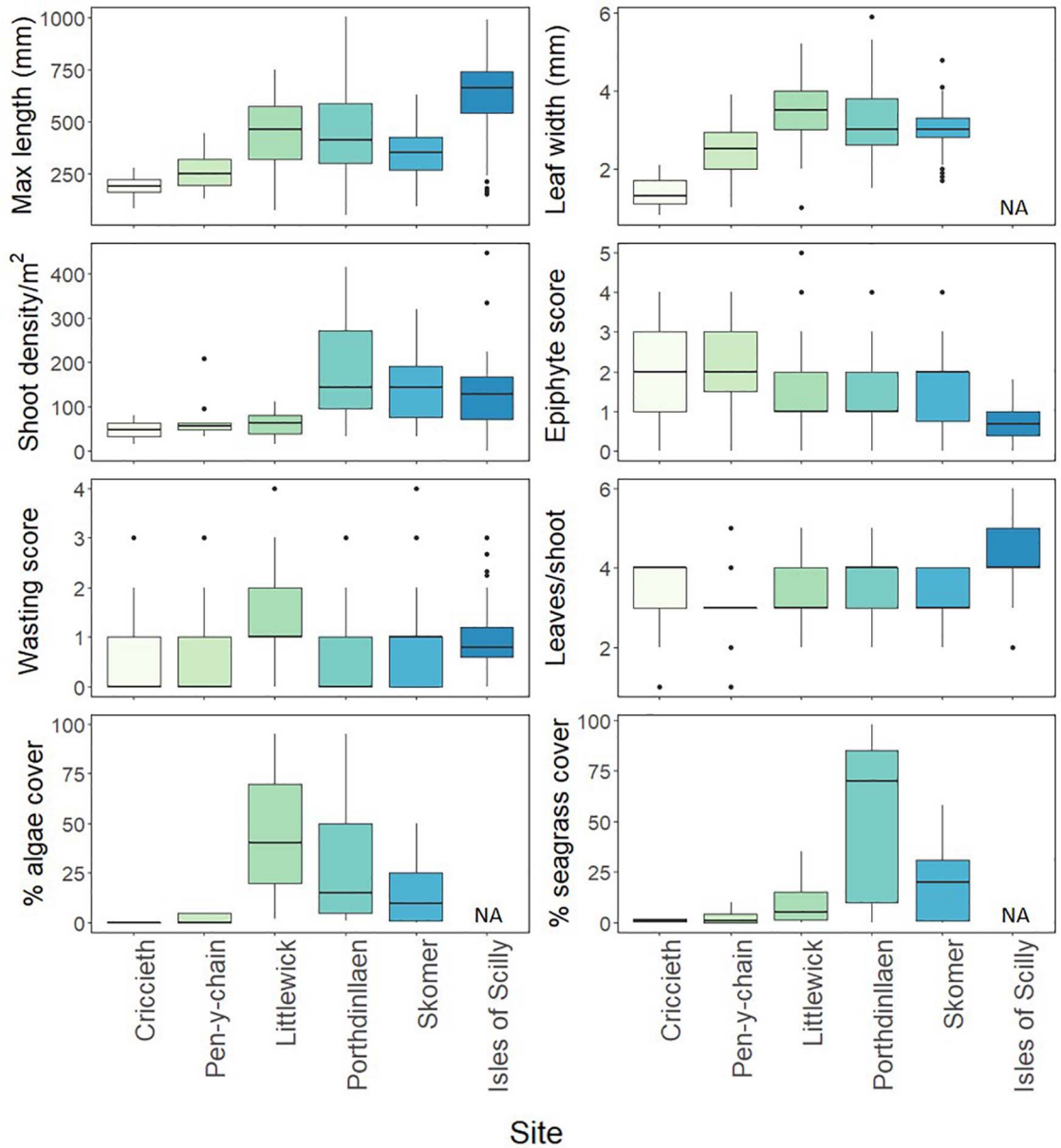

FIGURE 2 | Seagrass shoot and meadow characteristics measured at different seagrass sites. The box-whisker represents the median (line) and interquartile range (box) with additional 1.5 × interquartile range shown as whisker. Outliers are shown as points outside the box-whisker plots. Algae and seagrass cover taken from drop-down camera footage of quadrats taken at each site except Isles of Scilly ($n \geq 40$ per meadow except Criccieth where $n = 12$ due to poor visibility).

the higher wave exposure effecting the seagrass at Criccieth and Pen-y-chain when compared to average results for Skomer, Porthdinllaen, and Littlewick. Criccieth and Pen-y-chain were also found to be considerable shallower than other sites with higher turbidity.

Long-Term Changes

Shoot Density

Significant changes in shoot density with year were found at all sites except for Porthdinllaen (**Supplementary Table A.54**). For Littlewick, shoot density was found to be the highest in 1999 (141.39 ± 61.9, **Figure 4**). Shoot density has consistently decreased since surveys began (**Figure 4**) with the lowest density recorded in 2012 (**Figure 4** and **Supplementary Table A.6**). Pairwise comparisons show that all years measured have significantly lower shoot density than 1986 and 1999. Most recent surveys (2012, 2016, and 2018) are also significantly lower than in 2008 (**Supplementary Table A.5**). For Skomer, seagrass densities show a different pattern with densities significantly increasing between 1997 and 2006 (**Figure 4** and **Supplementary Table A.6**). The surveys in 2014 show the lowest overall density

TABLE 1 | Results from the elemental analysis of *Z. marina* leaf tissue taken from the study sites.

Site	%N	%P	C:N	$\delta^{15}N$	$\delta^{13}C$
Criccieth	2.23 ± 0.23	0.24 ± 0.03	15.87 ± 0.46	6.37 ± 0.33	-14.71 ± 0.22
Littlewick	2.27 ± 0.24	0.40 ± 0.04	18.98 ± 0.18	10.17 ± 0.1	-14.36 ± 0.31
Pen-y-chain	2.26 ± 0.13	0.29 ± 0.03	19.41 ± 0.82	7.60 ± 0.63	-13.69 ± 0.57
Porthdinllaen	2.22 ± 0.38	0.33 ± 0.04	21.09 ± 0.59	7.72 ± 0.05	-13.65 ± 0.59
Skomer	3.04 ± 0.19	0.33 ± 0.02	14.71 ± 0.18	8.03 ± 0.1	-16.90 ± 0.28
Isles of Scilly	2.76 ± 0.29	0.14 ± 0.01	20.56 ± 2.55	4.47 ± 0.97	n/a
Study average	*2.46 ± 0.36*	*0.29 ± 0.09*	*18.44 ± 2.44*	*6.71 ± 3.06*	*-14.66 ± 1.28*

The stable isotope values for $\delta^{15}N$ indicate the deviation of the isotopic composition relative to air. The isotope values for $\delta^{13}C$ indicate the deviation of the isotopic composition relative to the Vienna PeeDee Belemnite (VPDB) standard. All values are unitless.

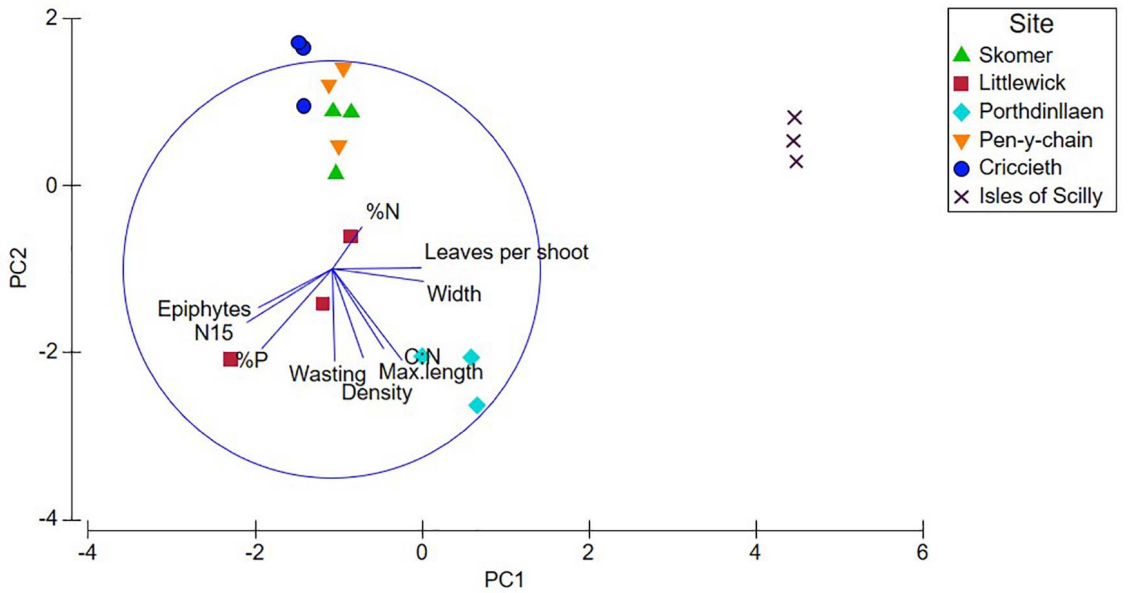

FIGURE 3 | Principal Components Analysis (PCA) plots for shoot, nutrient, and stable isotope data for each site, plotted with shoot density and metrics (max. leaf length, width, and leaves per shoot). Nutrient data for Isles of Scilly provided from Jones et al. (2018).

recorded (36.15 ± 22.04). Density was found to be highest in the 2016 survey (**Figure 4** and **Supplementary Table A.6**), although there is less variability between monitored years (**Figure 4**). Seagrass shoot density in Porthdinllaen has shown little variation through time, with year having no effect on density for the years measured (F = 0.9984), p = 0.41, df = 580, 584). For the annual Isles of Scilly surveys, year was found to be having a significant effect on density (F = 3.791, p < 0.001, df = 495, 516). The highest average shoot counts overall were recorded in 2003 (256.64 ± 199.76 shoots m^2) and the lowest shoot density was in 2015 (106.24 ± 93.17 shoots m^2). The pairwise comparison showed that only the years 2002, 2003, and 2004 (with the highest densities recorded) were significantly higher than other years, with only 14 out of 231 pairwise comparisons showing significance (**Supplementary Table A.5**). Most years did not show significant differences, and shoot density appears to be relatively stable over time (**Figure 5** and **Supplementary Table A.5**). The lowest shoot densities for Isles of Scilly were found to correlate with historic sunshine hour data taken the closest weather station data[1] (**Supplementary Figure A.2**). The continuous annual monitoring in the Isles of Scilly allowed us to undertake this analyse there but not at other sites.

Leaf Length

Leaf length data was the only other comparable metric monitored long-term, and only available for Littlewick and Porthdinllaen in Wales, and the Isles of Scilly whereby maximum leaf lengths are measured (**Figure 5**). Model comparison demonstrated that leaf length at all three sites showed significant changes with year (**Supplementary Table A.5**). Leaf length in Littlewick has changed significantly over time, with the biggest overall increase in lengths recorded in 1999 followed by the largest decline in 2012 (**Figure 5** and **Supplementary Table A.6**). The survey in 2016 did not record a significant change in leaf length, but 2018 data shows a significant increase (**Supplementary Table A.6**), back to similar lengths recorded in 1999 (**Figure 5**). For Porthdinllaen, since

[1]Metoffice.gov.uk

TABLE 2 | Results from the Principal Components Analysis (PCA) carried out using available data from Welsh sites and Isles of Scilly for nutrient data, shoot metrics, and density.

PCA1—Shoot data	PC1	PC2	PC3
Summary values			
Eigenvalues	**4.72**	**2.05**	**1.33**
Percent variation	47.2	20.5	13.3
Cumulative percent variation	47.2	67.7	80.9
Seagrass variables			
Max. leaf length	**0.333**	**–0.437**	0.074
Leaf width	**0.440**	**–0.058**	0.075
Epiphyte	**–0.355**	**–0.186**	0.145
Wasting	0.013	**–0.440**	0.163
Leaves per shoot	**0.428**	0.008	0.062
%N	0.145	0.204	**0.750**
%P	**–0.338**	**–0.381**	0.176
C:N	0.245	**–0.383**	**–0.493**
$\delta^{15}N$	**–0.411**	–0.254	0.023
Density	0.147	**–0.424**	**0.317**

Bold values show significant levels of eigenvalues (above 1 for principal components, and eigenfactors or variable coefficients ≤ –0.3, or ≥ 0.3).

2015 there is some decline in leaf length, with the biggest decline in 2018 (**Figure 5** and **Supplementary Table A.6**), but lengths have increased somewhat by 2019 with pairwise comparisons showing a significant increase in length from 2015 to 2018 (**Supplementary Table A.6**). The seagrass in the Scilly Isles is showing significant fluctuations in leaf length, with the longest since monitoring started (in 1996) being in 2009 (994.16 ± 265.43 mm), and the shortest in 2014 (534.63 ± 155.47mm). Over time, leaf length appears relatively stable (**Figure 5**), however the results of the pairwise comparison showed significant differences between most years (156 out of 231 pairwise comparisons, **Supplementary Table A.6**).

Leaf Condition

Long-term shoot condition data was only available for Littlewick and the Isles of Scilly. For Littlewick, both epiphyte and wasting disease showed significant temporal changes, with a decrease in epiphytes and an increase in wasting disease cover over each year (**Figure 6**). Changes in epiphyte cover between years for the Isles of Scilly site fluctuate but with a slight increase over time. Wasting disease shows little variation with the only significant increases shown between a few years (**Figure 6**).

DISCUSSION

Here we provide a unique analysis of bioindicators of seagrass at spatial (short-term) and temporal (long-term) scales. The spatial study allowed for the measurement of a wide range of seagrass characteristics which can provide evidence of environmental drivers affecting the variation in seagrass plasticity and condition between different locations. The long-term study involving the analysis of data from monitored seagrass sites provides insight into the relative stability or instability of the meadows studied.

The plasticity of seagrasses enables them to adapt to changes in environmental conditions and to a degree withstand or recover from some level of anthropogenic disturbance (Short and Wyllie-Echeverria, 1996; Maxwell et al., 2014). At sites in Wales and SW England environmental and anthropogenic factors were found to influence this plasticity as demonstrated in the large variation found across a suite of seagrass of indicators.

All the bioindicators measured were found to describe significant amounts of variation between sites. The morphological and physiological bioindicators enabled differentiation in Wales between sites, with the extensive meadow at Porthdinllaen appearing to be the healthiest reflected by shoot morphology, condition and leaf biochemistry. This meadow was found to have the highest shoot density and cover, with leaf nutrient bioindicators indicating a higher light environment and lower nutrient loading. The long-term data and earlier studies validate this finding with the seagrass community found to be stable between years (Edwards et al., 2003; Morris et al., 2009). Although the temporal range of data for Porthdinllaen is limited, evidence exists that this site remains a stable eelgrass bed showing similar shoot density to the Isles of Scilly site.

Relatively high wave energy and turbidity were recorded as the principle drivers of the two shallowest meadows at Criccieth and Pen-y-chain. These meadows had the shortest and narrowest leaves and lowest shoot densities, a possible response to increased wave motion and risk of uprooting. Average temperatures measured over the survey period were over 1°C higher in these two shallow meadows than the other sites surveyed which is likely to have an effect on the respiratory demand of the plants. Higher variability in temperature in shallower waters will be contributing to the dynamism of the localised environment. Eelgrass from Criccieth was found to be in the poorest condition due to low shoot C:N, $\delta^{13}C$, shoot density and high epiphyte cover. The PAR levels measured were found to be lowest in Criccieth presumably due to increased turbidity via the resuspension of sediments from high wave energy. However, shoot nutrient analysis indicates low nutrient input at this site suggesting natural processes are having the biggest impact on seagrass condition. Pen-y-chain was found to have the highest PAR levels most likely due to shallow depth and lower turbidity, reflected by high shoot C:N and $\delta^{13}C$. Criccieth has been previously recorded as a sparse meadow (Edwards et al., 2003), suggesting it is somewhat dynamic owing to its physical environment.

Our bioindicator approach found low light differentiated the meadow at Skomer from other localities (low PAR, C:N, $\delta^{13}C$) even though superficially shoot density was similar to Porthdinllaen and the Isles of Scilly. This prognosis is verified by the long-term instability in the system. Low light maybe a natural phenomenon driven by elevated nitrogen due to run-off from the colonies of breeding seabirds that nest on the surrounding cliffs from April to June (Wilkie et al., 2001). This regular seasonal input of nutrients appears to be causing periodic reductions in the local light environment, causing seagrass here to be relatively dense but with shorter and narrower leaves. The long-term data shows this meadow to be fluctuating significantly but there is no steady decrease which suggests these changes could be attributed

TABLE 3 | Abiotic and environmental data collected for each site collected in August-September 2016, averages ± standard deviation.

Site	Light (PAR)	Temp (C°)	Wave energy (N.m^2.s^{-1})	Turbidity—Secchi (m)	Max. depth (m)
Criccieth	391.42 ± 506.28	17.64 ± 0.31	160.45 ± 28.15	0.5 ± 0.01	2.5 ± 0.25
Littlewick	n/a	n/a	83.54 ± 46.49	1.65 ± 0.01	4 ± 0.45
Pen-y-chain	796.74 ± 875.16	17.76 ± 0.34	165.68 ± 39.1	1 ± 0.02	2.5 ± 0.32
Porthdinllaen	779.84 ± 702.83	16.53 ± 0.25	19.18 ± 9.1	5 ± 0.02	5.2 ± 0.39
Skomer	420.49 ± 324.84	16.07 ± 0.34	24.20 ± 3.2	6 ± 0.03	8.2 ± 0.46
Study average	595.89 ± 656.68	16.99 ± 0.78	90.61 ± 70.45	2.83 ± 2.23	4.48 ± 2.12

Light data for each site is a daily average of PAR logged every 10 min. Temperature was also logged every 10 min. Depths were adjusted to Chart Datum. Wave energy was averaged from data taken from EMODnet (https://www.emodnet-seabedhabitats.eu/access-data/launch-map-viewer/).

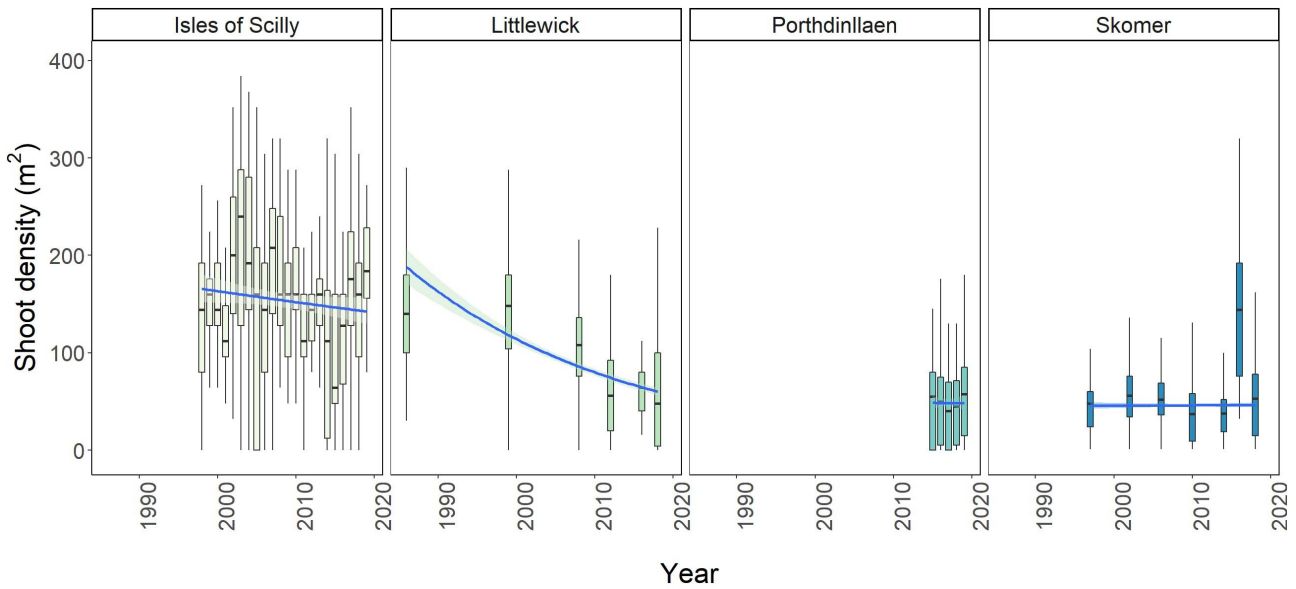

FIGURE 4 | Boxplots showing change in average shoot density per m^2 over time for Littlewick, Skomer, Porthdinllaen, and Isles of Scilly. The box-whisker represents the median (line) and interquartile range (box) with additional 1.5 × interquartile range shown as whiskers. Outliers not shown for clarity (data provided by NRW, Project Seagrass and Natural England respectively, with data from this study included for Skomer and Littlewick).

to natural fluctuations in yearly sunshine hours and short-term, seasonal light limitation from plankton blooms and epiphyte growth caused by nutrient run-off from seabird colonies.

By comparison, the bioindicators measured show the meadow at Littlewick is showing strong signs of anthropogenic impact. The shelter from wave action suggests the area should be conducive to seagrass growth, yet shoot densities are comparable to sites where wave action is much higher. The leaf condition and nutrient biondicators suggest that nutrient loading is impacting this meadow (highest δ^{15}N, %P and wasting score) despite leaf length and width being high. This meadow was also found to have the highest percentage cover of algae. Other studies looking at the effects of eutrophication in eelgrass beds have also found increases in leaf length and a reduction in shoot density as a response to increased shading from opportunistic algae (Moore et al., 1996; Short and Burdick, 1996; Schmidt et al., 2012). High inorganic nitrogen (N_i) in the water column can cause seagrasses to be more susceptible to infections from wasting disease as anti-microbial compounds are produced less to compensate for the synthesis of excess nitrogen in plant tissues (Short and Burdick, 1996; Burkholder et al., 2007). These factors combined strongly to imply that the seagrass meadow in Littlewick is under threat from eutrophic conditions and is undergoing a system shift from a seagrass dominated to macroalgae-dominated community. Long-term data for Littlewick supports this assumption, whereby leaf length has shown significant increases in most years, but shoot density is showing a steady significant decline. Wasting disease has also increased significantly since monitoring started.

Seagrass in Wales relative to the Isles of Scilly (IoS) as a reference site seagrass with limited anthropogenic impacts. Shoot densities and leaf widths in IoS are somewhat comparable with Skomer and Porthdinllaen, but the addition of shoot nutrient parameters (in this case C:N, δ^{15}N, and %P) results in huge dissimilarities between meadows. Leaf length is significantly longer in Isles of Scilly which has been previously recognised as the longest eelgrass found in United Kingdom waters (Den Hartog, 1970; Jones and Unsworth, 2016). The increased water clarity of this archipelago is caused by the granite substrate and sediments that settle rapidly (Jackson et al., 2011) and the

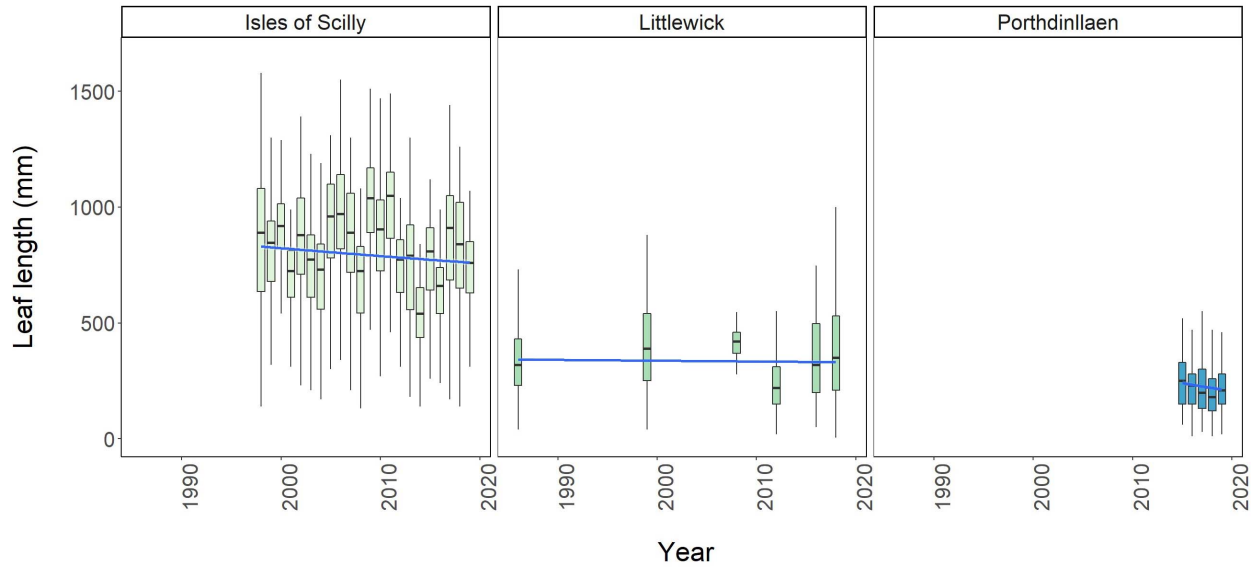

FIGURE 5 | Boxplots showing change in average leaf length over time for Littlewick, Porthdinllaen, and average maximum leaf length for Isles of Scilly. The box-whisker represents the median (line) and interquartile range (box) with additional 1.5 × interquartile range shown as whisker and a temporal trendline in blue (GLM smooth with Gamma family), grey area shows 95% confidence. Outliers not shown for clarity (data provided by NRW, Project Seagrass, and Natural England respectively, with data from this study included for Littlewick).

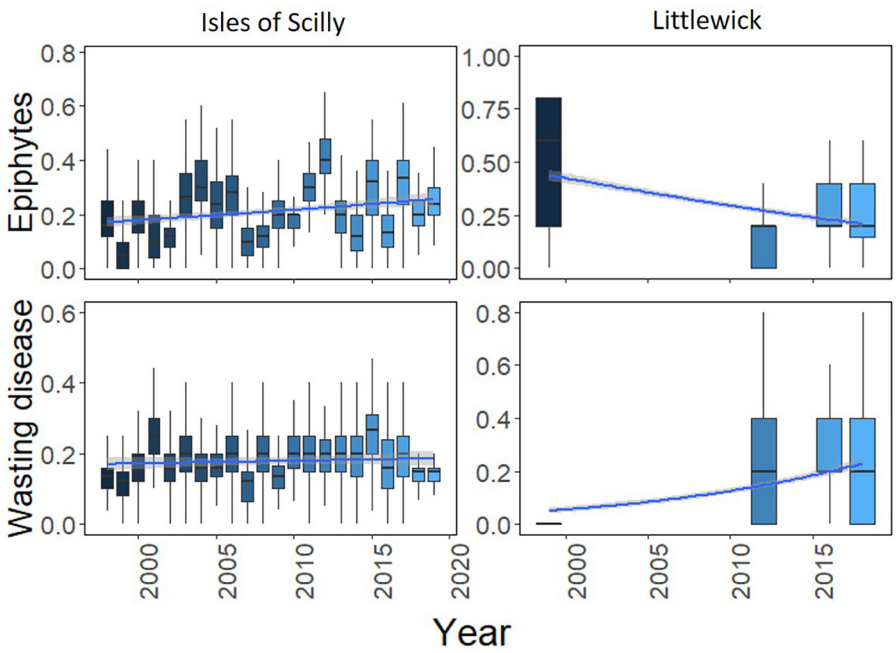

FIGURE 6 | Boxplots showing change in leaf condition (epiphyte cover and wasting disease) over time for Littlewick and the Isles of Scilly. The box-whisker represents the median (line) and interquartile range (box) with additional 1.5 × interquartile range shown as whisker. Scale is as a proportion based on the original scores, with temporal trendline in blue (GLM smooth with binomial errors for proportion data) with 95% confidence in grey either side. Outliers have been taken out for clarity (data provided by NRW, and Natural England, with data from this study included for Littlewick for 2016).

lack of large scale agriculture and urbanisation. This allows *Z. marina* to grow at greater depths with longer leaf lengths than other locations where turbidity reduces the maximum depth limit of seagrass growth (Nielsen et al., 2002). The lower impacts from terrestrial run-off are shown in the high C:N and lower %P and $\delta^{15}N$. The long-term yearly monitoring of the eelgrass meadows in the Isles of Scilly allows for fine-scale temporal changes to be shown. The main threats

to seagrass around these remote islands is physical damage caused by boat moorings, anchoring and storms (Jackson et al., 2013; Bull and Kenyon, 2015; Unsworth et al., 2017b), not necessarily water quality issues. The data used for this study comes from the site that was found to be the least impacted and provided a good control site for comparison of status. The yearly monitoring of the Isles of Scilly allows for better evidence-based projections of long-term trends and changes, with shoot density showing much more stability than canopy height over time. It is likely that fluctuations are caused by changes in sunshine hours or other natural processes, with sunshine hours showing a positive correlation with shoot density for the Isles of Scilly (**Supplementary Figure A.2**). The slower response of shoot density to environmental stresses than other metrics raises the alarm for systems that are seeing continuous declines.

Density of the seagrass *Zostera marina* overall is showing some decline over the last two decades, providing evidence that seagrass in the United Kingdom is still somewhat degraded in state with no measurable upward trend of recovery as seen in some species such as *Z. noltii* (Bernard et al., 2007; Bertelli et al., 2018). The lowest densities appear to have been recorded between 2012 and 2015 which could be a United Kingdom wide response to natural processes such as significant changes in average recorded sunshine hours.

We also present strong evidence of significant and consistent long-term decline of one of Wales' largest seagrass meadows at Littlewick in the Milford Haven Waterway. The increase in leaf length together with the reduction in density strongly indicate that Littlewick Bay is suffering from frequent and/or prolonged nutrient loading, to the point that natural environmental processes, such as fluctuations in sunshine hours, could be hidden. Milford Haven Waterway, which encompasses Littlewick, has been designated as being of moderate status and hypernutrified in terms of the WFD standards for nutrients (NRW, 2016). This is reflected in the high tissue nutrients found from the spatial study which explains this trend. By contrast, other sites have shown some increase in shoot density in the most recent years and an overall level of stability in density as seen in the Isles of Scilly, Porthdinllaen, and Skomer. Due to complexities of the factors influencing the resilience of seagrass meadows it is difficult to determine how close such a meadow is to a catastrophic tipping point, however, considerable long-term seagrass monitoring evidence globally indicates that once such a point is reached complete degradation and loss can be rapid (Waycott et al., 2009).

Shoot density is affected by numerous disturbances, including light limitation, nutrient loading, physical damage, temperature, or natural storm events, and therefore is one of the most important parameters that can be implemented into monitoring programmes. Consistent monitoring methods between sites can enable the identification of naturally occurring temporal trends that could be affecting structural responses or where trends are not consistent, indicate localised anthropogenic disturbances. Significant changes to shoot density should then justify the use of other robust bioindicators of stress to determine the causes of decline.

CONCLUSION

This study demonstrates the high levels of plasticity exhibited by eelgrass to environmental conditions and the need for regular, consistent long-term monitoring of seagrass sites for significant declines to be detected. Structural bioindicators or responses such as shoot density, cover, biomass and extent are often included (one or all) in general seagrass monitoring programmes but do not integrate the use of bioindicators.

Our evidence indicates that where significant changes are detected such biochemical indicators can become powerful metrics for determining sources of declines. For sites where there is a lack of monitoring data, a suite of bioindicators and abiotic factors can be measured to interpret environmental conditions and provide meaningful understanding as to the status of those seagrasses that are potentially indicative of long-term trends. Left unchecked seagrass meadows are highly susceptible to degradation and loss, principally due to the development of a phase shift from seagrass to an algal dominated state. Our study provides a warning that such shifts may be likely at some, particularly as their resilience to future stressors is compromised by poor water quality. In conclusion we find that long-term monitoring of seagrasses is critical for helping inform management of such meadows to prevent catastrophic changes from occurring.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analysed in this study. This data can be found here: Natural Resources Wales, https://naturalresources.wales/evidence-and-data/accessing-our-data/access-our-data-maps-and-reports/?lang=en and Natural England https://naturalengland-defra.opendata.arcgis.com/pages/accessing-data-services. Contains Natural Resources Wales information © Natural Resources Wales and database right. All rights reserved.

AUTHOR CONTRIBUTIONS

CB: contributed to the conceptualization, data curation, formal analysis, investigation, methodology, project administration, software, visualization, and writing—original draft preparation. LC-U and JB: contributed to the data resources and curation. RU: contributed to the conceptualization, supervision, validation, and writing—review and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.664523/full#supplementary-material

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Low Light Availability Reduces the Subsurface Sediment Carbon Content in *Halophila beccarii* From the South China Sea

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Eutrophication, dredging, agricultural and urban runoffs, and epiphyte overgrowth could reduce light availability for seagrass. This may affect "blue carbon" stocks in seagrass beds. However, little research is available on the effect of light intensities on carbon sequestration capacity in seagrass beds, especially small-bodied seagrasses. The dominant seagrass *Halophila beccarii*, a vulnerable species on the IUCN Red List, was cultured in different light intensities to examine the response of vegetation and sediment carbon in seagrass beds. The results showed that low light significantly reduced leaf length and above-ground biomass, while carbon content in both above-ground and below-ground tissues were not affected. Low light reduced both the above-ground biomass carbon and the total biomass carbon. Interestingly, while under saturating light conditions, the subsurface and surface carbon content was similar, under low light conditions, subsurface sediment carbon was significantly lower than the surface content. The reduction of subsurface sediment carbon might be caused by less release flux of dissolved organic carbon from roots in low light. Taken together, these results indicate that reduced light intensities, to which these meadows are exposed to, will reduce carbon sequestration capacity in seagrass beds. Measures should be taken to eliminate the input of nutrients on seagrass meadows and dredging activities to maintain the "blue carbon" storage service by enhancing light penetration into seagrass.

Keywords: seagrass, light availability, vegetative carbon, sediment carbon, *Halophila beccarii*

INTRODUCTION

Seagrasses are marine flowering plants, found on all continents except Antarctica (Hemminga and Duarte, 2000). Seagrass beds play a vital role in the ecosystem acting as one of the major primary producers with high productivity (Larkum et al., 2006; Valdez et al., 2020). Seagrasses provide multiple ecosystem services, including coastal protection, improved water quality through

the uptake of nutrients, provision of nursery habitat, and carbon sequestration (Hemminga and Duarte, 2000; Fourqurean et al., 2012). Seagrass beds occupy only a very small fraction of the coastal vegetation but contribute to almost 25% of the annual carbon sequestration of the coastal zone, acting as a sink of CO_2 (Duarte et al., 2013; Saderne et al., 2019). Organic carbon is not only stored in plant above- and below-ground compartments (i.e., shoots and roots), but is also stored in the sediment beneath seagrasses to a larger degree (Kennedy et al., 2010; Fourqurean et al., 2012; Jiang et al., 2017).

Underwater light intensity is one of the major factors that influence seagrass ecosystems, and seagrasses require nearly 11%–37% of the surface irradiance (Cussioli et al., 2020). Nevertheless, human activities in the catchment area and the coastal area can affect light availability for seagrass in the bottom habitats of the sea (Gattuso et al., 2006; Ralph et al., 2007). For example, eutrophication, dredging, agricultural and urban runoffs, and epiphyte overgrowth could reduce light availability for seagrasses (Ralph et al., 2007; York et al., 2015; Strydom et al., 2017; Yan et al., 2020). This undoubtedly inhibits seagrass photosynthesis, growth rate, and health status (Ralph et al., 2007). While most previous research has focused on the effect of light limitation on seagrass molecular and physiology (Dattolo et al., 2014; Schliep et al., 2015; Kumar et al., 2017; Davey et al., 2018; Griffiths et al., 2020), relatively little is known about the responses of carbon sequestration in seagrass beds (Serrano et al., 2014; Dahl et al., 2016).

The seagrass *Halophila beccarii* Asch is one of two species in the oldest lineage of seagrass distributed in the intertidal areas of the tropical Indo-Pacific region (Short et al., 2010; Aye et al., 2014; Jiang et al., 2020; Mishra and Apte, 2021). *H. beccarii* seems to be well adapted to the high light intensities when it gets exposed during low tides. *H. beccarii* often grows in river estuaries with large nutrient inputs. This results in higher epiphyte biomass attached to the leaves of *H. beccarii*, decreasing irradiance availability even further. Thus, *H. beccarii* has been declining at accelerating rates and is currently listed as a vulnerable species in the IUCN Red List of threatened species (Short et al., 2011; Jiang et al., 2020). Light limitation decreases seagrass carbon fixation and shoot density (Ralph et al., 2007; Ferguson et al., 2016), which might also reduce the amount of carbon available for root growth and root exudate formation (Jiang et al., 2018; Martin et al., 2018b). This may reduce the carbon stocks beneath seagrass meadows. While this has been confirmed by studying large-bodied species including *Posidonia* (Serrano et al., 2014) and *Thalassia* (Dahl et al., 2016), these effects have not been studied in small-bodied seagrass species, such as those in the *Halophila* genus.

Aiming to close some of these knowledge gaps, we conducted an indoor experiment to culture the seagrass *H. beccarii*, the dominant species in South China Sea, under different light intensities to examine the response of carbon storage in plant above- and below-ground compartments and associated sediments in seagrass beds. Two hypotheses were proposed. The first hypothesis was that low light reduces vegetative carbon stock due to decreased seagrass above-ground biomass. The second hypothesis was that carbon in the subsurface sediment (not including detritus) in seagrass beds was decreased by low light due to inhibited root growth and carbon allocation. Furthermore, we also estimated the change trend of vegetative carbon sequestration in *H. beccarii* beds in the South China Sea and globally caused by light limitation. The results obtained in the present study will undoubtedly enhance our understanding of the mechanisms controlling carbon storage in response to light. These will improve the management and conservation of these ecologically and economically important ecosystem engineers.

MATERIALS AND METHODS

Plant Materials and Experimental Design

H. beccarii plants were collected by hand in February 2019 with its natural sediment (6 cm sediment layer) using a smooth board (150 × 200 mm), at the intertidal zone of the monospecific seagrass bed in Yifengxi (116.903°E, 23.544°N; **Figure 1**), along the South China coast (Jiang et al., 2020). The shoot density of *H. beccarii* was about 7892 ± 744 shoots/m^2 in the collection site. The leaf length, leaf width, and root length were 1.20 ± 0.05 cm, 0.26 ± 0.01 cm, and 1.97 ± 0.28 cm, respectively. Water turbidity at the collection site is relatively high, due to local eutrophication, agricultural, and urban runoffs. Following collection, plants were taken to the laboratory and placed within nine glass tanks (150 × 170 × 200 mm) with seawater. Based on preliminary relative electron transport rates (ETR) performed using rapid light curves (using the MINI PAM), its minimum saturating light was 177.3 ± 15.5 μmol photons/m^2/s, approaching 200 μmol photons/m^2/s. In the laboratory (113.299°E, 23.096°N, Guangzhou; **Figure 1**), seagrasses were cultured with natural seawater from the collection site at 200 μmol photons/m^2/s for 1 week. This was for laboratory acclimation to minimize experimental error. After initial laboratory acclimation, three aquaria replicates were used for each of the three light treatments: the control saturating light (SL; 200 μmol photons/m^2/s), high light (HL; 600 μmol photons/m^2/s), and limited light (LL; 20 μmol photons/m^2/s) irradiance. SL and HL were in the optimal light range between the minimum saturating light and the minimum inhibiting light. The average air temperature and humidity in the room were 25°C and 60%, respectively. The seawater temperature, salinity, and pH were 20°C, 3, and 8.00, respectively. Pump velocity and air-stone flow rate were kept the same across all aquaria to ensure effective stirring of the water body and gaseous diffusion (**Figure 2**). After 1 month of treatment (**Supplementary Figure 1**), seagrass and sediment were collected for measuring morphology (leaf length, leaf width, and root length), biomass of seagrass living tissues and detritus above the surface sediment, and nutrients and stable carbon isotope values of seagrass and sediment. Seagrass plants from five different places in each tank were collected for measuring leaf length, leaf width, and root length. Seagrass mature leaves were selected for determination. Sediment (not including detritus) of 6 cm was sampled with a modified syringe (the diameter was 29.5 mm, **Supplementary Figure 2**) and cut into two layers denoting the surface layer and subsurface layer.

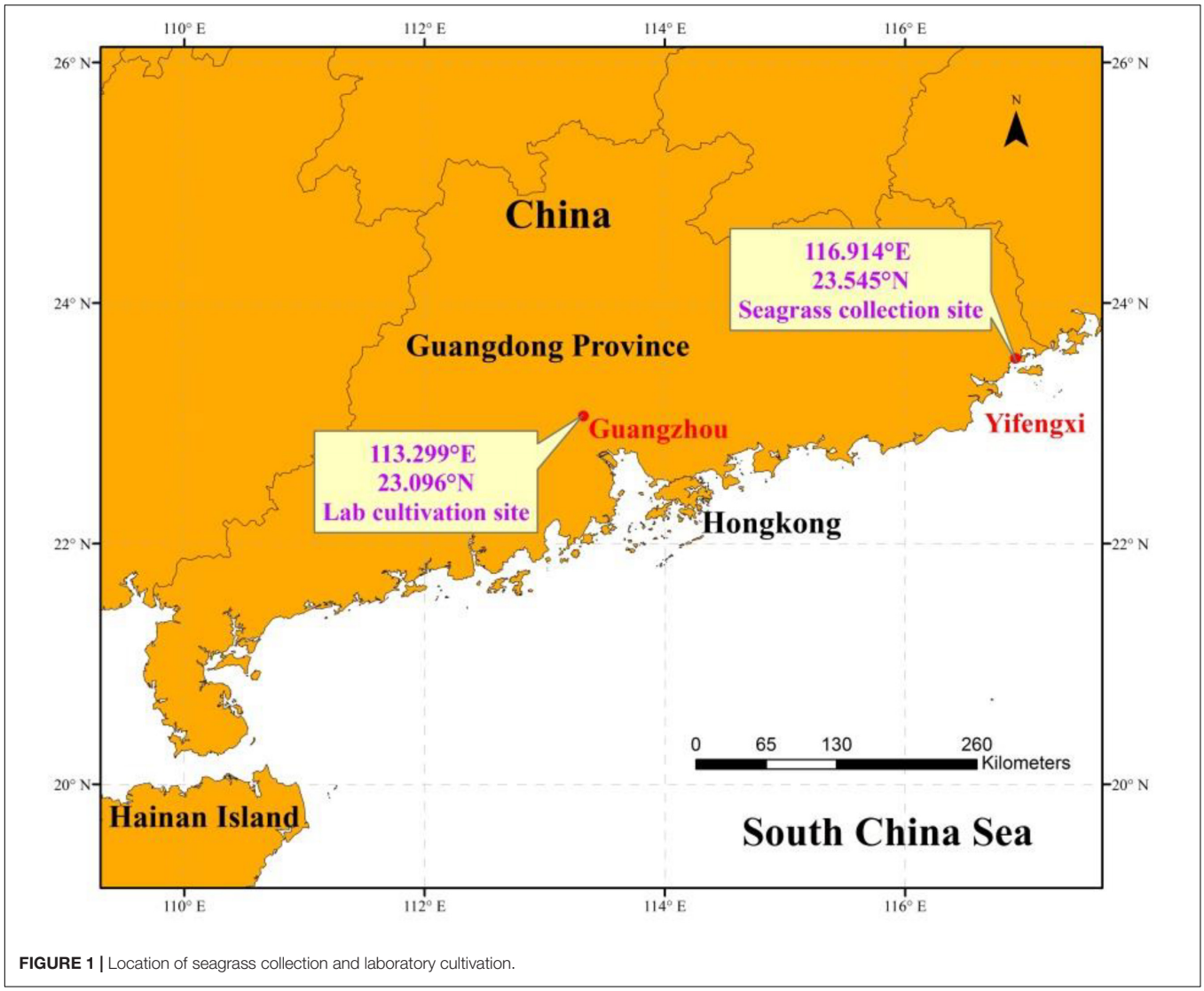

FIGURE 1 | Location of seagrass collection and laboratory cultivation.

The seagrass leaf length, leaf width, and root length were measured using a Vernier caliper. Seagrasses were carefully retrieved, separated into above-ground and below-ground tissue compartments, and subsequently dried at 60°C for 72 h until a constant weight was achieved. Seagrasses were then homogeneously powdered. The total carbon and nitrogen levels of seagrasses were analyzed using an Elementary Analyzer (Flash EA 3000 Thermo Fisher Scientific, Milan, Italy).

The sediment samples were freeze-dried, and sieved through a 500 μm screen to remove coarse materials, which were weighed so their mass could be accounted for in later calculations. Samples were ground and homogenized with a mortar and pestle. All samples were stored in a desiccator prior to analysis. The concentrations of sediment carbon were determined with a CHN analyzer (Elementar, Vario EL-III, Germany). We did not acidify sediment to remove inorganic carbon, since the sediment is mainly composed of organic carbon. $\delta^{13}C$ isotopes in seagrass and sediments were analyzed using a continuous-flow isotope-ratio mass spectrometer (Delta V Advantage, Thermo Fisher Scientific, Waltham, MA, United States). $\delta^{13}C = (R_{sample}/R_{standard}\text{-}1) \times 1000$, where R is the ratio of $^{13}C/^{12}C$. The reference standard for carbon was Vienna PeeDee Belemnite.

We estimated the total vegetative carbon stock of seagrass using the following equations (Howard et al., 2014; Lian et al., 2018):

Vegetative component carbon pool (Mg C/ha) = Carbon content (kg C/m^2) × (Mg/1,000 kg) × (10,000 m^2/ha).

Likewise, the total vegetative nitrogen stock of seagrass was also calculated.

Statistical Analysis

Statistical analysis was conducted using Minitab 17.0 Statistical software. The means and standard errors of all variables were calculated, and all the data were first tested to determine whether the assumptions of homogeneity and normality were met. Where these assumptions were not met, the raw data were

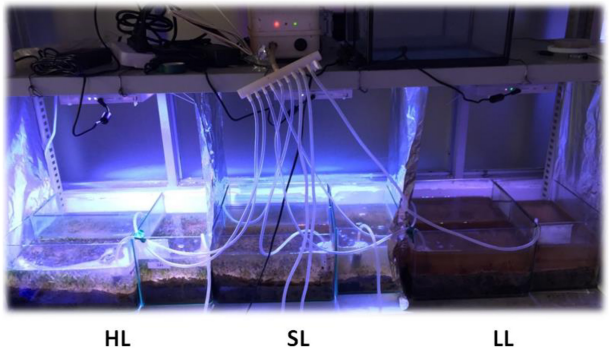

FIGURE 2 | Experimental set-up of the laboratory treatments. SL represents the control saturating light (200 μmol photons/m^2/s); LL represents low light (20 μmol photons/m^2/s); and HL represents high light (600 μmol photons/m^2/s). SL and HL were in the optimal light range between the minimum saturating light and the minimum inhibiting light.

transformed and a further statistical analysis was conducted using the dataset that fulfilled the assumptions. One-way ANOVA followed by Tukey's multiple comparisons tests were performed to determine whether the parameters of seagrass were significantly different among light treatments. Differences between mean values were considered to be significant at a probability of 5% ($p < 0.05$). Otherwise, Welch's t test was performed followed by Dunnett's T3's multiple comparisons tests for determining the significance ($p < 0.05$) (Giannios and Casanova, 2021) of seagrass parameters among light treatments. Two-way ANOVA was performed to investigate the significant difference of C and $^{13}C/^{12}C$ in sediments with respect to light stress and sediment layer.

TABLE 1 | Statistical analysis of effects of different light intensities on seagrass parameters.

Variable	Statistic (asymptotically F distributed)	df1	df2	*P*
Leave length	15.71	2	24.58	<0.05
Leave width	3.29	2	24.83	0.054
Root length	19.22	2	25.97	<0.05
Above-ground carbon	2.90	2	3.46	0.182
Below-ground carbon	3.14	2	3.04	0.182
Detritus carbon	0.87	2	2.77	0.509

Welch test, P < 0.05 at significant level.

TABLE 2 | Statistical analysis of effects of different light intensities on seagrass parameters.

Variable	df	*F*	*P*
Above-ground biomass	2	47.60	<0.01
Below-ground biomass	2	20.27	<0.01
Detritus biomass	2	0.25	0.787
Above-ground nitrogen	2	13.74	<0.01
Below-ground nitrogen	2	23.30	<0.01
Detritus nitrogen	2	1.94	0.223
Above-ground biomass carbon	2	78.07	<0.01
Below-ground biomass carbon	2	4.18	0.073
Detritus biomass carbon	2	0.43	0.668
Total biomass carbon	2	29.44	<0.01
Above-ground biomass nitrogen	2	70.41	<0.01
Below-ground biomass nitrogen	2	0.72	0.526
Detritus biomass nitrogen	2	0.53	0.611
Total biomass nitrogen	2	31.71	<0.01
Above-ground $\delta^{13}C$	2	477.02	<0.01

P < 0.05 (significant); P < 0.01 (highly significant).

RESULTS

Seagrass Morphology and Biomass

The seagrass morphology is depicted in **Figure 3**. A significant difference was found for leaf length and root length (**Table 1**). A declined trend was observed for leaf length and root length along the decreased light irradiance. Simultaneously, there were differences for biomass of above-ground and below-ground tissues among light treatments (**Table 2**). Biomass of above-ground and below-ground tissues both decreased along with decreased light irradiance (**Figure 4** and **Supplementary Figure 1**). Especially, above-ground biomass of SL and HL were about 5 times and 15 times of that of LL, respectively. Interestingly, the detritus biomass was higher in LL than in SL and HL, although there was no considerable difference.

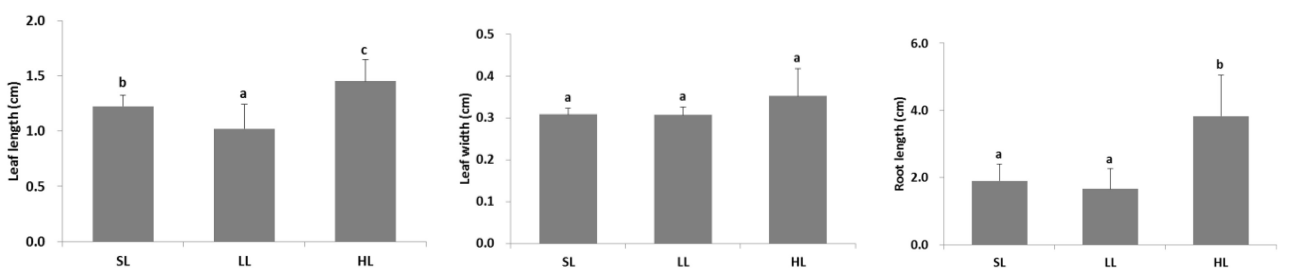

FIGURE 3 | Response of seagrass morphology to light treatments. Saturating light: SL, 200 μmol photons/m^2/s; low light: LL, 20 μmol photons/m^2/s; high light: HL, 600 μmol photons/m^2/s. SL and HL were in the optimal light range between the minimum saturating light and the minimum inhibiting light. Different letters on the bars indicate mean values for a particular light condition that significantly differed at ($p < 0.05$) (Mean ± SD, $n = 15$) analyzed by one-way ANOVA.

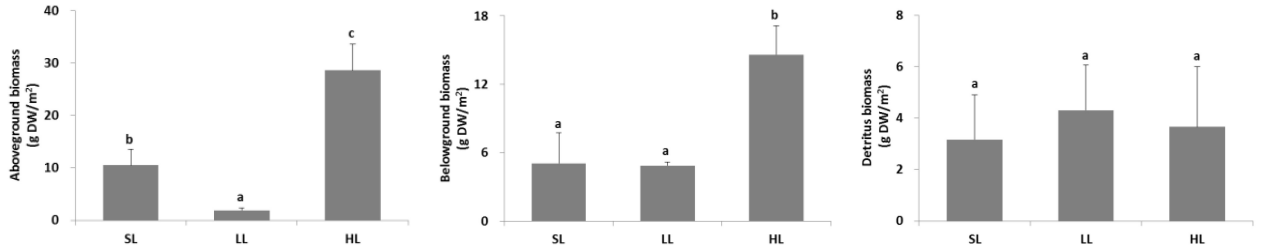

FIGURE 4 | Response of seagrass biomass to light treatments. Saturating light: SL, 200 μmol photons/m^2/s; low light: LL, 20 μmol photons/m^2/s; high light: HL, 600 μmol photons/m^2/s. SL and HL were in the optimal light range between the minimum saturating light and the minimum inhibiting light. Different letters on the bars indicate mean values for a particular light condition that significantly differed at ($p < 0.05$) (Mean ± SD, $n = 3$) analyzed by one-way ANOVA.

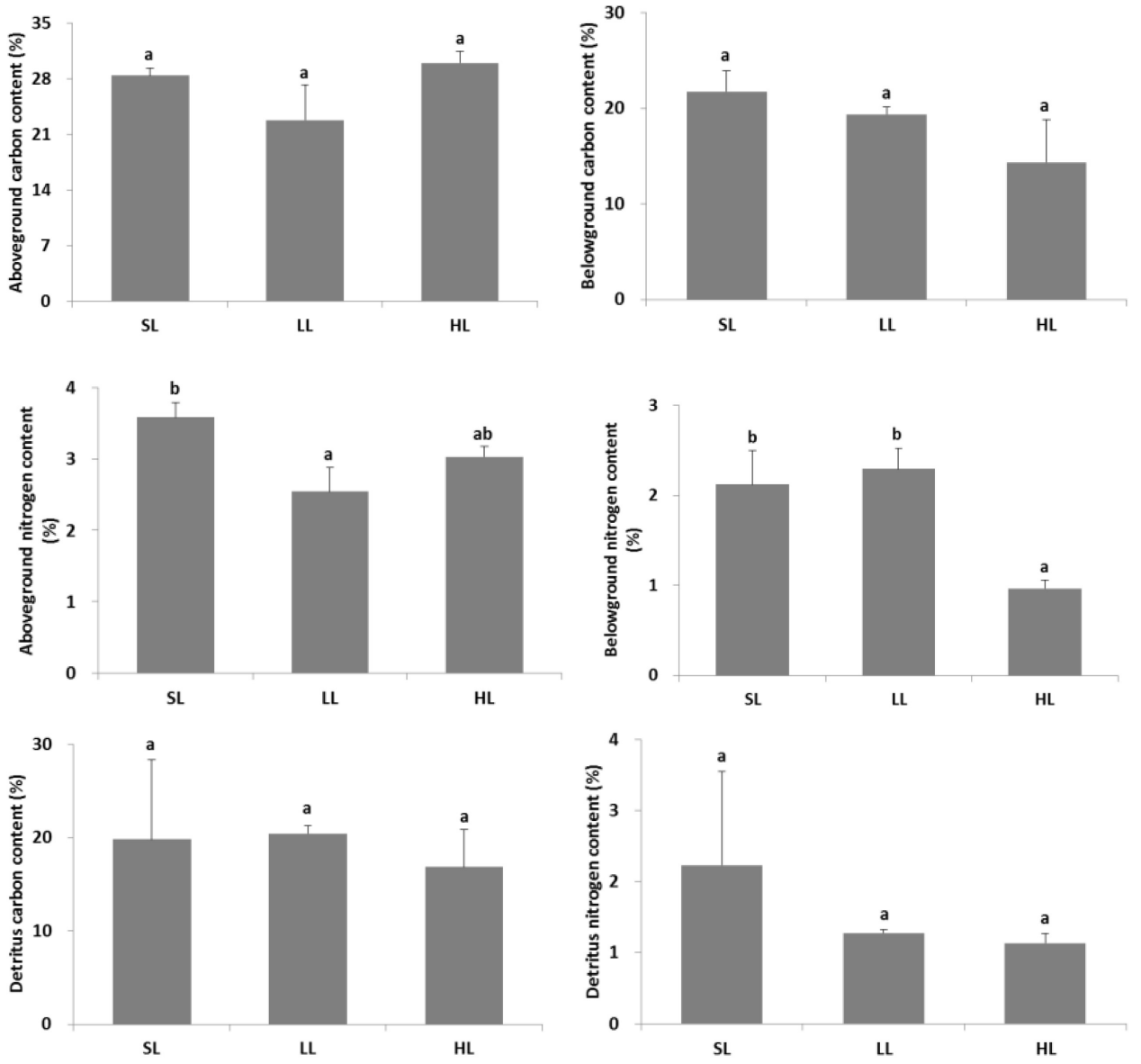

FIGURE 5 | Response of seagrass nutrients to light treatments. Saturating light: SL, 200 μmol photons/m^2/s; low light: LL, 20 μmol photons/m^2/s; high light: HL, 600 μmol photons/m^2/s. SL and HL were in the optimal light range between the minimum saturating light and the minimum inhibiting light. Different letters on the bars indicate mean values for a particular light condition that significantly differed at ($p < 0.05$) (Mean ± SD, $n = 3$) analyzed by one-way ANOVA.

Seagrass Carbon and Nitrogen

The response of seagrass nutrients to light treatments is depicted in **Figure 5**. There was no significant difference for seagrass carbon, while there was a marked difference for nitrogen in both above-ground and below-ground tissues (**Tables 1**, **2**). Carbon and nitrogen were the lowest in the above-ground tissue in the LL treatment. Interestingly, carbon content under HL was the highest in the above-ground tissue, while carbon content exhibited the lowest levels in the below-ground tissue. Simultaneously, detritus carbon and nitrogen, as well as nitrogen content in the below-ground tissues were the lowest under HL treatment (**Figure 5**). Furthermore, the difference of $\delta^{13}C$ content in the above-ground tissues was significant among light treatments (**Table 2**), with a higher value in the HL treatment (**Figure 6**).

The changes of plant carbon and nitrogen stock in response to light treatments are displayed in **Figure 7**. A significant difference was found for the living above-ground and total plant biomass carbon and nitrogen stock (**Table 2**). Biomass carbon in the above-ground, below-ground tissues, and combined plant compartments (i.e., the entire plant biomass) all exhibited a decreased trend along with decreased light intensities. A similar trend was also found for the living above-ground and total biomass nitrogen. Furthermore, the detritus biomass carbon was also higher in LL than in the other two light intensities.

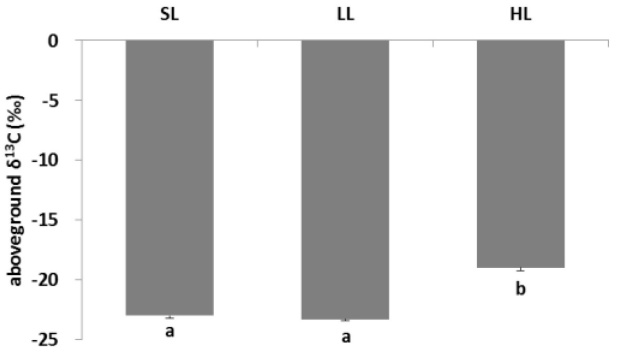

FIGURE 6 | Response of seagrass stable isotope carbon to light treatments. Saturating light: SL, 200 μmol photons/m^2/s; low light: LL, 20 μmol photons/m^2/s; high light: HL, 600 μmol photons/m^2/s. SL and HL were in the optimal light range between the minimum saturating light and the minimum inhibiting light. Different letters on the bars indicate mean values for a particular light condition that significantly differed at ($p < 0.05$) (Mean ± SD, $n = 3$) analyzed by one-way ANOVA.

Sediment Carbon

The effects of light intensities on sediment carbon are shown in **Figure 8**. The carbon content in the surface and subsurface of the sediments was found to be similar in the SL and HL treatment, while the carbon concentration in the subsurface sediment was significantly lower than in the surface sediment in the LL treatment (**Table 3**). It was similar for the sediment $\delta^{13}C$ among the light treatments and between layers.

DISCUSSION

The decrease in light availability is considered as the main anthropogenic disturbance to seagrass beds, causing lower carbon burial capacity (Schmidt et al., 2012; Dahl et al., 2016). The present study provided an opportunity to examine variation in plant and sediment carbon sequestration in seagrass beds across a wide range of light. The findings demonstrated that exposure to low light reduced vegetative carbon stock and subsurface sediment carbon in seagrass beds.

Low Light Decreased Vegetative Carbon Stock in Seagrass Beds

Morphological plasticity allow seagrasses to withstand changes in light availability (Ralph et al., 2007; Ferguson et al., 2016). The present study demonstrated that low light significantly reduced leaf length, above-ground biomass, and leaf densities (**Supplementary Figure 3**). Seagrasses are more sensitive to light reduction since high light is required to maintain a large quantity of non-photosynthetic tissue (Erftemeijer and Lewis Iii, 2006). Meanwhile, leaf density of *H. beccarii* (Ismail, 2014) and shoot density of *Zostera muelleri* (Ferguson et al., 2016) were also reduced by light limitation. Furthermore, light reduction even resulted in complete mortality for *Halophila ovalis* in a turbid environment (Yaakub et al., 2014). In contrast, increasing leaf length or area could allow seagrasses to acclimate to low light climates (Longstaff and Dennison, 1999; Collier et al., 2009; Yaakub et al., 2014; Azcárate-García et al., 2020; Winters et al., 2020). The difference might be caused by the fact that the low light condition in the present study was too limited to maintain a positive carbon balance for *H. beccarii*. Furthermore, low light decreased the energy for generation of ATP for both carbon fixation and HCO_3^- uptake (Ow et al., 2016). Low light induced less nitrogen content in the above-ground tissue, which might result in lower chlorophyll synthesis (Wen et al., 2019). This undoubtedly reduced seagrass carbon fixation. Similarly, low light also reduced the leaf starch of *H. ovalis* (Strydom et al., 2017). Thus, it would decrease the transport of photosynthetically derived non-structural carbohydrates to the root-rhizome system, leading to lower production of below-ground tissues (Duarte and Chiscano, 1999). Meanwhile, a reduction in non-structural carbohydrates also depleted carbon storage reserves that could be used when exposed to further stressors and might therefore reduce seagrass meadow resilience in the future (Alcoverro et al., 2001; Krause-Jensen et al., 2021). The decrease of biomass of above-ground and below-ground tissues also allowed light-limited plants to reduce carbon demands for respiration and maintain overall carbon balance (Lee et al., 2007). High shading also resulted in 45% lower carbon content in the below-ground tissue compared to control treatment (Dahl et al., 2016). Furthermore, detritus biomass was higher in low light, indicating that low light not only inhibited seagrass growth, but also induced leaf senescence to produce higher leaf detritus (York et al., 2013).

The biomass carbon and nitrogen stocks of living above-ground tissue were significantly reduced under lower light conditions compared to saturating light. Especially, the total biomass carbon stock of seagrass plants under low light was about

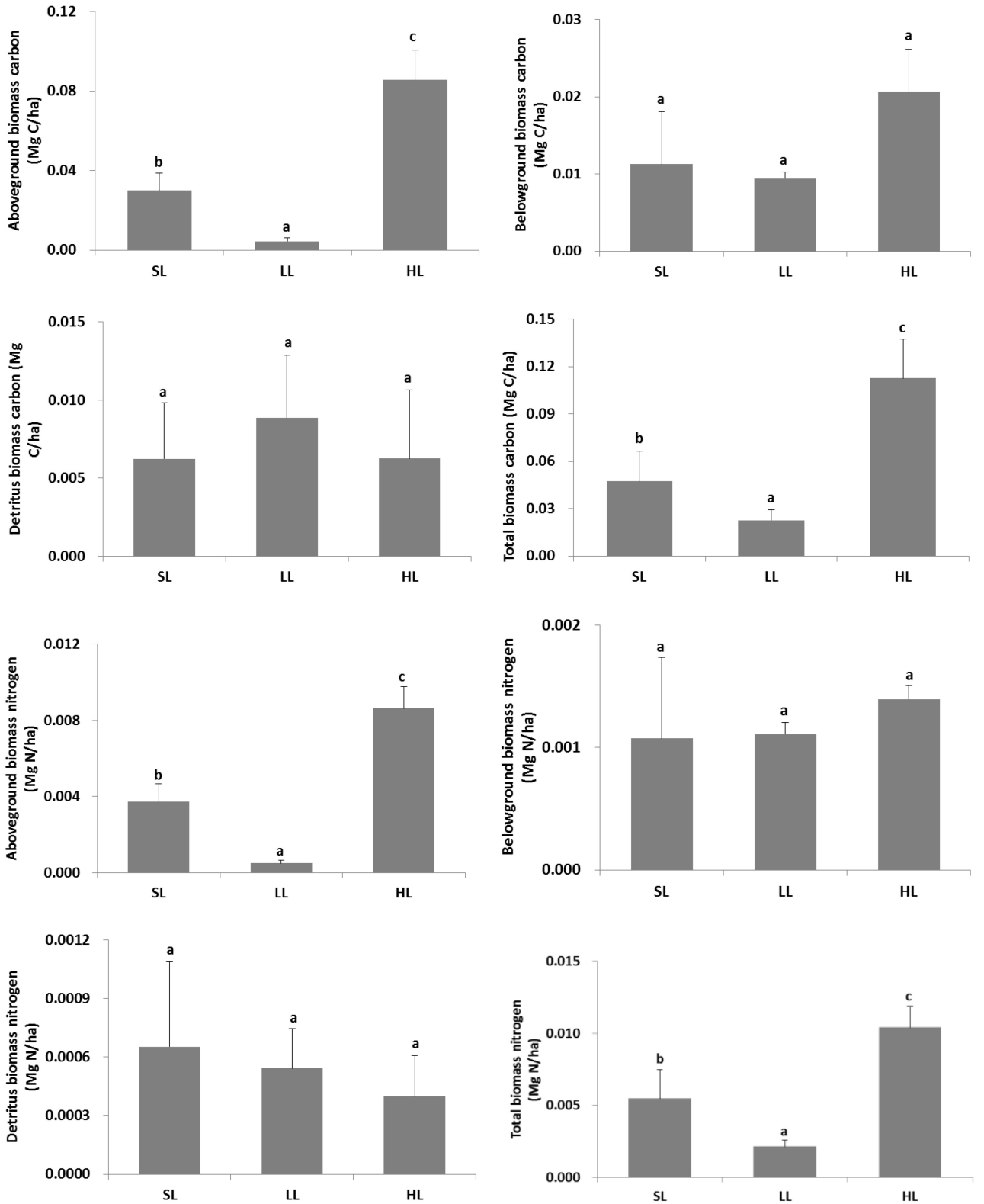

FIGURE 7 | Response of vegetative carbon and nitrogen stock to light treatments. Total biomass included living above-ground and below-ground tissues, and detritus above the surface sediment. Saturating light: SL, 200 μmol photons/m^2/s; low light: LL, 20 μmol photons/m^2/s; high light: HL, 600 μmol photons/m^2/s. SL and HL were in the optimal light range between the minimum saturating light and the minimum inhibiting light. Different letters on the bars indicate mean values for a particular light condition that significantly differed at ($p < 0.05$) (Mean ± SD, $n = 3$) analyzed by one-way ANOVA.

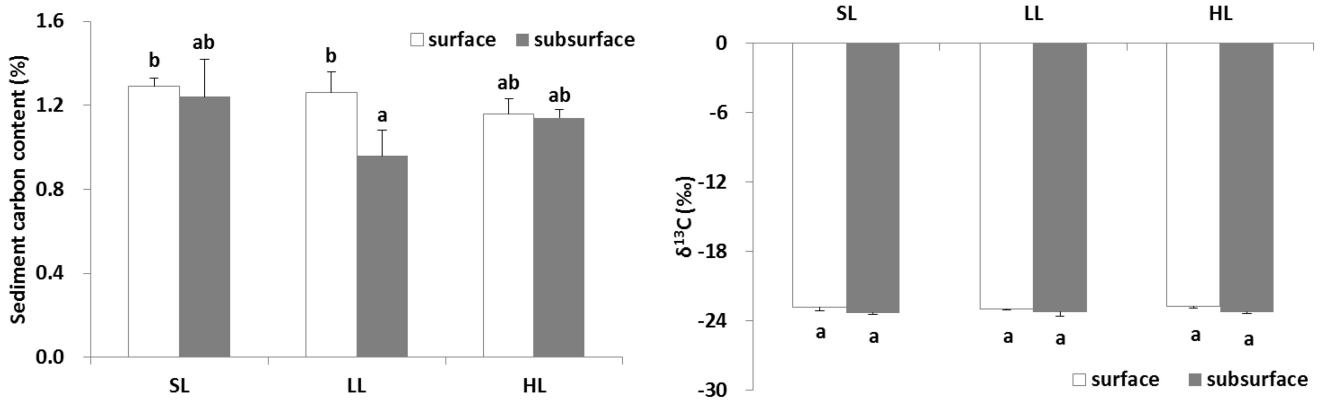

FIGURE 8 | Response of sediment carbon to light treatments. Saturating light: SL, 200 μmol photons/m^2/s; low light: LL, 20 μmol photons/m^2/s; high light: HL, 600 μmol photons/m^2/s. SL and HL were in the optimal light range between the minimum saturating light and the minimum inhibiting light. Different letters on the bars indicate mean values for a particular light condition that significantly differed at ($p < 0.05$) (Mean ± SD, $n = 3$) analyzed by two-way ANOVA.

TABLE 3 | Statistical analysis of effects of different light intensities on sediment parameters.

Parameters	**C%**					**$^{13}C/^{12}C$**				
Source	**DF**	**SS**	**MS**	***F***	***P***	**DF**	**SS**	**MS**	***F***	***P***
Light	2	0.075	0.038	3.54	0.062	2	0.031	0.016	0.29	0.75
Sediment layer	1	0.067	0.067	6.34	0.027	1	0.768	0.768	14.44	0.003
Light*sediment layer	2	0.074	0.037	3.49	0.064	2	0.036	0.018	0.33	0.723
Error	12	0.127	0.011			12	0.638	0.053		
Total	17	0.344				17	1.473			
R-Sq			R-Sq = 62.96%					R-Sq = 56.67%		

half of that under saturating light, indicating plant carbon stock decreased to a great extent under limiting light (**Figure 9**).

Based on the area (about 1158.74 ha) of *H. beccarii* in the South China Sea (Jiang et al., 2017, 2020; Huang et al., 2019), if the light availability for all *H. beccarii* beds was reduced to 20 μmol photons/m^2/s from 200 μmol photons/m^2/s by anthropogenic activities, its vegetative carbon and nitrogen stock would decrease by 28.74 Mg C and 3.86 Mg N, respectively. The global vegetative carbon and nitrogen stock of *H. beccarii* (the global area was estimated to be less than 2000 km^2 (Short et al., 2010), we calculated it using 2000 km^2) would also decrease by 4958.69 Mg C and 665.50 Mg N, respectively. Therefore, light limitation caused by anthropogenic activities would not only reduce the carbon sequestration in biomass, but also damage the ecological service of filtering the nutrients and bacteria within the water column (Lamb et al., 2017), a service that is estimated at 10 million $/year (Campagne et al., 2015) for this vast area covered by meadows.

In addition, higher $\delta^{13}C$ was observed in the above-ground tissue under HL, which might be induced by increased uptake of ^{13}C from the external C source (Grice et al., 1996). Interestingly, high light intensity exhibited the lowest carbon and nitrogen in the below-ground tissue of *H. beccarii*, while the biomasses of above-ground and below-ground tissues were the highest. This indicated that below-ground growth was enhanced by high light to dilute the nutrient content (Peralta et al., 2000). Furthermore, seagrasses in high light treatments were also shown to respond to these low nutrient conditions by increasing their root biomass in order to try and get more nutrients from the sediment (Abal et al., 1994; Grice et al., 1996).

Low Light Reduced Subsurface Sediment Carbon Contents Compared to Surface Sediment

The change of seagrass productivity and biomass caused by anthropogenic activities might result in the decreased flow of organic carbon sequestrated in the sediment (Dahl et al., 2016; Jiang et al., 2018). The release of root exudates might be of particular importance in subsurface sediment systems (Zhai et al., 2013). About 11% of total fixed carbon in *Halodule wrightii* was exuded into the sediment (Moriarty et al., 1986). Interestingly, the present study demonstrated that sediment carbon contents between surface and subsurface layers were similar in both saturating and high light, while subsurface sediment carbon was significantly lower (about 24%) than surface sediment carbon (**Figure 8**) under low light irradiance (**Figure 9**). This indicated that low light reduced subsurface sediment carbon contents compared to surface sediment. Meanwhile, depth also explained the carbon content in seagrass sediment, with lower carbon contents at deeper sites attributed to decreased light penetration

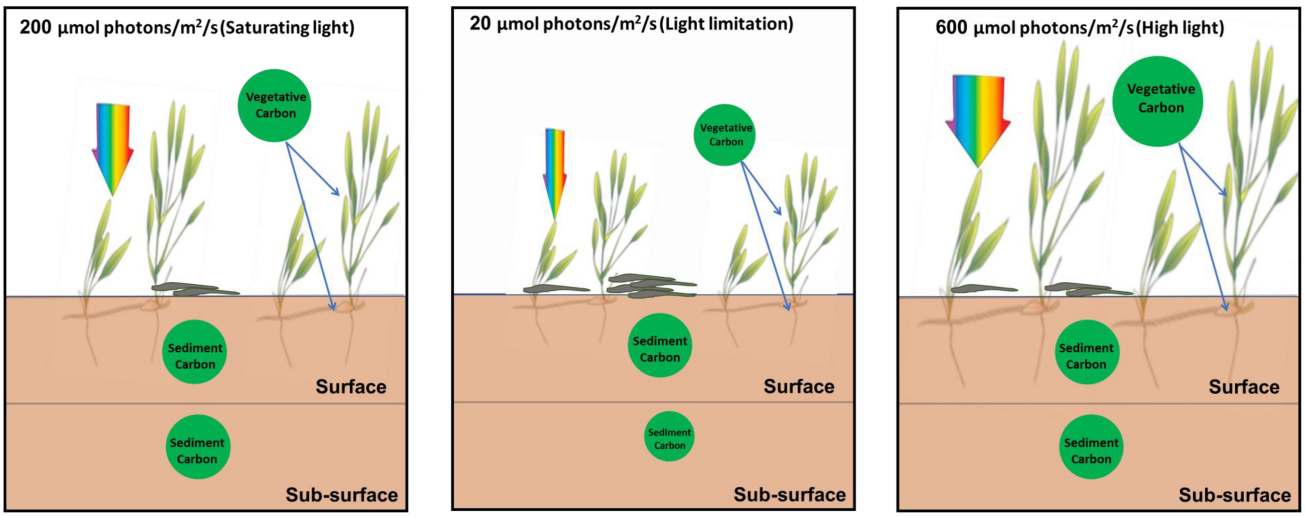

FIGURE 9 | Schematic pictures of the effects of light reduction on the carbon sequestration in seagrass beds.

(Serrano et al., 2014; Samper-Villarreal et al., 2016). Low light reduced root length and biomass (Martin et al., 2018b). The reduction of root biomass would decrease the flux of root exudation of dissolved organic carbon into sediment (Jiang et al., 2018). This undoubtedly lowered subsurface sediment carbon content. Similarly, the above-ground light reduction also invoked a cascade of changes from alterations in root exudation to a decrease in putative beneficial microorganisms (Martin et al., 2018a). However, no significant linear relationship between *Zostera marina*-dissolved organic carbon exudation and light treatment was observed (Kaldy, 2012). The difference might be caused by the fact that dissolved organic carbon exudation rates might be correlated to seagrass species-specific attributes. Nutrient enrichment also significantly reduced the sediment organic carbon content in a 6-21 cm layer around the seagrass root system of *Thalassia hemprichii* and *Enhalus acoroides* in Xincun Bay (Jiang et al., 2018). Therefore, eutrophication weakened subsurface sediment carbon sequestration by lowering light availability or enhancing toxic effect of nutrients on seagrasses.

Ecological Significance and Conclusion

The present study found that the reduction of light availability for seagrass caused by eutrophication and agricultural and urban runoff decreased the vegetative carbon of *H. beccarii* and subsurface sediment carbon content in seagrass beds (**Figure 9**). Meanwhile, low light availability also decreased the canopy complexity of *H. beccarii*. This would most likely trap less allochthonous organic matter in the seagrass canopy and be less efficient in the deposition of fine-grained particles, and thus might also have negative effects on the carbon sequestration capacity of *H. beccarii* (Agawin and Duarte, 2002; Hendriks et al., 2008; Samper-Villarreal et al., 2016; Gullström et al., 2018). The carbon stored in the sediment in seagrass beds is vulnerable to export and remineralization if shoot densities are reduced or seagrass cover is lost due to reduced irradiance (Pendleton et al., 2012; Dahl et al., 2016). *H. beccarii* is primarily distributed in river mudflats with large nutrient inputs in the South China Sea (Jiang et al., 2017, 2020). To ensure continued productivity and maintain the carbon sequestration capacity in *H. beccarii* beds in the future, the nutrient inputs and dredging activities should be reduced to improve water quality to enhance light penetration. In addition, removal of the epiphytes on seagrass leaves by using the combination of an acid treatment with moderate scraping without seriously damaging leaf substratum (Dauby and Poulicek, 1995) would also be a feasible measure to enhance carbon sequestration in seagrass beds.

The present study showed that light availability influenced the primary production as shown in the decreased above-ground biomass in the low light treatments. If above-ground biomass is reduced, photosynthesis will be impacted and seagrasses might as a response exude less photosynthates from their roots into the sediment which will in turn impact microbial communities (Ding et al., 2015; Dahl et al., 2016; Jiang et al., 2018; Martin et al., 2018a). Those microbial communities are essential in creating the precursors of stable organic matter which they do by using their host's root exudates (Cotrufo et al., 2013; Kallenbach et al., 2016). So if the seagrass host cannot supply their microbial communities with sufficient root exudates the carbon sequestration will be negatively affected. Therefore, future research should focus on examining the effect of low light on seagrass root exudates composition and rhizosphere bacterial communities, as well as their influence on sediment carbon transformation processes.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

ZJ and XH designed the study. CP, ZJ, JH, YF, QC, LC, YW, SL, ZC, and PV performed the experiments or analyzed the data. CP, ZJ, and XH wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.664060/full#supplementary-material

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Unusually Warm Summer Temperatures Exacerbate Population and Plant Level Response of *Posidonia oceanica* to Anthropogenic Nutrient Stress

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Posidonia oceanica is a key foundation species in the Mediterranean providing valuable ecosystem services. However, this species is particularly vulnerable towards high coastal nutrient inputs and the rising frequency of intense summer heat waves, but their combined effect *in situ* has received little attention so far. Here, we investigated the effects of *in situ* nutrient addition during an unusually warm summer over a 4-month period, comparing different morphological, physiological and biochemical population metrics of seagrass meadows growing in protected areas (Ischia) with meadows already exposed to significant anthropogenic pressure (Baia – Gulf of Pozzuoli). Our study highlights that the effects of warmer than usual summer temperatures on the population level of seagrass meadows can be exacerbated if the plants are already exposed to higher anthropogenic pressures. Morphological and population level indicators mainly changed over time, possibly impacted by season and the warmer temperatures, and displayed more pronounced reductions in seagrasses from impacted sites. The additional nutrient supply had even more deleterious effects, as shown by a decrease in approximately 67% in cover in fertilized plots at high impacted sites and 33% at low impacted sites. Moreover, while rhizome starch concentration showed a seasonal increase in plants from low impacted sites it displayed a trend of a 27% decrease in fertilized plots of the high impacted sites. Epiphyte biomass was approximately four-fold higher on leaves of plants growing in impacted sites and even doubled with the additional nutrient input. Predicting and anticipating stress in *P. oceanica* is of crucial importance for conservation and management efforts, given the limited colonizing and reproductive ability and extremely slow growth of this ecosystem engineer. Our results suggest that monitoring efforts should focus especially on leaf area index (LAI), carbohydrate concentrations in the rhizomes, and epiphyte cover on leaves as

indicators of the onset of stress in *Posidonia oceanica*, which can be used by decision makers to take appropriate measures before damage to the ecosystem becomes irreversible, minimize future human interference and strengthen the resilience of these important ecosystems.

Keywords: Mediterranean, global warming, nutrients, multiple stressors, warning indicators, seagrass degradation

INTRODUCTION

Coastal vegetated ecosystems are facing multiple local and global anthropogenic stressors that affect their health and their associated ecosystem services (Orth et al., 2006; Duarte et al., 2008; Hoegh-Guldberg and Bruno, 2010). Seagrasses are one of the most valuable marine ecosystems on Earth (Costanza et al., 2014). These marine ecosystems provide a range of ecosystem services including the provision of nursery habitats, filtration of nutrients, provision of food resources and habitat for herbivores as well as their ability to sequester large amounts of carbon aiding in climate change mitigation (de la Torre-Castro and Rönnbäck, 2004; Fourqurean et al., 2012; Ondiviela et al., 2014; Bakker et al., 2016). However, they are also among the marine habitats that are experiencing the most steep decline rates, estimated at 7% $year^{-1}$ (Waycott et al., 2009) even in relatively pristine areas (Marbà and Duarte, 2010; Arias-Ortiz et al., 2018). As a result, almost 14% of all seagrass species are endangered and 29% of the world's seagrass meadows have been lost (Waycott et al., 2009; Short et al., 2011).

Eutrophication on a local scale and climate change on a global scale are the most prominent threats to seagrass ecosystems (Burkholder et al., 2007; Halpern et al., 2007; Marín-Guirao et al., 2018).

The wide distribution of seagrasses along coastal urbanized areas make them particularly vulnerable to anthropogenic nutrient inputs, such as those from aquaculture farms as well as from sewage, industrial or agricultural runoff (Laubier, 2005). In particular, anthropogenic-derived nitrogen in the environment increased by almost 10-fold since 1850 as a consequence of the rising demand for reactive nitrogen in energy production, coastal aquaculture and agriculture (Galloway et al., 2014).

Excess nutrient loading promotes the proliferation of fast-growing species, including phytoplankton, macroalgae and epiphytic algae which ultimately limit light availability to the leaves of seagrasses through shading or overgrowth (Cardoso et al., 2004; Burkholder et al., 2007). While moderate increases in nutrient concentrations stimulate the production and growth of seagrasses (Short et al., 1990; Alcoverro et al., 1997; Udy et al., 1999), large amounts of nitrogen are lethal for the plants because they are unable to down-regulate nitrogen uptake (Rare, 1990; Touchette and Burkholder, 2000). Plant productivity and survival can be impacted by direct ammonium toxicity as well as by the depletion of plant carbon reserves caused by the increased energy demand for rapid ammonium assimilation (Burkholder et al., 1992; Van Katwijk et al., 1997; Invers et al., 2004). Additionally, high nutrient availability results in increased leaf nutritional quality, which stimulates seagrass grazing (Prado et al., 2010; Ravaglioli et al., 2017). A further consequence of eutrophication and organic matter enrichment to the sediment is the stimulation of bacterial respiration leading to hypoxic or anoxic sediments (Frederiksen et al., 2007; Marbà et al., 2007), which can negatively affect respiration, growth and nutrient uptake of seagrass roots (Hemminga, 1998; Pérez et al., 2007). More importantly, anoxic conditions stimulate sulfate reduction and the consequent accumulation of sulfide, a strong phytotoxin (Holmer and Bondgaard, 2001; Ontoria et al., 2019), limiting seagrass survival and expansion (Calleja et al., 2007; Frederiksen et al., 2007; Govers et al., 2014a).

Global warming is another significant threat that can lead to seagrass loss (Short et al., 2016; O'Hare et al., 2018), particularly given that the frequency and magnitude of local heat waves is expected to intensify as a consequence of climate change (Meehl et al., 2005; Oliver, 2019). Indeed, high mortality rates have been reported for temperate seagrass species after extreme temperature events, e.g., for *Zostera marina* in Chesapeake Bay, Virginia, for the period of 2004–2011 (Moore et al., 2014) and *Amphibolis antarctica* following the 2010/2011 heat wave in Shark Bay, Western Australia (Thomson et al., 2015).

The Mediterranean Sea ranks among the fastest-warming ocean regions, showing rates of seawater warming double or triple of those in the global ocean (Vargas-Yáñez et al., 2008). Summer surface temperatures of the Mediterranean have already increased by 1.15°C within the last three decades, with mean global sea-surface temperatures predicted to rise another 3–4°C by the end of this century (Levitus et al., 2001; Meehl et al., 2005; Marbà et al., 2015). An increasing occurrence of heat wave events have also been documented for the Mediterranean in recent years (e.g.1994, 2003, and 2009) (Coma et al., 2009), and die-offs were reported for *P. oceanica* in 2003 and 2006 (Marbà and Duarte, 2010). Seagrass shoot losses as well as impacts on the photosynthetic rates of adult *P. oceanica* plants and their seedlings are expected to occur when seawater temperature reaches 27°C for several days or weeks (Marbà and Duarte, 2010; Guerrero-Meseguer et al., 2017). Die-offs of *P. oceanica* meadows reveal the vulnerability of this species to intense warming events (Marbà and Duarte, 2010; Jordà et al., 2012) and can potentially lead to ecosystem regime shifts. *P. oceanica* is an extremely slow growing plant with shoots living for several decades and limited sexual reproduction (Marbà et al., 1996; Marbà et al., 2004; Díaz-Almela et al., 2009). Therefore, *P. oceanica* meadows need a long time to recover from these mortality events (Marbà and Duarte, 2010), and early successional seagrass species, invasive species, or macroalgae can colonize the free space if given the chance (McGlathery, 2001; Nowicki et al., 2017; Beca-Carretero et al., 2020).

Global warming and eutrophication rarely occur in isolation in the environment and these stressors may interact additively, synergistically, or antagonistically (Todgham and Stillman, 2013; Egea et al., 2018; Mvungi and Pillay, 2019). Thus, it is of highest importance to understand the response of seagrass species to combined stressors in their environment, to be able to give sound guidelines for adequately managing and restoring these endangered ecosystems.

Multiple stressors can have complex and unforeseen impacts on seagrass species and the few studies performed so far reported controversial outcomes. Additive effects for ocean warming and nutrient enrichment were reported for *Cymodocea nodosa* in the Mediterranean (Ontoria et al., 2019) and *Zostera marina* in the North Sea (Moreno-Marín et al., 2018), while only limited additive effects of those stressors were found in *Zostera capensis* from South Africa (Mvungi and Pillay, 2019) and even antagonistic effects for *P. oceanica* (Pazzaglia et al., 2020) as well as limited and antagonistic effects in three tropical seagrass species (Viana et al., 2020). Moreover, limited or no interaction between nutrient and temperature stress have been observed on seagrass seedlings of the tropical species *Enhalus acoroides* (Artika et al., 2020).

These studies make clear that predicting the impact on seagrass of one stressor in isolation could grossly under- or overestimate the combined effects that seagrass ecosystems will be experiencing in their environment. Moreover, other factors such as herbivory (Bakker et al., 2016) or mechanical stress through storm events (Oprandi et al., 2020) could worsen the effects of anthropogenic pressures on seagrasses, and these factors are not taken into consideration when individual seagrass plants are studied in mesocosm experiments. On the other hand, the whole seagrass ecosystem may be able to buffer negative effects that have been observed by single plants in mesocosm settings, or possibly, unforeseen biological interactions might emerge that generate positive or negative feedbacks within the system (Burnell et al., 2013).

We undertook this study to explore the combined effects of seasonal warming and nutrient enrichment in the water column and in the sediment on *P. oceanica* meadows in their natural environment. This study is part I of a parallel study that assesses the combined effects of temperature and nutrient stress on *H. stipulacea*, a small- bodied, fast- growing seagrass species (Helber et al., unpublished). In these combined studies, we aimed to compare these two species to expand our knowledge on the influence of growth strategy on the response of seagrasses under future eutrophication and climate scenarios that might lead to competitive interactions in areas where their distribution range overlaps. Our overarching aim was to identify warning indicators for the onset of stress in these seagrass species, including individual plant trait responses and population or community-level changes, to alert decision makers and provide an opportunity to take appropriate measures before irreparable damage to the ecosystem, such as seagrass die-off or catastrophic ecosystem regime shifts, can occur.

In part I, we hypothesized that maximum summer temperatures and additional nutrient enrichment will have more detrimental effects on *P. oceanica* meadows that are already heavily exposed to anthropogenic pressures. In contrast, we expect, that small- bodied, fast- growing seagrass species are able to adapt more quickly through especially morphological but also biochemical changes, and therefore might even benefit from the additional supply of nutrients. This is further tested in Part II of this study (Helber et al., unpublished). Moreover, we expect that in both species the combined effects of high temperatures and fertilization will be larger than the effects of one stressor alone.

MATERIALS AND METHODS

Study Site and Experimental Design

We compared the response of *P. oceanica* populations to thermal and nutrient stress at two locations exposed to significant anthropogenic nutrient load vs. in a Marine Protected Area subjected to relatively low anthropogenic pressures along the western Mediterranean Sea, off the coast of Naples, Italy (**Figure 1**). Four sites were chosen after verifying low within-meadow heterogeneity that would allow us to provide a good assessment of meadow status and to detect changes during the experiment.

The two impacted sites are located in the Gulf of Pozzuoli close to the city of Baia (S1: 40.7986°N, 14.0856°E and S2: 40.7961°N, 14.0865°E). This area is heavily influenced by anthropogenic activities, with sediments containing high levels of organic contaminants and pollutants (Celia Magno et al., 2012; Tornero and Ribera d'Alcalà, 2014). Additionally, this area is impacted by dense urban settlements, discharge of sewage without appropriate depuration, and intense maritime traffic as well as mussel aquaculture (Tornero and Ribera d'Alcalà, 2014; Appolloni et al., 2018). Some of the mussel aquaculture facilities rearing *Mytilus galloprovincialis* are located close to our two experimental sites with a distance of 500 m from the coast, covering an area of about 257 m^2 (Galletti et al., 2017).

Experimental sites S3 (40.7354°N, 13.9646°E) and S4 (40.7063°N, 13.9567°E) were located off the island of Ischia, which is a marine protected area since 2007 (Chiarore et al., 2019). However, Ischia attracts more than four million tourists per year with the majority visiting the island between May and September (Tomicic et al., 2001; Carlino et al., 2011). During this time, the number of people is fourfold higher than in winter, with the outdated wastewater system representing the major source of pollution (Tomicic et al., 2001). *P. oceanica* at the sites in Ischia is found in dense and continuous meadows that grow on a "matte," a dense mixture of rhizomes, roots and accumulated sediment (Boudouresque et al., 2016), while meadows in the Gulf of Pozzuoli do not possess the same vertical extent, growing directly on sediment (**Supplementary Figure 1**).

Six circular plots with a diameter of 2 m were established at each of the four sites, at least 10 m apart from each other. Plots were set up at water depths between 7.4 and 12.0 m in seagrass meadows around Ischia and at 6.6–7.9 m water depth in meadows around Baia. Three plots were used as control, while the other three were enriched with slow release fertilizer pellets (Osmocote® Pro: 19% N – 3.9% P – 8.3% K, ICL Specialty Fertilizers). Nitrogenous

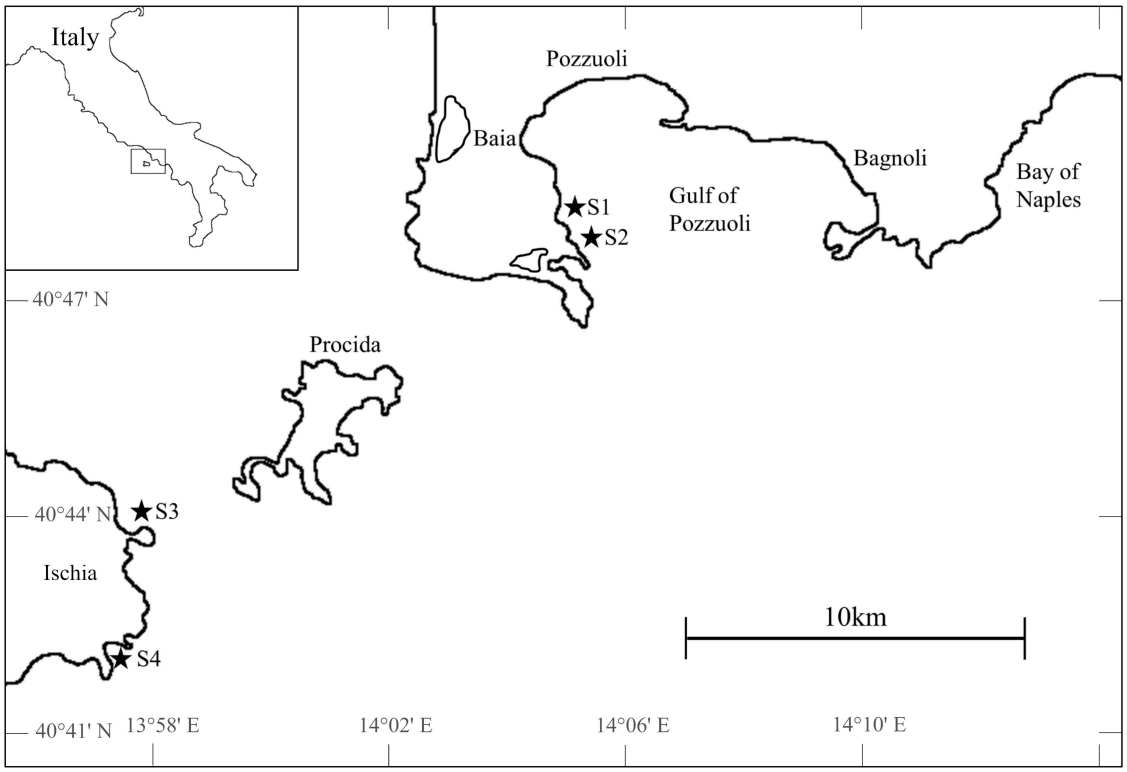

FIGURE 1 | Study sites in the urbanized Gulf of Pozzuoli (S1, S2) and in the Marine Protected Area "Regno di Nettuno" off the island of Ischia (S3, S4).

compounds were composed of nitrate (6.3%), ammonia (8.2%), and urea (4.5%). The nutrient treatment level was randomly assigned for each plot. Fertilizer pellets were filled in nine 0.5 m long punctured PVC tubes, resulting in an addition of approximately 1170 g of fertilizer per plot. PVC tubes were pushed approximately 20 cm into the sediment, delivering nutrients to the below-ground and above-ground tissues, thereby simulating the effects of long-term cultural eutrophication. From each plot, we measured a number of target variables in June and September 2019 for assessing different levels of biological organization, ranging from biochemical and morphological individual plant traits, to community level metrics as outlined below.

Shoot Density and Percent Cover

Shoot density and percentage cover were assessed at the start of the experiment in June 2019 during the seagrass growth season and right after the maximum summer temperatures in September 2019. Shoot density (number of shoots per m^{-2}) was measured by counting the number of shoots in triplicate 0.25 m^2 quadrats randomly placed within each plot. Meadow cover (as a percentage) was quantified by visually estimating the percentage of substrate occupied by *P. oceanica* shoots within one randomly placed 1 m^2 quadrat in each plot. To assess percent cover, the quadrat was positioned on the substratum, and seagrass leaves were moved aside to estimate the portion of underlying floor covered by shoots vs. by bare substratum.

Seagrass Collection

From each plot, nine shoots (when possible, orthotrophic) were randomly collected at different locations within the plot to reduce the likelihood that ramets were sampled from connected horizontal rhizomes (Rico-Raimondino, 1995). Shoot collections were done at the beginning and the end of the experiment. Three shoots were used to measure morphological parameters and epibiont biomass, while the other three shoots were used to quantify C and N nutrient content and $\delta^{13}C$ and $\delta^{15}N$ in leaves and rhizomes and the remaining three shoots to measure total non-structural carbohydrate reserves, such as starch and sugars in leaves and rhizomes.

Carbon, Nitrogen, and Phosphorous Tissue Content and Natural Stable Isotopes

Samples for total carbon (C), nitrogen (N), and phosphorous (P) content and $\delta^{13}C$ and $\delta^{15}N$ isotope analyses were collected from the second- and third-rank leaves and rhizome tissue of three randomly selected shoots per plot. Epiphytes were gently scraped off the leaves before freezing them at –20°C. Before analysis, the leaf and rhizome samples were freeze-dried for 48 h. The dried leaf and rhizome tissues were ground and total C and N content was measured using a Euro EA 3000 elemental analyser (EuroVector). Acetanilid 5 (Hexatech) was used as standard. Repeated measurements of internal standards

with known C and N concentrations (Low Soil Standard OAS 5; IVA) ensured measurement accuracy. Carbon and nitrogen content were expressed as a percentage of dry weight and the values used to calculate the C:N ratios.

Percent tissue phosphorus was analyzed using the wet alkaline persulphate digestion technique method on a TECAN M200Pro plate reader after Hansen and Koroleff (2009). Fichtennadel (1.69 mg P g^{-1}) and SRM1515 Apple leave (1.59 mg P g^{-1}) were used as standard reference materials. Repeated measurements of these reference materials ensured measurement accuracy. The coefficient of variation was always < 3.4%.

Stable isotopic ratios of carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) in samples were analyzed using a Finnigan Delta Plus mass spectrometer coupled with a Flash EA 1112 elemental analyzer. Results of isotopic composition in samples are expressed as following:

$$\delta X(‰) = [(R_{sample}/R_{reference}) - 1] \times 1000,$$

where X is either ^{15}N or ^{13}C, and R is the ratio of $^{15}N/^{14}N$ for nitrogen and $^{13}C/^{12}C$ for carbon. Reference materials IAEA N1 and N2 (nitrogen) and USGS 24 and NBS22 (carbon) from the International Atomic Energy Agency were used for calibration. The precision of the measurements was <0.06‰ for both carbon and nitrogen. All $\delta^{13}C$ and $\delta^{15}N$ values were normalized to the internal standards of wheat flour (carbon [$\delta^{13}C$]: – 27.21‰; nitrogen [$\delta^{15}N$]: 2.85‰) and high organic sediment (carbon [$\delta^{13}C$]: – 26.07‰; nitrogen [$\delta^{15}N$]: 4.4‰).

Morphological Parameters

The number of leaves was counted from three shoots that were randomly sampled within each plot in June and September 2019. The following parameters were measured: (i) number of leaves, (ii) length of all leaves and (iii) maximum leaf width. Subsequently, leaf area and leaf area per shoot as well as leaf area index (LAI) were determined. LAI was calculated as the product of leaf surface area per shoot and density of shoots per m^2.

Epiphyte Cover

Epiphytes were scraped off leaves from each shoot using a scalpel blade and frozen at –20°C in falcon tubes until further processing at the ZMT in Germany. In the laboratory, epiphytes were removed with distilled water from the falcon tubes and dried to a constant weight (60°C, 48 h) to determine their biomass [mg (DW) $shoot^{-1}$]. Epiphyte biomass was normalized to the leaf surface area of its shoot to quantify epiphyte load [mg (DW) cm^{-2}].

Carbohydrate Reserves

Triplicate subsamples of the rhizome and leaf samples were pooled and freeze-dried for 72 h. Subsequently, samples were ground with mortar and pestle. The dried powder (0.02–0.03 g) was suspended in 1.5 mL Milli-Q water and soluble sugars were extracted from the ground dry tissues by vortexing and shaking for 15 min. Samples were centrifuged (13000 rpm, 5 min) and the supernatant was used for soluble sugar determination via the Anthrone Assay (Viles and Silverman, 1949). Starch contents (total non-structural carbohydrates) in the remaining pellet were boiled in Milli-Q (10 min at 100°C) to gelatinize the starch. Subsequently, samples were hydrolysed by the enzyme alpha- amylase (80 min at 80°C). The supernatant containing oligosaccharides and/or glucose broken down by the enzyme was collected and, by boiling the sample material under acidic conditions (addition of 96% H_2SO_4) for 1.5 h at 100°C, the remaining polysaccharides were broken down to glucose molecules. Starch and sugar contents in the extracts were determined spectrophotometrically (620 nm) using an anthrone-sulphuric acid assay with a F200- Pro TECAN plate reader. Carbohydrate concentrations were quantified as sucrose equivalent using sucrose calibration curves (Standard sucrose 99%, from Sigma Aldrich). Samples of cellulose, glucose, and starch were used as reference samples.

Environmental Sampling

Approximately 30 mL water samples were taken at around 10 m depth in Ischia and around 7 m depth in Baia, above the seagrass canopy of each plot on each site for water column nutrient analysis. Additionally, porewater samples were taken by placing a syringe 5 cm deep in the sediment and carefully drawing out water from interstitial spaces of the sediment to determine nutrient concentrations. Porewater samples were not able to be measured in Baia at the first fieldtrip due to sediment characteristics- dense silty soil that blocked our porewater sampling lancets. For the second fieldtrip we used Metalplex hypodermic syringes. Water samples were taken with cleaned plastic syringes and immediately filtered using sterile syringe filters (LABSOLUTE®; cellulose acetate; 0.45 μm pore-size) into HDPE vials and stored on ice. HDPE vials were pre-rinsed twice with sample water. Samples were immediately frozen at –20°C upon arrival in the laboratory on the same day until further analyses. Nutrient measurements were performed spectrophotometrically with a TECAN plate reader (Infinite 200 pro Microplate reader; Switzerland) according to Laskov et al. (2007). The detection limits were 0.08, 0.32, 0.7, and 0.022 μM for NO_2^-, NO_x (NO_3^- and NO_2^-), NH_4^+, PO_4^-, respectively. The NO_x (NO_3^- and NO_2^-) and NH_4^+concentrations sum up to dissolved inorganic nitrogen (DIN). The coefficient of variation was always <3.4%.

The water quality of the different sites was assessed by taking additional water samples on top of the seagrass canopy with 5 × 5 L seawater pre-rinsed HDPE containers. After collection, containers were immediately stored in the dark on ice. A defined volume of water was vacuum- filtered onto pre- combusted (5 h, 450°C) Whatmann® GF/F filters. Filters for chlorophyll *a* (Chl-*a*) analysis were immediately stored at –80°C, filters for organic carbon (C_{org}) and C and N content were frozen at –20°C until further analysis at the Leibniz Centre for Tropical Marine Research. Filters for C_{org}, C and N content were divided in four equal pieces and one piece was used for analysis. For Chl-*a* analysis, filters were analyzed and extracted in 80°C hot Ethanol after Welshmeyer (1994). The supernatant was subsequently transferred into small vials and Chl-*a* concentrations were determined with a TD10AU-Fluorometer (Turner Designs). The detection limit of this method is 0.002 μg l^{-1}. Some water

parameters of the first fieldtrip could not be measured because filters got lost during the transport.

Salinity and pH were measured at the same time the water samples were taken at each site with a multi parameter probe (WTW Multiprobe). Moreover, temperature loggers (HOBO Water Temp Pro v2) were fixed to the main pole of plot 1 and plot 6 in the seagrass meadow to record the water temperatures hourly during the entire duration of the experiment.

Statistics

Statistical analyses were performed in R version 3.5.3 (R Core Team, 2019). For the statistical analysis, we combined data for Site 1 and 2 together as high impacted condition and Site 3 and 4 together as low impacted condition. Although, there were site-specific differences, we aimed to focus on large- scale patterns and therefore only distinguished between the environmental history of the two locations. Prior to analysis, we subset our data to values that have been collected in June 2019 (start of the experiment) and September 2019 (end of the experiment). The fertilization was only started in June and thus there was no nutrient treatment effect in June. We used two linear mixed effects models. One to determine if there were seasonal effects over the time of the experiment (1). For this analysis, we only looked for changes in the control plots over time and included a random intercept for site. The second linear mixed effects model was performed to look at differences between the conditions of the sites (high or low impacted) in June (2) and September (3), the effect of treatment- differences between fertilized and control plots (4) as well as their possible interactions (5). We performed both linear mixed effects models using the lme4 function in R (Bates et al., 2015). As fixed effects, we considered condition (high or low impacted), treatment (fertilization and control) and the interaction of condition and treatment. We added a random intercept of site to our linear mixed effects models to account for the variability in sites within areas of different conditions (high or low impacted). Differences in shoot densities among the sites were determined with a generalized linear mixed-effects model (GLMM), specifically a negative binomial model (link = log) because the data were counts and found to be over-dispersed (Zuur et al., 2009). The GLMM was fitted using the MASS package (Venables and Ripley, 2002). All models were validated visually with plots of model residuals (fitted values vs. absolute residuals (homogeneity of variance), a qqplot comparing the distribution of the standardized residuals to the normal distribution (normality), and a lag plot of the raw residuals vs. the previous residual (independence; Zuur et al., 2009). The significance of each independent variable (or interaction) in each model was assessed using the likelihood ratio (LR) test by comparing models with the variable of interest against the null or reduced model (Winter, 2013). To bolster the results found by LR test the models that best predicted the changes in seagrass indicators were identified by minimum Akaike Information Criterium with correction for small sample sizes (AICc), model ranking and weighing (Burnham et al., 2011; Symonds and Moussalli, 2011). Models with the variable of interest (or interaction) were compared to the null (or reduced) model and a model was considered superior model if it had the lowest AICc units (determining the model strength) as well as the highest Akaike weight (AICcWt) which defines the weight of evidence of each model relative to the null or reduced model (Arnold, 2010). All graphics were produced with the ggplot package (Wickham, 2016).

Water parameters were also combined after condition- low (Ischia) and high (Baia) impacted sites. Data were analyzed with the Kruskal–Wallis rank sum test function in R and subsequently a paired t-test with holms correction was performed.

RESULTS

Environmental Variables

Average daily temperatures above 27°C were recorded for 9 days at Site 1, for 20 days at Site 2, for 7 days at Site 3 and for 15 days at Site 4 between mid-June and September 2019 (**Supplementary Figure 2**). Temperatures were recorded at 7.4–12.0 m water depth in seagrass meadows around Ischia and at 6.6–7.9 m water depth in meadows around Baia. Maximum temperatures recorded during this time were 28.16°C for Site 1 and 28.26°C for Site 2 in Baia (Gulf of Pozzuoli) and 27.96°C for both sites in meadows around Ischia.

There were no significant differences in nutrient concentrations in the water column and porewater between the sites, even though DIN and NH_4^+ seemed to be higher in the porewater at Baia (**Supplementary Table 1**).

Water parameters differed significantly between locations that are exposed to different levels of anthropogenic stressors. High impacted sites in Baia (Site 1 + 2) had higher concentrations of organic carbon (Corg), C and N content as well as chla contents in the water column (**Supplementary Tables 1, 2**). CN ratios did not differ significantly between sites in Baia and Ischia. Values for organic carbon (Corg), C and N content were always under the limit of detection for Site 3 in Ischia.

Changes Between Seasons (June 2019 and September 2019) and With Fertilization

Percentage Cover and Shoot Density

Meadow cover in sites around Ischia was significantly higher with a mean of 84.6 ± 2.4% compared to sites of Baia with a mean of 58.3 ± 5.7% at the start of the experiment [**Figure 2**; $\chi^2(1) = 8.08$, $p = 0.0045$; **Supplementary Table 4A**]. From June to September seagrass cover decreased significantly in both locations [$\chi^2(1) = 8.42$, $p = 0.0037$; **Supplementary Table 5**], but still remained significantly higher in the two sites off Ischia compared to Baia in September [$\chi^2(1) = 16.42$, $p = 0.0001$; **Supplementary Table 4A**]. LME models additionally indicated that seagrass cover decreased even further with fertilization independent of meadow location [$\chi^2(1) = 14.77$, $p = 0.0001$; **Supplementary Table 4A**]. All significant effects reported from the LME models based on the LR tests were supported by the AICc based model comparison, meaning the model containing the tested variable (in this case condition or fertilization) was the model with the lowest AICc units and with the

highest AICcWt compared to the null (or other candidate) model(s) (**Supplementary Table 4A**). This holds for all further analysis reported below.

Shoot density was significantly higher in meadows around Ischia than in areas already under anthropogenic pressures [**Figure 3**; $\chi^2(1) = 10.56$, $p = 0.0012$], but similar at the different sites of each location in June 2019 (see **Supplementary Table 3** for data on all subsites). After the four month period, pristine sites around Ischia had still 1.6 times more shoots than impacted sites at Baia [$\chi^2(1) = 6.75$, $p = 0.0094$; **Supplementary Table 4B**]. However, fertilization had no significant effect on shoot density (**Figure 3**).

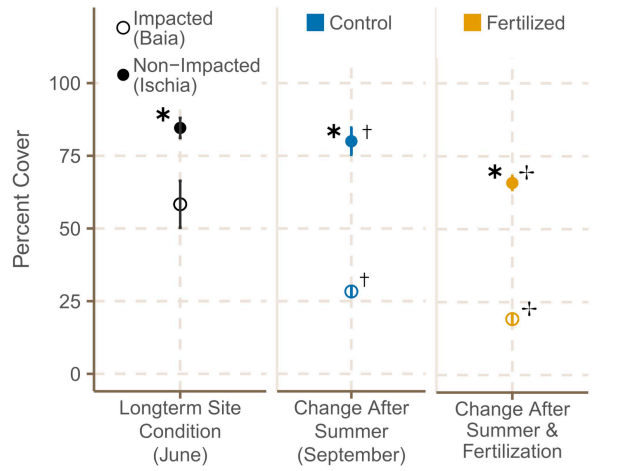

FIGURE 2 | Percentage meadow cover (mean ± SE) of fertilized and control plots in June 2019 and September 2019. ✱ Shows significant differences between impacted (Baia) and non- impacted (Ischia) areas. † Indicates if there seasonal differences between June and September and ✣ states if there are differences between control and fertilized plots.

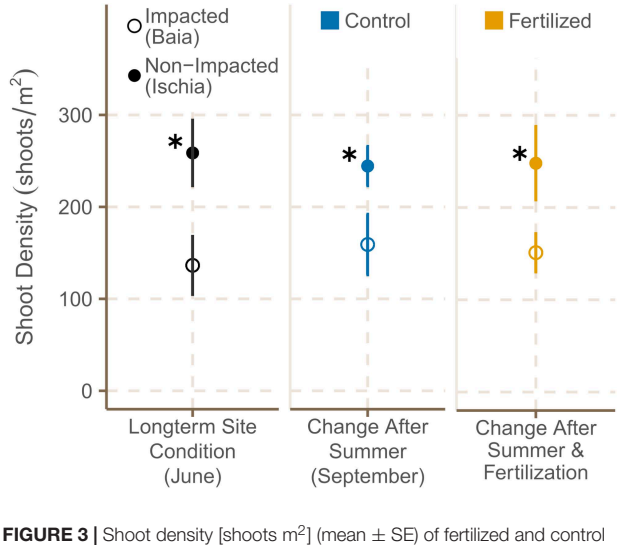

FIGURE 3 | Shoot density [shoots m^2] (mean ± SE) of fertilized and control plots in June 2019 and September 2019. ✱ Shows significant differences between impacted (Baia) and non- impacted (Ischia) areas.

Epiphyte Cover

Epiphyte biomass [mg (DW) epiphytes cm^{-2} $shoot^{-1}$] in June 2019 was approximately fourfold higher in seagrass meadows in Baia (4.48 mg cm^{-2} shoot, on average) than in meadows around Ischia (1.07 mg cm^{-2} shoot, on average) [**Figure 4**; $\chi^2(1) = 7.44$, $p = 0.0064$; **Supplementary Table 4C**]. By September 2019, epiphyte load on leaves of plants in high impacted sites was still significantly higher than the one in low impacted sites [$\chi^2(1) = 5.04$, $p = 0.0281$]. In contrast to Baia, epiphyte cover remained relatively low in sites around Ischia over the course of the experiment (**Figure 4**). All fertilized plots had a higher epiphyte biomass [**Figure 4**; $\chi^2(1) = 7.44$, $p = 0.0064$; **Supplementary Table 4C**] and epiphyte biomass from plants in high impacted sites even doubled. There was also a crossover interaction showing that epiphyte cover in low impacted sites increased slightly more in control plots rather than in fertilized ones [$\chi^2(1) = 7.79$, $p = 0.0053$; **Supplementary Table 4C**).

Morphological Trait Response

Plants growing in low impacted meadows around Ischia had fewer leaves when compared to seagrasses growing in the high impacted sites, in June [**Table 1**; $\chi^2(1) = 8.70$, $p = 0.0032$; **Supplementary Table 6A**]. However, the number of leaves per shoot decreased over the course of the experiment at both locations [$\chi^2(1) = 13.29$, $p = 0.0003$; **Supplementary Table 7**], but seagrasses growing off Ischia still had more leaves per shoot by September [**Table 1**; $\chi^2(1) = 9.62$, $p = 0.0019$]. According to predictions of the best model, plants from Ischia also had taller leaves [$\chi^2(1) = 10.83$, $p = 0.001$; **Supplementary Table 6B**] and a significantly higher leaf canopy height [**Table 1**; $\chi^2(1) = 7.55$, $p = 0.006$] than seagrasses in high impacted sites, in June. Leaf length [$\chi^2(1) = 69.77$, $p \leq 0.0001$] and the maximum

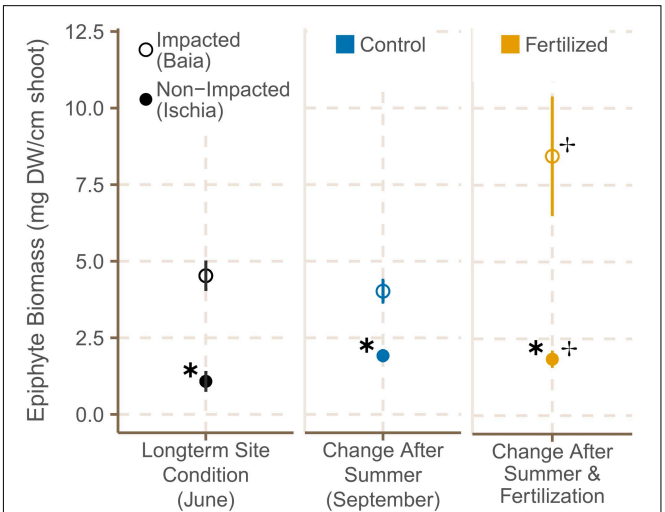

FIGURE 4 | Average epiphyte biomass (±SE) of *P. oceanica* shoots in control and fertilized plots at the different sites in June and September 2019. ✱ Shows significant differences between impacted (Baia) and non- impacted (Ischia) areas and ✣ indicates if there are differences between control and fertilized plots.

TABLE 1 | Mean (±SE) values of morphological traits from *P. oceanica* meadows (n = 12) from the anthropogenically impacted sites (Baia- Gulf of Pozzuoli) and low impacted sites (Ischia) in control (n = 6) and fertilized (n = 6) plots.

Morphological traits	Area	June 2019	September 2019	
			Control	Fertilized
No. leaves per shoot	Baia	5.56 ± 0.12	3.67 ± 0.18	3.61 ± 0.23
	Ischia	4.61 ± 0.15	4.72 ± 0.21	4.22 ± 0.17
Leaf height	Baia	549.71 ± 16.70	273.03 ± 27.08	274.05 ± 27.72
	Ischia	704.87 ± 22.12	435.84 ± 36.48	370.88 ± 33.73
Max. leaf canopy height	Baia	825.08 ± 17.12	490.17 ± 35.80	512.67 ± 33.84
	Ischia	961.39 ± 28.69	824.72 ± 32.15	694.44 ± 36.42
LAI	Baia	3.32 ± 0.24	1.47 ± 0.19	1.64 ± 0.17
	Ischia	7.44 ± 0.47	3.79 ± 0.36	3.36 ± 0.29
Leaf width	Baia	10.0 ± 0.0	11.03 ± 0.23	11.20 ± 0.22
	Ischia	9.11 ± 0.12	9.78 ± 0.19	9.69 ± 0.23
Leaf area	Baia	549.71 ± 60.70	301.78 ± 30.46	311.22 ± 32.09
	Ischia	646.33 ± 21.44	423.60 ± 35.59	353.70 ± 32.33
Leaf area per shoot	Baia	2394.33 ± 85.98	1017.24 ± 120.44	1080.31 ± 107.18
	Ischia	2813.03 ± 135.03	1540.36 ± 129.67	1334.43 ± 108.38

Fertilization did not show any effects on morphological parameters except for maximum leaf canopy height, which was lower in fertilized plots in seagrasses around Ischia. Leaf height, maximum leaf canopy height, leaf width, leaf area, and leaf area per shoot are expressed in mm, while LAI is expressed as m^2 leaf area per shoot x shoot density per m^2.

canopy height [$\chi^2(1) = 32.82$, $p \leq 0.0001$; **Supplementary Table 7**] showed a significant seasonal reduction from June to September in both locations (**Table 1**). *P. oceanica* shoots in low impacted sites still had significantly taller leaves [$\chi^2(1) = 9.12$, $p = 0.0025$) and a higher leaf canopy at the end of the experiment [$\chi^2(1) = 11.39$, $p = 0.0001$; **Supplementary Table 6C**] than plants growing in highly impacted sites.

According to predictions of the best model, leaves of plants growing in Baia were wider than leaves from plants in Ischia [$\chi^2(1) = 5.14$, $p = 0.0234$; **Supplementary Table 6D**]. However, leaf width significantly increased over the course of the experiment independent of their location [**Table 1**; $\chi^2(1) = 21.79$, $p \leq 0.0001$; **Supplementary Table 7**], but leaves were still wider in plants from Baia in September [$\chi^2(1) = 5.96$, $p = 0.0146$]. LME models further indicated that leaf area, leaf area per shoot as well as LAI were significantly larger in seagrasses from low impacted sites than in plants from Baia in June as well as in September 2019 (**Supplementary Tables 6E,F**). However, LAI [$\chi^2(1) = 23.26$, $p \leq 0.0001$] as well as leaf area [$\chi^2(1) = 53.60$, $p \leq 0.0001$] and leaf area per shoot [$\chi^2(1) = 47.86$, $p \leq 0.0001$; **Supplementary Table 7**] did decrease over the time of the experiment independent of their location (**Table 1**).

Fertilization did not result in major changes in morphological traits (**Table 1**). Only maximum canopy height was significantly reduced in low impacted sites off Ischia as a response to the additional nutrient input [$\chi^2(1) = 4.99$, $p = 0.001$; **Supplementary Table 6C**].

Carbon, Nitrogen and Phosphorous Content and Stable Isotope ($\delta^{13}C$ and $\delta^{15}N$) Composition

Leaves

Carbon content was not significantly different in leaves of plants from the four sites at the start of the experiment (**Table 2**), but increased significantly over time at both locations [$\chi^2(1) = 20.05$, $p \leq 0.0001$; **Supplementary Table 9**]. Nitrogen content in leaves was significantly lower in plants growing around Ischia at the start [$\chi^2(1) = 5.92$, $p = 0.0149$] and at the end of the experiment [$\chi^2(1) = 12.00$, $p = 0.0005$; **Supplementary Table 8C**]. Consequently, C:N ratios in leaf tissues of seagrasses around Ischia were higher than those of plants in Baia at the beginning [$\chi^2(1) = 4.72$, $p = 0.0298$] and at the end of the experiment [$\chi^2(1) = 12.69$, $p = 0.0004$; **Supplementary Table 8A**]. According to predictions of the best model, fertilization significantly reduced Leaf C:N ratios at both locations (**Table 2** and **Supplementary Table 8A**).

Leaf P content was approximately three times higher in plants from Baia as in plants from Ischia in June [$\chi^2(1) = 7.75$, $p = 0.0054$; **Supplementary Table 8D**] and slightly decreased over the course of the experiment [$\chi^2(1) = 0.8625$, $p = 0.03$; **Supplementary Table 9**]. However, leaf P content did not differ significantly between locations in September probably due to the high site- specific variability (see **Supplementary Table 3** for subsite variation). Plants from impacted areas had significantly reduced $\delta^{13}C$ values in their leaves by September when compared to seagrasses from Ischia [$\chi^2(1) = 5.70$, $p = 0.0170$; **Supplementary Table 10A**].

Rhizomes

Carbon and phosphorous contents in rhizomes of plants from both locations were not significantly different and did also not change with the addition of fertilizer (**Supplementary Tables 8G,H**). However, nitrogen contents in rhizomes of seagrasses from Baia were significantly higher than the ones of plants off Ischia in June [$\chi^2(1) = 9.73$, $p = 0.0018$; **Table 2**] as well as in September [$\chi^2(1) = 5.83$, $p = 0.00157$; **Supplementary Table 8F**]. Seagrasses growing in low impacted sites had

TABLE 2 | Mean (±SE) values of nutrient content in leaf and rhizome tissues from *P. oceanica* meadows (n = 12) from the anthropogenically impacted sites (Baia-Gulf of Pozzuoli) and low impacted sites (Ischia) in control (n = 6) and fertilized (n = 6) plots.

Biochemical traits	Area	June 2019	September 2019	
			Control	Fertilized
Leaf C	Baia	31.72 ± 0.30	34.90 ± 0.73	35.00 ± 0.54
	Ischia	31.87 ± 0.18	33.81 ± 0.41	34.76 ± 0.43
Rhizome C	Baia	39.75 ± 0.40	42.30 ± 1.46	39.80 ± 0.25
	Ischia	39.84 ± 0.23	41.13 ± 0.39	42.42 ± 1.53
Leaf N	Baia	1.79 ± 0.06	2.10 ± 0.25	2.20 ± 0.20
	Ischia	1.18 ± 0.08	1.02 ± 0.10	1.31 ± 0.10
Rhizome N	Baia	3.08 ± 0.29	5.10 ± 0.38	3.70 ± 0.38
	Ischia	1.64 ± 0.17	2.26 ± 0.18	3.26 ± 0.40
Leaf $\delta^{13}C$	Baia	−12.73 ± 0.20	−14.10 ± 0.48	−13.40 ± 0.29
	Ischia	−13.20 ± 0.26	−12.28 ± 0.53	−12.94 ± 0.30
Rhizome $\delta^{13}C$	Baia	−12.90 ± 0.17	−13.20 ± 0.11	−12.90 ± 0.18
	Ischia	−13.29 ± 0.14	−12.71 ± 0.22	−13.11 ± 0.12
Leaf $\delta^{15}N$	Baia	6.54 ± 0.22	5.70 ± 0.28	4.90 ± 0.23
	Ischia	6.13 ± 0.35	4.80 ± 0.52	4.17 ± 0.34
Rhizome $\delta^{15}N$	Baia	6.58 ± 0.12	6.20 ± 0.07	5.80 ± 0.22
	Ischia	6.00 ± 0.24	5.37 ± 0.14	5.25 ± 0.25
Leaf C:N ratio	Baia	14.38 ± 1.50	17.80 ± 1.87	16.70 ± 1.43
	Ischia	28.50 ± 1.98	34.42 ± 2.83	27.43 ± 2.12
Rhizome C:N ratio	Baia	17.98 ± 0.72	8.60 ± 0.86	11.50 ± 1.42
	Ischia	28.22 ± 3.95	18.77 ± 1.52	14.05 ± 1.75
Leaf P	Baia	1038.25 ± 113.28	904.80 ± 304.93	727.00 ± 67.10
	Ischia	618.08 ± 40.48	654.00 ± 69.42	722.83 ± 67.10
Rhizome P	Baia	647.00 ± 123.47	1790.70 ± 219.08	1496.30 ± 257.00
	Ischia	571.83 ± 76.87	1183.17 ± 74.93	1685.83 ± 201.48

C and N content are expressed as percentages, while $\delta^{13}C$ and $\delta^{15}N$ are in parts per thousands. P content is in $\mu g\ g^{-1}$.

consequently significantly higher C:N ratios in their rhizomes compared to seagrasses from Baia at the beginning [$\chi^2(1) = 7.56$, $p = 0.0059$] and at the end of the experiment [$\chi^2(1) = 4.80$, $p = 0.0284$; **Supplementary Table 8E**]. Nitrogen [$\chi^2(1) = 12.80$, $p = 0.0001$], carbon [$\chi^2(1) = 47.86$, $p \leq 0.0001$] as well as phosphorous content [$\chi^2(1) = 27.63$, $p \leq 0.0001$; **Supplementary Table 9**] significantly increased in rhizome tissues of seagrasses from both locations over the course of the experiment. In contrast, C:N ratios in rhizomes decreased significantly from June to September in all plots independently from their location [$\chi^2(1) = 6.89$, $p = 0.0087$; **Supplementary Table 9**].

The addition of fertilizer had opposing effects on rhizome nutrient contents depending on the location of the seagrass meadows. Plants in the high impacted sites of Baia had significantly lower rhizome N contents in fertilized plots, while the %N in rhizomes of seagrasses in the low impacted sites increased with fertilization [$\chi^2(1) = 12.39$, $p = 0.0004$; **Supplementary Table 8F**]. Additionally, plants in fertilized plots in Baia had higher C:N ratios in rhizomes, while fertilization resulted in reduced C:N ratios in rhizomes of seagrasses from low impacted sites [$\chi^2(1) = 9.51$, $p = 0.0020$; **Supplementary Table 8E**].

There were no differences in $\delta^{15}N$ and $\delta^{13}C$ isotope values neither in leaf nor in rhizome tissues of seagrasses from Ischia and Baia in June 2019. Fertilization only resulted in lower $\delta^{15}N$ content in leaf tissues of seagrasses from both locations, with a more pronounced reduction in plants from Ischia (**Supplementary Table 10B**). Moreover, rhizome $\delta^{15}N$ values declined significantly over the course of the experiment [$\chi^2(1) = 5.63$, $p = 0.0177$; **Supplementary Table 11**], resulting in significantly lower $\delta^{15}N$ values in rhizomes of plants from Ischia by the end of the experiment [$\chi^2(1) = 6.59$, $p = 0.0103$].

Non-structural Carbohydrates

Leaves

Starch concentration in the leaves of *P. oceanica* plants were significantly higher in low impacted sites at the start of the experiment [$\chi^2(1) = 5.97$, $p = 0.0146$; **Supplementary Table 12B**]. From June to September, starch concentration in leaf tissue was significantly reduced in all sites independently from their location [**Figure 5**; $\chi^2(1) = 6.22$, $p = 0.0126$; **Supplementary Table 13**]. The addition of fertilizer reduced the starch contents in leaves from seagrasses in Ischia even further, whereas plants from Baia had significantly increased starch concentrations when nutrients were added [$\chi^2(1) = 6.46$, $p = 0.0110$; **Supplementary Table 12B**].

In contrast, according to predictions of the best model, leaf sugar concentration showed a slight trend of increasing over the course of the experiment in all sites (**Supplementary Table 13**) and leaves of seagrasses from low impacted sites around Ischia had significantly higher leaf sugar concentration than plants from Baia by the end of the experiment [**Figure 5**; $\chi^2(1) = 4.53$, $p = 0.0333$; **Supplementary Table 12A**].

Rhizomes

Starch concentrations in the rhizomes of *P. oceanica* meadows did not differ significantly among the sites in Baia (Gulf of Pozzuoli) and sites around Ischia at the start of the experiment (**Figure 6**). However, by September, the rhizome starch concentration of *P. oceanica* in the high impacted sites was significantly lower than those of the Ischia plants [$\chi^2(1) = 7.49$, $p = 0.0062$; **Supplementary Table 12D**].

The average concentration of soluble sugars was significantly higher in rhizomes in the plants from Ischia compared to the ones of Baia in June 2019 [**Figure 6**; $\chi^2(1) = 7.46$, $p = 0.0063$], and according to predictions of the best model showed a trend of declining over the time of the experiment in all plots at all sites (**Supplementary Table 13**). Rhizome sugar content was still significantly higher in seagrasses from low impacted sites by September 2019 [$\chi^2(1) = 5.63$, $p = 0.0062$; **Supplementary Table 12C**].

The short- term fertilization had no statistically significant effect on starch or sugar content even though there was a different trend in the response to fertilization between the high impacted and low impacted sites as shown by the low deviance explained the models (**Supplementary Table 12**). Seagrass rhizomes of high impacted sites showed a slight decrease in rhizome starch concentration with fertilization, while plants in low impacted sites showed a trend of increasing their rhizome starch concentration with fertilization (**Figure 6**).

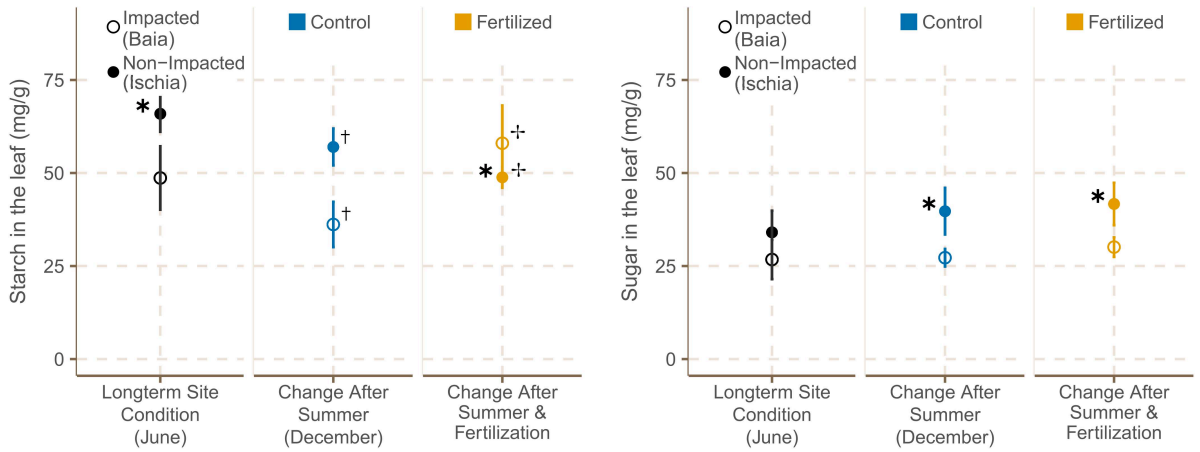

FIGURE 5 | Starch and sugar concentrations [mg g^{-1}] in leaves (mean ± SE) of *P. oceanica* plants (n = 6) in fertilized and control plots from different sites in June and September 2019. ✱ Shows significant differences between impacted (Baia) and non- impacted (Ischia) areas. † Indicates if there seasonal differences between June and September and ✣ states if there are differences between control and fertilized plots.

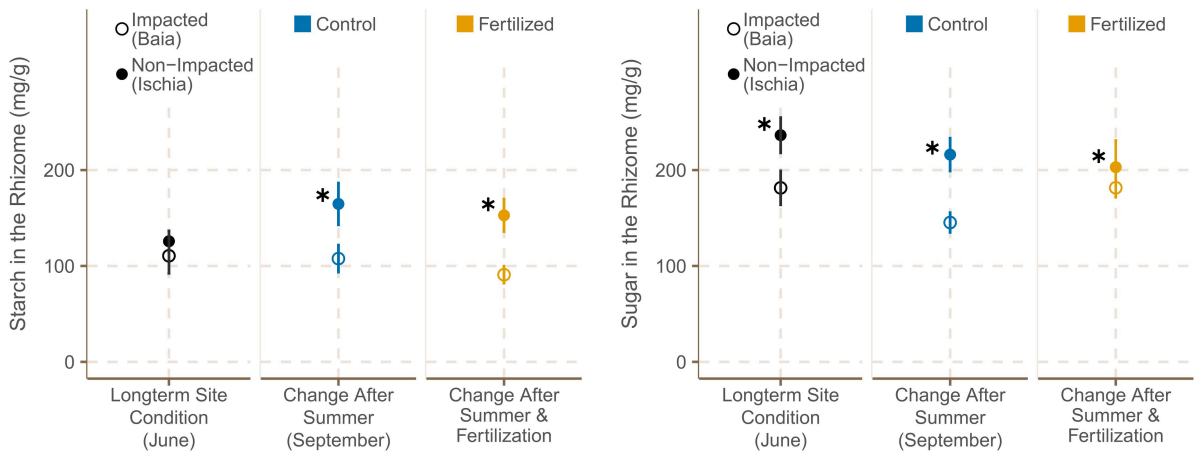

FIGURE 6 | Starch and sugar concentrations [mg g^{-1}] in rhizomes (mean ± SE) of *P. oceanica* plants (n = 6) in fertilized and control plots from different sites in June and September 2019. ✱ Shows significant differences between impacted (Baia) and non- impacted (Ischia) areas.

Comparison of Relative Changes With Season and Fertilization Across Population and Individual Plant Traits

To better compare and visualize differences across all population level metrics and individual plant morphological and biochemical traits due to season and to fertilization effects, we calculated the relative percent differences of all responses in September compared to in June (**Table 3**). Morphological traits were mainly impacted by season than by the additional fertilization. All morphological parameters got significantly reduced over time with more pronounced negative responses in plants from Baia. Population metrics of seagrasses generally displayed negative trends as well, except for epiphyte load which mainly increased over time and with fertilization. Biochemical traits showed a mix of either negative or positive trends, depending on the interaction of fertilization and on the level of impact of the sites.

DISCUSSION

Our results show a significant impact of local anthropogenic nutrient pressure on *Posidonia oceanica* meadows in the mid-western Mediterranean. With a larger number of spatial or temporal replicates, it is likely that the effects of fertilization would be reflected in more significant changes in seagrass indicators, but due to both time and logistic constraints further replication was not possible in this study. Despite these limitations, our findings identify useful indicators of nutrient and temperature stress for meadows of *Posidonia oceanica,* which provides us with a good example of a typical response of a large-bodied, slow growing seagrass undergoing various levels of local anthropogenic stressors (see **Table 4** for simplified summary of indicators). In contrast to the small-bodied, fast growing species *Halophila stipulacea*, from Part II of our study, which seemed to be able to adapt to eutrophic conditions (Helber et al., unpublished), *Posidonia oceanica* from the impacted

TABLE 3 | Relative percent change (mean ± SE) over the season (shown under Control) and relative percent difference (mean ± SE) with the interactive effects of season and fertilization (as shown under Fertilized) in the measured parameters in rhizome and leaf tissues of seagrass plants in control (n = 6) and fertilized (n = 6) plots from the anthropogenically impacted sites (Baia, Gulf of Pozzuoli) and low impacted sites (Ischia) in June compared to control (n = 6) and fertilized (n = 6) plots in September.

Traits	Site	Changes until September 2019 (%)	
		Control	Fertilized
Population level			
Cover	Baia	**−54.05 ± 2.70**	**−65.15 ± 2.80**
	Ischia	**−2.04 ± 5.70**	**−24.76 ± 2.72**
Shoot density	Baia	+29.71 ± 15.48	+0.60 ± 8.00
	Ischia	−6.24 ± 8.23	−4.12 ± 15.43
Epiphytes	Baia	−11.03 ± 8.77	**+86.16 ± 41.86**
	Ischia	+55.65 ± 13.25	**+82.23 ± 27.71**
Morphological			
No. leaves per shoot	Baia	**−32.65 ± 3.32**	−36.28 ± 4.07
	Ischia	**+1.62 ± 7.86**	−7.32 ± 6.56
Leaf height	Baia	**−6.18 ± 4.66**	+0.16 ± 4.84
	Ischia	**+1.15 ± 4.56**	−1.75 ± 4.11
Max. leaf canopy height	Baia	**−38.92 ± 4.46**	−39.52 ± 3.99
	Ischia	**−14.93 ± 5.75**	**−26.36 ± 6.69**
LAI	Baia	**−49.73 ± 6.39**	−55.75 ± 4.69
	Ischia	**−47.76 ± 8.59**	−55.05 ± 6.73
Leaf width	Baia	**+10.28 ± 2.30**	+ 12.00 ± 2.16
	Ischia	**+7.94 ± 3.64**	+6.40 ± 4.29
Leaf area	Baia	**−6.85 ± 4.54**	+0.16 ± 4.84
	Ischia	**+1.27 ± 4.86**	−1.78 ± 4.32
Leaf area per shoot	Baia	**−56.45 ± 5.16**	−55.95 ± 4.37
	Ischia	**−42.82 ± 8.34**	−53.48 ± 6.54
Biochemical			
Leaf C	Baia	**+11.51 ± 2.33**	+9.05 ± 1.67
	Ischia	**+6.05 ± 1.27**	+9.13 ± 1.34
Rhizome C	Baia	**+7.14 ± 3.70**	−0.72 ± 0.63
	Ischia	**+3.14 ± 0.97**	+6.55 ± 3.84
Leaf N	Baia	+15.71 ± 13.80	+23.09 ± 11.21
	Ischia	−9.05 ± 9.15	+5.41 ± 8.19
Rhizome N	Baia	**+70.80 ± 12.82**	**+15.37 ± 11.92**
	Ischia	**+65.66 ± 13.10**	**+71.01 ± 21.21**
Leaf $\delta^{13}C$	Baia	+10.10 ± 3.75	+5.98 ± 2.31
	Ischia	−5.35 ± 4.08	**−3.49 ± 2.24**
Rhizome $\delta^{13}C$	Baia	−0.38 ± 0.85	+0.27 ± 1.44
	Ischia	−4.66 ± 1.68	**−1.11 ± 0.91**
Leaf $\delta^{15}N$	Baia	−9.60 ± 4.34	**−27.23 ± 3.46**
	Ischia	−12.45 ± 9.50	**−38.48 ± 5.08**
Rhizome $\delta^{15}N$	Baia	**−4.10 ± 1.07**	−12.67 ± 3.24
	Ischia	**−8.95 ± 2.31**	−14.15 ± 4.07
Leaf C:N ratio	Baia	+1.04 ± 10.65	**−9.32 ± 7.77**
	Ischia	+12.73 ± 9.26	**+3.92 ± 8.03**
Rhizome C:N ratio	Baia	**−44.33 ± 5.53**	**−13.23 ± 10.71**
	Ischia	**−45.50 ± 4.42**	**−36.07 ± 7.94**
Leaf P	Baia	**−11.64 ± 29.78**	−30.93 ± 19.48
	Ischia	**+10.54 ± 11.73**	+12.15 ± 10.41
Rhizome P	Baia	**+310.39 ± 50.21**	+74.47 ± 29.96
	Ischia	**+130.64 ± 14.61**	+167.31 ± 31.95
Leaf sucrose	Baia	+13.53 ± 9.75	−1.97 ± 8.87
	Ischia	+31.81 ± 20.74	+9.77 ± 14.98
Leaf starch	Baia	**−40.17 ± 9.81**	**+57.07 ± 27.03**
	Ischia	**−13.22 ± 7.59**	**−26.09 ± 4.30**
Rhizome starch	Baia	+11.07 ± 14.38	−26.98 ± 7.14
	Ischia	+25.43 ± 16.53	+26.60 ± 13.99
Rhizome sucrose	Baia	−20.89 ± 5.58	+1.29 ± 5.52
	Ischia	−12.69 ± 6.91	−9.83 ± 12.33

Statistically significant values are in bold.

sites showed negative responses while enduring unusually high summer temperatures that were exacerbated even more by the additional nutrient enrichment provided in our study. This was shown by the decline in percentage cover, leaf area index (LAI), rhizome starch and sugar concentrations and the drastic increases in epiphyte biomass. Short-term effects of high temperature exposure during the summer months were also found in the less impacted sites, but with mixed effects of nutrient enrichment.

The most responsive indicators we detected for nutrient stress in this seagrass can be used to monitor further impacts of nutrient inputs to these seagrass meadows and provide management options to minimize future human interference on these important ecosystems.

Locally Driven Environmental Impacts Across Sites

Nutrient Concentrations and Water Quality

Nutrient concentrations in the water column and in the porewater were found to give inconclusive results, especially since sampling was only done at two time points. This was reported in previous studies: rapid dilution processes as well as fast transfer through the food web to higher trophic levels through phytoplankton grazing can make it difficult to detect signs of eutrophication via dissolved nutrients (Dalsgaard and Krause-Jensen, 2006; Holmer et al., 2008; Pitta et al., 2009). In addition, nutrients are often rapidly taken up by epiphytes of *P. oceanica* leaves as well as macroalgae in the seagrass meadow (Ruiz et al., 2010; Apostolaki et al., 2011). Micro- and macroalgae bioassays were proposed by Dalsgaard and Krause-Jensen (2006) as a reliable detection method for higher nutrient loads caused by fish farming activities. Similarly, in our study, epiphyte cover may be used as such a proxy (but see discussion below).

Epiphyte Cover as an Indicator of Short- and Long-Term Nutrient Enrichment and Thermal Stress

The epiphyte community of sites in Baia was characterized by large development of brown algae as reported for sites subjected to human pressure, whereas the sites off Ischia showed a mature epiphyte community consisting of red algae (Lepoint et al., 2007; Balata et al., 2008; Giovannetti et al., 2010). Nutrient enrichment and exposure to sewage was shown to drastically change the species composition of epiphytes (Balata

TABLE 4 | Significant differences in the most important investigated parameters caused by time (season and/or temperature) and by the addition of fertilizer as well as their interactive effects.

Indicator	Seasonal differences		Effect of fertilization		Interactive effects	
	Baia	Ischia	Baia	Ischia	Baia	Ischia
Meadow cover	↓	↓	↓	↓	↓	↓
Epiphyte cover	↓	↑	↑	↓	↔	↔
LAI	↓	↓	↔	↔	↔	↔
Maximum leaf canopy height	↓	↓	↔	↓	↔	↓
Leaf C:N ratio	↔	↔	↓	↓	↔	↔
Rhizome N content	↑	↑	↓	↑	↕	↑
Rhizome C:N ratio	↓	↓	↑	↓	↕	↓
Rhizome P	↑	↑	↔	↑	↔	↑
Leaf $\delta^{15}N$	↔	↔	↓	↓	↔	↔
Leaf starch	↓	↓	↑	↓	↕	↓
Leaf sugar	↑	↑	↓	↔	↔	↔
Rhizome starch	↔	↑	↓	↔	↔	↔
Rhizome sugar	↓	↓	↔	↓	↔	↔

*Statistically significant effects on seagrasses from the high impacted sites in Baia are depicted in brown color, while statistically significant effects on plants from the low impacted sites around Ischia are represented in green colors. The direction and size of the arrows represent the direction and strength of the effects (according to **Table 3**), respectively. The gray arrows represent no significant effects in either direction.*

et al., 2008; Prado et al., 2008; Giovannetti et al., 2010). In addition, previous studies (Frankovich and Fourqurean, 1997; Ferdie and Fourqurean, 2004; Kružić, 2008; Balata et al., 2010) confirmed that increased dissolved nutrient concentrations can promote epiphyte overgrowth on seagrass leaves. However, the reliability of epiphyte load as an indicator for eutrophication depends strongly on the interaction with herbivory on epiphytes, as increased grazing pressure can control the abundance of epiphytes (Ruiz et al., 2001; Heck et al., 2006). Herbivores have been observed to prefer adult leaves with a higher epiphyte load and higher nitrogen content (Peirano et al., 2001). Although we did not directly quantify herbivory on epiphytes in our study, it did not seem to have much effect on epiphyte biomass which was significantly higher in sites already subjected to anthropogenic stress in Baia and also differed between fertilized and control plots. The continuous nutrient input received by the seagrass meadows in Baia over a longer time scale possibly outweighs herbivory and as a result shifted the control of epiphytes from top–down to bottom–up.

Temperature and light availability are assumed to be the main drivers that facilitate the summer increase in epiphyte load (Prado et al., 2008; Apostolaki et al., 2011; Peirano et al., 2011). Here, we observed a slightly higher increase in epiphyte load from June to September as recorded in previous studies

(Prado et al., 2008; Apostolaki et al., 2011; Peirano et al., 2011), but the epiphyte biomass of seagrass leaves in Baia was almost five-fold higher in fertilized plots in September 2019 than the one found in plants off Ischia. While a modest epiphyte cover protects the leaves from damage due to ultraviolet radiation (Trocine et al., 1981; Nelson and Waaland, 1997), an increase in epiphyte biomass has several negative impacts on the seagrass plant. Epiphytes reduce light reaching the leaf surface and block leaf nutrient uptake outcompeting seagrass plants for nutrients (Ruiz et al., 2001; Cornelisen and Thomas, 2004; Apostolaki et al., 2012). In a related study, the photo-chemical performance in *P. oceanica* was shown to decrease in seagrasses from both low and high impacted locations during nutrient enrichment in mesocosm experiments (Pazzaglia et al., 2020); thus reduced light availability possibly due to epiphyte growth could have been one of the reasons. This can impact seagrass growth rates and negatively influence the carbon budget of plants exposed to eutrophication (Ruiz et al., 2001; Cornelisen and Thomas, 2004; Apostolaki et al., 2012).

Overall, our study confirms that epiphyte biomass in *P. oceanica* is more sensitive to changes in nutrient availability compared to the host plants or the whole community and is therefore an ideal indicator to identify seagrass meadows in which plant stress is likely to occur.

P. oceanica Population Response to Nutrient and Thermal Stress

The percentage cover of *P. oceanica* meadows already experiencing high anthropogenic pressure dropped drastically from June to September 2019 regardless of further nutrient enrichment in comparison to less impacted sites which showed only minor changes. Similar low shoot densities as the one in the Gulf of Pozzuoli have been observed for meadows in the vicinity of fish farms (Delgado et al., 1999; Ruiz et al., 2010). The lower shoot density recorded in a few plots within the low impacted sites, could possibly be the result of anchor damage (S. Helber, personal observation). Percent cover was also affected in all fertilized plots compared to non-fertilized plots within each site, particularly at the impacted location. Sewage input as well as high summer temperatures, have been identified in other studies for a decrease in shoot density (Delgado et al., 1999; Mayot et al., 2005; Marbà et al., 2014).

Shoot density was significantly higher in the low impacted sites of Ischia compared to the high impacted sites in Baia during both sampling periods. No significant change in shoot density over the experimental period could been detected, likely due to high variability in shoot counts as observed in other studies (Kletou, 2019; Zulfikar and Boer, 2020). This is not surprising, since shoot density in meadows at 10 m depth does not show strong seasonality (Peirano et al., 2011), and can remain fairly constant over long time periods (Kletou, 2019). In the present study, the lower response in shoot density to additional fertilization compared to percent cover shows that this measure is less reliable as an indicator for detecting nutrient stress in *P. oceanica*.

Posidonia oceanica meadows often contain two types of shoots, the most common ones being vertical shoots and the less frequent ones being horizontal or apical shoots (Hemminga and Duarte, 2000). Interestingly, we could barely find any vertical shoots in September 2019 at Site 1 in the plots in Baia. This might further indicate that the plants are stressed and the condition of the meadow will deteriorate further. Plants try to spread horizontally to recover and find more favorable conditions (Meinesz and Lefevre, 1984). Indeed, stressed meadows will prioritize apical shoots survival in order to secure population persistence and spread to more favorable habitats (Ruocco et al., 2020). In order to respond to temperature stress, plants can perform escape strategies other than favoring the growth of apical shoots, and can invest resources in flowering, seeking for spreading through sexual reproduction (Ruiz et al., 2018; Marín-Guirao et al., 2019). Nevertheless, flowering was not observed during the experimental period.

Rhizomes of plants from Baia were more brittle, easy to break and had a distinct hydrogen sulfide smell, whereas rhizomes from seagrasses around Ischia were firm and hard to break off (S. Helber, personal observation). Shoot mortality in *P. oceanica* as well as impacts on the photosynthetic rates of adult *P. oceanica* plants and their seedlings have been shown to increase during periods of high temperature in the summer months, in particular when temperatures reach 27°C for several days or weeks (Mayot et al., 2005; Díaz-Almela et al., 2009; Marbà and Duarte, 2010; Guerrero-Meseguer et al., 2017). Our data show that seagrass plants were exposed to temperatures over 27°C for 1–3 weeks at our experimental sites. Moreover, maximum temperatures recorded at 10 m depth were as high as 28.26°C, similarly to temperatures previously recorded for the extremely hot summer in 2003 (Garrabou et al., 2009).

Overall, our results confirm other studies which found increases in shoot mortalities of *P. oceanica* plants when seawater temperatures exceeded 27°C (Marbà and Duarte, 2010). Other studies showed that a one month exposure of *P. oceanica* seedlings above 29°C led to a seedling mortality rate of 33% and to a leaf mortality rate of 60% (Guerrero-Meseguer et al., 2017). Previous heat waves in the Mediterranean caused already high shoot mortalities from which plants in deeper areas were not able to recover within years (Marbà and Duarte, 2010). Future heat waves and warming are expected to reach even higher temperatures thereby posing a significant threat to *P. oceanica* populations (IPCC., 2007, 2014; Giorgi and Lionello, 2008). Our study further highlights that the overall magnitude of thermal stress effects on the population level can be exacerbated by the degree of anthropogenic nutrient supply, as those sites with higher impact were more significantly affected by the summer heat wave compared to low impacted sites.

Individual Plant Responses as Stress Indicators

Elemental (C, N, and P) and Stable Isotope ($\delta^{13}C$ and $\delta^{15}N$) Tissue Content

Tissue content of nitrogen, carbon and phosphorous are valid descriptors of the nutritional status of seagrasses. The

nitrogen content in leaves and rhizomes of seagrasses provides information about their long-term nutrient exposure and has been found to be higher in *P. oceanica* tissues at sites that receive higher nutrient inputs (Udy and Dennison, 1997; Invers et al., 2004; Fourqurean et al., 2006). In our study, an increase in %N content was first visible in the rhizomes of plants in fertilized plots growing in low impacted areas, highlighting the role of the rhizomes as a main location for N storage in this species (Invers et al., 2004; Fourqurean et al., 2006). Moreover, %P content increased significantly in the rhizomes of the seagrasses at both sites from June to September and the increase was even more pronounced in fertilized plots of meadows around Ischia suggesting that seagrasses might have been P limited.

Changes in C:N ratios of plants from both locations were caused by differential uptake of nitrogen as carbon content in rhizome and leaves of seagrasses at both locations remained constant. The same results have been observed by Pazzaglia et al. (2020) indicating that seagrass plants from pristine sites and sites receiving high nutrient inputs in the Gulf of Pozzuoli (Baia) developed different nutrient-balance strategies. Additional fertilization showed no effect in nitrogen content of seagrass tissues in plants growing under eutrophic conditions, possibly because nutrient imbalances might already exist in those plants, which may respond by downregulating their nitrogen uptake (Burkholder et al., 1992; Udy and Dennison, 1997; Ruocco et al., 2018; Pazzaglia et al., 2020). *P. oceanica* from the high impacted sites seemed to have already taken up and stored inorganic forms of N following their persistent nutrient exposure as indicated by their higher N content in leaf and rhizome tissues. Uptake and assimilation of nitrate or ammonium are energetically costly and require energy for nitrate reduction as well as C skeletons to produce amino acids (Van Katwijk et al., 1997; Touchette and Burkholder, 2000). Thus, C skeletons needed to produce carbohydrate reserves are diverted for the amino acid production (Invers et al., 2004; Pazzaglia et al., 2020). In the present study, we measured lower carbohydrate reserves in *P. oceanica* rhizomes from Baia and a trend towards a further decrease of carbohydrate reserves in fertilized plots, confirming this notion.

Conversely, plants from Ischia showed a decline in their leaf starch content in the fertilized treatments, indicating a mobilization of their carbon reserves from leaves to cope with the nutrient addition, confirming what has been previously reported in a mesocosm experiment (Pazzaglia et al., 2020). Plants in fertilized plots off Ischia showed an increase of N and P content in leaf and rhizome tissues, suggesting N and P limitation for *P. oceanica* at these sites. Thus, in response to the supply of the fertilizer, these plants showed an opportunistic nutrient strategy, assimilating N and P as soon as it became available (Marín-Guirao et al., 2018; Ruocco et al., 2018).

$\delta^{13}C$ and $\delta^{15}N$ values obtained in this study are in the same order of magnitude as those reported in previous studies for the same species (Vizzini et al., 2003; Lepoint et al., 2004; Fourqurean et al., 2007; Mateo et al., 2010), and ranged from –10.76 to –15.48 ‰ for $\delta^{13}C$ and from 2.24 to 8.12 ‰ for $\delta^{15}N$. However, $\delta^{15}N$ values were at the upper limit or slightly above those previously reported, suggesting that meadows both in Baia and in Ischia may be influenced by untreated or not properly treated sewage outflows, which are known to be enriched in $\delta^{15}N$ (Tomicic et al., 2001; Lepoint et al., 2004; Fernandes et al., 2009). At the same time, seagrass leaves of plants from fertilized plots showed a decrease in their $\delta^{15}N$ values, confirming that the plant incorporated the additional nutrients provided by the artificial fertilizer, which have $\delta^{15}N$ signatures close to 0‰ (Fourqurean et al., 2005).

Morphological Traits

Plants from the low impacted sites of Ischia had a higher LAI than seagrasses in Baia. This difference was even more pronounced after the summer peak in temperature. Thus, plants in Ischia possess a higher photosynthetically active area, being able to produce more substrates for growth, carbohydrate, and storage. Overall, seagrasses growing in Baia had smaller leaves and a lower leaf canopy. A reduction in leaf length has been previously observed in nutrient enrichment experiments with *P. oceanica* and *Z. marina* (Short et al., 1995; Leoni et al., 2006) as well as for plants growing in natural environments exposed to urban effluents and fish farms (Delgado et al., 1999; Ruiz et al., 2001; Balestri et al., 2004). Changes in sediment biogeochemistry (e.g., anoxia or sulphidic conditions; Delgado et al., 1999), imbalances in the internal nutrient budget or ammonium toxicity (Burkholder et al., 1992; Van Katwijk et al., 1997; Invers et al., 2004) might be responsible for the smaller leaf size in the impacted sites. Leaf canopy height was reduced in fertilized plots in meadows of Ischia, which would support the hypothesis that reduced leaf length was the result of internal nutrient imbalances. Additionally, reduced leaf length could be caused by an enhanced overgrowth of epiphytes. Leaf apexes might become more fragile and as a result break off more easily (Harlin, 1980).

Reduction in leaf growth was shown to be related to an epiphyte-induced decrease of available light (Short et al., 1995; Moore and Wetzel, 2000; Leoni et al., 2006). To compensate for the increased epiphyte overgrowth and the associated declining photosynthetic rates, seagrasses increased their number of leaves as an adaptive response to maximize their photosynthetic leaf surface (Dalla Via et al., 1998; Balestri et al., 2004; Leoni et al., 2006). In our study, plants in the impacted sites indeed had higher number of leaves per shoot than plants growing in meadows around Ischia. However, after the summer peak in temperature, the number of leaves per shoot declined in plants of Baia. The extremely high summer temperatures could have been responsible for the reduction in leaf numbers per shoot. Similarly, in *P. oceanica* leaf numbers per shoot were observed to experience a 20% reduction immediately after a disturbance occurred (Guidetti, 2001).

Morphological descriptors could be used to reveal short-term stress in seagrass meadows, but their limits of use have to be considered in terms of sampling times and reproducibility. The most valid morphological indicators for stress in our study were maximum leaf canopy height and LAI, a combined measure of leaf area, number of leaves, and shoot density, all of which had been found to decrease in other studies

investigating anthropogenic impacts on seagrasses (Guidetti, 2001; Leoni et al., 2006; Lopez y Royo et al., 2011; Kletou, 2019; Zulfikar and Boer, 2020).

By combining these individual plant traits with population level metrics, such as shoot density, we might be able to make predictions about changes in primary production on the ecosystem level as these functional traits have been linked to this important ecosystem function in aquatic and terrestrial plant systems (Gustafsson and Norkko, 2018; Karamfilov et al., 2019; Zulfikar and Boer, 2020). In terrestrial ecosystems, plant height, LAI and leaf coverage had all direct positive effects on ecosystem primary production (Asner et al., 2003; Gustafsson and Norkko, 2018). LAI was often found to be the main determinant of forest gross or net primary production and is considered one of the most significant parameters in most Terrestrial Biosphere Models (Asner et al., 2003; Barr et al., 2004; Saigusa et al., 2005; Wang et al., 2019). Thus, negative changes in seagrass canopy height, LAI and shoot density might in turn have long-lasting and devastating effects on important ecosystem functions, including the carbon sequestration capacity of the seagrass system.

Carbohydrates

Rhizome starch content of *P. oceanica* plants around Ischia was significantly higher than the one from plants in Baia and increased from June to September, whereas the rhizomes of plants in Baia did not show any seasonality. The seasonality in carbohydrate concentration found for plants in Ischia might be crucial for survival during winter, as *P. oceanica* has a distinctive asynchrony between its carbon use (respiration and growth) and its carbon fixation through photosynthesis (Alcoverro et al., 2001). The plant accumulates carbohydrate reserves in their rhizomes during summer, which are used to survive the winter season and to support growth in early spring when solar radiation is low (Alcoverro et al., 2001). In Spring, seagrass plants take advantage of their stored starch reserves and of the high water nutrient concentrations to meet their respiratory demands and to support leaf growth (Alcoverro et al., 2001). This permits them to build a large photosynthetic biomass until summer, when irradiances are high but nutrient concentrations in the water column (Romero, 1985) as well as in the porewater are at their lowest (Alcoverro et al., 1995; 1997).

The summer months of 2019 were particularly hot with maximum temperatures reaching 28.26°C at 6.6–7.9 m water depth in meadows in the Gulf of Pozzuoli (Baia) and at 7.4–12.0 m depth 27.96°C in meadows around Ischia. These hot temperatures could have led to the lower starch concentrations in the rhizomes of the already impacted seagrass meadows while the plants in less impacted areas seemed to be able to take advantage of the additional nutrients provided by the fertilization and use it to store even more starch compounds in their rhizomes. While starch content in leaves decreased in fertilized plots of low impacted sites, it increased in fertilized plots in high impacted sites. This suggests that the strategy of starch allocation and the stress coping mechanisms of the seagrasses differ with fertilization in high impacted sites compared to low impacted sites. Plants from high impacted sites seem to store energy as starch in their leaves for the direct use of new leaf growth whereas plants from low impacted sites use the additional fertilization to store higher amounts of starch in their rhizomes.

Seagrass plants require energy in the form of carbon skeletons (e.g., sugars and starch) for the assimilation of nitrogen, which can lead to a decrease in their carbohydrate reserves of up to 50% under conditions of high nitrogen availability (Burkholder et al., 1992; Van Katwijk et al., 1997; Invers et al., 2004). This decrease has been observed to be most pronounced during autumn and winter (Invers et al., 2004) for which we have no data. However, from June to September there was a tendency towards a more pronounced decline in starch content for the fertilized plots in Baia, suggesting that future nutrient input might further threaten the persistence of these meadows. High starch concentrations in the rhizome of *P. oceanica* do not only ensure winter survival, but also increase their resilience in case of disturbances since these resources can be readily mobilized in the event of extreme climatic or grazing events (Peterson et al., 2002; Fraser et al., 2014; Nowicki et al., 2017).

The lower starch concentrations in rhizomes of plants at the impacted sites could compromise seagrass health. While seagrasses from Ischia increased their starch concentrations from June to September, there was no difference in starch concentrations from plants in Baia. Thus, they might not be able to mobilize any reserves for overwintering and regrowth when their internal carbon budget is depleted. Therefore, starch content in rhizomes is considered to be a useful indicator of health status and survival probability of seagrass plants (González-Correa et al., 2008).

During heat waves, respiration of seagrasses increases which in turn might reduce the storage of carbohydrate reserves and lead to higher mortality rates, when under high temperature stress, *P. oceanica* plants, in fact, mobilize their starch reserve from rhizomes to leaves, to cope with higher energetic demand (Marín-Guirao et al., 2018). Therefore, heat stress might have a more detrimental impact on carbohydrate reserves than nutrient availability, as indicated by our study that could not detect significant effects of fertilization on carbohydrate concentrations in the rhizomes. Higher shoot mortalities have been reported in *P. oceanica* meadows in the Mediterranean in the winter months after the heat wave in the summer 2003 and could have been the consequence of depleted carbohydrate reserves (Díaz-Almela et al., 2009). In addition, it has been demonstrated that low irradiance resulted in a decline of carbohydrate concentrations in the rhizome and also in a drop in shoot density (Romero et al., 1996; Alcoverro et al., 2001). Thus, it will properly indicate when the carbon balance of the seagrass plants starts to be compromised through heat stress and/or nutrient pollution and is thus a particularly suitable indicator of the plants metabolic status.

Ecological Implications

Posidonia oceanica meadows might tolerate further heat waves in the Mediterranean, but are likely to experience substantial cutbacks in their photosynthetic performance as well as in their carbohydrate allocation for storage and growth. This weakens the plant ability to compete with other macrophytes, that are not only able to survive, but also to photosynthesize and grow

within a wider range of temperatures (Klein and Verlaque, 2008; Boudouresque et al., 2009; Rotini et al., 2017; Marín-Guirao et al., 2018; Beca-Carretero et al., 2020). Additionally, further increases in ambient nutrient concentrations as well as the rising input of ammonium due to the growing demand for agriculture and mariculture off the Mediterranean coasts (Karakassis and Hatziyanni, 2000; Pusceddu et al., 2007), pose a threat to the growth and survival of *P. oceanica* meadows. Persistent genera such as *Posidonia*, *Amphibolis,* and *Thalassia* spp. are particularly vulnerable to coastal eutrophication showing often a lack of recovery following nutrient reduction (Burkholder et al., 2007; Govers et al., 2014b; Fernandes et al., 2019). On top of this, the recovery of seagrass meadows that are already exposed to local anthropogenic stressors, might be further impeded by ocean warming as evidenced by the current and previous studies (Moreno-Marín et al., 2018; Ontoria et al., 2019). Our study confirms that overgrowth of epiphytes can be a sensitive indicator of a change in ecological water quality over large spatial scales, better than any *P. oceanica* structural indicator as it shows a faster response (Delgado et al., 1999; Giovannetti et al., 2010; Kletou, 2019). Epiphyte cover on leaves is also an easy to implement and low cost indicator for monitoring programs that was already proposed as an early warning indicator for deteriorating water quality as its response is independent of climate zone (Giovannetti et al., 2010; Marbà et al., 2013; Nelson, 2017). We further identify plant indicators that monitoring efforts should focus on, and especially recommend LAI and carbohydrate concentrations (starch and sugars) in the rhizomes as indicators of stress in this seagrass species.

It is of most importance to predict and anticipate stress in this sensitive key foundation species as *P. oceanica* has very limited colonizing abilities (Meinesz and Lefevre, 1984) and is highly vulnerable due to its extremely slow growth rates and low reproductive capacity (Kendrick et al., 2005; Holon et al., 2015; Noè et al., 2020). Therefore, the recovery of *P. oceanica* in denuded areas might take decades to centuries if even possible and conservation strategies should be given the utmost priority.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

SH, GP, HR, and MT conceived and designed the experiments. EB and MS contributed in designing the experiments. UC, AS-S, and SB assisted with the fieldwork and during sampling campaigns. SH performed the field experiment, developed the methodologies, and performed all the other laboratory analyses together with SB. EB did the statistical analysis. All the authors wrote and reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.662682/full#supplementary-material

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frontiers in Plant Science

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Nutrient History Affects the Response and Resilience of the Tropical Seagrass *Halophila stipulacea* to Further Enrichment in Its Native Habitat

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Eutrophication is one of the main threats to seagrass meadows, but there is limited knowledge on the interactive effects of nutrients under a changing climate, particularly for tropical seagrass species. This study aimed to detect the onset of stress in the tropical seagrass, *Halophila stipulacea*, by investigating the effect of *in situ* nutrient addition during an unusually warm summer over a 6-month period. We measured a suite of different morphological and biochemical community metrics and individual plant traits from two different sites with contrasting levels of eutrophication history before and after *in situ* fertilization in the Gulf of Aqaba. Nutrient stress combined with summer temperatures that surpassed the threshold for optimal growth negatively affected seagrass plants from South Beach (SB), an oligotrophic marine protected area, while *H. stipulacea* populations from North Beach (NB), a eutrophic and anthropogenically impacted area, benefited from the additional nutrient input. Lower aboveground (AG) and belowground (BG) biomass, reduced Leaf Area Index (LAI), smaller internodal distances, high sexual reproductive effort and the increasing occurrence of apical shoots in seagrasses from SB sites indicated that the plants were under stress and not growing under optimal conditions. Moreover, AG and BG biomass and internodal distances decreased further with the addition of fertilizer in SB sites. Results presented here highlight the fact that *H. stipulacea* is one of the most tolerant and plastic seagrass species. Our study further demonstrates that the effects of fertilization differ significantly between meadows that are growing exposed to different levels of anthropogenic pressures. Thus, the meadow's "history" affects it resilience and response to further stress. Our results suggest that monitoring efforts on *H. stipulacea* populations in its native range should focus especially on carbohydrate reserves in leaves and rhizomes, LAI, internodal length and percentage of apical shoots as suitable warning indicators for nutrient stress in this seagrass species to minimize future impacts on these valuable ecosystems.

Keywords: Red Sea, global warming, eutrophication, seagrass degradation, warning indicator, pollution, multiple stressors, Gulf of Aqaba (Eilat)

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INTRODUCTION

Seagrass meadows are becoming increasingly threatened, as their distribution along coastal areas makes them especially vulnerable toward local anthropogenic pressures (Orth et al., 2006; Wilkinson and Salvat, 2012). Seagrasses are disappearing four times faster than tropical rainforests (Masakazu et al., 2019), causing great concern considering the biodiversity associated with seagrass meadows, alongside the wide range of valuable ecosystem services and functions that these meadows provide. In addition to being vital for adjacent ecosystems (e.g., coral reefs), many of these services and functions also have important impacts on local economies and human health. Tropical seagrasses benefit neighboring coral reefs by increasing water quality, decreasing sediment resuspension and particulate matter loading (Orth et al., 2006; Gillis et al., 2014), mitigating the effects of ocean acidification (Unsworth et al., 2012; Bergstrom et al., 2019) and by reducing pathogen concentrations that would also have negative impacts on humans (Lamb et al., 2017).

Halophila stipulacea (Forsskål) Ascherson is a tropical seagrass species native to the Indian Ocean, Red Sea and Persian Gulf (Lipkin, 1975). It invaded the eastern Mediterranean through the Suez Canal some 150 years ago (Lipkin, 1975; Sghaier et al., 2011), but has also expanded into the Caribbean, where it outcompetes the native seagrass species *Syringodium filiforme*, *Halodule wrightii,* and *Halophila decipiens* (Ruiz and Ballantine, 2004; Willette and Ambrose, 2012; Willette et al., 2014; Smulders et al., 2017; Winters et al., 2020). Contrary to other seagrass species, *H. stipulacea* is able to survive under a broad range of temperatures (11 to 38°C), shows a high photo-physiological plasticity and has been found to thrive under eutrophic conditions, even in sulfidic sediments (Sharon et al., 2009; van Tussenbroek et al., 2016; Rotini et al., 2017; Wesselmann et al., 2020; reviewed by Winters et al., 2020). The ability of *H. stipulacea* to rapidly acclimate to a wide range of environmental conditions paired with its fast growth rates, leaf turnover and high fragment dispersal are possible reasons for its worldwide colonization success and, hence, sparked interests in its population dynamics as well as its resilience toward anthropogenic pressures (Weatherall et al., 2016; O'Brien et al., 2018b).

Research efforts in the Gulf of Aqaba (GoA), northern Red Sea, have mainly focused on local coral reefs (e.g., Rinkevich, 2005; Fine et al., 2013, 2019), even though an extensive area of approximately 707,000 m^2 along the coastline is covered by seagrass meadows (Winters et al., 2017). *H. stipulacea* is the most dominant and widespread seagrass species in the GoA (Winters et al., 2017), where it forms large, discontinuous meadows in both shallow and upper mesophotic zones (1–51 m depth) (Sharon et al., 2011; Kramer et al., 2019). These seagrass meadows along the coastline of Eilat, Israel, provide important ecosystem services that are estimated to exceed US$ 2,000,000 per year (Winters et al., 2017). However, the importance of these ecosystems is still not fully recognized, not only by scientists but also by policy makers and local stakeholders (Winters et al., 2017, 2020).

The semi- enclosed nature of the Red Sea makes it particularly vulnerable towards eutrophication and global warming. It has been shown that annual sea surface temperatures (SSTs) in the northern GoA are increasing at rates of 0.254 ± 0.058°C $decade^{-1}$ (Fine et al., 2013; Nguyen et al., 2020), slightly faster than the global coastal SST trend of 0.17 ± 0.11°C $decade^{-1}$ (Liao et al., 2015). The cities of Eilat and Aqaba, located on the northernmost part of the GoA, are the largest, fast growing population centers in the region, both of which attract millions of tourists annually. In both these cities, adjacent coastal ecosystems face numerous threats due to significant infrastructure development as one of the Red Sea's major tourist destinations (Fine et al., 2019). In addition to global threats of ocean warming and acidification, the main regional threats to local marine ecosystems include enhanced nutrient inputs (i.e., eutrophication) from sewage, mariculture and agricultural runoff (Loya, 2004; Winters et al., 2017). There is no natural terrestrial run off into the Red Sea since it is surrounded by deserts and only nutrient- depleted Red Sea surface water can enter the GoA through the Straits of Tiran making the Red Sea world famous for its oligotrophic, clear waters (Stambler, 2005; Wurgaft et al., 2016). Due to the GoA being a semi- enclosed ocean body, its waters are characterized by a particularly long residence time of 3–8 years (Loya and Kramarsky-Winter, 2003; Winters et al., 2017). This long residence time means that the effects of land-associated sources of pollution, such as high nutrient inputs from poorly or non-treated sewage as well as municipal discharge, mari- and agricultural runoffs, and severe flash flood events, are intensified several folds. These local anthropogenic stressors may, in combination with global warming, affect seagrass ecosystems even more dramatically than previously predicted by experiments that have been conducted with only one stressor in laboratory facilities without including the whole seagrass ecosystem (Egea et al., 2018; Mvungi and Pillay, 2019; Nguyen et al., 2020; Pazzaglia et al., 2020). Studies investigating the effect of multiple stressors on *H. stipulacea* meadows *in situ* within its native range are particularly scarce, yet these studies will be crucial if we want to understand how environmental and anthropogenic pressures affect the state and population dynamics of this seagrass species. Moreover, multiple stressor studies on *H. stipulacea* in its native range will inform us how to improve management efforts for its conservation in the northern GoA since establishing marine protected areas (MPAs) alone has already been shown to be insufficient for protecting seagrass meadows (Eklöf et al., 2009; Quiros et al., 2017). Identifying how different stressors impact these ecosystems is of fundamental importance if we are to take timely targeted management actions.

The aim of our study was to identify warning indicators for nutrient stress in *H. stipulacea* seagrass meadows growing in both anthropogenically low and high impacted sites. For this we compared the effects of nutrient history on seagrass responses to *in situ* nutrient enrichment, created by using fertilizer addition over 6 months, capturing as well the high summer temperature peak. Signs of stress were investigated on individual plant as well as on the population level by measuring structural community metrics (% cover, shoot density, biomass) and morphological (leaf width, length, leaf area, leaf area index, % apical shoots, no. of leaves, internodal distance) and biochemical (tissue nutrient content and carbohydrates) plant traits identified as potential

stress response indicators (Roca et al., 2016). This study is part 2 of a parallel study on *Posidonia oceanica* in the Mediterranean (Helber et al., 2021). Through both of these studies, we aim to compare responses of *H. stipulacea*, a fast-growing, small-bodied seagrass, to *P. oceanica*, a slow-growing, large-bodied seagrass, in order to better understand how these very different seagrasses may respond under future nutrient and climate scenarios and potentially interact in areas where *H. stipulacea* occurs within *P. oceanica* meadows (Beca-Carretero et al., 2020b).

MATERIALS AND METHODS

Study Sites

The response of *H. stipulacea* populations to thermal and nutrient stress were compared between two sites that are already heavily exposed to anthropogenic pressures (North Beach – NB) and two sites in a marine protected area with relatively low anthropogenic pressures (South Beach – SB) along the western coast of the northern Gulf of Aqaba (Eilat, Israel; **Figure 1**). The South Beach area is characterized by a steep slope, gravel sized sediment and a high cover of neighboring corals. In terms of light penetration, this site has been shown to have a low photosynthetic active radiation (PAR) diffuse attenuation coefficient (K_d PAR), entailing a relatively high Secchi disk depth and relatively small seagrass leaves (Mejia et al., 2016; Beca-Carretero et al., 2020a). Lastly, this site falls within a local MPA with little tourist infrastructure, no buildings at the beaches, but is a popular diving spot. In contrast, seagrass meadows on the North Beach are in close proximity to a dense strip of hotels and a marina. Extensive boating activities occur right over the meadows. The North Beach site is characterized by a small slope and fine, muddy sediment, and hardly any corals. The light attenuation (K_d) determined by measurements of PAR at the water surface and sediment depth, is much higher than in the South Beach site (Beca-Carretero et al., 2020a), and secchi disk depth has been found to be much shallower due to less light penetrating the water, and the leaves of local seagrass plants are much larger (Mejia et al., 2016; **Supplementary Figures 1**, **2**). Although removed some 10 years ago, gilthead sea breams (*Sparus aurata*) were cultivated in cages in this area for approximately 20 years, which led to nutrient enrichment of the water column and underlying sediments, and a die-off of the underlying seagrass meadows (Oron et al., 2014). It was estimated that the North Beach area received annually more than 250 tons of N and 50 tons of P as a result of the fish farming activities (fish excretion and undigested feed pellets) by the end of the 1990s and beginning of the 2000s (Lazar et al., 2008). This area is also exposed to sewage run-offs, the effects of flash floods, and the Kinnet Canal that bring nutrients into the local meadows (Mejia et al., 2016; Winters et al., 2017). Sewage run off contributed to approximately 150 tons of N per year during the 1980s and 1990s, but almost stopped in 1995 (Lazar et al., 2008). Recent stable nitrogen isotope work by Beca-Carretero et al. (2020a) found inputs of anthropogenic-origin nutrients ($\delta^{15}N$) and higher levels of NH_4^+ in seawater in the NB than in the SB alongside a higher annual signal of $\delta^{15}N$ in *H. stipulacea* plants growing in the more impacted site (NB) compared with the more pristine site (SB). These results indicate a potential anthropogenic input of N in the seawater, which is reflected in the plant content in the impacted site (NB), and together with the light measurements and leaf morphometrics (detailed above; Mejia et al., 2016; Beca-Carretero et al., 2020a) demonstrate a difference between the two sites.

Experimental Set-Up

Six circular plots with a diameter of 2m were established at each one of the four sites (S1–S4), at least 10 m apart from each other. Three of these plots were used as control, while the other three were enriched with slow-release fertilizer pellets (Osmocote® Pro: 19% N – 3.9% P – 8.3% K, ICL Speciality Fertilizers). The fertilizer contained nitrogenous compounds that were composed of nitrate (6.3%), ammonia (8.2%) and urea (4.5%). Fertilizer pellets were filled in 0.5 m long PVC tubes, which were hammered approximately 20 cm into the sediment, delivering nutrients to the below-ground and above-ground tissues (Worm et al., 2000). Nine fertilizer filled PVC tubes were inserted in each nutrient treatment plot resulting in an addition of approximately 1,170 g of fertilizer per plot (**Figure 2**). All PVC tubes used in treatment and control plots were previously drilled throughout their entire length with 2mm holes to allow the flushing of seawater through the tubes into the surrounding environments.

From each of the plots at all four sites, we measured a number of target variables in July and December 2019 for assessing different levels of nutrient concentrations as well as biological organization, ranging from biochemical and morphological individual plant traits, to community level metrics as outlined below.

Environmental Sampling

Water samples were taken with acid rinsed plastic syringes (approximately 30 mL) at around 10 m depth above the seagrass canopy of each plot on each site for nutrient analysis of the surrounding water. Porewater samples were taken by placing a syringe 5 cm deep into the sediment and carefully drawing out water from interstitial spaces of the sediment to determine porewater nutrient concentrations. Once on shore, water samples were immediately filtered into HDPE vials using sterile syringe filters (LABSOLUTE®; cellulose acetate; 0.45 μm pore size) and stored on ice. Samples were frozen at −20°C upon arrival to the laboratory on the same day of sampling and stored for further analyses. Nutrient measurements for samples taken in July were performed spectrophotometrically with a TECAN plate reader (Infinite 200 Pro microplate reader; Switzerland) according to Laskov et al. (2007). The detection limits were 0.08, 0.32, 0.7, and 0.022 μM for NO_2^-, NO_x (NO_3^- and NO_2^-), NH_4^+, PO_4^{3-}, respectively. The NO_x (NO_3^- and NO_2^-) and NH_4^+ concentrations sum up to dissolved inorganic nitrogen (DIN). The coefficient of variation was always <3.4%. The lower nutrient concentrations from samples taken during December 2019 were analyzed using a continuous flow injection analyzing system (Skalar SAN+++System). The detection limits were 0.043, 0.094, 0.290, and 0.042 μM for NO_2^-, NO_x (NO_3^- and NO_2^-), NH_4^+, and PO_4^{3-}, respectively. The coefficient of variation was always <3.4%.

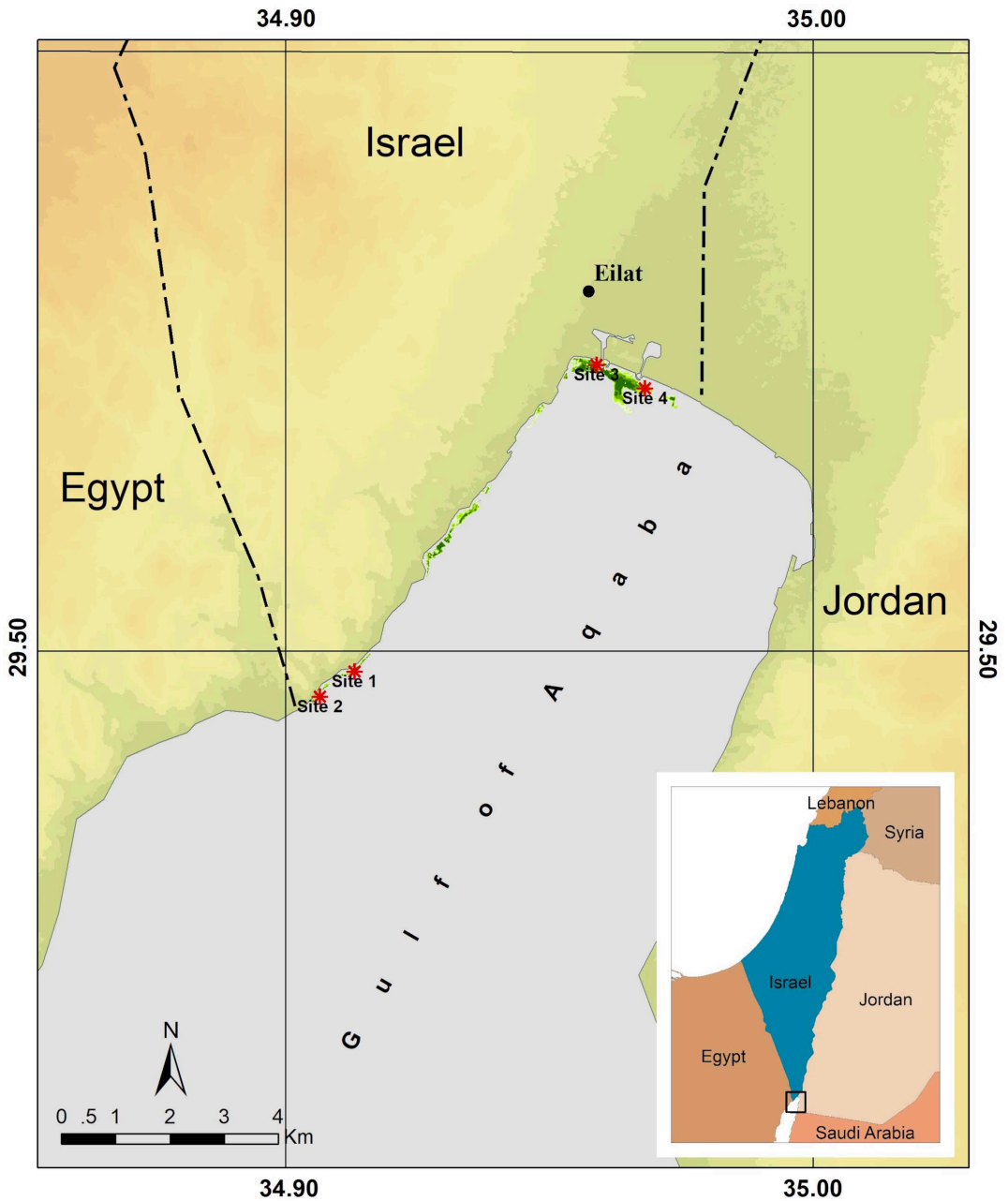

FIGURE 1 | Overview of the northern Gulf of Aqaba with our study sites. Shown here are the study sites within the marine protected South Beach area (S1 and S2) and the study sites at the North Beach area (S3 and S4), which are exposed to anthropogenic pressures. S3 is close to a marina and S4 is close to an outlet draining local agricultural fields and land-based fish farms (Winters et al., 2017). Seagrass meadow extent is indicated in green (adapted from Winters et al., 2017).

In addition to water samples taken within the plots, the water quality of the different sites was assessed by taking water samples directly above the seagrass canopy with a pre-rinsed 5-L Niskin (HDPE) bottle. Collected seawater was immediately stored in the dark on ice. Environmental parameters, such as salinity, temperature, oxygen and pH were measured directly after collection with a multi parameter probe (WTW Multiprobe). Moreover, temperature loggers (HOBO Water Temp Pro v2) were fixed to the main pole of plots 1 and 6 in the seagrass meadows (ca. 10 m water depth) to record the water temperatures hourly during the entire time of the experiment.

A defined volume of water was vacuum-filtered onto pre-combusted (5 h, 450°C) Whatman® GF/F filters on return to the laboratories of the Interuniversity Institute for Marine Sciences in Eilat (IUI). Filters for chlorophyll *a* (Chl *a*) analysis were immediately stored at −80°C, filters for organic carbon (C_{org}), total carbon (C) and nitrogen (N) content were frozen at −20°C until further analysis at the Leibniz Centre for Tropical Marine

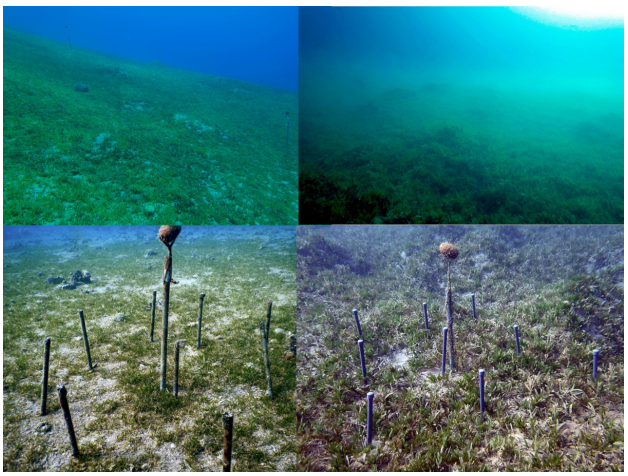

FIGURE 2 | Overview of the study sites within the MPA South Beach **(Left side)** and the North Beach **(Right side)** exposed to anthropogenic pressures. The lower panel shows the set- up of the plots. Pictures: Upper panel, Dr. MiS; lower panel left, MaS; lower panel right Julia Cerutti.

Research (ZMT) in Germany. Filters for suspended particulate matter (SPM) determination were weighed prior to filtering. Subsequently, filters were dried at 40°C for 48 h and the dried filters were weighed again. Filters for C_{org}, C and N content were divided in 4 equal pieces and one quarter was used for each analysis. For Chl *a* analysis, filters were analyzed and extracted in 80°C hot ethanol, following Welshmeyer (1994). The supernatant was subsequently transferred into small vials and Chl *a* concentrations were determined with a TD10AU-Fluorometer (Turner Designs). The detection limit of this method is 0.002 $\mu g\, l^{-1}$.

Percent Cover

Percent cover was measured by randomly placing a 25 cm × 25 cm quadrat in each plot and taking a picture with an underwater camera. Pictures were analyzed with Coral Point Count for Excel (CPCe; Kohler and Gill, 2006). 100 points were randomly overlaid onto the images. Points intersecting with the occurrence of seagrasses were counted as "hit" and used to quantify the percentage of seagrass cover (0–100%).

Shoot Density, Biomass, and Morphological Parameters

A biomass corer was used to sample above ground (AG) and below ground (BG) seagrass biomass in July and December 2019 at SB and NB sites. Samples were taken with an open- ended, standard PVC drainpipe with a diameter of 12.5 cm and a length of 0.5 m. In each plot, biomass cores were taken by twisting and hammering the corer approximately 10–15 cm into the sediment. The contents of the biomass cores were transferred underwater into diving mesh-bags and sediment was removed carefully underwater. Upon arrival at the laboratory facilities of the IUI in Eilat, biomass samples were carefully cleaned and all sand, sediment particles and epiphytes were carefully removed. Plant material from the core samples was separated into AG (leaves) and BG (rhizomes and roots) plant compartments. Shoot density was determined by counting the total number of shoots in each biomass core sample and then normalizing these counts per m^2. Shoots that were examined for the presence of male or female reproductive structures were counted separately and the flowering percentage (no. of flowering shoots/total no. of shoots × 100) was calculated (Malm, 2006; Nguyen et al., 2018). AG and BG material was dried at 60°C for 48 h and then weighed to determine AG and BG biomasses (g DW m^{-2}) and the above/below-ground biomass ratios (AG:BG).

Additionally, leaf and rhizome samples were taken from each biomass core to evaluate shoot morphology. Internodal distance of rhizome was measured for each plot (9 subsamples per plot). From each subsample, intact and representative leaf samples including the smaller, youngest shoot (3–14) were used to measure leaf length, leaf width, leaf area and leaf area index (LAI). The leaves were either scanned (Canon Lide 110 digital scanner) or photographed. Leaf descriptors were measured with ImageJ software (version 1.53a; Abramoff et al., 2004).

Plant Collection for Biochemical Parameters

Several ramets were randomly collected at different locations within each plot. AG and BG tissues were separated and immediately frozen at −20°C until further analysis for AG and BG nutrient content, stable carbon and nitrogen isotope analyses ($\delta^{13}C$ and $\delta^{15}N$), as well as total non-structural carbohydrate (NSC) reserves such as starch and sugars.

Carbon and Nitrogen Tissue Content and Natural Stable Isotopes

Before analysis, the leaf and rhizome samples were freeze-dried for 48 h at the ZMT's facilities. The dried leaf and rhizome tissues were ground and total C and N content was measured using a Euro EA 3000 elemental analyzer (EuroVector) with Acetanilid 5 (Hexatech) used as standard. Measurement accuracy was ensured by repeatedly measuring standards with known C and N concentrations (Low Soil Standard OAS 5; IVA). Carbon and nitrogen contents were expressed as a percentage of dry weight and the values were used to calculate the *C*:*N* ratios. Percent tissue phosphorus was analyzed using the wet alkaline persulphate digestion technique method on a TECAN M200Pro plate reader after Hansen and Koroleff (2009).

Stable isotopic ratios of carbon ($\delta^{13}C/\delta^{12}C$) and nitrogen ($\delta^{15}N/\delta^{14}N$) in samples were analyzed using a Finnigan Delta Plus mass spectrometer coupled with a Flash EA 1112 elemental analyzer. Results of isotopic composition in samples were expressed as following:

$$\delta X(‰) = [(R_{sample}/R_{\text{reference}}) - 1] \times 1000, \quad (1)$$

where *X* is either $\delta^{15}N$ or $\delta^{13}C$, and R is the ratio of $\delta^{15}N/\delta^{14}N$ for nitrogen and $\delta^{13}C/\delta^{12}C$ for carbon. Reference materials IAEA N1 and N2 (nitrogen) and USGS 24 and NBS22 (carbon) from the International Atomic Energy Agency were used for calibration.

The precision of the measurements was <0.06‰ for both C and N isotopes. All $\delta^{13}C$ and $\delta^{15}N$ values were normalized to the international standards of wheat flour (carbon [$\delta^{13}C$]: −27.21‰; nitrogen [$\delta^{15}N$]: 2.85‰) and high organic sediment (carbon [$\delta^{13}C$]: −26.07‰; nitrogen [$\delta^{15}N$]: 4.4‰).

Carbohydrate Reserves

Triplicate subsamples of the rhizomes were pooled and freeze-dried for 72 h. Subsequently, the samples were ground with mortar and pestle. The dried powder (0.02–0.03 g) was suspended in 1.5 mL Milli-Q water and soluble sugars were extracted from the ground dry tissues by vortexing and shaking for 15 min. Samples were centrifuged (13,000 rpm, 5 min) and the supernatant was used for soluble sugar determination via the Anthrone Assay (Viles and Silverman, 1949). Starch contents (total non-structural carbohydrates) in the remaining pellets were boiled in Milli-Q (10 min at 100°C) to gelatinize the starch. Subsequently, samples were hydrolyzed by the enzyme alpha-amylase (80 min at 80°C). The supernatant containing oligosaccharides and/or glucose broken down by the enzyme was taken and by boiling the sample material under acidic conditions (addition of 96% H_2SO_4) for 1.5 h at 100°C, the remaining polysaccharides were broken down to glucose molecules. Starch and sugar contents in the extracts were determined spectrophotometrically (620 nm) using an anthrone-sulphuric acid assay with a F200-Pro TECAN plate reader. Carbohydrate concentrations were quantified as sucrose equivalents (Viana et al., 2020) using sucrose calibration curves (Standard sucrose 99%, from Sigma-Aldrich). Samples of cellulose, glucose and starch were used as reference samples.

Sediment Natural Stable Isotope Composition

From each plot of the four sites, surface layer sediments were collected in July and December 2019 in polystyrene Biological Specimen Containers. After collection, samples were stored at −20°C until further analysis.

Upon arrival at the ZMT, samples were dried at 60°C for 48 h and dry sediments were subsequently ground with a ball mill. Ground samples were then acidified with 37% HCl vapor to remove carbonates and stable isotopic composition measured as described under Section 2.7.

Statistics

Statistical analyses were performed in R (R Core Team, 2019). For the statistical analysis, we combined data for Site 1 and 2 (SB sites) together as low impacted condition and Site 3 and 4 (NB sites) together as high impacted condition. Although, there were site- specific differences, we aimed to focus on large-scale patterns and therefore only distinguished between the environmental history of the two locations (i.e., SB vs. NB). Prior to analysis, we subset our data to values that were collected in July 2019 (start of the experiment) and December 2019 (end of the experiment). Since the *in situ* fertilization treatment started in July, it was assumed there was no nutrient treatment effect in July. We used two linear mixed effects models (LMM). One to determine if there were seasonal effects over the time of the experiment (1). For this analysis, we only looked for changes in the control plots over time and added site as a random effect. The second linear mixed effects model was performed to look at the differences between the conditions of the sites (high [NB] and low [SB] impacted sites) in July (2) and December (3), the effect of treatment- differences between fertilized and control plots (4) as well as their possible interactions (5). We performed both linear mixed effects models using the lme4 function in R (Bates et al., 2015). As fixed effects, we considered condition (high or low impacted), treatment (fertilization and control) and the interaction of condition and treatment. We added site as a random effect to our linear mixed effects models to account for the variability in sites within areas of different conditions (high or low impacted). Differences in shoot densities and leaf numbers among the sites were determined with a generalized linear mixed-effects model (GLMM), specifically a negative binomial model (link = log) because the data were counts and found to be over-dispersed (Zuur et al., 2009). The GLMM was fitted using the MASS package (Venables and Ripley, 2002). The results of the models were considered to be significant with a p value < 0.05. All models were validated visually with plots of model residuals [fitted values vs. absolute residuals (homogeneity of variance), a quantile–quantile (q–q) plot (i.e., probability plot), comparing the distribution of the standardized residuals to the normal distribution (normality), and a lag plot of the raw residuals vs. the previous residual (independence; Zuur et al., 2009)]. We assessed the significance of each independent variable (or interaction) in each model by using the likelihood ratio (LR) test that compared models with our variables of interest to the null or reduced model (Winter, 2013). To further support the results obtained by the LR test, we identified the models that best predicted the changes in seagrass indicators by minimum Akaike Information Criterium with correction for small sample sizes (AICc), model ranking and weighing (Burnham et al., 2011; Symonds and Moussalli, 2011). Models were considered the superior model if they had the lowest AICc units (determining the model strength) as well as the highest Akaike weight (AICcWt) which defines the weight of evidence of each model relative to the null or reduced model (Arnold, 2010).

Environmental parameters were analyzed separately for July and December 2019 with the ANOVA aov() or Kruskal–Wallis function in R, and a subsequent paired *t*-test with holms correction (*post hoc* test) was performed to look for significant differences between low (S1 + S2) and high (S3 + S4) impacted sites.

RESULTS

Environmental Variables

Salinity ranged between 40.4 and 40.6 psu and pH ranged between 8.22 and 8.23 with no significant differences between sites (**Table 1**). Daily average water temperatures reached its maximum on August 15th–16th 2019 for SB sites at 29.14 ± 0.03°C SE and for NB sites at 29.07 ± 0.47°C SE at approximately 10 m water depth (**Figure 3**). Temperatures over

TABLE 1 | Environmental quality parameters in the seawater.

Variable	n	July 2019				December 2019			
		S1 SB	S2 SB	S3 NB	S4 NB	S1 SB	S2 SB	S3 NB	S4 NB
Seawater									
Chl*a*	4	0.08 ± 0.0	0.13 ± 0.0	0.17 ± 0.0✱	0.36 ± 0.1✱	n.a	n.a	n.a	n.a
SPM	4	32.7 ± 0.6	33.7 ± 0.4	37.6 ± 4.3✱	35.8 ± 0.2✱	32.3 ± 0.5	32.0 ± 0.4	32.6 ± 0.1✱	34.2 ± 1.0✱
Corg	4	46.1 ± 2.6	42.9 ± 2.0	46.0 ± 6.6	58.0 ± 6.0	107.2 ± 5.4	116.0 ± 4.4	130.7 ± 11.4	117.2 ± 6. 5
C	4	211.2 ± 39.2	167.6 ± 35.1	187.3 ± 17.5	393.1 ± 142.0	124.5 ± 7.0	133.4 ± 9.6	177.6 ± 25.0	119.7 ± 6.0
N	4	26.7 ± 5.0	20.3 ± 4.1	22.7 ± 2.4	44.5 ± 15.9	13.7 ± 0.8	12.2 ± 1.0	22.0 ± 2.7✱	16.0 ± 0.6✱
C:N	4	24.2 ± 2.3	24.9 ± 1.6	24.9 ± 1.1	26.3 ± 0.9	9.1 ± 0.3	11.3 ± 1.8	8.1 ± 0.2✱	7.5 ± 0.4✱
Salinity	1	40.5	40.6	40.4	40.5	40.5	40.6	40.4	40.4
pH	1	8.230	8.224	8.220	8.214	8.230	8.220	8.220	8.220

Water parameters from the GF/F filter samples (Chla, SPM, Corg, C and N) are reported in $\mu g\ l^{-1}$*. Salinity is reported in psu.* ✱ *indicates significant differences between impacted (NB) and non-impacted (SB) sites.*

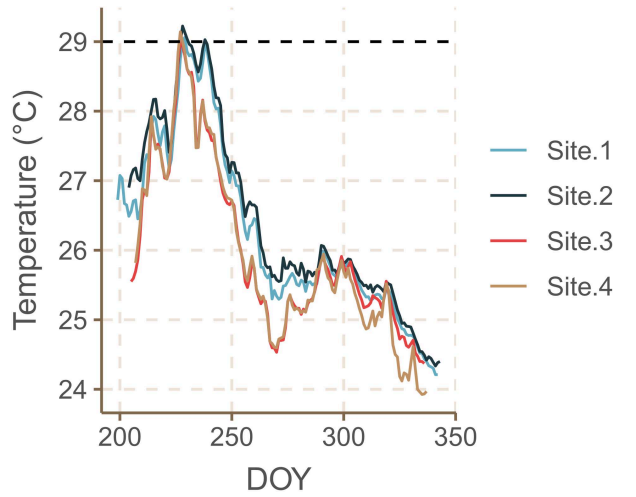

FIGURE 3 | Daily average water temperatures measured at the South Beach (sites 1 and 2) and North Beach (sites 3 and 4) sites at approximately 10 m water depth recorded from 18.07–09.12.2019 (DOY = Day of the Year). The dashed line at 29°C indicates the physiological threshold, beyond which *H. stipulacea* plants were found to reduce growth rates, leaf sizes and the loss of shoots (Nguyen et al., 2020; Viana et al., 2020).

29°C persisted for 15 consecutive days in SB Site 1, for 30 days in SB Site 2, for 8 days in NB Site 3 and for 6 days at NB Site 4. From mid-August onward, temperatures began to gradually decrease, reaching their minimum values of approximately 24°C in December 2019.

In July 2019, there were no significant differences in N and C content of water column particulates. However, both chl*a* and SPM concentrations were higher in the NB sites (**Table 1** and **Supplementary Table 1**). In December, NB sites had significantly higher particulate N content in the water column and lower C:N than SB sites (**Table 1** and **Supplementary Table 2**). Moreover, both NB sites had significantly higher concentrations of SPM in the water column (**Table 1** and **Supplementary Table 2**).

TABLE 2 | Inorganic nutrient concentrations in the seawater and porewater in control and fertilized plots, expressed in $\mu mol\ l^{-1}$.

Nutrients	Site	July 2019	December 2019	
			Control	Fertilized
SEAWATER				
DIN	North Beach	1.34 ± 0.08	0.17 ± 0.06	0.35 ± 0.10
	South Beach	0.86 ± 0.06	0.55 ± 0.14	0.92 ± 0.30
NH_4^+	North Beach	0.89 ± 0.28	0.17 ± 0.06	0.27 ± 0.07
	South Beach	0.69 ± 0.05	0.37 ± 0.11	0.52 ± 0.22
NO_X	North Beach	0.45 ± 0.05	0.10 ± 0.07	0.08 ± 0.06
	South Beach	0.17 ± 0.03	0.13 ± 0.09	0.53 ± 0.22
NO_2	North Beach	0.17 ± 0.03	0.01 ± 0.02	0.01 ± 0.02
	South Beach	0.11 ± 0.03	0.03 ± 0.02	0.03 ± 0.02
PO_4^{3-}	North Beach	0.19 ± 0.03	0.22 ± 0.03	0.21 ± 0.02
	South Beach	0.15 ± 0.02	0.19 ± 0.02	0.23 ± 0.06✱
Si	North Beach	n.a	0.47 ± 0.10	0.35 ± 0.11
	South Beach	n.a	0.00 ± 0.00	0.00 ± 0.00
POREWATER				
DIN	North Beach	6.00 ± 0.13	1.95 ± 0.26	5.38 ± 0.49
	South Beach	7.50 ± 0.18	3.68 ± 0.37	5.18 ± 0.44
NH_4^+	North Beach	5.69 ± 0.13	1.48 ± 0.26	4.72 ± 0.49
	South Beach	6.89 ± 0.17	6.53 ± 0.54	4.28 ± 0.49
NO_X	North Beach	0.30 ± 0.03	0.47 ± 0.10	0.67 ± 0.09
	South Beach	0.60 ± 0.05	0.73 ± 0.16	1.28 ± 0.14✱
NO_2	North Beach	0.40 ± 0.04	0.05 ± 0.03	0.07 ± 0.02
	South Beach	0.65 ± 0.05	0.08 ± 0.05	0.15 ± 0.06
PO_4^{3-}	North Beach	1.52 ± 0.09	0.56 ± 0.09	0.56 ± 0.09
	South Beach	2.35 ± 0.09	1.15 ± 0.12	2.35 ± 0.32✱
Si	North Beach	n.a	26.57 ± 0.71	26.12 ± 0.66
	South Beach	n.a	13.35 ± 0.47	14.58 ± 0.34

✱ *indicates significant differences between impacted (NB) and non-impacted (SB) sites.*

There were no significant differences in nutrient concentrations in the water column and the porewater between SB and NB sites (**Table 2**). However, fertilized

plots in the SB sites were significantly enriched in porewater P (β = 1.19; *SE* = 0.51; df = 16; *t* = 2.32; *p* = 0.0338) and N compounds (NOx; β = 0.38; *SE* = 0.22; df = 19; *t* = 1.744; *p* = 0.0973) as well as P in the water column (β = 0.02; *SE* = 0.01; df = 19; *t* = 2.44; *p* = 0.0249), confirming that our fertilization was successful.

Sediment Natural Stable Isotope Composition

There were no significant differences in $\delta^{13}C$ isotopic signatures of sediment contents between low (SB) and high (NB) impacted sites in July and December 2019 (**Table 3**). However, $\delta^{15}N$ isotopic signatures of sediment contents were lower in the SB sites in both, July and December 2019 (**Supplementary Table 5**). $\delta^{15}N$ isotopic signatures of Site 4 (NB) were even two to three times higher than the SB sites (**Supplementary Table 4**).

Sediment $\delta^{13}C$ isotopic contents increased significantly from July to December (**Supplementary Table 5**). Fertilization had no effects on $\delta^{13}C$ and $\delta^{15}N$ isotopic signatures in sediments.

Changes Between Seasons (July 2019 and December 2019) and With Fertilization

Percentage Cover, Shoot Density, and Biomass

Seagrass cover was higher at the SB meadows in July 2019, but increased significantly from July to December at both locations (**Figure 4** and **Supplementary Tables 6A**, **7**). Shoot density did not differ significantly between meadows in the South and North Beach in July 2019 (**Supplementary Table 3**). However, shoot densities showed a trend of being higher in NB sites in both July and December. From July to December 2019, shoot density was declining within both locations in control plots (**Supplementary Table 7**), but increased significantly in plots that had been fertilized (**Figure 5** and **Supplementary Table 6B**).

Aboveground (AG) biomass was significantly higher in NB meadows in July 2019 (**Supplementary Table 7C**). However, both the AG and belowground (BG) biomasses decreased significantly over the course of the experiment at NB and SB sites (**Figure 6** and **Supplementary Table 7**). By December 2019, AG and BG biomass were both significantly higher at NB sites (**Supplementary Tables 6C,D**). Moreover, fertilization led to a significant increase in AG and BG biomass with a more pronounced increase at NB sites. The AG:BG ratio was lower in SB meadows in July 2019, but did not change over the course of the experiment or with fertilization (**Supplementary Table 6E**).

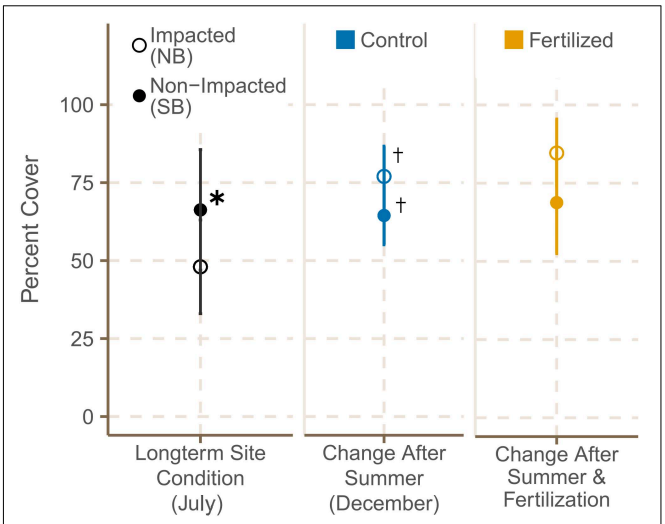

FIGURE 4 | Meadow cover (%) (mean ± SE) of fertilized and control plots in July 2019 and December 2019 and * shows significant differences between impacted (NB) and non-impacted (SB) sites. † indicates if there were significant seasonal differences between July and December.

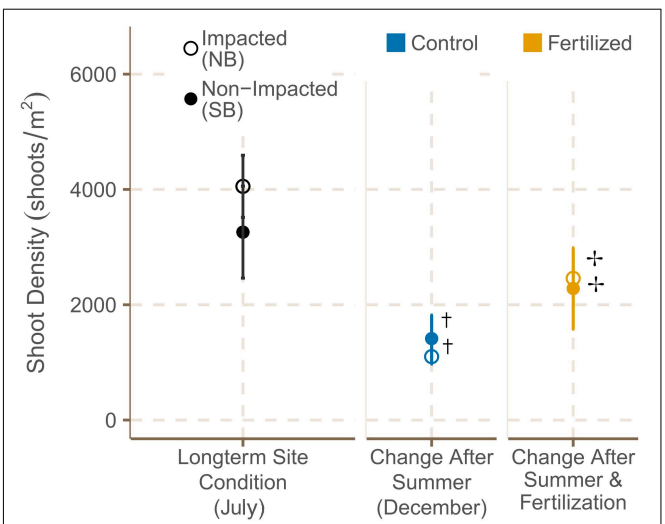

FIGURE 5 | Shoot densities [shoots m^2] (mean ± SE) of fertilized and control plots in July 2019 and December 2019. † indicates if there were significant seasonal differences between July and December and ✢ indicates if there were significant differences between control and fertilized plots.

TABLE 3 | Mean (±SE) values of sediment isotope composition from the anthropogenically impacted sites (North Beach) and low impacted sites (South Beach) in control (*n* = 6) and fertilized (*n* = 6) plots.

Isotopic composition	Site	July 2019	December 2019	
			Control	Fertilized
δ15N	North Beach	3.32 ± 0.35	3.43 ± 0.45	3.51 ± 0.49
	South Beach	1.85 ± 0.30✱	1.08 ± 0.36✱	0.83 ± 0.46✱
δ13C	North Beach	−16.00 ± 1.00	−15.12 ± 0.26†	−15.03 ± 0.15
	South Beach	−14.77 ± 0.66	−14.45 ± 0.12 †	−15.21 ± 0.47✱✢

$\delta^{13}C$ and $\delta^{15}N$ are expressed as parts per thousands. ✱ indicates significant differences between impacted (NB) and non-impacted (SB) sites. † indicates significant seasonal differences between July and December, and ✢ indicates significant differences between control and fertilized plots.

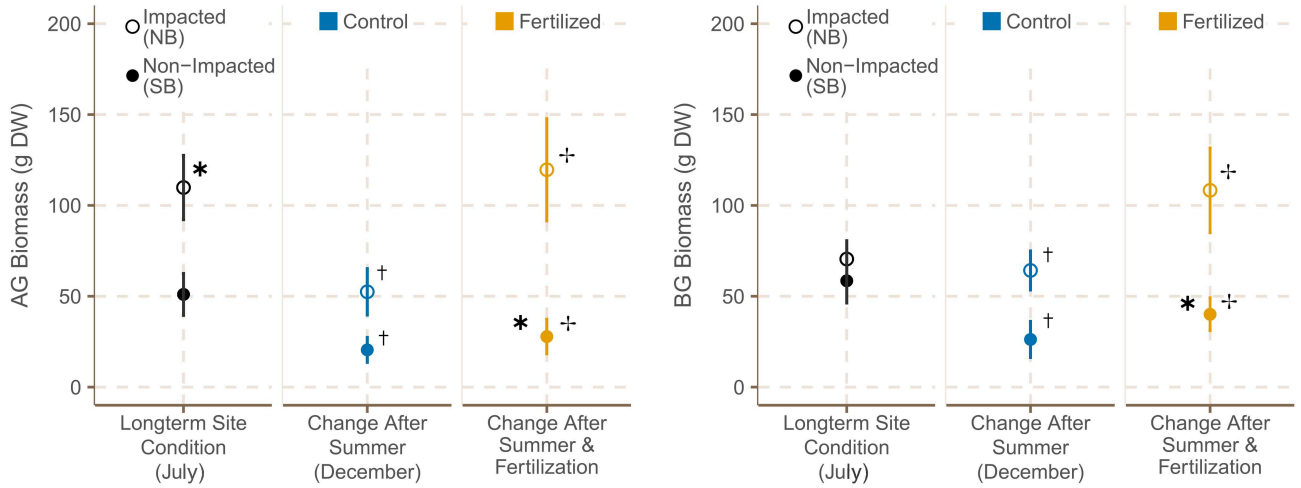

FIGURE 6 | Average below and above-ground biomasses (±SE) from the *H. stipulacea* biomass cores (n = 12) of control (n = 6) and fertilized plots (n = 6) in July 2019 and December 2019. ✱ indicates significant differences between impacted (NB) and non-impacted (SB) sites. † indicates significant seasonal differences between July and December, and ✣ indicates significant differences between control and fertilized plots.

TABLE 4 | Mean (±SE) values of morphological traits from *H. stipulacea* biomass cores (n = 12) in the anthropogenically-impacted sites (North Beach) and low impacted sites (South Beach) in control (n = 6) and fertilized (n = 6) plots.

Morphological traits	Site	July 2019	December 2019	
			Control	Fertilized
Number of leaves per m^2	North Beach	9180.79 ± 869.42	2716.21 ± 311.12†	5595.39 ± 1010.49✣
	South Beach	8571.04 ± 1542.55	3558.24 ± 1025.06†	5622.56 ± 1628.50✣
% apical shoots	North Beach	13.30 ± 0.85	23.72 ± 1.72	15.10 ± 2.39
	South Beach	28.23 ± 2.01✱	22.55 ± 5.63	25.24 ± 3.46
Leaf length	North Beach	4.32 ± 0.05	4.12 ± 0.13	4.67 ± 0.08✣
	South Beach	2.80 ± 0.06✱	2.86 ± 0.10✱	2.86 ± 0.07✱✣
Leaf width	North Beach	1.06 ± 0.02	1.09 ± 0.04	1.25 ± 0.03✣
	South Beach	0.73 ± 0.02✱	0.71 ± 0.03✱	0.63 ± 0.03✱✣
Leaf area	North Beach	2.92 ± 0.06	2.82 ± 0.15	3.45 ± 0.10✣
	South Beach	1.19 ± 0.05✱	1.28 ± 0.07✱	2.32 ± 0.32✣
LAI	North Beach	3.29 ± 0.06	3.17 ± 0.17	3.80 ± 0.13✣
	South Beach	1.34 ± 0.02✱	1.44 ± 0.08✱	2.60 ± 0.36✣
Internodal distance	North Beach	10.44 ± 0.14	9.61 ± 1.18†	9.52 ± 0.28✣
	South Beach	8.44 ± 0.19✱	9.40 ± 0.35✱†	9.05 ± 0.37✱✣
% reproductive shoots	South Beach- S1	41.73 ± 9.93	0.00 ± 0.00	0.00 ± 0.00
	South Beach- S2	8.31 ± 2.34	0.00 ± 0.00	0.00 ± 0.00
	North Beach- S3	7.74 ± 5.99	0.00 ± 0.00	0.00 ± 0.00
	North Beach- S4	6.13 ± 1.34	0.00 ± 0.00	0.00 ± 0.00

Leaf length, leaf width, and leaf area per shoot are expressed in cm and internodal distance in mm, while LAI is expressed as m^2 leaf area per shoot × shoot density per m^{-2}. ✱ indicates significant differences between impacted (NB) and non-impacted (SB) sites.† indicates significant seasonal differences between July and December, and ✣ indicates significant differences between control and fertilized plots.

Morphological Parameters

Low impacted sites (SB) had a higher percentage of apical shoots in their shoot population in July 2019 (**Table 4** and **Supplementary Table 8D**). The number of leaves per m^2 was not significantly different in seagrass meadows from SB and NB sites in July 2019, but decreased significantly in both locations by December 2019 (**Table 4** and **Supplementary Table 9**). However, fertilization resulted in a significant increase in the number of leaves independent of their location, but had no significant effects on percentage of apical shoots (**Table 4** and **Supplementary Tables 8A,D**).

Seagrass plants growing in meadows at SB had significantly smaller and thinner leaves as well as a smaller leaf area and LAI than plants growing in NB in July and in December 2019 (**Supplementary Table 9**). The additional nutrient input resulted in even smaller and thinner leaves in plots at the SB

sites, whereas fertilization had a positive impact on leaf length and width of plants in plots at the high impacted NB sites (**Supplementary Tables 8B,C**).

Seagrasses from the SB sites had significantly smaller internodal distances at the beginning and at the end of the experiment (**Supplementary Table 8F**). Over the course of the experiment, internodal distances were significantly reduced in all plants from both locations (**Supplementary Table 9**). Internodal distances of seagrasses from SB decreased even further due to fertilization. This is contrary to plants from the NB, where internodal distances increased in response to *in situ* fertilization treatment (**Table 4** and **Supplementary Table 8F**).

Reproductive shoots were only observed in July 2019 with higher percentages in the SB Site 1 (**Table 4**). Male reproductive shoots (0.01% of 41.73% reproductive shoots) were only found in SB Site 1.

Carbon, Nitrogen, and Phosphorous Content and $\delta^{13}C$ and $\delta^{15}N$ Stable Isotopes

There were no differences in leaf C, N, P, and *C*:*N* of *H. stipulacea* plants between the SB and NB meadows in July and later in December 2019 from control plots (**Table 5**). Leaf C content and leaf *C*:*N* decreased significantly over the time of the experiment in both locations (**Supplementary Tables 10A,B**).

Rhizome tissue of seagrasses in the low impacted SB sites had significantly lower nitrogen content and higher *C*:*N* ratios in July 2019 (**Supplementary Tables 10E,G**). Moreover, plants from the SB had lower P contents in their rhizomes in the beginning and in the end of the experiment (**Supplementary Table 10H**).

Rhizome N content increased significantly over the course of the experiment and as a response the *C*:*N* of rhizomes decreased significantly in all sites (**Supplementary Table 11**). Plants from the low impacted sites had still significantly higher rhizome *C*:*N* as well as higher C contents in December than their *H. stipulacea* counterparts from the NB site (**Supplementary Tables 10E,F**). Fertilization resulted only in significantly lower *C*:*N* in rhizome tissues of seagrasses from both locations (**Supplementary Table 10E**).

Plants from SB meadows had significantly lower $\delta^{13}C$ isotopic signatures in leaves and significantly lower $\delta^{15}N$ isotopic signatures in rhizomes than seagrasses from the NB sites in July 2019 (**Table 5** and **Supplementary Tables 12A,D**). There were no significant differences in $\delta^{13}C$ and $\delta^{15}N$ isotopic contents in both leaf and rhizome tissues between plants growing in the SB and NB sites in December 2019 (**Table 5**). However, leaf and rhizome $\delta^{13}C$ as well as leaf $\delta^{15}N$ isotopic contents significantly decreased from July to December within both sites (**Supplementary Table 12E**). Fertilization resulted in a significant decrease of $\delta^{15}N$ isotopic signatures in both leaf and rhizome tissues in SB and NB sites.

Non-structural Carbohydrates

The main form of NSC was sucrose in the rhizome tissues, whereas starch was the more dominant NSC in the leaf tissues.

Leaves

Plants growing in the SB sites had significantly higher starch and sugar contents in their leaf tissues at the beginning and at the end of the experiment (**Figure 7** and **Supplementary Tables 13A,B**).

Rhizomes

Sugar and starch contents did not differ between seagrass meadows from NB and SB sites at the start of the experiment. However, sugar was the dominant form of NSC in rhizome tissues of NB plants whereas SB plants had higher starch contents in their rhizomes tissue by December. Starch content in rhizome tissues of seagrasses growing in the SB sites was significantly higher at the end of the experiment than the one of plants growing in the NB (**Figure 7** and **Supplementary Table 13D**). Fertilization resulted in a significant reduction of sugar contents in the rhizomes of seagrasses from both locations (**Table 5** and **Supplementary Table 13C**). Moreover, starch concentrations in the rhizomes of SB plants also showed a decreasing trend with fertilization (**Supplementary Table 4**).

Principal Component Analysis (PCA)

Principal component analysis were performed for the two different sampling months, July and December. In July, the first two components of the PCA explained roughly 50% of the variability (**Figure 8**). Seagrass plots that were assigned to the two treatments (fertilized and control) did initially not differ from each other at the start of the experiment in July 2019. However, there was a distinct difference between the two locations, NB and SB. Also, plots from each of the four sites clustered together, but the two sites within one location (S1 + S2 and S3 + S4) slightly overlapped, showing that they are similar to each other. SB sites were characterized by higher porewater nutrient concentrations, percent cover, starch content in their rhizomes and roots and higher $\delta^{13}C$ isotopic contents in their sediments. NB sites were characterized by larger leaf length, width, LAI and internodal distances as well as increased nutrient content (%N, %C, and P content) and sugar concentrations in their rhizomes and roots. Moreover, plants in the NB sites were related with increased AG and BG biomass, higher AG:BG ratios and were enriched in $\delta^{15}N$ isotopic contents in their rhizomes as well as in their sediments.

In December, SB and NB sites were clearly separated as well as the control and the fertilized plots, although these latter plots showed some slight overlap (**Figure 9**). The first two components of the PCA explained more than 50% of the variability. Plants growing in the SB were related with higher sugar concentrations in their rhizomes and roots and higher $\delta^{13}C$ isotopic contents in their sediments. SB plants from control plots were also characterized by higher starch concentrations in their rhizomes and higher $\delta^{15}N$ isotopic contents in their rhizomes, while SB plants in fertilized plots were associated with a higher percentage of apical shoots in their shoot populations and higher porewater P and N compounds.

On the other hand, plants from fertilized plots in the NB sites, were positively correlated with increased AG and BG biomass and higher P content in their rhizome and roots as well as larger leaf length, width and LAI. Seagrasses from fertilized plots in the impacted NB sites were positively correlated with higher AG:BG ratios as well as increased shoot densities and percent cover. Seagrasses from control plots in both sites were positively correlated with higher rhizome and root tissue nutrient content (%C and %N).

TABLE 5 | Mean (±SE) values of biochemical traits from *H. stipulacea* shoots from the anthropogenically impacted sites (NB) and low impacted sites (SB) in control (n = 6) and fertilized (n = 6) plots.

Biochemical traits	Site	July 2019	December 2019	
			Control	Fertilized
Leaf C	North Beach	23.60 ± 0.58	20.35 ± 0.30†	20.47 ± 0.41
	South Beach	24.76 ± 0.41	21.18 ± 2.47†	23.95 ± 2.07
Rhizome C	North Beach	27.15 ± 0.28	24.81 ± 0.49	24.37 ± 0.94
	South Beach	26.04 ± 0.72	26.36 ± 2.75✱	20.69 ± 0.40
Leaf N	North Beach	1.03 ± 0.02	1.02 ± 0.03	1.19 ± 0.04
	South Beach	1.04 ± 0.04	1.10 ± 0.22	1.27 ± 0.18
Rhizome N	North Beach	0.53 ± 0.03	0.95 ± 0.12†	1.31 ± 0.17
	South Beach	0.36 ± 0.02✱	0.93 ± 0.50†	0.64 ± 0.08
Leaf $\delta^{13}C$	North Beach	−6.80 ± 0.12	−7.88 ± 0.30†	−8.07 ± 0.29
	South Beach	−6.85 ± 0.13✱	−8.22 ± 0.99†	−9.57 ± 1.16
Rhizome $\delta^{13}C$	North Beach	−7.40 ± 0.13	−7.73 ± 0.28†	−8.20 ± 0.35
	South Beach	−7.34 ± 0.16	−8.51 ± 0.96†	−7.66 ± 0.13
Leaf $\delta^{15}N$	North Beach	2.09 ± 0.26	1.16 ± 0.38†	−2.38 ± 0.40✣
	South Beach	1.37 ± 0.18	0.33 ± 0.93†	−0.52 ± 1.77✣
Rhizome $\delta^{15}N$	North Beach	1.28 ± 0.24	0.58 ± 0.38	−4.15 ± 0.46✣
	South Beach	0.75 ± 0.16✱	0.68 ± 1.15	−4.62 ± 0.50✣
Leaf *C:N* ratio	North Beach	22.99 ± 0.52	20.02 ± 0.70†	17.25 ± 0.57
	South Beach	24.17 ± 0.65	20.50 ± 1.62†	19.50 ± 1.07
Rhizome *C:N* ratio	North Beach	52.59 ± 2.88	28.69 ± 4.42†	20.63 ± 3.30✣
	South Beach	74.76 ± 3.57✱	49.54 ± 8.72✱†	35.07 ± 4.30✱✣
Leaf P	North Beach	1499.22 ± 133.35	1515.46 ± 162.22	1580.18 ± 236.69
	South Beach	1171.68 ± 132.54	1310.87 ± 191.34	1244.18 ± 249.36
Rhizome P	North Beach	1704.03 ± 50.10	1854.39 ± 208.95	1837.99 ± 198.91
	South Beach	986.41 ± 69.35✱	793.81 ± 131.69✱	802.34 ± 197.99✱
Leaf starch	North Beach	56.46 ± 3.74	44.31 ± 4.10	40.99 ± 2.99
	South Beach	88.47 ± 6.20✱	82.75 ± 23.34✱	62.08 ± 3.32✱
Leaf sucrose	North Beach	25.00 ± 1.78	22.80 ± 1.49	23.00 ± 1.76
	South Beach	52.28 ± 5.29✱	37.67 ± 4.23✱	34.00 ± 3.69✱
Rhizome starch	North Beach	74.46 ± 4.79	44.41 ± 4.71	49.11 ± 4.61
	South Beach	80.31 ± 4.01	83.05 ± 14.71✱	68.71 ± 4.41✱
Rhizome sucrose	North Beach	121.00 ± 8.31	88.15 ± 18.97	55.00 ± 14.20✣
	South Beach	113.88 ± 8.01	109.77 ± 26.41	72.00 ± 17.58✣

C and N contents are expressed as percentages, while $\delta^{13}C$ and $\delta^{15}N$ are in parts per thousands. P content is shown in $\mu g\ g^{-1}$ and starch as well as sugar contents are expressed as sucrose equivalent mg per g dry weight. ✱ *indicates significant differences between impacted (NB) and non-impacted (SB) sites.*† *indicates significant seasonal differences between July and December, and* ✣ *indicates significant differences between control and fertilized plots.*

DISCUSSION

Our experimental design investigating changes in morphological, biochemical, structural and population level traits proved useful for the identification of indicators for nutrient stress in the tropical seagrass *H. stipulacea* in its native range (northern GoA). It is likely that the effects of fertilization would have resulted in more significant changes in our chosen seagrass indicators if further temporal and spatial replication would have been possible. Despite these limitations, our approach was successful for highlighting the different responses to eutrophication among *H. stipulacea* meadows growing under different levels of anthropogenic pressures (Mejia et al., 2016; Winters et al., 2017), while enduring summer temperatures that were higher than normal, and which potentially surpassed the threshold for *H. stipulacea*'s optimal growth (Nguyen et al., 2020). Our findings provide a good example of the response of a fast-growing opportunistic seagrass species undergoing various levels of local anthropogenic stressors and clearly show the difference to responses of a large-bodied, slow growing seagrass species in a previous study (Helber et al., 2021).

To better compare and visualize the changes in population level metrics and individual plant morphological and biochemical traits that occurred over the season and with the fertilizer treatment, we summarized the most important indicators in **Table 5**. Population metrics of plants from both locations showed generally negative trends. However, the effects of fertilization were strongly dependent on the meadows' location and/or contrasting levels of eutrophication history.

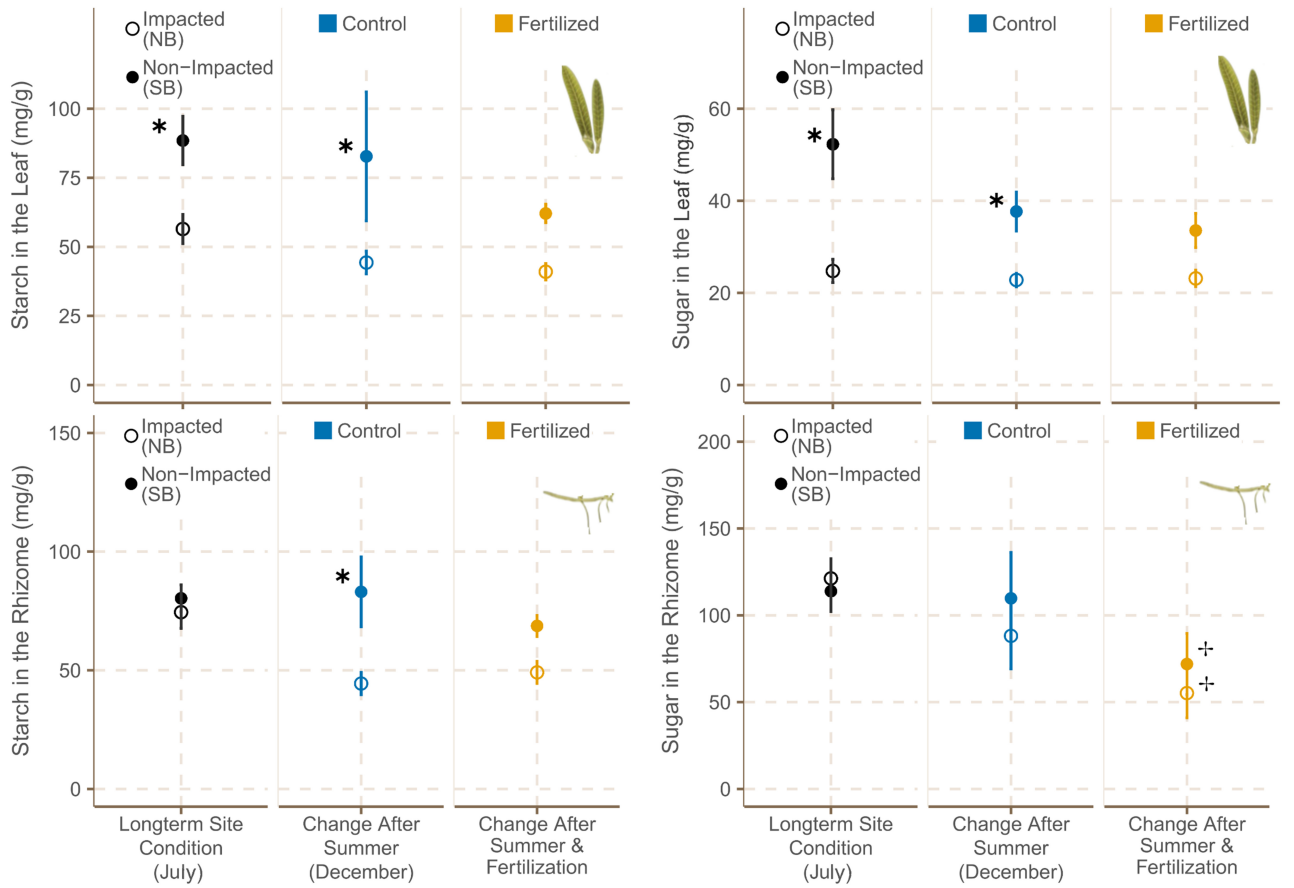

FIGURE 7 | Starch and sugar concentrations [sucrose eq mg g (DW)$^{-1}$] in leaves (mean ± SE) and rhizomes (mean ± SE) of *H. stipulacea* in fertilized (n = 6) and control plots (n = 6) from different sites in July and December 2019. ✱ shows significant differences between impacted (NB) and non-impacted (SB) areas and ✣ states if there are differences between control and fertilized plots.

Fertilization had mainly positive effects on plants from the NB, often resulting in antagonistic effects from seasonal differences, possibly due to temperature stress. In contrast, fertilization negatively affected seagrasses from SB, reinforcing the negative trends of seasonal changes or temperature stress (**Table 6**).

Halophila stipulacea populations from the NB and SB sites showed different responses to *in situ* fertilization even though these two sites are geographically rather close (6–8 km). The *in situ* fertilization treatment, simulating chronic eutrophication had mainly negative effects on seagrass plants from the SB sites, a marine protected area, while *H. stipulacea* populations from the anthropogenically impacted NB sites benefited for the most part from the additional nutrient input (**Table 6**). Lower AG and BG biomasses, reduced LAI, smaller internodal distances, high sexual reproductive effort and the increasing occurrence of apical shoots in seagrasses from SB sites might indicate that the *H. stipulacea* plants are stressed or not growing under optimal conditions at the SB sites. The addition of fertilizer in the SB sites seemed to have pushed plants even further away from their optimal conditions as shown by the reductions in AG and BG biomass and internodal distances. Indeed, these differences between the two sites might suggest yet again that the SB region might not represent the ideal environment for *H. stipulacea* plants, as hypothesized by Mejia et al. (2016) and Beca-Carretero et al. (2020a). While a population genetics study has so far not been conducted in the northern GoA, genotypic selection of *H. stipulacea* plants that are able to grow within eutrophic conditions in the NB sites might have resulted in the locally adapted populations from the NB as demonstrated for other seagrasses growing in areas under high disturbances (Diaz-Almela et al., 2007; Arnaud-Haond et al., 2010; Connolly et al., 2018).

However, plants from the NB sites might experience negative effects by the reduced light penetration in the future as an indirect effect of increasing nutrient pollution. First signs of a compromise in the plants' carbon balance could be detected by a strong decrease in their rhizome's starch contents. Thus, we identified carbohydrate reserves in leaves and rhizomes, LAI, internodal length, reproductive effort and percentage of apical shoots as suitable indicators for stress in this seagrass species in its native range. Additionally, light penetration at sites that receive high nutrient inputs should be monitored. Even more important would be to monitor the maximal depth

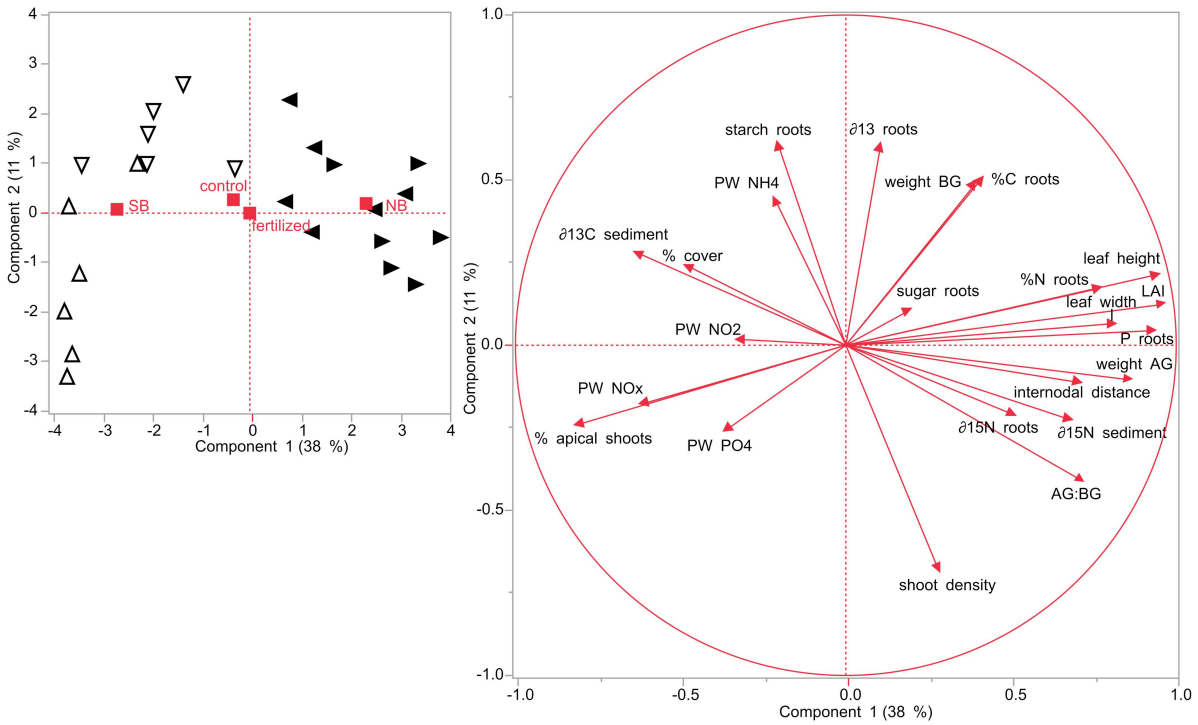

FIGURE 8 | Principal component analysis (PCA) performed on seagrasses from impacted (NB) and non-impacted (SB) sites at the start of the experiment in July 2019. Different symbols refer to the different sites: Δ site 1 (SB), ∇ site 2 (SB), ◀ site 3 (NB) and ▶ site 4 (NB).

boundaries, which would be directly related to the local water quality (Nielsen et al., 2002).

Environmental Conditions Across Sites

Unexpectedly, we did not find any significant differences in water and porewater nutrient concentrations between the SB and NB sites. Higher nutrients at the SB could be related to the neighboring coral reefs, which may add additional nutrients to the area (Silverman et al., 2007; Mejia et al., 2016). Water column nutrient concentrations might not have shown differences between the sites because of the prevailing current regimes. The current in the GoA moves southwards on the western coast leading to a faster dilution of nutrients in the North Beach and at the same time accumulating a higher amount of nutrients in the South Beach (Abelson et al., 1999). Moreover, biogeochemical differences in sediment characteristics (NB, muddy and dense; SB, sandy and loose) and different composition in sedimentary benthic faunal communities might have influenced nutrient dynamics at both sites (Gilad et al., 2018). While we did not find any proof of nutrient enrichment in the water samples of the fertilized NB plots, we did, however, find that fertilized plots in the SB sites were significantly enriched in porewater P and N compounds as well as P in the water column, confirming that our fertilization was successful. The fact that the δ^{15}N isotopic signatures in the tissue samples decreased in fertilized plots in the NB and SB sites, further confirms that the fertilization was successful in both sites (detailed below; **Table 5**). Previous studies (Armitage and Fourqurean, 2009; Teichberg et al., 2013) measured lighter δ^{15}N isotopic signatures in macroalgae and seagrasses in response to the addition of artificial fertilizer, which have δ^{15}N isotopic signatures close to 0‰ (Fourqurean et al., 2005). These results suggest that sampling of water column and porewater nutrients would have probably required a more substantial spatial and temporal sampling in order to detect the effects of the fertilizer addition. It has been shown in previous studies that rapid dilution processes and high phytoplankton grazing resulting in a fast transfer of dissolved nutrients through the food web to higher trophic levels makes it harder to detect signs of eutrophication (Dalsgaard and Krause-Jensen, 2006; Pitta et al., 2009).

The highest temperatures were observed in August with daily average values of 27.88 ± 0.07°C SE at the NB sites and 28.33 ± 0.07°C SE at the SB sites at around 10 m water depth. *In situ* monitoring of water temperature measured at 2 m depth on the IUI's pier confirms that August 2019 water temperatures were 1.0–2.5°C warmer than temperatures normally measured during this month over the last 11 years (the Israel National Monitoring Program at the Gulf of Eilat – Available Data, 2020).

The Morphological and Community-Level Responses of *H. stipulacea* Populations to *in situ* Fertilization

Before we started our experiment in July 2019, there were no significant differences in shoot densities between seagrasses from

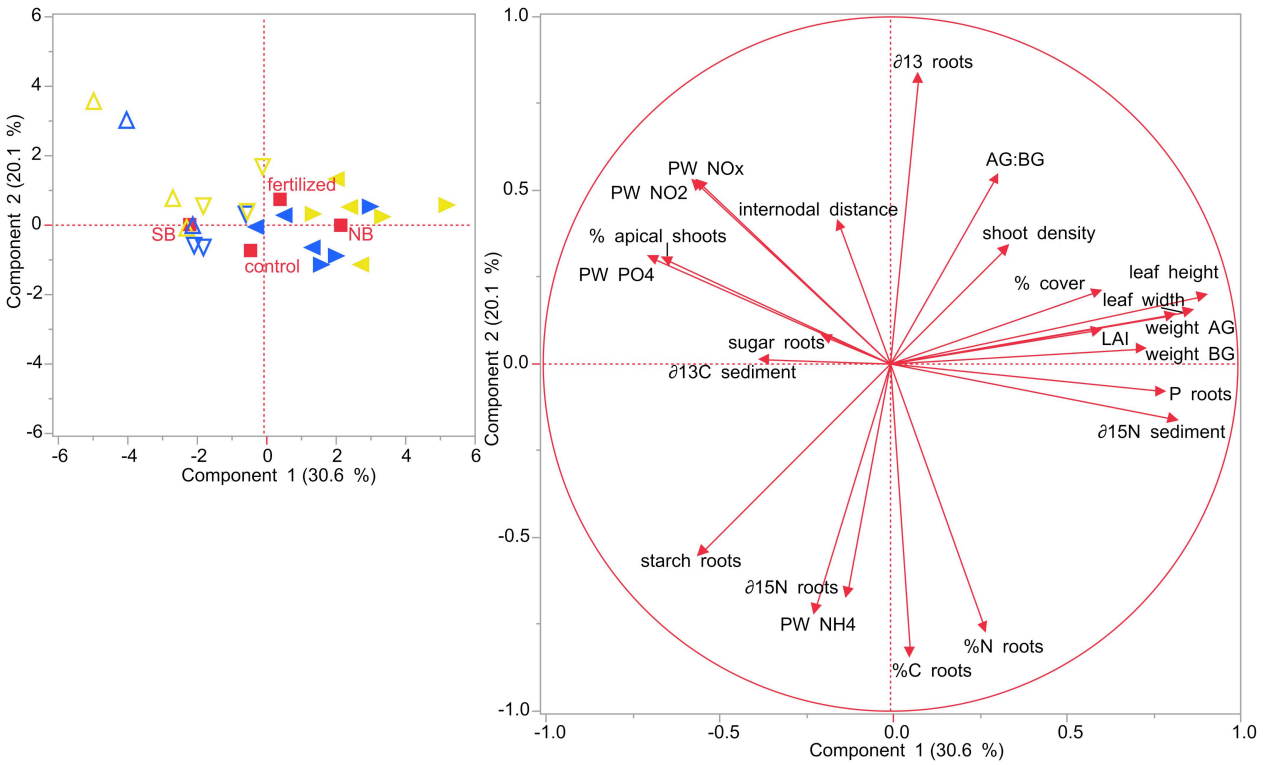

FIGURE 9 | PCA performed on seagrasses from impacted (NB) and non-impacted (SB) sites at the end of the experiment in December 2019. Different symbols refer to the different sites: Δ site 1 (SB), ∇ site 2 (SB), ◀ site 3 (NB) and ▶ site 4 (NB). Yellow colored symbols depict fertilized plots while blue colored symbols show control plots.

SB and NB sites. However, meadow cover was higher in SB sites in the beginning of the experiment, but by December there were no differences in meadow cover between SB and NB sites. Plants from the NB sites produced more AG biomass than SB plants, most likely caused by the larger leaves of plants growing in the NB sites (2.92 ± 0.06 cm^2) compared with the leaf sizes of plants growing in the clearer waters of the SB sites (1.19 ± 0.05 cm^2) (**Table 4**; Mejia et al., 2016; Rotini et al., 2017). Seagrasses from NB populations have adapted to their low irradiance environment and high water turbidity and as a response produced longer and wider leaves resulting in a higher leaf surface area and LAI, as also reported in recent studies (Mejia et al., 2016; Azcárate-García et al., 2020; Beca-Carretero et al., 2020a). This adaptive efficacy helps to improve their carbon balance by increasing their photosynthetically active surfaces (Erftemeijer and Stapel, 1999; Ralph et al., 2007). Indeed, starch content was high in leaf tissues indicating that *H. stipulacea* and especially the NB populations store energy in the form of starch in their leaves, which they can use for new leaf growth and for enlarging their photosynthetically active surfaces.

The addition of fertilizer had different effects on seagrass meadows from low and high impacted sites. Percent cover and shoot density significantly increased in both sites as a response to the additional nutrient input compared to control plots. However, while the additional nutrient input resulted in a significantly enhanced biomass production in plants from the NB, there was only a small trend of an increase in biomass of seagrasses growing in fertilized plots in the SB sites.

Individual Plant Responses as Indicators of Stress

Morphological Traits

Internodal distances of plants growing in the SB sites were smaller than the ones of seagrasses from the NB, and decreased further with fertilization, probably as a response to nutrient stress. Shorter internodal distances have been shown to be a common response to environmental stress and might serve as a proxy of increased shoot and/or root densities (Kilminster et al., 2008; Holmer et al., 2011; Viana et al., 2020). This is in accordance with seagrasses from the SB having shorter internodal distances and investing more energy in roots than in leaf formation, as shown by their reduced AG:BG ratio. Seagrasses from the SB had a higher percentage of apical shoots as well as reproductive shoots in their shoot population in July 2019. Reproductive shoots were only found in July and seagrass populations at both locations were female-biased, confirming previous studies (Malm, 2006; Nguyen et al., 2018). In their review across thirty-two studies for 11 seagrass species, Cabaço and Santos (2012) showed that an increase in sexual reproductive efforts were strongly associated with responses to stress or when plants are growing in unfavorable environments. Furthermore,

TABLE 6 | Summary of significant differences in responses of the most important indicators affected by season and by the addition of fertilizer as well as their interactive effects.

	Seasonal differences		Effect of fertilization		Interactive effects	
Indicator	**NB**	**SB**	**NB**	**SB**	**NB**	**SB**
Meadow cover	↑	↑	↑	↑	↑	↑
Shoot density	↓	↓	↑	↑	↕	↕
AG biomass	↓	↓	↑	↓	↕	↓
BG biomass	↓	↓	↑	↓	↕	↓
Number of leaves per m^2	↓	↓	↑	↑	↕	↕
Leaf length	↔	↔	↑	↓	↔	↔
Leaf width	↔	↔	↑	↓	↔	↔
Leaf area	↔	↔	↑	↑	↔	↔
LAI	↔	↔	↑	↑	↔	↔
Internodal distance	↓	↓	↑	↓	↕	↓
Leaf C content	↓	↓	↔	↔	↔	↔
Leaf *C:N* ratio	↓	↓	↓	↓	↓	↓
Rhizome N content	↓	↑	↔	↔	↔	↔
Rhizome *C:N* ratio	↓	↓	↓	↓	↓	↓
Leaf starch	↓	↓	↓	↓	↓	↓
Leaf sugar	↓	↓	↑	↓	↔	↔
Rhizome starch	↓	↑	↑	↓	↑	↑
Rhizome sugar	↔	↔	↓	↓	↔	↔

Statistically significant effects on seagrasses from the high impacted sites in NB are represented by brown color while statistically significant effects on plants from the low impacted sites in the SB, are represented by the green colors. The direction and size of the arrows represent the direction and strength of the effects, respectively. Gray arrows represent trends that were not statistically significant ($p > 0.05$).

the predominant presence of apical shoots might also indicate that the plants are not growing under optimal conditions at the SB sites (as discussed above). There was a trend of a further increase in apical shoots with fertilization at the SB sites, while the opposite trend was observed in seagrasses from NB (**Table 4**). It has been shown that plants exposed to stressors have a higher number of apical shoots in their shoot population to secure the persistence of their population (Ruocco et al., 2020). In this way, seagrasses are able to spread and find more favorable habitats (Meinesz and Lefevre, 1984; Ruocco et al., 2020).

Moreover, seagrasses from SB had smaller and thinner leaves and a smaller LAI. LAI combines individual plant traits (leaf area and number of leaves) with population level metrics (shoot density) that have been shown to decrease in response to anthropogenic pressures (Guidetti, 2001; Leoni et al., 2006; Karamfilov et al., 2019; Kletou, 2019; Zulfikar and Boer, 2020). LAI was found to be the most significant determinant of ecosystem primary production and negative changes might thus have devastating impacts on seagrass ecosystems such as impairing their carbon sequestration capacity (Asner et al., 2003; Barr et al., 2004; Saigusa et al., 2005; Wang et al., 2019).

Nutrient Content and Isotopic Signatures ($\delta^{15}N$ and $\delta^{13}C$)

Seagrasses from both NB and SB sites seem to be N- limited in the oligotrophic conditions of the GoA, since %N in leaves and rhizomes was well below 1.8% DW as previously reported (Duarte, 1992; Cardini et al., 2018; Beca-Carretero et al., 2020a). The N as well as P contents were also significantly lower in rhizomes of plants from the two SB sites, suggesting that NB sites receive higher nutrient inputs than the protected SB sites. Additionally, seagrass tissues from NB sites were markedly enriched in $\delta^{15}N$ in both their leaf and rhizome tissues (**Supplementary Table 4**). Seagrasses from fertilized plots in December were markedly depleted in $\delta^{15}N$ in both their leaf and rhizome tissues (**Supplementary Table 4**), further

confirming the fertilization success as synthetic fertilizers have $\delta^{15}N$ signatures ranging from −2‰ and 2‰ (Bateman and Kelly, 2007). Sedimentary $\delta^{15}N$ was shown to be a more reliable indicator for changes in anthropogenic pressures and the exposure to sewage effluents was reflected in $\delta^{15}N$ values of 4–6‰ which were measured in sediments of Site 4 in the NB area (**Supplementary Table 4**; Ruiz-Fernández et al., 2002; Lapointe et al., 2004; Román et al., 2019). In contrast, plants from the two SB sites had significantly lower $\delta^{15}N$ isotopic signatures in their underlying sediments. This further indicates that the NB plants might be exposed to land-based anthropogenic nutrient inputs [suggested also by Winters et al. (2017) as N content in seagrass tissue was shown to be an indicator for their long-term nutrient exposure (Udy et al., 1999; Fourqurean et al., 2006; Mejia et al., 2016; Beca-Carretero et al., 2020a)]. This area is also exposed to sewage run-off, the effects of flash floods, and the Kinnet Canal that bring nutrients into the local meadows (Mejia et al., 2016; Winters et al., 2017). In July as well as in December we found higher SPM concentrations in the water column of the NB meadows. Higher nutritional content of leaves resulted often in increased consumption rates of herbivores and leaf palatability was observed to be greater under chronic nutrient pollution and in combination with warming and acidification (Jiménez-Ramos et al., 2017; Campbell et al., 2018; Ravaglioli et al., 2018). However, mesograzers (such as small crustaceans) were shown to buffer eutrophication effects by consuming epiphytic algae that would otherwise overgrow seagrass leaves and compete with the plants for light and nutrients (Heck et al., 2006; Reynolds et al., 2014). We did not measure grazing pressure and/or quantify herbivore communities at SB and NB sites. However, grazing activity might be indeed higher at NB sites as those seagrasses had a significantly higher percentage of leaves with lost apex (Mejia et al., 2016).

The long-term exposure to nutrients in the NB area, caused not only by the proximity of the hotel strip and marina, but also by the sporadic sewage run-offs and winter flash floods (Katz et al., 2015; Winters et al., 2015), might have resulted in creating a rather nutrient "spoilt" *H. stipulacea* population, that is always in demand for even more nutrients. Plants growing in those sites seem to benefit from the additional nutrients since their N demands are not met in their environment. Our results are in agreement with previous studies in the Caribbean showing that *H. stipulacea* is able to form extremely dense mats under increased nutrient conditions even in sulfidic sediments (van Tussenbroek et al., 2016).

Carbohydrates

Plants from SB had higher starch concentrations in their rhizomes by December 2019. In contrast to plants from NB, fertilization seemed to negatively impact seagrasses from SB, resulting in a small decrease of SB plant's rhizome starch content of around 14%. The considerable depletion of rhizome starch contents (reduction of 35–40%) 5 months after the summer heat peak in *H. stipulacea* populations in NB might have been the result of the constrained light penetration during the summer months on these sites as evidenced by the higher water turbidity (**Supplementary Figure 1**) and the higher chl *a* concentrations measured (Roca et al., 2016; Krause-Jensen et al., 2020; **Supplementary Figure 1**). Under low light availability, plants do not meet their energy demands through photosynthesis and need to use up their carbohydrate reserves (Alcoverro et al., 2001; Ruiz et al., 2001). Additionally, future warming (already occurring in the northern GoA; Fine et al., 2013; Nguyen et al., 2020) will likely further increase respiration rates (Ryan, 1991; Rasmusson et al., 2020), thereby increasing the light requirements of *H. stipulacea* populations in NB. This might cause a negative C budget which might lead in the future to a further depletion of their carbohydrate reserves (Lee et al., 2007; Beca-Carretero et al., 2018; Krause-Jensen et al., 2020). In addition to the remobilization of carbohydrates, seagrasses are able to cope with reduced light availability by increasing their rate of carbon fixation per unit biomass as shown in the morphological adaptations (longer and wider leaves) and by the increased photosynthetic pigment content of plants growing in NB sites (Mejia et al., 2016; O'Brien et al., 2018a). These populations receive less light than seagrasses growing in the SB; however, the light reduction at 10 m is not too limiting as the meadows reach down to 30 m depths (Winters et al., 2017) and our plots were established at around 10 m depth.

Seagrasses growing in the low impacted SB sites also experienced a depletion of their leaf sucrose content by 28–35%, which has been considered a general indicator for stress in different seagrass species (Roca et al., 2016). Recent climate change simulations based on mesocosm studies have shown that *H. stipulacea* populations from NB experienced temperature stress and reduced fitness after a 2 weeks exposure to temperatures of beyond the possible thermal threshold of 29°C, resulting in reduced growth rates, leaf sizes and the loss of shoots (Nguyen et al., 2020). Additionally, it was shown in a mesocosm experiment with *H. stipulacea* from the Indian Ocean that 31°C is above its thermal optimum (Viana et al., 2020). Maximum temperatures close to those (29.7–30.4°C) have been reached at 10 m depth over the summer months (6–30 consecutive days depending on the site) in 2019 in the northern GoA and may be also a reason for the observed decrease in their rhizome starch contents.

ECOLOGICAL IMPLICATIONS

Known for its ability to grow in a wide range of light conditions (i.e., depths), salinities, temperatures, and substrates [reviewed by Winters et al. (2020)], our findings show that *H. stipulacea* is also able to acclimate to environments with different levels of nutrients confirming its high plasticity (Viana et al., 2020; Winters et al., 2020). This plasticity was mostly associated with morphological and biochemical responses of *H. stipulacea* to the experienced stressors. In addition to the response to the nutrients themselves (i.e., the *in situ* fertilization treatment; discussed above), changes in structural and demographic/population-level traits occurred also as a response to different seasons. Nutrient enrichment, as shown in our study, combined with summer temperatures that surpassed the threshold for optimal growth, had only negative effects on performance of plants from the more

pristine SB sites, located within a marine protected area. Seagrass plants growing in the protected SB sites showed first signs of stress under the simulated eutrophication and high summer temperatures by reduction in NSC, sugar (decline of 37%) and starch (decline of 15%) contents of their rhizomes. Furthermore, plants in SB demonstrated declining internodal distances and an increase in the frequency of their apical shoots within their shoot population, which are confirmed indicators for stress in this and other seagrass species (Kilminster et al., 2008; Holmer et al., 2011; Ruocco et al., 2020; Viana et al., 2020). Moreover, LAI of NB populations was approximately twice as high as the ones of *H. stipulacea* growing in SB at the same depth. LAI in low impacted sites decreased even further with fertilization, indicating that seagrasses from SB sites might suffer impairments in their ecosystem functions, such as their carbon sequestration capacity, under future scenarios that include eutrophication of the oligotrophic GoA. In contrast, the limited response to nutrient stress of *H. stipulacea* populations from NB suggests that these populations are well adapted to the conditions in this environment (low irradiance, high water turbidity and high nutrients). The fact that NB populations perform even better under the additional nutrient inputs further supports previous suggestions of Cardini et al. (2018) and Beca-Carretero et al. (2020a) that *H. stipulacea* living in these vast NB meadows is actually N limited.

However, future studies should look into the genotypic and genetic diversity of local meadows, as it has been shown that reduced genotypic and genetic diversity might lead to constraints in the future adaptive potential of plants, resilience to stress, and sexual reproductive efforts (Linhart and Grant, 1996; Ehlers et al., 2008; Tiffin and Ross-Ibarra, 2014). Rhizome starch contents were significantly depleted (reduction of 35–40%) after the summer heat peak in NB seagrasses, which might have been the result of the constrained light penetration during the summer months on these sites as indicated by the high water turbidity and higher chl *a* concentrations in comparison to SB sites (Roca et al., 2016; Krause-Jensen et al., 2020; **Supplementary Figure 1**). With the GoA being one of the region's most popular tourist attractions, alongside it being one of its strongest economic growth engines, large coastal development projects are taking place on both sides of the Gulf[1]. These ongoing and increasing human activities so close to local *H. stipulacea* meadows may further deteriorate water quality and light penetration. This needs to be closely monitored as temperature maxima in the GoA and their duration will increase in the future (Fine et al., 2013; Nguyen et al., 2020) close to values that are not only above their optimum growth temperatures, but even beyond their upper thermal tolerance. Thus, nutrient loading from human related activities might make seagrasses at the NB sites even more sensitive to global warming in the future.

Together with recent mesocosm simulated climate change experiments (Nguyen et al., 2020), the results of our study demonstrate that global warming and increased coastal nutrient pollution might lead to vast reductions of *H. stipulacea* populations from the SB area. In contrast, seagrasses from the NB area seemed to be mainly unaffected or performed even better under the enriched conditions. These differences between a stress sensitive population vs. a stress resilient population, are similar to recent work on native (from the Red Sea) vs. invasive *H. stipulacea* populations from Greece and Cyprus (Nguyen et al., 2020; Wesselmann et al., 2020). Using a common garden simulated thermal stress experiment, Nguyen et al. (2020) showed that native *H. stipulacea* plants were negatively affected in photo-physiological and growth responses by thermal stress, while the invasive plants did not suffer and might have even benefited from it.

Recent work by Chiquillo et al. (2018) used 2bRAD genotyping (Wang et al., 2012) and found that genotypic diversity of the invasive Mediterranean *H. stipulacea* populations was lower than in the native populations in the GoA [further indicating to a genotypic selection of the invasive population as was also shown for *Zostera muelleri* in Moreton Bay, Australia (Padfield et al., 2016; Connolly et al., 2018; Molina-Montenegro et al., 2018)]. Invasive *H. stipulacea* populations in the Mediterranean were even able to adapt their thermal niche to the colder winter temperatures experienced in their new habitat (Wesselmann et al., 2020). The ability for this rapid evolutionary adaptation and to sexually reproduce in its invasive habitat (Gerakaris and Tsiamis, 2015; Nguyen et al., 2018) makes *H. stipulacea* an exceptionally strong competitor for other macrophytes.

It was shown in recent mesocosm and field experiments (Pazzaglia et al., 2020; Helber et al., 2021) that eutrophication and higher temperatures will have detrimental effects on *P. oceanica*, the dominating key foundation seagrass species in the Mediterranean. Nitrogen load thresholds for this endemic seagrass were identified to range between 0.8 and 1.1 t N km^{-2} over 6 months (Fernandes et al., 2019). Especially in areas where plants were already exposed to higher anthropogenic pressures, *P. oceanica* activated strategies to cope with the additional nutrient input that probably were energetically costly as indicated by cutbacks in their carbohydrate reserves (Pazzaglia et al., 2020; Helber et al., 2021). In contrast, *H. stipulacea* populations from NB in the current study seem to thrive under the additional nutrient supply, similar to invasive populations in the Caribbean (Willette et al., 2014; van Tussenbroek et al., 2016; Winters et al., 2020).

Thus, while future ocean warming and the rising frequency of heat waves and high cultural eutrophication will most likely lead to further losses in *P. oceanica* meadows (Coma et al., 2009; Marbà and Duarte, 2010; Jordà et al., 2012; Chefaoui et al., 2018), emerging new habitats might be available for the colonization by better adapted macrophytes, especially the invasive *H. stipulacea* (Klein and Verlaque, 2008; Montefalcone et al., 2010; Sghaier et al., 2011; Beca-Carretero et al., 2020b). Indeed, it was predicted that climate change and anthropogenic impacts might make around 85% of the Mediterranean coastline suitable for the colonization by *H. stipulacea* (Beca-Carretero et al., 2020b). The resulting regime shift in the seagrass community might not only have far reaching consequences on ecosystem functions, but also lead to massive economic losses due to the loss of ecosystem services provided by *P. oceanica*

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(Vassallo et al., 2013; Campagne et al., 2014; El Zrelli et al., 2020). Our results highlight the high acclimation potential of *H. stipulacea* found growing in high nutrient environments, giving it an advantage over other seagrasses less tolerant to nutrient stress. These results have important implications to management and conservation efforts, not only in shallow coastal areas where *H. stipulacea* is the native species, but also (and maybe even more) in both its historical (Mediterranean) and the new invasive (Caribbean) habitats.

DATA AVAILABILITY STATEMENT

The original contributions generated for this study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

SH, GW, HR, and MT conceived and designed the experiments. EB and MiS contributed in designing the experiments. MiS, MaS, and SB assisted with the fieldwork and during sampling campaigns. SH performed the field experiments, developed methodologies, and performed all the other laboratory analyses together with SB. EB did the statistical analysis. EB and MaS did the graphs and worked on the manuscript. All authors wrote and reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.678341/full#supplementary-material

Supplementary Figure 1 | Monthly averages of sechi disk depth [m] and chlorophyll a concentration [$\mu g\ l^{-1}$] in 2019 at South Beach and North Beach sites. The data have been obtained from Israel's National Monitoring Programme (NMP) of the Gulf of Eilat (http://iui-eilat.ac.il/Research/NMPMeteoData.aspx; accessed 03/06/2021).

Supplementary Figure 2 | Yearly averages of sechi disk depth [m] and chlorophyll a concentration [$\mu g\ l^{-1}$] from 2010 to 2019 at South Beach and North Beach sites. The data have been obtained from Israel's National Monitoring Programme (NMP) of the Gulf of Eilat (http://iui-eilat.ac.il/Research/NMPMeteoData.aspx; accessed 03/06/2021).

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How Does Ocean Acidification Affect the Early Life History of *Zostera marina*? A Series of Experiments Find Parental Carryover Can Benefit Viability or Germination

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Elevated partial pressure of carbon dioxide (pCO_2) as a concomitant of global climate change may facilitate the establishment of future seagrass meadows and subsequently its benefit could be incorporated into techniques to increase restoration success. In five manipulative experiments, we determined how increased CO_2 affects the maturation of flowers, and the development of seeds and seedlings for the foundation species *Zostera marina*. Experiments tested the development from both seeds collected from non-treated flowering shoots (direct) and seeds harvested from flowering shoots after CO_2 exposure (parental carryover). Flowering shoots were collected along the western coast of Sweden near the island of Skafto. The seeds produced were used in experiments conducted at Kristineberg, Sweden and Dauphin Island, AL, United States. Experiments varied in temperature (16, 18°C) and salinity (19, 33 ppt), as well as duration and magnitude of elevated CO_2 exposure. Flowering maturation, spathe number, seed production, and indicators of seed quality did not appear to be affected by 39–69 days of exposure to CO_2 conditions outside of natural variability (pCO_2 = 1547.2 ± 267.60 μatm; pH_T = 7.53 ± 0.07). Yet, seeds produced from these flowers showed twofold greater germination success. In another experiment, flowering shoots were exposed to an extreme CO_2 condition (pCO_2 = 5950.7 ± 1,849.82 μatm; pH_T = 6.96 ± 0.15). In this case, flowers generated seeds that demonstrated a fivefold increase in an indicator for seed viability (sinking velocity). In the latter experiment, however, germination appeared unaffected. Direct CO_2 effects on germination and seedling production were not observed. Our results provide evidence of a parental CO_2 effect that can benefit germination or seed viability, but early benefits may not lead to

bed establishment if other environmental conditions are not well suited for seedling development. Outcomes have implications for restoration; CO_2 can be supplied to flowering shoot holding tanks to bolster success when the purpose is to redistribute seeds to locations where beds are extant and water quality is adequate.

Keywords: CO_2, parental investment, seedlings, seagrass restoration, seed viability

INTRODUCTION

Seagrass meadows serve as important ecosystem engineers hallmarked for their ability to sequester carbon and provide habitat for a diversity of marine organisms (Heck and Orth, 1980; Hemminga and Duarte, 2000; Hernán et al., 2017). Seagrasses are in decline due to anthropogenic stressors, like eutrophication and ocean warming, which have accelerated global decline in coverage while simultaneously increasing susceptibility to natural stressors like disease (Orth et al., 2006; Waycott et al., 2009; Zimmerman et al., 2015). Loss of highly productive seagrass beds represents a significant disruption to global carbon cycling. In the context of changing ocean carbonate chemistry, seagrasses serve as valuable carbon sinks utilizing carbon dioxide for photosynthesis and burying carbon in their root and rhizome mass. Efforts to restore seagrass beds serves as a possible adaptive strategy for protecting coastal systems from disruptions in marine carbonate chemistry (Unsworth et al., 2012).

The ocean absorbs carbon dioxide (CO_2) from the atmosphere increasing seawater (SW) concentrations of inorganic carbon [DIC] and CO_2 while simultaneously decreasing pH in a process referred to as ocean acidification (OA). Carbon in its dissolved form [DIC] can exist as one of three species: aqueous carbon dioxide [$CO_{2(aq)}$], bicarbonate (HCO_3^-), and carbonate (CO_3^{2-}) (Doney et al., 2009). OA has resulted in bicarbonate rather than carbonate dominating in the water column; reducing the amount of carbonate available for marine calcifers (Sabine et al., 2004; Caldeira and Wickett, 2005). The reduction in carbonate is also linked to an excess release of H^+ decreasing pH and leading to OA (Doney et al., 2009; Hall-Spencer and Harvey, 2019). Surface ocean average pH has decreased by 0.1 units since the beginning of the industrial revolution with a further decline (0.06–0.32 units) projected over the next century (Ciais et al., 2013).

Under current ocean conditions HCO_3^- is already widely available and CO_2 is the smallest pool of DIC. CO_2 will have the greatest percent increase in the next century (Koch et al., 2013). Seagrasses can use bicarbonate, but preferentially use CO_2 for photosynthesis with rates experimentally increasing in response to elevated partial pressure CO_2 (pCO_2) (Invers et al., 1997; Jiang et al., 2010; Koch et al., 2013; Cox et al., 2015). Indirect responses to elevated pCO_2 have been described as increases in above and below ground biomass (Zimmerman et al., 1997; Russell et al., 2013) as well as increases in flowering frequency (Palacios and Zimmerman, 2007). Increasing pCO_2 may counteract the negative impacts of rising sea surface temperature (e.g., reduced photosynthesis, reduced growth, increased physiological stress, increased rate of mortality). Although the mechanism is unclear, Zimmerman et al. (2015) hypothesize that elevated pCO_2 provides additional carbon reserves necessary to promote physiological repair despite thermal stress, thus inferring that increased pCO_2 may improve landscape level resiliency (Zimmerman et al., 2015, 2017). For climax species like *Zostera marina*, it is still unclear how short-term changes in plant performance under high pCO_2 fit into long-term carbon budgets or how elevated pCO_2 will integrate into the life history of the plant (Campbell and Fourqurean, 2013; Takahashi et al., 2015; Cox et al., 2016). OA has the potential to stimulate seagrass parental investment into the production and quality of seeds to ultimately facilitate seedling establishment and increase population growth.

Remarkably little is known about how conditions experienced during the flowering stage influence phenotypic and genetic diversity of seagrass populations at large (Höckerstedt et al., 2021). A handful of published studies indicate a positive effect of OA on flowering frequency, seed quality, germination, and seedling carbon gains (Palacios and Zimmerman, 2007; Burnell et al., 2014; Hernán et al., 2016). Seeds generated at a high pCO_2 (>1,550 μatm) may have higher carbon content and stored sucrose; thus, allowing an advantage in establishment and growth (Hernán et al., 2016). Additionally, when *Ruppia maritima* seeds were passed through fish digestive tracks (pH < 7), germination was facilitated. Therefore, low pH may trigger the rupture of the seed coat (Agami and Waisel, 1988). This is also observed in terrestrial species where low sediment pH (<7) induces germination for species like *Stylosanthes humilis* (Pelacani et al., 2005). Although low pH may enhance germination, studies focused on this topic are largely confined to the terrestrial realm with limited published material available for marine angiosperms.

Initiatives using seeds for seagrass restoration have primarily focused on small scale dispersal efforts except for the Chesapeake Bay, United States where large scale efforts resulted in the most successful restoration effort worldwide (Orth and McGlathery, 2012). Recent evidence suggests an upswing in bed surface coverage for some regions in Europe and southeast Florida, United States (Tomasko et al., 2018; de los Santos et al., 2019). Increases in coverage are associated with improved water quality and may be indirectly linked to alterations in carbonate chemistry as result of global change. More available (DIC) decreases dependency of improved water quality and provides more substrate available for photosynthesis (Zimmerman et al., 1997; Invers et al., 2002). Carbon enrichment may provide additional below ground carbon stores to bolster response to thermal stress (Zimmerman et al., 2015, 2017; Wilson and Lotze, 2019). This, in combination with hypothesized increases in parental investment, may serve to bolster restoration efforts and facilitate the establishment of meadows in locations now extant.

The aims of this study were twofold: (1) determine how OA influences flowering, seed production, seed quality, and seedling development of *Zostera marina* and (2) develop novel techniques geared toward rearing healthier, more viable seeds. We hypothesize that flowering shoots exposed to a short-term exposure of elevated pCO_2 will increase spathe production and maturation, seed production, and investment into seed quality to ultimately enhance seedling development. Short term exposure to elevated pCO_2 was used as a preliminary step to understand how low pH-high pCO_2 will influence germination and seedling development as well as to determine if it is a feasible restoration technique. Secondly, we tested whether low pH-high pCO_2 has direct effects on germination and seedling development. Previous evidence demonstrates the efficacy of low pH/salinity shocks to stimulate germination (Pelacani et al., 2005). We hypothesize an overall positive effect of increased pCO_2 on the production and development of seedlings.

MATERIALS AND METHODS

Five experiments were done to test for CO_2 effects on flowering maturation, seed production and quality, and seedling development. An overview of experimental setup, images, and conditions can be found in **Figure 1**, **Table 1**, and **Supplementary Figure 1**.

Flowering Shoot and Ambient Seed Collection

Reproductive shoots of *Zostera marina* were harvested from two bays located on the West coast of Sweden, Gåsö (58°13′48.3N 11°23′43.7) on 17-July-2017 and Skallhavet (58°11′58.3N 11°26′34.6) on 9-August-2018. After harvesting, shoots were transported to the Kristineberg Marine Research Station. Shoots were used for two main tasks, (i) to test the effect CO_2 on flowering maturation and seed development and (ii) to later test the effect of CO_2 on seed germination and seedling development.

Shoots harvested for seed collection developed outdoors in a seawater (SW) flow-through 1,500 L tank until September (pH_T 7.97 ± 0.06, temperature: 25.77°C ± 1.51; salinity: 21.96 ppt ± 3.98). The tank was aerated with compressed air to increase water mixing and prevent hypoxia. Shoots were covered with a PVC frame to submerge shoots just below the water to avoid desiccation [see Infantes and Moksnes (2018) for full description]. In September of both years, seeds were siphoned from the bottom of the tank and stored in the dark at 4°C, 32 ppt. Studies conducted at the Kristineberg Marine Research Station have shown that these conditions prevent germination (Infantes et al., 2016). Additional shoots, harvested to test the effect of elevated pCO_2 on seed development, were held overnight and immediately used in laboratory experiments.

Experiment 1: Effect of CO_2 on Spathe and Seed Development

The effect of CO_2 was tested by exposing flowering shoots to two treatments (n = 5): a low (∼380 μatm; pH_T = 8.1) and elevated pCO_2 (∼1,800 μatm; pH_T = 7.5). In the fjords of Sweden, pH can have a diel variability of 0.9 units (minimum pH_T ∼ 7.6) (Dorey et al., 2013). The low pH treatment is therefore outside the present range of variability and within range projected for 2,100 [−△ 0.3 pH units relative to the present natural variability following the RCP 8.5 scenario (Schwalm et al., 2020)].

One hundred and twenty flowering shoots were randomly distributed across ten 3-L containers with each container populated by 12 shoots. Shoots had an overall mean length of 61 ± 15.8 cm. The ten containers were arranged into pairs (n = 5) under a light bank (18:6 h light: dark cycle, 82 μmol m^{-2} s^{-1} at the canopy) with temperature ranging from 16.1 to 18.3°C and salinity ranging from 24–32 ppt. Light was reduced 20% from field observations, but this reduction was compensated for by using an elongated growing period (Björk et al., 2021). Seawater passed through a 500 μm filter entering the bottom of each container and continuously overflowed averaging a minimum of 5 turnovers per hour. Air was bubbled into both ambient and treated containers to prevent hypoxia and enhance water mixing. For clarity, individual flowers are referred to as spathes. Like seeds, spathes go through stages of development with earlier stages (1–3) being comparatively immature to later stages (5–6) (Infantes and Moksnes, 2018; **Supplementary Figure 2**). Prior to pH manipulation, spathes in stages 4 and 5 (seeds that are mature or have been released) were removed to ensure that the flowers matured, and seeds developed under experimental conditions for 69 days. Spathes continue to develop and drop seeds through September in temperate environments, therefore we carried the experiment through until all seeds developed and dropped (Infantes and Moksnes, 2018).

Five AquaMedic pH stat systems (controller, probe, CO_2 tank) were utilized for each carbon treatment and controlled the delivery of pure CO_2 into the containers. pH_T was measured twice weekly in containers using a glass potentiometric probe (Metrohm 827 pH Meter) calibrated on total scale with TRIS and AMP buffers (Université de Liege) at a salinity of 35 ppt. The stat system was adjusted at each measuring interval to maintain targeted experimental conditions. At weekly timepoints, 80 mL of seawater was collected from each mesocosm, filtered through a Whatman G/F filter, and refrigerated. Samples were analyzed for Total Alkalinity (A_T) using an SI Analytics TitroLine alpha plus. A_T and pH_T were used to calculate the carbonate chemistry of the system using CO2SYS v2.1 with dissociation constants set to Mehrbach et al. (1973) refit by Dickson and Millero (1987) (Lewis and Wallace, 2006).

Measurements of flowering maturation were initiated 1 week after the commencement of the experiment and assessed on nine to 10-day intervals for a total of 39 days following the methods of Infantes and Moksnes (2018). Under normal conditions, spathe development occurs at approximately one stage per week, thus our measuring intervals correspond to spathes naturally moving through development (Infantes and Moksnes, 2018). Number of spathes and number of developing and mature seeds within each spathe were measured on every flowering shoot. Flowering stage was also recorded for all spathes from each shoot as described by De Cock (1980). Flowering stages were classified as: (1) styles are erect from the spadix, (2) styles bent back after pollination, (3) pollen released from the anthers, (4) seed maturation commencing at 4–5 weeks, and (5) seeds are released (see **Supplementary Figure 2**). Any seeds observed

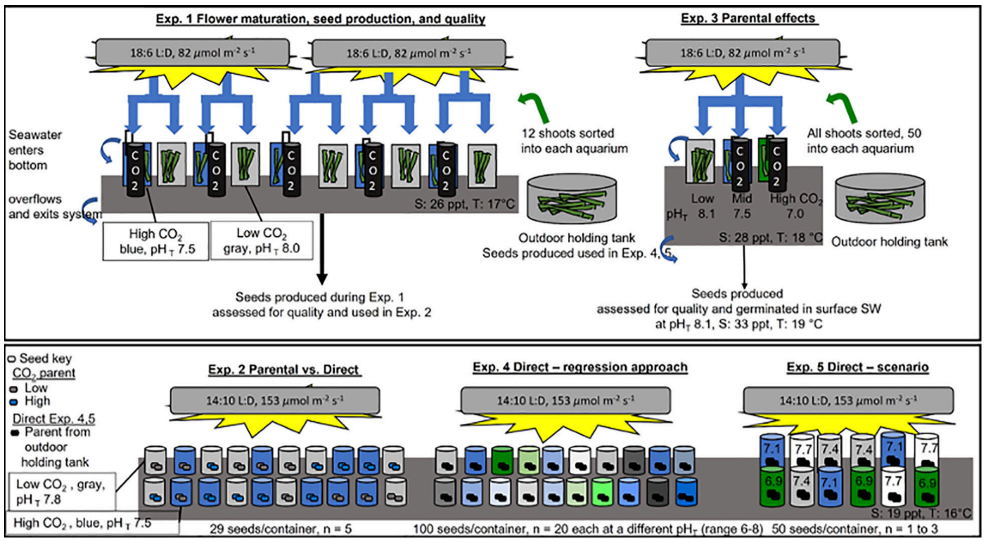

FIGURE 1 | Schematic of experimental design for experiments (Exp.) 1–5. See **Table 1** and see section "Materials and Methods" for more information. Exp 1 and 3 (top box) were done at Kristineberg Marine Research Station under a light bank, within a flow through system where surface supplied seawater (SW) entered the bottom of the containers, over flowed and exited the system (water flow shown by blue arrows). Flowering shoots of *Zostera marina* were collected on 17 July 2017 (Exp 1) and 9 August 2019 (Exp 3) near the station. Shoots were held overnight in an outdoor holding tank and then immediately sorted into replicate containers. Containers were held at appropriate CO_2 treatments with a controller that sensed pH (black line) and regulated bubbling of CO_2 (black cylinders) into seawater. Exps. 2, 4, and 5 (bottom box) were done at Dauphin Island Sea Lab in closed system placed within a cold room (16 C), under a light bank. SW at appropriate treatment levels was replaced in containers every 1–2 days.

at the bottom of each container were collected and counted. Seeds were held within the container of origin, inside a labeled submerged open-top watch glass. Because seeds fell on different dates, we maintained each batch in separate open-top watch glasses labeled with the date of collection.

Experimentally raised seeds were tested for viability using the "squeeze" method. Viable seeds develop a hard seed coat and do not compress with a pinch from tweezers. We also estimated seed quality from sinking velocities. A large 500 mL Erlenmeyer flask was filled with SW, marked, and then seeds were dropped one by one into the flask. A handheld stopwatch was used to time seed fall from the mark to the bottom (30 cm). Seeds with a falling velocity greater than 5.5 cm s^{-1} have a 95% chance of germination (Infantes and Moksnes, 2018). Seed quality may be related to seed size or mass. A microscope or zooscan was used to image all seeds at high resolution (105 pixels to mm). A scale bar was included in each image. These images were analyzed in ImageJ using a calibrated line and measure tool to determine seed length and diameter (width). Repeated measures on the same seed by data collectors showed reproducibility within 0.05 mm. Seed volume was calculated based on the volume of an ellipsoid π /6 L D2 following methods of Delefosse et al. (2016). Seeds not used in other experiments were placed in a drying oven at 60°C until dry and weighed to the nearest milligram on a microbalance.

Experiment 2: Parental vs. Direct Effects of CO_2 on Seedling Development

A two-level factorial experiment was designed to evaluate the impact of seed seawater treatment (low vs. high CO_2) and parental seawater treatment (low vs. high CO_2) on seedling production. Seeds developed from the previous experiment were pooled into two groups (high and low CO_2) and shipped to the Dauphin Island Sea Lab (DISL) in Mobile, Alabama (United States).

Large volumes of seawater from Mobile Bay were coarse filtered, aerated, and held in a cold room (16°C) until use in experiment. The mean pH_T of this seawater for the area was 7.84 $\pm$ 0.05 with a salinity of 19.19 ppt. For clarity, the control treatment in this experiment was not modified from natural seawater carbonate chemistry found in Mobile Bay. In the carbon enriched seed treatment, a CO_2 gas cylinder bubbled pure gas into seawater until the target pH_T of 7.5 was reached. pH was measured using a glass potentiometric probe (InLab Routine Pro) calibrated on total scale using Dickson Certified Reference Tris (Batch 30) at a salinity of 33.4 ppt. After calibration, pH_T and temperature was measured every other day prior to water replacement. Two 120 mL aliquots of seawater were collected from the holding tank, filtered on a Whatman GF/F filter (Riebesell et al., 2011), and immediately inoculated with 72 µL of 33% saturated mercuric chloride solution ($HgCl_2$) and stored until analyzed for A_T. A standard provided by Dickson (Batch 157) was used to check precision and accuracy (A_T, 3.9 and 0.1 µmol kg^{-1}, respectively; n = 7). The carbonate chemistry was calculated using pH_T and A_T using CO2SYS v2.1 with dissociation constants set to Mehrbach et al. (1973) refit by Dickson and Millero (1987) (Lewis and Wallace, 2006).

Seeds were sorted. An equal number of seeds (n = 29) that developed early, mid, or later in time were placed into 20–125 mL clear glass jars filled with seawater (19 ppt) and

TABLE 1 | Experimental setup and conditions.

Experiment	*N*	Number in each container	Duration (days)	CO_2 Condition	$pH_{Totalscale}$	pCO_2 (μatm)	Temperature (°C)	Salinity (ppt)	Irradiance Cycle, Intensity h light:dark cycle, μmol m^{-2} s^{-1}
1. Effect of CO_2 on spathe and seeds	5	12 shoots	39, 69	Low	7.97 ± 0.06	508.4 ± 86.5	17.1 ± 0.7	26 ± 2.0	18:6, 82
				High	7.53 ± 0.07	1,547.2 ± 267.6	17.3 ± 0.6	26 ± 2.0	
2. Parental vs. direct effects of CO_2 on seedling development	5	29 seeds	32	Parent Low × Seed Low	7.82 ± 0.03	717.8 ± 44.5	16	19.2	14:10, 153.7 ± 89.4
				Parent Low × Seed High	7.56 ± 0.05	1,311.8 ± 146.3			
				Parent High × Seed Low	7.85 ± 0.05	711.9 ± 90.0			
				Parent High × Seed High	7.53 ± 0.03	1,452.6 ± 104.7			
3. Parental effects of CO_2 on seedling development	1	50 shoots	55	High Parent	8.13 ± 0.08	327.7 ± 62.8	17.8 ± 0.7	27.7 ± 1.6	18:6, 82
				Mid Parent	7.54 ± 0.24	1,603.1 ± 893.0	18.3 ± 0.7	27.5 ± 0.5	
				Low Parent	6.96 ± 0.15	5,950.7 ± 1,849.82	18.0 ± 1.0	27.5 ± 0.5	
		13 seeds	30	Seeds	8.10 ± 0.06	356.2 ± 58.7	18.6 ± 0.6	33.2 ± 0.3	18:6, 82
4. Direct effects of CO_2 on seedling development–regression approach	20	100 seeds	41	Experimental range	6.00–7.99	433.0–51,605.9	16	19.2	14:10, 153.7 ± 89.4
5. Direct effects of CO_2 on seedling development—scenario approach	1–3	50 seeds	30	7.7	7.68 ± 0.15	1,000.5 ± 0.0	16	19.2	14:10, 153.7 ± 89.4
				7.4	7.40 ± 0.02	1,953.1 ± 76.6			
				7.1	7.10 ± 0.00	3,944.4 ± 5.4			
				6.9	6.88 ± 0.01	6,668.4 ± 182.8			

sealed. The jars were divided with respect to flowering treatment and carbonate chemistry (n = 5). The experiment continued for 32 days, and seedling development was assessed at the end (see **Supplementary Figure 3**). Seeds need approximately 30 days to germinate and move through development (Xu et al., 2016).

Experiment 3: Parental Effects of CO_2 on Seedling Development

To isolate how parental investment influences germination and seedling development, flowering shoots were kept at three CO_2 conditions and resulting seeds were allowed to develop under ambient pCO_2. One hundred and fifty flowering shoots (see section "Materials and Methods," *Flowering shoot and ambient seed collection*) were assigned to three 50 L containers at Kristineberg Marine Research Station. Before assignment, spathes in development stages 4 and 5 were cut from the rhipidia ensuring all seeds developed under treatment conditions. Each container was populated with 50 intact shoots. These plants were arranged, held, and maintained within the same flow-through system as described in the first experiment. Containers with submerged plants were exposed to one of three distinct CO_2 seawater treatments: high, mid, and low CO_2 condition corresponding to a pH_T of 6.5, 7.4, 7.9 for 55 days. Two AquaMedic pH stat systems (see first experiment) were used to maintain pH_T in the mid and high CO_2 treatments, however, pCO_2 conditions fluctuated in the low treatment (ambient) in the seawater flow-through system. The pH_T was monitored and water was collected from containers for A_T determinations at weekly intervals using the same protocols as in experiment 1. Titrations for A_T and calculations for carbon speciation were done using the same protocol in experiment 1.

Seeds were collected from the bottom of each of the tank and tested for viability. Seeds were then stored separately at 4°C for 120 days to simulate a period of winter dormancy (Infantes et al., 2016; Infantes and Moksnes, 2018). Seeds were sorted into fifteen

1-L jars (13 seeds per jar) resulting in 5 replicates per parental CO_2 condition. Seeds placed in containers developed for 30 days under ambient flow-through conditions under a saturating light field (18:6 h light: dark cycle, 82 μmol m^{-2} s^{-1} at the canopy) at 14°C (Dennison and Alberte, 1982). Seed germination and seedling development were assessed weekly and unlike earlier experiments, viability of ungerminated seeds was assessed at each time point using the squeeze method. Remaining seeds not used in experimentation were tested for viability by assessing sinking velocities.

Experiment 4: Direct Effects of CO_2 on Seedling Development—Regression Approach

To test the effect of high pCO_2/low pH on germination, control seeds (developed in flow-through, outdoor holding tanks) were transported from Kristineberg Marine Research Station to DISL and randomly sorted into jars filled with seawater (19 ppt) and sealed. Twenty jars (125 mL) each received 100 seeds. Four targeted CO_2 levels were selected: 900, 1,800, 4,800, and 7,700 μatm (corresponding to a pH_T of 7.8, 7.5, 7.1, 6.9) with 5 replicates each. However, biological respiration in experimental units lowered pH from targets leading to differences between replicates. pH was monitored rigorously (up to twice daily), and water changes were frequent (1–2x daily), but the carbonate chemistry did vary through time so that each jar had different pH-pCO_2 environment. Therefore, the response of seeds/seedlings in each jar (n = 20, no longer n of 5 at selected levels) with its corresponding measured carbon enriched conditions was used in a regression approach. Seeds were sorted on December 18, 2017, and experimental conditions lasted for 41 days. Development was assessed on four intervals: day 9, 17, 29, 40.

Experiment 5: Direct Effects of CO_2 on Seedling Development—Scenario Approach

The experiment was conducted using 12–250 mL jars with each replicate receiving 50 seeds. The larger volume and reduced seed numbers were used to carefully control pH and robustly test for direct effects of CO_2 on germination and seedling development. The same CO_2 conditions as in experiment 4 were targeted (corresponding to pH_T 7.7, 7.4, 7.1, 6.9) with 3 replicates each. Carbon enriched conditions are not representative of ocean acidification projections within the next century but were selected to examine replicable physiological responses for enhancing restoration. Furthermore, the experiment was started December 23, 2017, and continued for 27 days. Development was assessed at day 22.

For both experiments testing direct pH effects (experiment 4 and 5), seeds were kept in a closed system (jars) at 16°C under a fluorescent light bank (153.7 ± 89.4 μmol m^2 s^{-1} as measured across replicate jars) on a 14:10 h light: dark cycle. These light conditions were selected to mimic spring conditions at a shallow depth (∼1.5 m) in the fjords where flowering shoots were collected. To remove any location bias, jars were haphazardly rotated in position within a cold room daily. seawater at the specified pH_T was replaced in jars once to twice daily to ensure treatment conditions were maintained and water remained oxygenated. Seawater (19 ppt) was collected at DISL mesocosm facility from Mobile Bay and manipulated using the system described above. pH_T was measured daily within the jars. Aliquots of seawater (n = 1–5 per measuring interval) were collected periodically from the mesocosm facility holding tanks, filtered, dosed with $HgCl_2$, and used for A_T determinations at end of study. A_T and carbonate chemistry calculations follow the protocol outlined in experiment 2.

Assessing Seedling Development Across Experiments

At each interval, the stage (0–6) in seedling development was also noted for each seed or seedling in a replicate jar. We followed the schematic outlined by Xu et al. (2016) which can be observed in **Supplementary Figure 3**: stage 0: intact seed coat; stage 1: germination as the emergence of the cotyledon and hypocotyl; stage 2: pre-seedling stage with no true leaf or adventitious roots, but the cotyledon and hypocotyl are elongated; stage 3: seedling stage reached with the emergence of first true leaf; stage 4: development of adventitious root; stage 5: development of second true leaf; and finally stage 6: intact seedling lacking cotyledonary blade and develops a third leaf. The length of each seedling (stages 3–6), from root tip to longest leaf height was also assessed to the nearest mm.

Statistical Analyses

Prior to all parametric analyses, residuals were plotted and examined for normality. Prior to all t-tests and ANOVAs, data were also tested for homogeneity of variance using the Levene's test.

Data on sinking velocity from experiment 1 were natural log transformed to meet parametric requirements prior to use. Data were averaged to produce one value per replicate experimental unit. To analyze the dataset from the first experiment (flower maturation and spathe production), a series of repeated measures two-way ANOVAs with the container as a random effect and date and treatment (and their interaction) as fixed factors were used to test for differences in (1) the number of spathes in development stages (1–5), (2) the number of spathes produced, and (3) the number of mature seeds in the spathes. Holm–Sidak multiple comparison procedure was used to identify pairwise differences when a main effect was found. A series of t-tests were applied to examine seed quality (viability with squeeze method, sinking velocity, volume, and dried weight) and quantity of seeds produced. In addition, a t-test was used to test for differences in percentage of population with reduced chance of germination (sinking velocity below 5.5 cm/s).

A series of two-way ANOVAs tested whether parental condition and the water condition surrounding seeds and seedlings, and the interaction, contributed to (1) the number of seeds which germinated, (2) maximum stage reached in seedling development, and (3) the total number of seedlings (stages 3–6) produced at the end of the study. Differences in seedling height between treatments could not be robustly tested because of

the limited replicates within the same stage (4, 5, or 6) at the end of the study.

For the dataset generated from experiment 3, differences in germination success (percentage of viable seeds entering stage 1) and germination rate among the three treatments were examined using a two-way repeated measures ANOVA with time (weekly sampling interval) and treatment as fixed factors with container as a random subject factor. Lastly, we considered each seed a replicate from one of the three container populations when testing for the effects on sinking velocity. Holm–Sidak multiple comparison test was used to determine pairwise differences when a main effect was found. One-way ANOVA (on ranks) due to failing the assumptions of normality was then used to test for differences in sinking velocity among the three CO_2 treatments, followed by a Dunn's multiple comparison procedure to determine pairwise differences.

To test the direct effects of CO_2 on seed germination, seedling production, and seedling size, a series of orthogonal least squares linear regressions were done with pH to ease data visualization. The mean pH_T and lowest pH_T measured for jars from the start of the experiment to the sampling interval (1, 2, and 3) was regressed with the non-cumulative count of seeds that germinated (stage 1) between intervals. This approach was taken to account for the cumulative pH-CO_2 conditions that seeds experienced and possible delays in effects on germination. At the end of the study (interval 4), the mean pH and lowest pH of jars during the entire course of study was used to predict (separately) the cumulative number of germinated seeds, and seedlings produced. Separate general linear models with seedling stage as a covariate was used to test the effects of CO_2 (mean pH_T and lowest pH_T from initiation of experiment through interval 4) on seedling height at the end of study.

In experiment 5, using larger volumes and fewer seeds to maintain chemistry, separate one-way ANOVAs were used to test for differences in the cumulative number of germinated seeds and the total seedlings produced at the end of the study. To ensure treatments were discrete, when the deviation in pH overlapped with another treatment, the data from that replicate was removed prior to any analyses. This resulted in an n of 1–3 for each CO_2 level. Differences in seedling height were not analyzed because of the few numbers of replicate jars with seedlings within the same developmental stage by the end of the experiment duration.

RESULTS

Experiment 1: Effect of CO_2 on Spathe and Seed Development

Low (pH_T = 7.97 ± 0.06, pCO_2 = 508.4 ± 86.46 μatm) and high (pH_T = 7.53 ± 0.07, pCO_2 = 1547.2 ± 267.60 μatm) CO_2 conditions were maintained for 69 days and monitored for the first 39 days (**Supplementary Table 1**). The low CO_2 condition has a carbonate chemistry within the present range of variability reported in local surface waters (Dupont et al., 2013). All values are reported as means (±SD).

Flowering maturation, number of spathes produced, and the number of seeds in development within spathes did not differ significantly between CO_2 conditions (**Table 2** and **Figures 2**, **3**). The flowers developed though time following a typical maturation pattern (**Figure 2** and **Table 2**). The number of spathes in stage 1 was greatest on day 1 (low CO_2 condition: 51.8 ± 8.2, high CO_2 condition: 53.8 ± 13.2) and declined through time, while the number of spathes in stage 3 was highest on day 9 (low CO_2 condition: 56.0 ± 12.3, high CO_2 condition: 54.4 ± 9.9). The highest number of spathes in stage 4 (low CO_2 condition: 101.4 ± 18.5, high CO_2 condition: 98.6 ± 26.5) and in stage 5 (low CO_2 condition: 77.2 ± 16.8, high CO_2 condition: 66.2 ± 11.9) occurred on day 19 and 39, respectively.

The number of spathes also increased significantly through time (**Figure 3A** and **Table 2**). The number of spathes produced increased from day 1 (low CO_2 condition: 107.8 ± 24.7, high CO_2 condition: 112.4 ± 18.6) to day 9 (low CO_2 condition:150.8 ± 19.0, high CO_2 condition: 148.8 ± 22.1) and declined in number from day 29 (low CO_2 condition: 154.6 ± 27.5, high CO_2 condition: 160.8 ± 26.5) to day 39 (low CO_2 condition: 129.0 ± 26.9, high CO_2 condition: 123.8 ± 31.0). This is congruent with the observation that many spathes became brittle and broke off from shoots in later stages of development and were not counted.

TABLE 2 | Results of repeated measures two-way ANOVA testing for the effects of treatment condition (degrees of freedom, df = 1), time (df = 4), and their interaction (df = 4) on flower maturation (tested for differences in stages 1–5) and, spathe and seed production (Experiment 1).

Parameter	Factor	*F*	*p*-value
Number of spathes in stage 1			
	Treatment	0.451	0.52
	Time	105.07	< 0.001
	Treatment × Time	0.555	0.70
Number of spathes in stage 2			
	Treatment	0.828	0.39
	Time	6.34	< 0.001
	Treatment × Time	22.030	0.50
Number of spathes in stage 3			
	Treatment	0.0002	0.99
	Time	85.14	< 0.001
	Treatment × Time	0.105	0.98
Number of spathes in stage 4			
	Treatment	0.000	1.0
	Time	140.32	< 0.001
	Treatment × Time	0.559	0.69
Number of spathes in stage 5			
	Treatment	1.858	0.21
	Time	107.27	< 0.001
	Treatment × Time	0.709	0.59
Number of spathes produced			
	Treatment	0.009	0.93
	Time	66.03	< 0.001
	Treatment × Time	0.853	0.50
Number of seeds within spathes			
	Treatment	0.013	0.91
	Time	142.59	< 0.001
	Treatment × Time	0.310	0.87

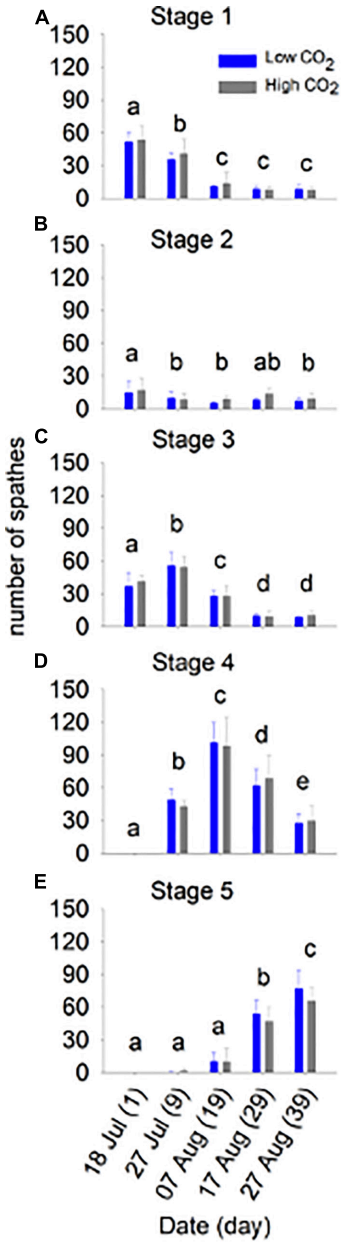

FIGURE 2 | Flower maturation at low and high CO_2 conditions for 39 days (Experiment 1). The number of spathes (mean ± SD) in stages 1–5 (top to bottom) are shown for each day (n). The different letters above bars represent statistical differences between times as specified by the results of a Holm–Sidak pairwise comparison test following a repeated measured two-way ANOVA, **Table 2**.

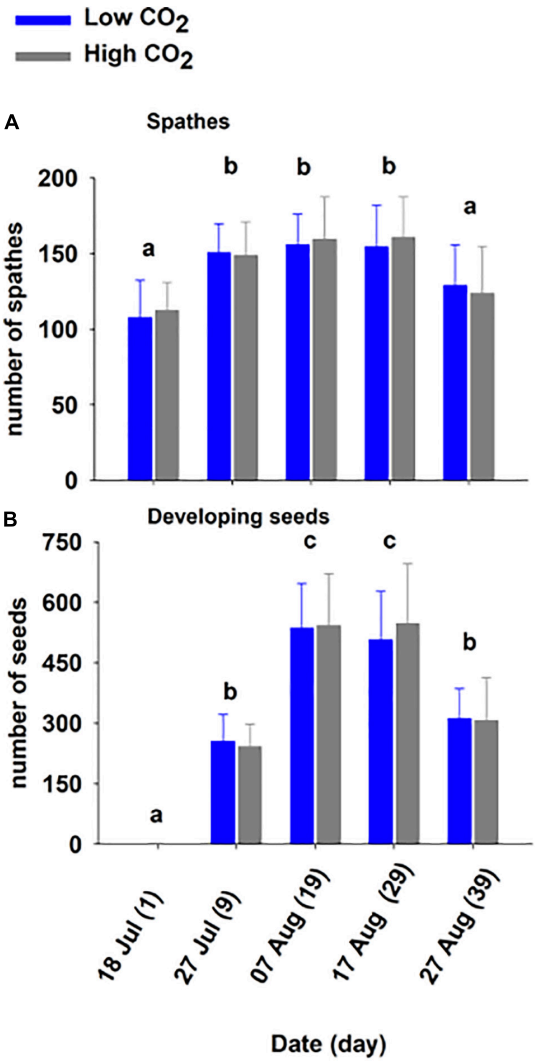

FIGURE 3 | Mean (± SD) spathes **(A)** and seed number **(B)** on shoots held at low and high CO_2 condition for 39 days (Experiment 1). The different letters above bars represents statistical differences between time as specified by the results of a Holm–Sidak pairwise comparison test following a repeated measure two-way ANOVA, **Table 2**.

The number of seeds developing reflected flowering maturation (**Figure 3B**) and was significantly impacted by time ($p < 0.001$; **Table 2**). The number increased from July—day 1 and 9 (low CO_2 condition: 256.0 ± 66.1, high CO_2 condition: 242.2 ± 55.9) to early August—day 19 and 29 (low CO_2 condition: 536.4 ± 110.9, high CO_2 condition: 542.4 ± 128.4) and the mean number (low CO_2 condition: 311.6 ± 74.6, high CO_2 condition: 306.8 ± 106.5) was lower at the end of August—day 39, as seeds matured and were released.

After 69 days a pooled total of 2,017 seeds were released from plants and 226 of these seeds were deemed non-viable using the "squeeze" method. Plants from the high and low CO_2 conditions produced a similar number of seeds (**Table 3**). Furthermore, all measured metrics (number non-viable, sinking velocity, volume, and dried weight) indicated seeds formed under high CO_2 conditions were no different in quality than those developed at low CO_2 conditions. Overall seeds (n = 1,903) sunk to 30 cm in 4.5 ± 0.6 s with a mean velocity of 6.67 cm s^{-1}, seed length and width (n = 2,010) were 4.42 ± 0.377 and 1.96 ± 0.217 mm, respectively, and seed dried weight (n = 1,001) was 3.136 ± 0.853 mg.

TABLE 3 | Mean (± SD) of seed quantity and metrics of seed quality produced from flowering shoots maintained in low and high CO_2 conditions for 69 days (Experiment 1).

	Low CO_2	High CO_2	*t*	*p*-value
Seeds produced (#)	200 ± 84	204 ± 60	−0.09	0.93
Non-viable seeds (#)	5.2 ± 1.7	6.2 ± 2.9	−0.67	0.53
Dried weight (mg)	3.186 ± 0.376	3.068 ± 0.216	0.61	0.56
Volume (mm^3)	0.0319 ± 0.005	0.0328 ± 0.001	−0.37	0.72
Sinking velocity (cm/s)	6.64 ± 0.21	6.65 ± 0.16	−0.01	0.99
Percentage with velocity < 5.5 (cm/s)	7.9 ± 3.6	4.9 ± 1.0	1.60	0.15

Results (t and p-value) are from two-tailed t-tests (n = 5) with 8 degrees of freedom. Percentage velocity was ln transformed prior to testing to meet parametric requirements.

Experiment 2: Parental vs. Direct Effects of CO_2 on Seedling Development

Seawater chemistry was maintained for the entirety of the experiment (32 days): low CO_2 condition (pH_T = 7.84 ± 0.05; pCO_2 = 714.85 ± 67.28 µatm) and high CO_2 condition (pH_T = 7.55 ± 0.05; pCO_2 = 1381.9 ± 125.50 µatm) (for parental CO_2 condition, see **Supplementary Table 1**; for more complete carbonate chemistry, see **Supplementary Table 2**). Overall germination success (mean 6.9% ± 5.5) and seedling production (total of 18 seedlings out of 580 total seeds) was low within the 32-day period. Despite low values, seed germination was significantly (∼2x) greater when the parental generation was from the high CO_2 condition (3.2 ± 1.7 vs. 1.5 ± 1.0, **Figure 4** and **Table 4**). Greater germination success translated into 2x greater mean number of seedlings (high CO_2 parents 1.2 ± 0.9 vs. low CO_2 parents 0.6 ± 0.7) and an increased mean maturation as gauged by maximum stage reached at end of the study (high CO_2 parents 3.7 ± 1.9 vs. low CO_2 parents 2.3 ± 1.8, **Figure 3**). However, there was high variation among replicate jars and these metrics (i.e., seedlings produced and maturation) were not found to be statistically different between treatments. There was also no indication of an effect of CO_2 on germination, nor an interaction of parent and seed water condition, on germination success, seedlings produced, and maximum stage reached (**Table 4**). It is anecdotal, but worth nothing that at the end of the study, seedlings in stage 5 and 6 within high CO_2 condition were taller (Stage 5 = 3.7 cm; Stage 6 = 4.3 cm) than those in the low CO_2 condition (Stage 5 = 2.2 cm; Stage 6 = 3.5). Statistical analysis could not be performed due to limited number of replicate seedlings within the same stage of development (**Supplementary Figure 3**).

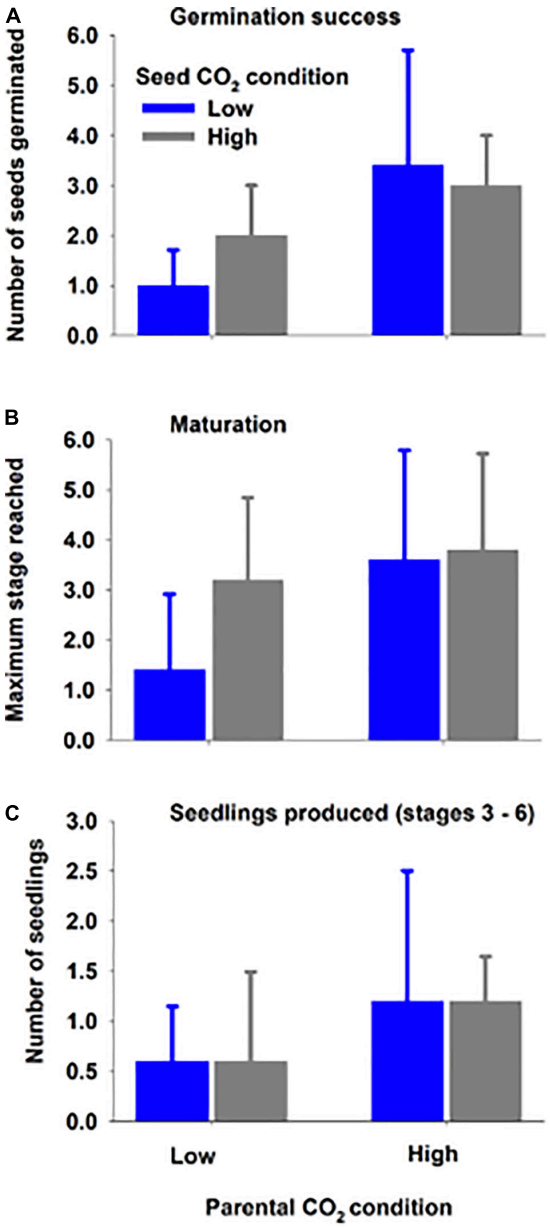

FIGURE 4 | Mean (± SD) germination success **(A)**, seedling maturation **(B)**, and seedlings produced **(C)** at the end of study for seeds developed (parent) under high or low CO_2 condition and placed (seed) within water with high or low CO_2 condition for 32 days (Experiment 2). Results of two-way ANOVAs (**Table 4**) indicate that the high parental CO_2 condition resulted in greater germination success.

Experiment 3: Parental Effects of CO_2 on Seedling Development

Three treatments corresponding to high, mid, and low parental CO_2 condition (pH_T = 6.96 ± 0.15, 7.54 ± 0.24, 8.13 ± 0.08) were maintained for 55 days (additional parameters are reported in **Supplementary Table 3**). Once seeds were harvested and transferred for germination, ambient water (pH_T 8.1 ± 0.06) from Gullmarsfjord was used (**Supplementary Table 4**). There were 102 seeds produced from flowering shoots (45, 35, and 27 seeds from the high, mid, and low parental CO_2 treatments, respectively). Seeds sunk at a velocity of 6.12 ± 1.3, 5.6 ± 1.6, 3.7 ± 1.8 cm s^{-1} for the populations, respectively (**Figure 5**). 75.0, 68.2, and 14.3 % of the seed population from the high, mid, and low parental CO_2 condition sunk at a velocity greater than 5.5 cm s^{-1} a cutoff indicating greater than 95% chance of germination. There was a significant effect of parental condition on sinking velocity (ANOVA: df = 2, H = 16.0, $p < 0.001$).

TABLE 4 | Results of two-way ANOVAs testing for parental (parent) and direct (seed) CO_2 effects on germination success, seedling maturation (as maximum stage reached), and number of seedlings produced (Experiment 2).

Parameter	Factor	*F*	*p*-value
Germination success			
	Parent	7.410	0.015*
	Seed	0.231	0.637
	Parent × Seed	1.256	0.279
Maximum stage reached			
	Parent	2.09	0.108
	Water	1.48	0.241
	Parent × Water	0.95	0.345
Number of seedlings			
	Parent	2.880	0.109
	Water	0.320	0.579
	Parent × Water	2.880	0.109

**Significance at statistical alpha < 0.05 with 1 degree of freedom for each factor.*

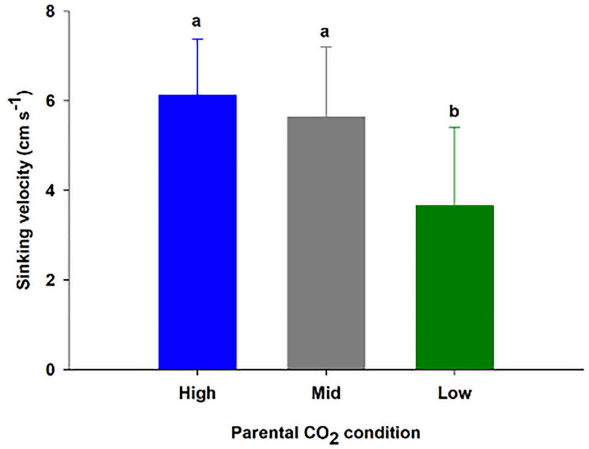

FIGURE 5 | Sinking velocity (mean ± SD) of 14–24 seeds produced from flowering shoots kept at three CO_2 conditions (high, mid and low) for 55 days (Experiment 3). Conditions correspond to a pH_T of 6.96, 7.54, 8.13 (**Supplementary Table 3**). The different letters above bars represent statistical differences between conditions as specified by the results of a Tukey's pairwise comparison test following significance from a one-way ANOVA.

Thus, low parental CO_2 condition produced a seed population with distinctively slower sinking velocities compared to the other two with carbon enriched parents ($p < 0.05$). Yet, percent germinated out of viable seeds (**Figure 6**) did not vary by parental CO_2 condition (two-way ANOVA: df = 2, $F = 1.24$, $p = 0.32$, treatment × time: df = 8, $F = 0.39$, $p = 0.32$). Instead, germination increased through time (ANOVA: df = 4, $F = 36.1$, $p < 0.001$). A greater number of seeds germinated on day 23 and day 30 than on day 1, 8, and 16. Out of the total seeds that germinated (25.6 % ± 9.5) only 2 seeds developed and survived into seedling stage 3 by the end of the experimental period.

Experiment 4: Direct Effects of CO_2 on Seedling Development—Regression Approach

On average 15.2 % ± 4.1 of the seeds in jars (100 per jar) germinated to produce a total of 191 seedlings (out of 2,000 used) in 41 days. Seawater treatments were reported in intervals (see **Supplementary Tables 5, 6**) and integrated to capture the dynamic carbonate chemistry driven by biological activity experienced by the seeds and seedlings over the course of the experiment The pH_T of the experiment ranged from 6.00–7.99 across experimental units for the duration of the experiment (**Supplementary Tables 5, 6**). The target pH for each treatment (which negatively correlated with mean pCO_2) repeatedly failed to predict number of germinated seeds (intervals 1–3), the cumulative number germinated (at end of study), and the number of seedlings produced at the end of the study. This was evident from the high, non-significant *p*-values (0.29–0.77) and very low R^2 values (ranging from 0.0 to 7.9%) reported from orthogonal least squares linear regression models (**Supplementary Table 7** and **Supplementary Figure 4**). Germination and seedlings produced were also not related to the maximum pCO_2 (minimum pH_T) that seeds experienced (**Supplementary Table 7** and **Supplementary Figure 4**). Furthermore, seedling height significantly differed with stage in development, yet it was not related to CO_2 conditions (**Supplementary Figure 5**, ANCOVA mean pH, stage df = 1, $F = 17.2$, $p < 0.001$, pH df = 19, $F = 1.3$, $p = 0.27$; ANOVA lowest pH, stage df = 1, $F = 13.11$, $p = 0.001$, pH df = 15, $F = 0.91$, $p = 0.56$). Seedlings in stage 3 of development had a height of 2.60 ± 0.65 cm. Seedlings in stage 4, 5, and 6 of development were larger, increasing in size with development stage, 2.83 ± 0.42, 3.00 ± 0.66, and 3.42 ± 0.66 cm respectively.

Experiment 5: Direct Effects of CO_2 on Seedling Development—Scenario Approach

Three out of twelve jars with variable conditions (pH ≥ 1.5 SD) were removed from this dataset (**Supplementary Figure 6**) to ensure the carbonate chemistry was tightly controlled and results could be contrasted with the earlier experiment where the carbonate chemistry was variable through time. It should be noted that the overall outcome and conclusions drawn from the full dataset are congruent to those reported with the reduced dataset. In the reduced dataset seawater treatments had minimal deviation: pH_T 6.88 ± 0.01, 7.10 ± 0.00, 7.40 ± 0.02, 7.68 ± 0.15 (for a more complete characterization, see **Supplementary Table 8**). The total number of seeds germinated was 13.3 % ± 4.0 out of 50 seeds used and the mean number of seedlings produced was 3.2 ± 1.5 per jar. The number of seeds germinated statistically differed between treatments (**Supplementary Figure 6**, df = 2, $F = 10.9$, $p = 0.015$) yet, pairwise comparisons did not show any trend with increasing CO_2 (**Supplementary Figure 6**). Furthermore, the number of seedlings produced did not differ between CO_2 conditions (df = 2, $F = 2.2$, $p = 0.21$). Seedling height was plotted and can be found in **Supplementary Figure 7**.

DISCUSSION

Our results provide a better understanding of how increased CO_2 affects three potential bottlenecks (seed production, germination,

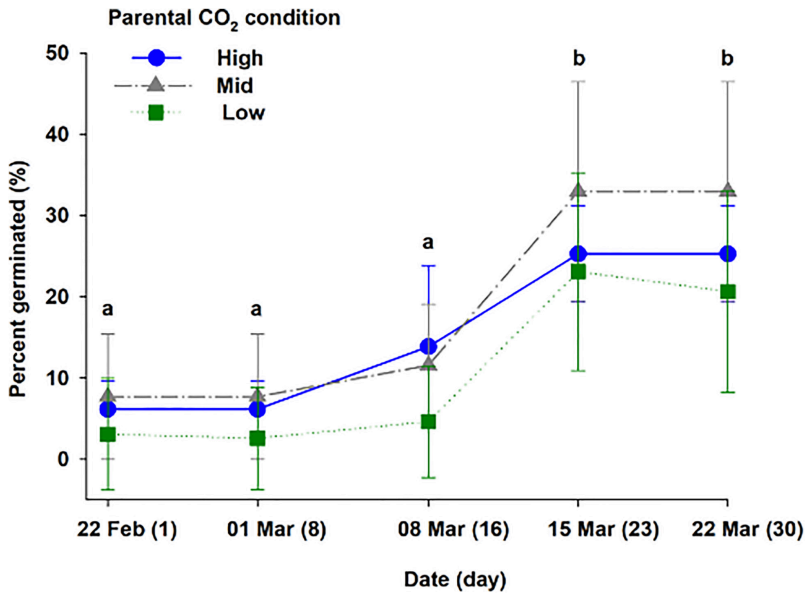

FIGURE 6 | Percent germinated out of viable seeds harvested from flowering shoots kept at three CO_2 conditions (high, mid, and low), corresponding to a pH_T of 6.96, 7.54, 8.13 (Experiment 3, **Supplementary Table 3**). The different letters above bars represents statistical differences between days of study as specified by the results of a Holm–Sidak pairwise comparison test following a repeated measure two-way ANOVA. The ANOVA model indicated a main effect for time ($p < 0.001$) but, not for treatment level nor for an interaction of time × treatment.

and seedling production) in eelgrass establishment from seed (**Table 5**). Firstly, results indicate that flowering shoots exposed to brief periods of high CO_2 produce a greater proportion of viable seeds (experiment 3, **Table 5**). Secondly, results indicate that the germination success of *Z. marina* is greater in seeds produced by parents maintained in high CO_2 (experiment 2 and 3, **Table 5**). Thirdly, results provide insight into the mechanism driving this response. Increased germination does not appear to be directly related to the conditions which surround the seed but rather stem from a carryover effect from parental exposure. A common practice for bed restoration is to harvest flowers, develop seeds, and then distribute seeds to the targeted location to allow for germination and bed development. Therefore, once flowering shoots have been collected and returned to laboratory settings, bubbling CO_2 into the seawater holding tanks could help bolster germination success and facilitate bed recovery. However, we found that exposure to elevated CO_2 conditions did not significantly translate into seedling success (experiment 2–5, **Table 4**). The small percentage of seedlings established in the present study highlight the vulnerability of this life stage. Our results give further evidence for the precarious nature of seed germination and seedling development. Efforts in the future must be made to standardize experimental conditions to make more direct comparisons and to clarify the role of other environmental metrics in these outcomes.

Resource availability (e.g., light and ammonium) can influence spathe and seed production for *Zostera marina* (van Lent and Verschuure, 1994; Jackson et al., 2017; Johnson et al., 2017). A yearlong study of carbon enrichment found flowering to occur earlier, maturation to occur faster, and an increased percentage of flowering to vegetative shoots for *Z. marina,* with significant affects occurring at extreme OA (pH_T = 6.4) treatment (Palacios and Zimmerman, 2007). Increased flowering production is also described as a response to environmental stressors (e.g., turbidity, temperature, salinity) (Phillips et al., 1983). Our study intended to test the use of elevated pCO_2 as a dose injection to bolster seed production or seed quality to ultimately increase seedling biomass. We found flowering maturation patterns to reflect field observations (Infantes and Moksnes, 2018) and we did not find any statistical

TABLE 5 | Summary of outcomes for experiments (Exp.) 1–5 testing direct or parental CO_2 effects on flower, seed, and seedling metrics.

	Exp. 1	Exp. 2		Exp. 3	Exp. 4	Exp. 5
Metric assessed	**Direct**	**Direct**	**Parent**	**Parent**	**Direct**	**Direct**
Flower						
Maturation	=					
Spathes number	=					
Developing seeds	=					
Seeds						
Number	=					
Quality	=			+		
Germination		=	+	=	=	=
Seedling						
Number		=	=	=	=	=
Development		=	=		=	=

An equal sign (=) shows the metric did not differ between tested CO_2 conditions. A positive sign, highlighted in a blue box, notes high CO_2 benefits the metric assessed. A gray box occurs when a metric was not tested.

evidence supporting the effect of CO_2 on flowering development or seed production. However, duration and magnitude of resource availability (e.g., light, ammonium) need to be considered since both variables are important in seagrass allocation from vegetative growth to sexual reproduction (Johnson et al., 2017; Qin et al., 2021). Indeed, in experiment 5, under a larger CO_2 gradient, seed quality increased with increasing pCO_2.

Positive parental CO_2 effects for early seedling development were observed in two experiments, yet the metric affected (germination vs. sinking velocity) was different. A large part of the seed tissue is of maternal origin and thus, significant increases in seed mass (proxied by sinking velocity) are important indicators for increased investment in offspring (Wulff, 1995). Seeds developed at extreme CO_2 condition ($pCO_2 > 1{,}600$ μatm, experiment 3) had faster sinking velocities and provide evidence for greater parental investment (*via* increased rate of photosynthesis corresponding to more stored carbon) into seed quality. Increased germination was observed in experiment 2 when more moderate enrichment ($pCO_2 < 1{,}600$ μatm) was applied to flowering shoots. Presumably this increased germination is also the result of greater parental allocation into seed quality, even though statistical testing indicated the metrics assessed (e.g., volume, weight, sinking velocity) were unaffected. These two experiments were conducted at different temperatures and salinities both of which are important environmental factors to consider for germination success (Agami and Waisel, 1988; Tanner and Parham, 2010). Experiments conducted at DISL were conducted at 19.19 ppt, 16°C and showed a significant increase in germination compared to experiments performed at the Kristineberg Marine Research Station where conditions were 33.25 ppt, 19.5°C. Orth and Moore (1983) report that experiments conducted in cooler, less haline environments have higher germination rates. An additional study conducted by Niu et al. (2012) demonstrated that the ideal temperature range for seedling development and establishment is between 16 and 17°C. Experiment 2 not only had the highest germination rate, but also the highest percentage of seeds developing into seedlings (62.8%). This is presumably due to ideal conditions for the early life stage of *Z. marina*.

Sinking velocity and other measured metrics of seed quality are not consistent indicators for germination. Maternal environment in conjunction with abiotic variables (e.g., pCO_2, salinity, temperature) are powerful covariates in predicting germination success (Jarvis and Moore, 2015). Because of our comparatively small sample sizes, our results may also be attributed to Type II error. When germination success favored seeds from high CO_2 parents (experiment 2), it corresponded to a greater mean proportion of seeds from low CO_2 parents with sinking velocities below 5.5 cm s^{-1}. Furthermore, in experiment 3, when sinking velocity differed among CO_2 treatments, seeds from mid and high CO_2 parents had a greater mean germination success than seeds from low CO_2 parents. The content of seeds was not directly measured in the present study (dried seeds were lost in a laboratory fire) and we suggest future studies examine seed content to confirm the described mechanism benefitting germination. We also suggest future experiments standardize environmental conditions and repeat experiments using greater sample sizes to clarify disparate outcomes between predictors of germination and measured germination success.

Increased CO_2 appears to have allowed shoots to allocate more carbon into seed content to facilitate viability and germination. *Zostera marina* seeds on average are composed of 52% starch, 13% protein and 4% minerals with variation in these ratios influencing development (Delefosse et al., 2016). Heavy seeds correlate to increased sinking velocities and contain more microgram per gram carbon in the form of starch to benefit germination (Delefosse et al., 2016; Jorgensen et al., 2019). Energy and nutrients stored within the hypocotyl is consumed during germination and higher energy reserves assist in the development and emergence of the radicle and leaf primordium (Kuo and Kirkman, 1992). Seeds with sinking velocities less than 4 cm s^{-1} do not have the energy or nutrients available to undergo germination. Current research demonstrates that 5.5 cm s^{-1} velocity is a threshold delineating heavier, viable seeds from light, nutrient poor seeds (Delefosse et al., 2016; Infantes and Moksnes, 2018). Our results can be used to explain increased bed establishment observed by Zimmerman et al. (2017). They found that following the reproductive season, vegetative shoots in carbon enriched treatments (pH_{NIST} = 6.1, 6.5) nearly quadrupled compared to ambient conditions. We hypothesize that there were more viable seeds of better quality available to germinate and increase aboveground biomass.

Increases in seed quality and germination success are not density dependent and can scale up significantly to alter the landscape level (Orth et al., 2003). Field studies have recorded germination rates as remarkably low; averaging only 3.6% at the landscape level (Statton et al., 2017). In a typical meadow in Shinnecock Bay, New York (40.856678, −72.454090), an approximate calculation estimates the reproductive output of 1 m^{-2} of intact eelgrass at approximately 835 unaborted seeds (Parameters for dimensional analysis provided by Jackson et al., 2017).

$$\frac{40\ \textit{reproductive shoots}}{\textit{meter}} \times \frac{2.9\ \textit{rhipidia}}{\textit{shoot}} \times \frac{2\ \textit{spathes}}{\textit{rhipidia}} \times \frac{3.6\ \textit{unaborted seeds}}{\textit{spathe}}$$

If we are to use our results from experiment 5, 75% of the seeds raised at pH_T = 6.9 will have > 95% chance of germination. Thus, if these same 40 shoots (40 shoots × 2.9 rhipidia × 2 spathes × 3.6 unaborted seeds × 75% viable seeds) are raised in carbon enriched conditions, it will generate an estimated 626 seeds. Whereas those same shoots (40 shoots × 2.9 rhipidia × 2 spathes × 3.6 unaborted seeds × 14% viable seeds) raised in low CO_2 conditions (pH_T = 7.9) may only produce as many as 120 viable seeds. This represents a fivefold increase in the production of viable seeds by altering only one parameter during development. This in combination with a twofold increase in germination may have substantial effects on the community at large. Successful sexual reproduction can increase genotypic variation, thereby increasing resiliency (McDonald et al., 2016).

Beds of high genotypic diversity have been associated with greater stability and productivity (Hughes and Stachowicz, 2004; Macreadie et al., 2014; Jarvis and Moore, 2015; McDonald et al., 2016). Indeed, large scale improvements in seed quality will have demonstrable effects on *in situ* seed banks providing an additional bolster to erratic environmental conditions concurrent with climate change. Increasing genetic resiliency must be prioritized to ensure long term habitat sustainability.

The work conducted on the reproductive patterns of perennial meadows of *Zostera marina* have demonstrated the appreciable role of seeds in the configuration and expansion of established meadows (Furman et al., 2015). Viable seeds have a markedly higher chance of populating well beyond their natal patches and significant increases in viability will further increase colonization success (Sumoski and Orth, 2012). This is also particularly beneficial given that future ocean carbon scenarios predict that coastal acidification (a biogeochemical integration of eutrophication and ocean acidification) will result in more severe diurnal swings in pH with predictions fluctuating as much as 1 unit (Hofmann et al., 2010; Wallace et al., 2014; Pacella et al., 2018). Determining how coastal acidification will integrate into the reproductive history of *Z. marina* is critical to predicting long term ecological resilience.

Germination is a bottleneck for population growth from seeds, but our results also highlight the vulnerability of seedlings as they develop. Considering all five experiments, less than 10% of seeds developed into photosynthetically active seedlings (>stage 3). This limited seedling production reflects both the life history of *Z. marina* and emphasizes the complex interplay of biogeochemical interactions in influencing development (Probert and Brenchley, 1999; Valdemarsen et al., 2010). For example, the reproductive output of *Zostera marina* can be as high as 10^4 seeds m^{-2} with only a small fraction resulting in seedling establishment and survival (5–15%) (Harrison, 1993; Orth et al., 2003). In the present study, with typical germination success (enhanced by parental exposure to high CO_2), only a fraction of the germinated seeds resulted in seedlings: 62.8, 4.0, 48.3, and 38.2 % of germinated seeds for experiments 2–5, respectively. When the seed coat lyses, the cotyledon acts as a proxy for the developing embryo to sense the outside environment. As the cotyledon elongates during the first and second stage of development, it not only prepares to unfurl the first primordial leaf, but it also serves as a siphon to harvest nutrients from the sediment and water column (Sugiura et al., 2009). Unfavorable environments can halt development and cause mortality. Indeed, for perennial meadows in mid-Atlantic, seedling establishment, not germination serves as the reproductive bottleneck for *Z. marina* (Churchill, 1983; Jarvis and Moore, 2015).

It should be noted that the small percentage of seedlings produced in experiment 3 (4% of seed germinated) is possibly an artifact of experimental duration. Development from germination to seedling establishment (stage 6) is reported to occur within 4–6 weeks (Alexandre et al., 2006; Salo and Pedersen, 2014). Therefore, the experimental durations in the present study should have allowed for the growth of seedlings into later stages (3–6). However, germination rates in experiment 3, greatly increased in the last two sampling intervals and we might have had greater seedling production with more time.

Once seedlings emerge, they are vulnerable to dynamic and sometimes adverse environmental conditions and thus survival rate varies widely among habitats (Churchill, 1983; Harrison, 1993; Greve et al., 2005; Boese et al., 2009). Hydrodynamic forces (Valdemarsen et al., 2010), light attenuation (from sediment burial, degraded water quality, and competition) (Hauxwell et al., 2001; Mills and Fonseca, 2003), as well as hypoxia (Hauxwell et al., 2003), are reported to cause seedling mortality (Valdemarsen et al., 2010; Salo and Pedersen, 2014). Other than mobilization of metabolic reserves in seeds, temperature, low dissolved oxygen, low organic matter, and burial depths are important predictive factors that correlate with increased seedling growth (Jarvis and Moore, 2015). Low salinity (>20 ppt) and lack of sediment used in the present study may have altered seedling investment in aboveground growth by failing to provide a source of additional nutrients and preventing the seedling from anchoring to its surroundings (Marion and Orth, 2010). Although low salinity significantly increases germination (Niu et al., 2012), seedlings are damaged by salinity less than 20 ppt due to osmotic stress generated by low salinity (Xu et al., 2016). Both salinity and temperature are confirmed factors influencing seedling aboveground morphology and biomass and must be considered for successful restoration.

A major concern for bed establishment from seeds is that CO_2 availability increased germination but did not statistically increase seedling production nor survival. For *Posidonia oceanica* seedlings, CO_2 enrichment increases the rate of the dark reaction in photosynthesis resulting in increased growth and storage capacity, carbon reserves in the below-ground tissues and lowered nitrogen content in leaves (Artika et al., 2021). Direct effects of CO_2 may be masked by epiphytic growth, herbivory, or simultaneously co-occurring heat or light stress. Shading from epiphytic filamentous algae negates positive net growth associated with available [DIC] as both seedlings and algae benefit from increases in available metabolic substrate (Burnell et al., 2014). In the present study, we observed microscopic algae growing on developing seedlings presumably stunting development. Seedlings must also contend with how elemental changes to their leaf material will influence their palatability. *P. oceanica* seedlings grown under increased pCO_2 shift their elemental leaf structure to favor carbon-based sucrose over nitrogen. Increased sugar production increases palatability to herbivores (Arnold et al., 2012; Hernán et al., 2016). Additionally, although there is a clear correlation between seagrass metabolism and temperature (Escolano-Molto et al., 2021), an emerging body of evidence suggests that seedlings are not as robust as adults and are therefore more vulnerable to heat stress (Guerrero-Meseguer et al., 2017; Zimmerman et al., 2017).

Much of our results are derived from highly variable conditions representing a potentially realistic portrayal of future variability. Although a potential cause for contention, our carbonate chemistry is reflective of the rapid biogeochemical restructuring of the Swedish waterways with areas along the Skagerrak Sea predicting pH_T variations moving from present day values (8.7–7.6 units) to future conditions (8.3–7.2 units)

in 2100 (Dorey et al., 2013). Our results infer germination response in the context of intense environment flux indicative of climate change, eutrophic events, sewage drainage and industrial effluence. Continued effort must be invested into determining how *Z. marina* will respond to the rapid restructuring of global oceans. Additional field experiments are needed to determine how sediment geochemistry, temperature and PAR influence germination and growth dynamics *in situ*.

Our data indicates that there is a significant and meaningful effect of CO_2 at the parental level, and we accept our hypotheses. This research provides fundamental groundwork for laboratory-based adaptation strategies to increase parental investment and augment restoration success. Short-term injections of CO_2 into flowering holding containers while seeds are developing is a feasible technique to bolster germination. Restoration strategies should be multitiered with site selection considering substrate (muddy sediment, >1% organic matter), light attenuation, and proximity to established meadows for both the adult plants but also the developing seedling (Bintz and Nixon, 2001; Jarvis and Moore, 2015). Carbon enriched seeds should be nested in burlap bags to anchor seeds in sediment while preventing from >5 cm burial depth (Jarvis and Moore, 2015). Using burlaps bags has resulted in nearly 100% retention of seed used for restoration (Harwell and Orth, 1999). Successful recovery of *Z. marina* in large systems is dependent upon seed production and dispersal and generating resilience, diverse eelgrass bed capable of withstanding impending environmental flux (Olesen and SandJensen, 1994).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

AL collected and analyzed data as well as wrote the manuscript. EI and SD assisted with analysis and experimental design. LW, LP, CH, and KR assisted in data collection. BP and JC assisted in manuscript preparation. TC designed, analyzed, and assisted in writing the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.762086/full#supplementary-material

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Spatial Patterns of *Thalassia testudinum* Immune Status and *Labyrinthula* spp. Load Implicate Environmental Quality and History as Modulators of Defense Strategies and Wasting Disease in Florida Bay, United States

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Seagrass wasting disease, caused by protists of the genus *Labyrinthula*, is an important stressor of the dominant macrophyte in Florida Bay (FB), United States, *Thalassia testudinum*. FB exhibits countervailing gradients in plant morphology and resource availability. A synoptic picture of the *Thalassia-Labyrinthula* relationship was obtained by assessing the activity of four immune biomarkers in conjunction with pathogen prevalence and load [*via* quantitative PCR (qPCR)] at 15 sites across FB. We found downregulated immune status paired with moderate pathogen load among larger-bodied host phenotypes in western FB and upregulated immunity for smaller-bodied phenotypes in eastern FB. Among the highest immune response sites, a distinct inshore-offshore loading pattern was observed, where coastal basins exposed to freshwater runoff and riverine inputs had the highest pathogen loads, while adjacent offshore locations had the lowest. To explain this, we propose a simple, conceptual model that defines a framework for testable hypotheses based on recent advances in resistance-tolerance theory. We suggest that resource availability has the potential to drive not only plant size, but also tolerance to pathogen load by reducing investment in immunity. Where resources are more scarce, plants may adopt a resistance strategy, upregulating immunity; however, when physiologically challenged, this strategy appears to fail, resulting in high pathogen load. While evidence remains correlative, we argue that hyposalinity stress, at one or more temporal scales, may represent one of many potential drivers of disease dynamics in FB. Together, these data highlight the complexity of the wasting disease pathosystem and raise questions about how climate change and ongoing Everglades restoration might impact this foundational seagrass species.

Keywords: immunocompetence, host-pathogen interactions, hyposalinity stress, opportunistic pathogens, environmental fluctuation, anthropogenic influences, resistance, tolerance

INTRODUCTION

Seagrass meadows form the benthic habitat of some of the most globally significant and ecologically diverse communities in the aquatic realm (Robblee et al., 1991; Zieman et al., 1999; Orth et al., 2006). As autogenic and allogenic engineers, seagrasses are foundation species whose meadows provide a broad array of ecosystem services, including erosion control, carbon and nutrient cycling, and water column, as well as sediment oxygenation (Costanza et al., 1997; Waycott et al., 2009; Duarte et al., 2010). They also contribute directly and indirectly to secondary production that supports important commercial and recreational fisheries and ecotourism (Kitting et al., 1984; Heck et al., 1995). However, high light requirements, shallow coastal distributions, and proximity to human development make seagrasses especially vulnerable to ecological perturbation (Onuf, 1996; Ralph et al., 2007; Biber et al., 2009), so much so that they often serve as bio-indicators of ecosystem function and resilience (Hackney and Durako, 2004).

As the impacts of climate change intensify, predictions can be made regarding the persistence of seagrass-dominated communities across time and space (Short and Neckles, 1999). For example, growing evidence suggests that increased environmental fluctuation and recurrence of extreme weather events (i.e., marine heatwaves and altered precipitation; Donat et al., 2013) will negatively impact seagrasses through degraded water quality and physiological constraints (Waycott et al., 2009; Fraser et al., 2014; Peñalver et al., 2020). Another prediction is that climatic changes will lead to pathogen emergence, increased pathogen virulence, and/or the spread of pathogens to naïve host populations (Harvell et al., 1999; Burge et al., 2014; Cohen et al., 2018). This poses a significant mass mortality risk to densely packed seagrass monocultures (Waycott et al., 2009), similar to trends already detected in terrestrial systems (Fey et al., 2015). Further, the well-documented histories of environmentally- (Robblee et al., 1991; Hall et al., 2016) and disease-driven die-offs (Tutin, 1938; Short et al., 1987) suggest that understanding host-pathogen dynamics will be critical for managing seagrass resilience in the decades to come.

Protistan stramenopiles of the genus *Labyrinthula* include a set of opportunistic pathogens that infect seagrasses worldwide with a lesion-forming "seagrass wasting disease" (SWD) that reduces productivity (Durako and Kuss, 1994) and contributes toward host mortality (e.g., Vergeer and den Hartog, 1994; McKone and Tanner, 2009; Bull et al., 2012; Martin et al., 2016; see Sullivan et al., 2013, 2018 for comprehensive literature review). In the tropical and sub-tropical Caribbean and Gulf of Mexico, *Labyrinthula* spp. cause wasting disease in the long-lived, climax seagrass species, *Thalassia testudinum* (turtlegrass; Robblee et al., 1991; Blakesley et al., 2002; Trevathan-Tackett et al., 2013). Recent manipulative work has shown that parasite virulence is a complex function of host competency, physiological stress, and immune status (Brakel et al., 2014, 2019; Trevathan-Tackett et al., 2015; Bishop et al., 2017; Duffin et al., 2020), making the *Thalassia-Labyrinthula* pathosystem an attractive model for testing host-parasite interactions across a range of environmental scenarios. Using newly-developed quantitative PCR (qPCR) and immunoassay techniques (Duffin et al., 2020), we examined the relationship between the immune status of *T. testudinum* and *Labyrinthula* spp. loading along well-documented resource (i.e., phosphorus and sediment depth), morphological (i.e., leaf area and above-ground biomass), and stress (i.e., salinity regime) gradients in Florida Bay (FB), Florida, United States.

Florida Bay is a semi-enclosed estuary bordered by the Everglades and Florida Keys, opening westward into the Gulf of Mexico and eastward through a series of cuts into the Atlantic Ocean (Schomer and Drew, 1982; Hall et al., 2006; Bricker et al., 2011; Herbert et al., 2011). Within the bay, a network of shallow carbonate mud-banks limits wind and tidal currents to create idiosyncratic basins at a variety of spatiotemporal scales (Nuttle et al., 2000; Rudnick et al., 2005; Hall et al., 2006; Lee et al., 2006). However, broad northeast-to-southwest gradients in tidal range, sediment depth, and phosphorus availability – a function of sediment availability and proximity to the Gulf of Mexico (Rudnick et al., 1999; Herbert and Fourqurean, 2009) – oppose a prominent southwest-to-northeast gradient in freshwater delivery (Boyer et al., 1997; Peterson and Fourqurean, 2001). Perpendicular to the alongshore gradients is the rapidly attenuated influence of runoff from the Florida mainland (McIvor et al., 1994; Nuttle et al., 2000).

The resultant salinity regime reflects a long history of upstream changes to the Everglades watershed, resulting in altered freshwater, nutrient, and carbon flows into FB (Hunt and Nuttle, 2007; Kelble et al., 2007; Stabenau and Kotun, 2012). Reductions in freshwater input have led to a contraction of estuarine conditions and associated biota (Tabb et al., 1962; Schmidt and Davis, 1978; Marshall et al., 2014), a process termed the "marinization" of FB that has driven increases in the distribution and dominance of *T. testudinum* monocultures, even within coastal basins.

Today, FB supports some of the most extensive seagrass meadows in the world (Fourqurean and Robblee, 1999; Hackney and Durako, 2004; Madden et al., 2009; Bricker et al., 2011), including *Thalassia testudinum*, *Halodule wrightii*, and *Syringodium filiforme* (Hall et al., 2006; Cole, 2017). *T. testudinum* is by far the dominant canopy-forming species, occupying some 2000 km^2 of the bay's 2,100 km^2 (Zieman et al., 1989). Across FB, it responds to resource and stress gradients with remarkable phenotypic plasticity in above-ground biomass, canopy height, and C:P ratios (Frankovich and Fourqurean, 1997; Bricker et al., 2011). During the last half-century, large, dense *T. testudinum* meadows in west-central FB have undergone several mass mortality events (e.g., 1974: Schmidt and Luther, 2002; 1987: Robblee et al., 1991; and 2015: Hall et al., 2016), each driven by a combination of high bottom-water temperature, hypersalinity, and stratification leading to widespread anoxia, photosynthetic stress, and sulfide toxicity (Carlson et al., 1994; Hall et al., 1999; Zieman et al., 1999; Borum et al., 2005; Koch et al., 2007; Johnson et al., 2018). Also, during this time, varied wet/dry season intensity and tropical weather systems have resulted in fluctuating *T. testudinum* densities in the coastal basins due to hyposalinity and light limitation (Wilson et al., 2020).

Labyrinthula infection is thought to be widespread in FB and likely factors into lost productivity and mortality that follow periodic reductions in light availability

(e.g., sediment resuspension events and algal bloom) and other physiological challenges presented to FB seagrasses (e.g., Robblee et al., 1991; Carlson et al., 1994; Durako and Kuss, 1994; Blakesley et al., 2002; Hall et al., 2016). Such challenges allow opportunistic pathogens, such as *Labyrinthula* spp., to capitalize on compromised host immunity (Bishop et al., 2017; Duffin et al., 2020). Given current climate predictions of the region (e.g., increased temperatures, altered precipitation, more frequent hurricanes, and sea level rise; Pederson et al., 2012; Koch et al., 2015; Carlson et al., 2018), and the ongoing restoration of the greater Everglades ecosystem (i.e., the Comprehensive Everglades Restoration Plan, CERP; Everglades Forever Act, 1994; Florida Forever Act, 2000; Water Resources Development Act, 2000) aimed at bringing increased freshwater delivery to the upper portions of the FB, it is likely that *Thalassia-Labyrinthula* interactions will play an important role in the resilience of *T. testudinum*-dominated meadows.

As an initial step toward understanding the dynamics of this complex pathosystem, we collected baseline data regarding host resistance (immune status) and *Labyrinthula* spp. load across the well-documented ecophysiographic landscape of FB (Zieman et al., 1989; Boyer et al., 1999; Herbert et al., 2011; Briceño et al., 2013). Our collections at the end of May 2015 coincided with the late dry season, during a period of advancing hypersalinity that is characteristic of FB in early summer (Kelble et al., 2007). However, long-term monitoring stations in the northeast region of the bay detected a brief freshwater pulse in the weeks leading up to our sampling. The timing of this event and coincidence with our synoptic survey captures a unique perspective of *Thalassia-Labyrinthula* associations in a natural system. Using antecedent environmental trajectories, host morphometric data, and spatial patterns in immuno-status and pathogen loading, we build a conceptual model of the *Thalassia-Labyrinthula* pathosystem rooted in prevailing theories of resistance and tolerance in phytopathology. While aspects of this model remain speculative, it provides a compelling framework to generate testable hypotheses for a wide variety of future explorations in this seagrass pathosystem.

MATERIALS AND METHODS

Turtlegrass Sampling

A survey of 15 *T. testudinum* beds was conducted across FB in late May, 2015 (**Supplementary Table S1**), in tandem with the spring 2015 Fisheries Habitat Assessment Program (FHAP) survey of the region (see next section for details). Ten turtlegrass shoots were haphazardly collected (roughly 8–10 m apart) along each permanent FHAP transect site. Individual specimens were bagged, temporarily stored on ice, and processed on land no more than 4 h post-collection. For processing, the third rank blade of each sample was split longitudinally; one half was stored in −80°C for use in immune activity assays and the other was preserved in Drierite® (Sigma Aldrich, Darmstadt, Germany) desiccant for qPCR procedures.

Long-Term Survey Data

Turtlegrass Morphometric Surveys

Turtlegrass abundance surveys were conducted every spring and fall (May and September/October, respectively), in coordination with the end of the dry and wet seasons of FB by FHAP, a component of the Monitoring and Assessment Plan (MAP) of Restoration, Coordination, and Verification (RECOVER) within the CERP (Everglades Forever Act, 1994; Florida Forever Act, 2000; Water Resources Development Act, 2000). Starting at the coordinates for each of the 15 sites included in this investigation (see **Supplementary Table S1**), a 50-m transect line was haphazardly subsampled 10 times within a 0.25-m^2 quadrat for macrophyte cover-abundance data (using a modified Braun-Blanquet technique; see Fourqurean et al., 2003), shoot density, standing crop, epiphyte biomass, and leaf morphometrics for 10 individuals, chosen haphazardly across quadrats. We focused on leaf morphometric data (specifically mean total area, in cm^2, per shoot) for the scope of our investigation as it is considered an important aspect of disease ecology (Burdon and Chilvers, 1982). Although these surveys have been conducted biannually since 2006, only the data collected between spring 2010 and spring 2015 were considered, as this time frame most directly reflects the conditions of the current standing crop of *T. testudinum*.

FB Salinity Monitoring Data

Salinity is often regarded as the most influential driver of environmental conditions in FB (Stabenau and Kotun, 2012), while also affecting *Labyrinthula* sp. *in vitro* and *in planta* dynamics (Martin et al., 2009; Bishop et al., 2017). We used two long-term water quality monitoring programs to characterize salinity trends at two temporal scales: monthly South Florida Water Management District (SFWMD) grab samples[1] and daily mean summaries of Everglades National Park (ENP) continuous monitoring data [South Florida Natural Resources Center (SFNRC), n.d.].

The short-term scale featured daily salinity means over a 2-month period leading up to the 2015 sampling (April 1st to May 30th 2015). Ideally, all salinity measurements would be obtained from the SFWMD database, as the monitored regions coordinate perfectly with 13 of our 15 study sites (the remaining two, CAR and MAN, featured significant gaps in the SFWMD data). However, as this survey records only monthly values, we substituted in daily salinity data from the SFNRC DataForEver monitoring stations at locations matching 10 of our 15 sample sites to analyze the short-term temporal scale as follows: six of the ENP stations (BS, MK, JB, JK, LM, and MB) matched perfectly or were very close to our sampling locations (BLK, GAR, JOE, JON, LIL, and MAN, respectfully); another four sites (DK, LB, BK, and WB) were located close enough that we considered them a proxy for our sites (DUC, LON, RAN, and WHP; **Supplementary Figure S1**).

The long-term scale spanned ~4.5 years antecedent to our sampling point (January 2010 to May 2015), and was measured

[1]https://www.sfwmd.gov/science-data/dbhydro

at roughly 1-month intervals. FHAP permanent transects are co-located with the SFWMD stations, so we relied on the SFWMD-DBHYDRO database to capture salinity (psu) measures at the permanent transect sites on a monthly basis, weather permitting. The protocol measures a suite of water quality parameters including salinity *in situ*, with a combination salinity-conductivity-temperature probe (Orion Model 140) 10 cm beneath the surface. Our investigation led us to consider whether more local and/or recent historical salinity conditions helped explain an intriguing dichotomy we observed in pathogen loading among a group of sites of the "East" immune class (see Materials and Methods section "Immune Classes" below). One site, MAN, is not included in the SFWMD survey, so we extracted values from the SFNRC data (which does sample at this location) for all dates when other sites were sampled.

Labyrinthula spp. Load

qPCR Strain Specificity

Our group recently developed a new qPCR primer set in an effort to accurately quantify *Labyrinthula* spp. loading in seagrass tissue (Duffin et al., 2020). Notably, these primers were designed to target putatively pathogenic *Labyrinthula* spp. (*sensu*: Martin et al., 2016), irrespective of *Labyrinthula* phylotype or host seagrass species origin (e.g., the turtlegrass pathosystem hosts more than one phylotype). Our initial specificity test surveyed 37 *Labyrinthula* sp. isolates, reliably amplifying 100% of putatively pathogenic strains and only 15% of non-pathogenic strains (Duffin et al., 2020). In this current study, we were interested in providing more conclusive evidence that our protocol is indeed strongly biased toward pathogenic *Labyrinthula* spp. This was accomplished by evaluating several sets of additional criteria including: (1) relative Cq values of pathogenic vs. non-pathogenic isolates; (2) lower DNA concentrations (per reaction) reflective of field sample pathogen loading; (3) presence of "background" DNA; and, (4) simulation of a scenario where a non-pathogenic phylotype (specifically, one consistently amplifying in the previous study; Duffin et al., 2020) is found in a higher concentration compared to a pathogenic phylotype.

First, summary information was compiled for the average cycle quantification value (Cq, also referred to as Ct) of amplified strains across previous qPCR runs (conducted before and after publication of the Duffin et al., 2020 pilot study) with varying DNA template concentrations, to assess whether the qPCR assay amplified putatively pathogenic isolates more readily (i.e., at a lower Cq value, on average) than non-pathogenic isolates. In the pilot study, we tested strains using very high concentrations of DNA isolated from pure *Labyrinthula* sp. cultures. Thus, secondly, we adjusted the concentration of starting *Labyrinthula* sp. template DNA to 25 cells per reaction (converted from *Labyrinthula* sp. cells per mg dry seagrass tissue), to better match realistic concentrations in the field. This reflects a cell count greater than the load present in >95% (and within 1 SD of the highest load detected) of our FB-collected *T. testudinum* samples with detectable levels of the pathogen (this study). Third, we accounted for the possibility that non-specific binding may occur when using pure non-pathogenic *Labyrinthula* sp. cultures as the only template, so we introduced UltraPure™ Salmon Sperm DNA Solution (Invitrogen™) as background DNA in our diagnostic qPCR assays (i.e., to mimic host "background" DNA). Fourth, we evaluated bias for the case of pathogenic types being outnumbered by non-pathogenic. Specifically, we tested pathogenic isolate "8b" and non-pathogenic isolate "98b," both of which reliably amplified in Duffin et al. (2020), but with notably different melt curve peaks at 76°C and 78.5°C, respectively, under several template DNA conditions: (1) pure *Labyrinthula* sp. culture at 1x concentration (2 µl 8b at 1x ≈ 646.8 cells per reaction; 2 µl 98b at 1x ≈ 1522.5 cells per reaction); (2) *Labyrinthula* sp. DNA equivalent of 25 cells per reaction with salmon sperm DNA to bring template volume to 20 ng total (i.e., 1 ng/µl reaction volume); and (3) both *Labyrinthula* sp. DNA loaded in a single reaction at a 1:70 ratio (~5.36 cells of 8b; ~375.2 cells of 98b per reaction), brought to 20 ng total with salmon sperm DNA. Each reaction was run in duplicate.

Quantitative Real-Time PCR Procedure

DNA Extraction

Dry longitudinal half-leaf sections were pulverized to a fine powder with a tissue homogenizer. DNA was then extracted from a ~5 mg subsample using an Invisorb® Spin Tissue Mini Kit (Stratec Molecular, Berlin, Germany). The resulting eluent was purified using a OneStep™ PCR Inhibitor Removal Kit (Zymo Research, Irvine, CA, United States). Prior to assaying, DNA concentration was quantified *via* spectrophotometer, allowing sample standardization at 10 ng μl^{-1}. See Duffin et al. (2020) for additional details.

Cell Counts/Standard Curve

We utilized the same standard curve protocol and template samples as in Duffin et al. (2020), derived from pure cultures of *Labyrinthula* sp. "E" isolate 8b, a strain isolated from and known to readily infect *T. testudinum* (Trevathan et al., 2011; Martin et al., 2016; Bishop et al., 2017). Briefly, cell concentrations from pure cultures were estimated using a Neubauer-improved hemocytometer, and the genomic DNA extracted as described above. This stock solution was then used to generate aliquots of DNA corresponding to *Labyrinthula* sp. cell concentrations (ranging from 320, 81, 5.0, 0.31, 0.02, and 0.0012 cells μl^{-1}) for use in the standard curve for all qPCR runs.

qPCR Protocol

Primers (LabPathITS1-3F: 5'-CAA CTC AAT GAA TAT CTT GGT TTC C-3', and LabPathITS1-3R: 5'-CCG CTT ATT GAT ATG CTT AAA TTC-3') targeted the ITS region of the ribosomal RNA gene complex (Duffin et al., 2020). Quantitative PCR (qPCR) reactions were prepared with the following final concentrations: 1 ng μl^{-1} of DNA template, 0.025µM of each primer, 2.7 ng μl^{-1} of BSA, 1X of iTaq SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, United States), and nuclease free water up to 20 µl. Reactions were run in triplicate on a CFX Connect thermal cycler (Bio-Rad) with the following cycle parameters: 5min at 95°C, followed by 45 rounds of 30 s at 95°C and 60 s at 63°C.

Reactions were terminated with a melting curve analysis (65–95°C, at 0.5°C increments). Results are reported as the number of Labyrinthula spp. cells per mg starting seagrass tissue (dry weight, ~5 mg). See Duffin et al. (2020) for additional details.

Pathogen Load Trends in FB

Prevalence and Severity by Site

We defined pathogen prevalence as the proportion of individuals infected at a given site (presence/absence of *Labyrinthula* spp. cells) and pathogen severity as the number of cells present in a sample (averaged across three qPCR assay replicates) or group of samples (arithmetic mean across samples) in cells per mg dry weight of seagrass tissue.

Severity Classes

Pathogen severity values were averaged across the 10 individuals from each of the 15 sites and then grouped into three pathogen severity classes by first plotting a histogram of mean pathogen severity values by site, and then using visual cues to classify sites into three roughly equally sized groups. After verifying that the new severity classes were highly statistically different from one another, they were used in downstream analyses and visualizations.

Host Immunity

Immunological Assay Procedures

Several common, well-studied immune activity enzymes were recently adapted for use with turtlegrass, including peroxidase (POX), exochitinase (EXOC), polyphenol oxidase (PPO), and a group of enzymes with lysozyme-like activity (LYS). The reader is referred to Duffin et al. (2020) for additional background and methodological details. Briefly, a small section (~0.1 g) of each *T. testudinum* blade (3rd rank, split longitudinally – with one half for use in qPCR, below) was homogenized and assayed. POX activity was measured using a modified guaiacol assay and presented as the change in absorbance at 470 nm min^{-1} μg^{-1} of total crude protein. EXOC activity was quantified using the enzymatic conversion of 4-methylumbelliferyl N-acetyl-β-D-glucosaminide into 4-methylumbelliferone (MU) and expressed as nmol of MU released per μg of total crude protein. PPO activity followed the conversion of L-DOPA to an oxidized dopachrome product and was quantified as a change in absorbance at 490 nm min^{-1} μg^{-1} of total crude protein. Finally, LYS activity was measured as the degree to which crude sample extract inhibited growth of *Micrococcus luteus*, given as percent inhibition per μg of total crude protein added as compared to uninhibited growth of the bacteria.

Immune Biomarker Levels by Site

Activity levels at each of the four biomarkers (POX, EXOC, PPO, and LYS) were compared among the 15 FB sites using a series of bubble plots. More specifically, bubbles were scaled following a linear relationship between mean sitewide biomarker level and bubble diameter, and then superimposed over a base map of the region to examine spatial patterns with respect to the geography of the bay.

Immune Classes

Multivariate analyses performed in PRIMER (v6) were utilized to group sites according to their overall immune status (simultaneously incorporating POX, EXOC, PPO, and LYS activity levels). First, we performed a hierarchical cluster analysis (CLUSTER routine), producing a dendrogram of similarity among sites based on immune status. This was followed by a similarity profile routine (SIMPROF), which is a permutation procedure intended to prevent over-dissection of subgroups and assess significance (Clarke et al., 2008). We also conducted non-metric multidimensional scaling (MDS) analysis with data from the four immune markers; the MDS groupings corroborated the site groupings of the CLUSTER analysis, so the results are not shown. These immune "classes" were used in downstream analyses and visualizations.

Host Morphology and Immune Classes

We characterized turtlegrass morphology as a function of immune status by generating box plots to visually summarize mean leaf area (cm^2) per shoot for each of three immune classes. While the original FHAP data set surveyed sites biannually (spring/fall), we detected no significant patterns between seasonal changes in leaf area and our factor of interest (immune grouping), so the results represent yearly averages of the two sampling points. The main box in the plot represents the interquartile range (IRQ) of the data, and the whiskers signify minimum and maximum spread of the data outside of the IRQ.

Statistics

All statistical tests were performed with 95% confidence intervals ($\alpha = 0.05$) on data that were tested for normality and computed using RStudio software (RStudio Team, 2018), unless otherwise stated. When plotting the immune activity data together for comparison, values were standardized through *Z*-score conversion in Microsoft Excel (v16.16.19). All error bars represent SEM, unless otherwise stated. Differences between groups were globally tested using a one-way ANOVA, followed by Tukey's test for *post-hoc* analysis; alternatively, the non-parametric Kruskal-Wallis test was used when the data could not be transformed to meet assumptions of normality.

RESULTS

qPCR Strain Specificity

A series of experiments were performed to further demonstrate that our qPCR assay (Duffin et al., 2020) preferentially amplifies pathogenic isolates of *Labyrinthula* spp. Pathogenic strain 8b and non-pathogenic strain 98b DNA, isolated from pure culture, both amplified in assays when high concentrations of either template were provided (**Supplementary Figures S2A,B**). However, average Cq values for 8b (20.16) were much lower than that of 98b (36.60), despite loading over twice as many cells in the 98b reaction as the 8b reaction. This pattern was representative of a larger trend: among qPCR assays with a

standardized addition of *Labyrinthula* sp. DNA template (10 ng/µl input concentration), the average (±SEM) Cq value for pathogenic strains (26.54 ± 0.69, n = 18) was significantly lower (p < 0.001) than that of non-pathogenic strains (39.02 ± 1.12, n = 7).

Further, when templates were reduced to low concentrations (DNA isolated from ~25 *Labyrinthula* cells per reaction) and supplemented with background DNA (salmon sperm) to deter non-specific binding, the representative non-pathogenic strain failed to amplify in either replicate, while the pathogenic strain amplified readily (results not shown). Finally, both isolate DNA templates were analyzed simultaneously at a 1:70 ratio favoring the non-pathogenic strain and in the presence of background DNA. The amplified product featured a strong melt peak at 76°C, matching the expected peak temperature of 8b, with no indication of a secondary peak corresponding to 98b at 78.5°C (**Supplementary Figure S2C**).

Labyrinthula spp. Load Trends in FB

Prevalence and Severity

We surveyed 15 sites in FB that captured the geomorphic, hydrological, and morphological variability of turtlegrass communities in the system. **Figure 1** summarizes *Labyrinthula* spp. prevalence and severity values quantified in turtlegrass samples collected from each site, with each section of the pie representing one *T. testudinum* individual. Site prevalence, or percent of individuals with detectable levels of *Labyrinthula* spp. according to our qPCR assay, was 46% of individuals pooled across all 15 sites (69 of 150 samples). Site prevalence (n = 10 per site) ranged from 0% (DUC) to 100% (JOE; **Figure 1; Supplementary Table S2**). The severity of *Labyrinthula* spp. infection, or the quantified number of *Labyrinthula* spp. cells per mg dry seagrass tissue (i.e., putative pathogen load), was analyzed at both the individual level (pie wedge shading in **Figure 1**) and the site-wide level (**Supplementary Table S2**). The average load among all 150 samples collected was 40.05 cells mg^{-1}; among those with detectable levels, the average was 87.07 cells mg^{-1}. The maximum and minimum (non-zero) loading values among all individuals were 1009.08 and 4.89×10^{-9} cells mg^{-1}, from JOE and CAR, respectively. Further, five of the top 10 most infected samples were collected from JOE; the remaining were from TER (n = 2), LON (n = 2), and BLK (n = 1). Site-wide severity averages ranged from 0.00 to 280.55 cells mg^{-1} (**Supplementary Table S2**). The pathogen was present with varying degrees of severity across all main geographic regions of the bay (western, central, and eastern; **Figure 1**).

Severity Classes

We further categorized sites into three classes according to their average (relative) pathogen severity for downstream analyses. Four sites (JOE, LON, TER, and MAN) fell under the "high" pathogen loading class, coded as "3"; seven sites (JON, RAN, RKB, LIL, TWN, GAR, and WHP) were characterized by "moderate" pathogen loading, coded as "2"; the remaining four sites (BLK, EAG, CAR, and DUC) had "low" (or zero) pathogen loading and were coded as the number "1" (**Supplementary Table S2**). The average (±SEM) pathogen loading of the severity classes were as follows: Individuals of Class 3 (high load; n = 40) harbored 143.14 ± 36.13 *Labyrinthula* spp. cells mg^{-1} tissue; plants in Class 2 (moderate load; n = 70) had an average of 4.03 ± 1.60 cells mg^{-1}; and finally, individuals belonging to severity Class 1 (low load; n = 40) harbored 0.0017 ± 0.0013 cells mg^{-1}. The overall differences between pathogen loading values across severity groups were highly significant (Kruskal-Wallis test; p < 0.001).

Turtlegrass Immune Activity in FB

Biomarker Activity by Site

We assessed the immune status of FB-collected individuals at four biomarkers to provide a broad physiological perspective: POX, EXOC, PPO, and LYS. **Figure 2** depicts activity of each of the four immune markers, averaged across individuals collected at each of the 15 sites (n = 10 per site). The size of the bubble represents relative activity at a given biomarker. Sites significantly differed from one another, overall, at the EXOC, POX, and PPO biomarkers at α = 0.01 (p < 0.0001), but the differences between sites were not significant at the LYS biomarker (p = 0.159). Generally, enzymatic trends followed an east-to-west gradient with low activity in the west and higher activity in the east (**Figure 2**). EXOC activity at the centrally-located site EAG represented an outlier to this trend, but was driven by only two individuals with notably high EXOC-substrate conversion rates (**Figure 2B**).

Immune Classes

We used hierarchical clustering to group sites together based on similarities at all four immune biomarkers (**Supplementary Figure S3**). The clustered divisions were supported by SIMPROF at α = 0.05; further, in an ANOVA analysis, the groupings differed significantly from one another at the EXOC, POX, and PPO biomarkers at α = 0.01 (p < 0.0001 each), and at the LYS biomarker at α = 0.05 (p = 0.042). The three distinct immune classes generated through these analyses (arranged lowest to highest response: A, B, and C) also distributed geographically, and thus further designated with the FB region they grouped in, where: A = "West" sites JON, TWN, LIL, and RKB; B = "Central" sites GAR, RAN, TER, and WHP; and C = "East" sites BLK, EAG, CAR, LON, JOE, DUC, and MAN (**Supplementary Figure S3**). Our multivariate clustering results reinforced trends seen at the site-level (**Figure 2**) in that the West and Central classes were more similar to one another than either were to the East. Importantly, this distinction was due to an elevation of biomarker activity in the East immune class as compared to both the West and Central classes (p < 0.05 at all pairwise comparisons except West-East LYS activity; **Supplementary Figure S4**).

Morphology and Immunity

Turtlegrass morphology varies remarkably across different basins of FB and is likely a plastic response to heterogeneous selective pressures across the bay (Hackney and Durako, 2004; Bricker et al., 2011). We examined potential relationships

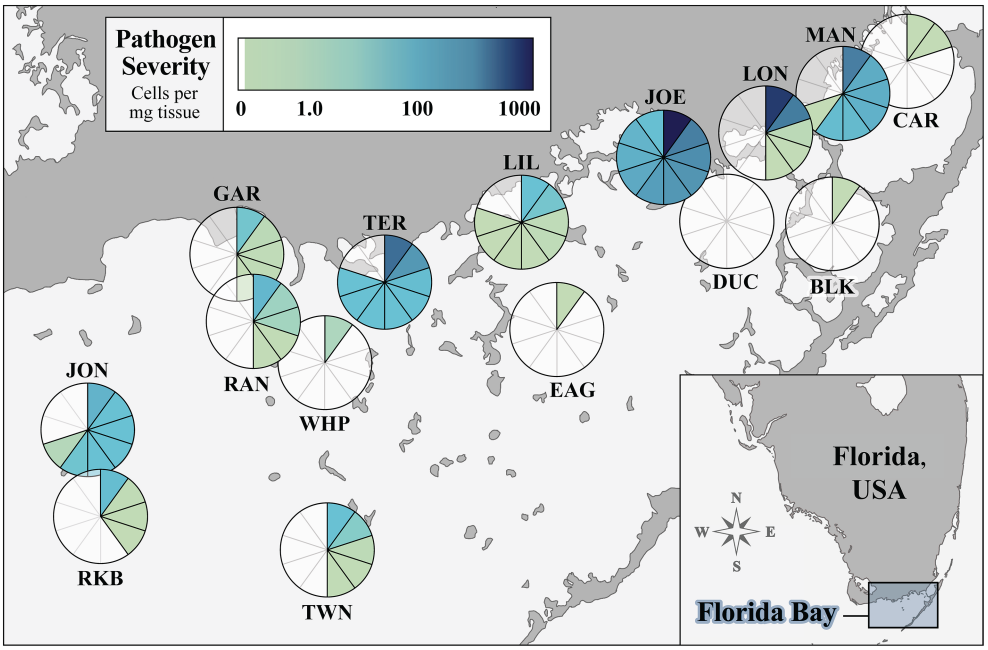

FIGURE 1 | Prevalence and severity of *Labyrinthula* spp. in turtlegrass among 15 Florida Bay study sites. The pie chart layered over the location of a site represents disease prevalence (percent of samples out of 10 infected with quantifiable pathogen load) at that site. Each pie segment represents one *Thalassia testudinum* individual and is shaded according to pathogen severity (*Labyrinthula* spp. cells per mg dry weight of seagrass tissue). Sample size n = 10 individuals per site. Inset map shows study region relative to the mainland peninsula of Florida, United States.

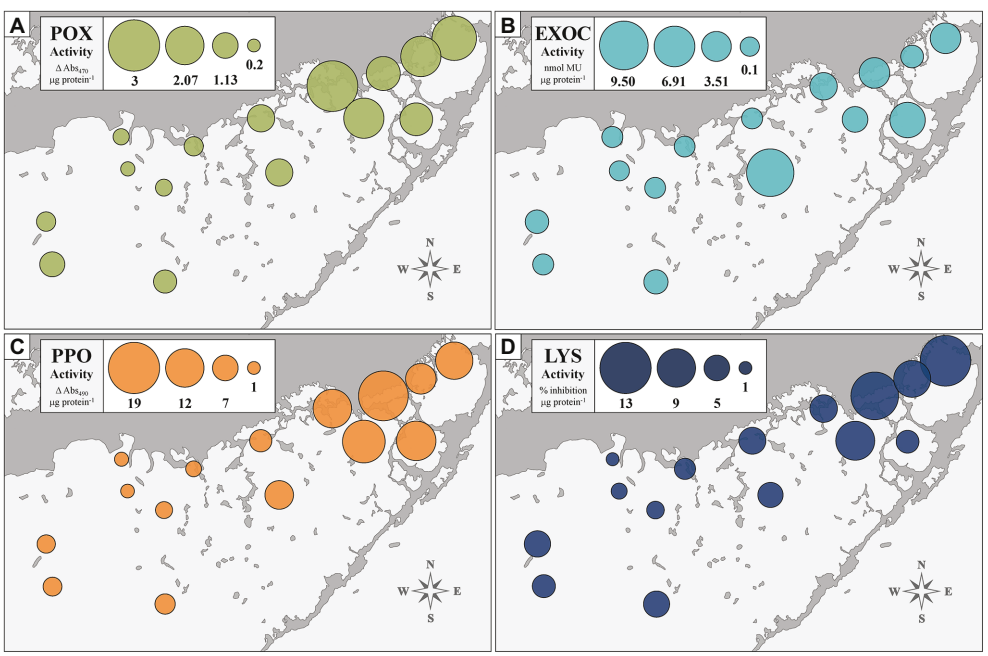

FIGURE 2 | Immune enzyme activities of four biomarkers (POX, EXOC, PPO, and LYS: **A–D**) in turtlegrass among 15 Florida Bay study sites. Sizes of bubble plots layered over site locations represent magnitude of biomarker activity averaged across the 10 individuals collected at that site.

between *T. testudinum* morphology and immune activity by summarizing variability in mean leaf area (cm^2) across sites clustered into the three immune classes (**Figure 3**). These differences were highly significant in a global ANOVA analysis ($p < 0.0001$); further, pairwise differences were significant between the East class and both the Central and West classes

(Tukey's *post-hoc*: $p < 0.0001$), but not between the West and Central classes (Tukey's *post-hoc*: $p = 0.841$; **Figure 3**). Indeed, the interquartile range of the East class fell below both the mean (29.3 cm^2) and median (20.5 cm^2) values pooled across FB (fine and coarse dashed lines, respectively; **Figure 3**).

Geography, Morphology, and Immunity

Over the past several decades, several attempts have been made to partition the bay into discrete regions based on biogeochemical characteristics and ecological gradients (e.g., Wanless and Tagett, 1989; Boyer et al., 1999; Bricker et al., 2011; Briceño et al., 2013). We reviewed and compared these biogeographical/ecological models to the spatial patterns observed in our data (**Figures 1, 2**). One model, a projected reorganization of seagrass communities based on changes in freshwater inflow described in Herbert et al. (2011), largely coincided with the geographical distribution of the immune classes we established in this study (**Supplementary Figure S3**). Superimposing our data over Herbert et al. (2011), we created a layered map of FB (**Figure 4**) where biogeochemical zone delineations were outlined in black, and shaded according to the immune classes that coincided with these regions (West, Central, and East; shaded in dark blue, light blue, and yellow, respectfully; **Figure 4**), now representing "immune zones." A third layer of data, pathogen severity class, was incorporated into the zone map by adding the numerical label for each pathogen severity class (low, moderate, and high severity: "1," "2," and "3," respectively) at each site location (**Figure 4**).

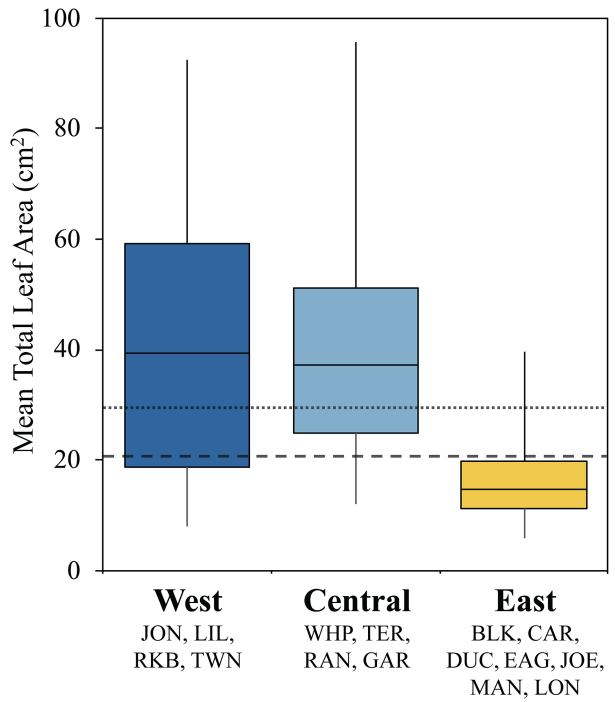

FIGURE 3 | Box plots displaying turtlegrass mean leaf area per shoot (cm^2) collected biannually from January 2010 to May 2015 as a function of immune class. Sample sizes as follows: West, $n = 56$; Central, $n = 53$; and East, $n = 97$. Boxplots represent the interquartile range (IRQ) centered around the median of the data. Whiskers signify the minimum and maximum spread of the data outside of the IRQ. Fine and coarse dashed lines represents the mean (29.26 cm^2) and median (20.50 cm^2) values across all sites, respectively. Data obtained through Fisheries Habitat Assessment Program (FHAP) survey database (M. Durako, pers. comm.).

Generally, immune activity increased from west to east in FB, in a roughly inverse relationship with leaf area (cm^2), which decreased along the same gradient (**Figure 4**). Further, eight of the nine sites within the West or Central zone had moderate ("2") pathogen loading, excluding site TER in the Central zone (**Figure 4**). This is in contrast with pathogen load in the East zone, where sites always fell within one of the two extremes; the three sites closest to the mainland (JOE, LON, and MAN) all had high ("3") loading, whereas the four sites more distal to the shoreline (EAG, DUC, BLK, and CAR) had low ("1") loading (**Figure 4**).

Antecedent Salinity and Pathogen Load

One driving aim of CERP is to manipulate freshwater output from the South Florida watershed to restore historic conditions which favored a more diverse species assemblage in seagrass communities (Everglades Forever Act, 1994; Florida Forever Act, 2000; Water Resources Development Act, 2000). Thus, we were interested in relating temporal patterns of salinity to *Labyrinthula* spp. loading in host turtlegrass tissue. First, we examined daily salinity trends over a 2-month period leading up to the 2015 sampling date (**Figure 5A**). Sites with moderately severe ("2") pathogen load generally experienced the most saline conditions in the 2 months leading up to the sampling period, while sites falling in the low and high pathogen severity classes ("1" and "3," respectively) experienced lower salinities (**Figure 5A**). The majority of sites spent most of April and May of 2015 outside the host optimal salinity range (25–35 psu), and likely the pathogen salinity range as well (see Discussion section). One site with high pathogen load, JOE, stands out due to the significant dip in salinity beginning around May 1, 2015, approximately 1 month before tissue collection (**Figure 5A**). This site is also distinct in terms of pathogen load, even among other sites of the "High" pathogen severity class; pathogen prevalence at JOE was 100% and average pathogen severity was nearly double that of the next highest site, LON (280.6 ± 88.8 vs. 122.8 ± 86.7 cells mg^{-1}, respectively; **Supplementary Table S2**).

Finally, we further investigated the stark dichotomy displayed by the pathogen severity classes of sites in the East immune zone, noting that "High" pathogen severity-grouped sites in this zone were located within semi-enclosed coastal basins, while "Low" sites were all more distal to the coast and in relatively open water (**Figure 4**). Specifically, we examined longer-term salinity trends in the East zone by plotting monthly salinity data by site from 2010 to 2015 (color coded by pathogen severity class), with local regression (loess) smoothing lines defining average trends shared by sites in either pathogen severity class (**Figure 5B**). While salinity fluctuations in "Low" severity class sites were often confined within the upper and lower margins of the host optimal range, conditions in "High" severity class sites often dipped well below the lower threshold optima of

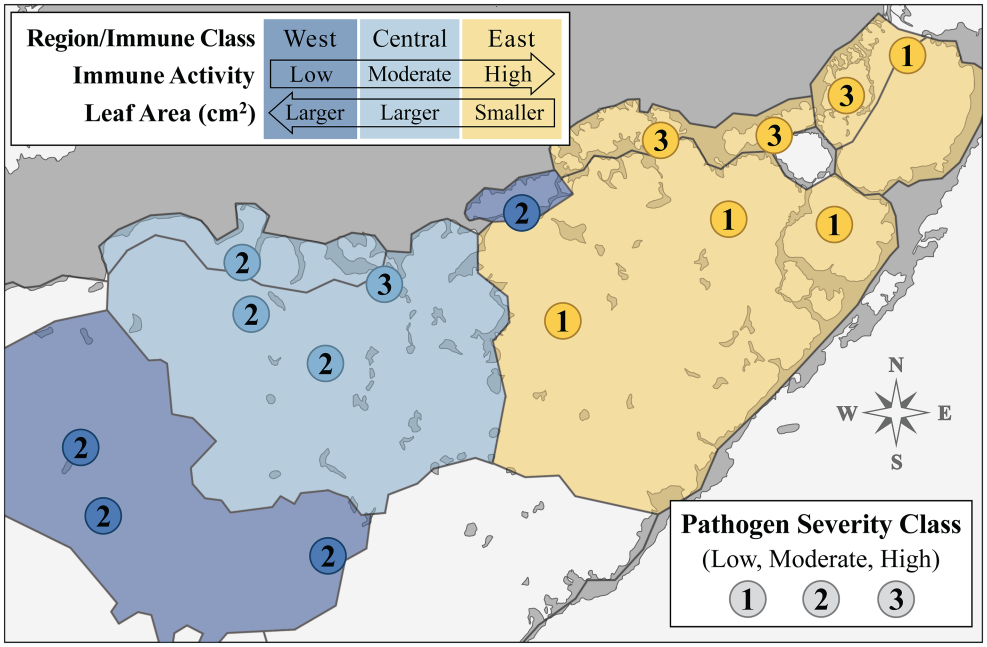

FIGURE 4 | Map displaying site-level immune activity (color) and pathogen load (number label) classes layered over the biogeochemical segmentation of Florida Bay (RECOVER, 2010; Herbert et al., 2011) to effect designation of overall "immune response zones." Numbers labeling each site represent pathogen severity class. Biogeochemical segments shaded according to associated immune class sites. Legend arrows indicate directional trends observed in composite enzyme measures (immune activity) and morphology (leaf area in cm^2) across the spatial gradient of sampled Florida Bay regions.

25 psu (**Figure 5B**). Extreme hyposaline conditions (between 0 and 10 psu) were not uncommon among high-load coastal sites JOE, LON, and MAN, but never occurred during the ~4.5 year span among offshore low-load sites BLK, CAR, DUC, and EAG (**Figure 5B**). In addition to experiencing frequent extreme hyposaline conditions, sites of the "High" severity class experienced greater fluctuations in salinity, with hypersaline conditions comparable to that of the "Low" severity class sites (**Figure 5B**). For example, we note extreme fluctuation amplitudes (trough to crest) in high-load sites measuring between ~20 and ~38 over an approximate 6 month period (late 2010 to mid 2011; **Figure 5B**).

DISCUSSION

Florida Bay turtlegrass meadows are characterized by a well-documented phenotypic gradient with larger-bodied high-density *T. testudinum* shoots in the West and Central regions, and smaller low-density shoots in the East (this study; Bricker et al., 2011). We leveraged this, along with the known environmental gradients and homogeneous distribution of genotypes across the region, for comparative purposes. Our composite measure of host immune metrics displayed a comparable but opposing gradient to that of phenotype, exhibiting the lowest enzymatic responses in the West and highest in the East. We further characterized FB turtlegrass by surveying both pathogenic *Labyrinthula* spp. prevalence and load *in situ*; to our knowledge, this component of the study was the first of its kind for *T. testudinum*. Additionally, we build off of a previous study (Duffin et al., 2020) in providing more evidence that the primers used also serve as a universal qPCR-based method to quantify pathogenic *Labyrinthula* spp. loading in other seagrass species. Specifically, we confirmed that qPCR bias heavily favors seagrass-pathogenic clade phylotypes (*sensu*: Martin et al., 2016) when used in an ecological context (i.e., when at realistic concentrations, and with ample background DNA – as from host tissue; **Supplementary Figure S2**), even when outnumbered (1:70) by a non-pathogenic phylotype. Overall, *Labyrinthula* spp. were detected at 14 of the 15 sites, showed a prevalence of 46% for individual shoots, and exhibited a strong spatial pattern related to host immunity and morphology, and the ecophysiography of FB. Below, we unite our data observations with previous FB characterizations of resource availability and resistance-tolerance theory, deconstructing this complex pathosystem into a simple, conceptual model that serves as a framework for future hypothesis-driven work. In summary, we posit that FB turtlegrass defenses present with a strategy of constitutive immune resistance in more stressful environments but adopt a strategy of tolerance when resources are more plentiful.

Immunity – Geography and Morphology

Immune enzyme activities were used to reflect the functional status of host defense pathways. Our results showed spatially coherent clusters that were based upon enzyme (POX, EXOC, PPO, and LYS) activities: lowest in western and central FB and highest in the east (**Figure 2** and **Supplementary Figure S4**; hereafter: West, Central, and East classes). Because immune status aligned well with the ecophysiographic delineations

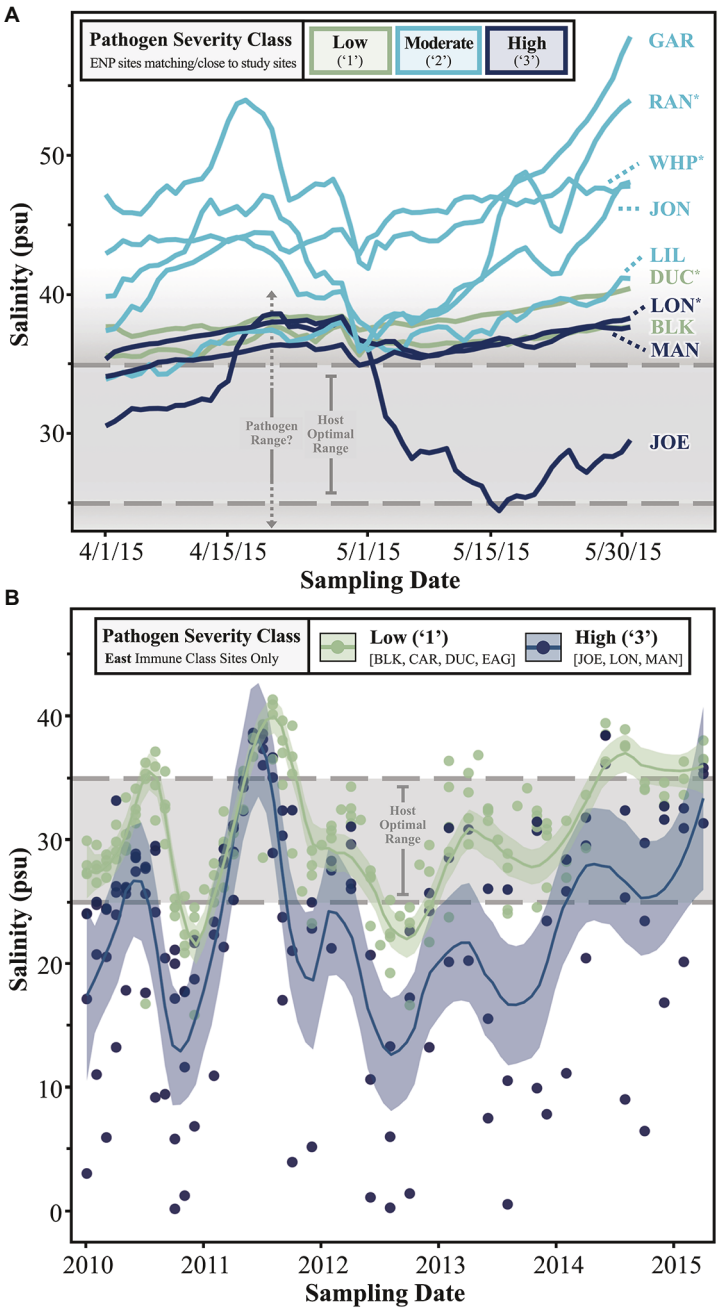

FIGURE 5 | Line graphs depicting trends in **(A)** short- and **(B)** long-term salinity measurements collected from either **(A)** all ENP sites in close proximity to our study sites or **(B)** permanent FHAP transects/our study sites falling within the "East" immune class. In part **(A)**, the data represents ENP station sampling points but are named according to our study sites/FHAP basins they pair with. Asterisks (*) denote study sites with nearby, but not perfectly complementary, ENP stations. See **Supplementary Figure 1** for details.

described in Herbert et al. (2011), we interpreted them as "immune response zones" (**Figure 4**) with a linkage between plant immunity and the biogeochemistry of the bay. It was noted that individuals of the East immune class had much lower total leaf area than individuals of either Central or West regions (**Figure 3**), as expected from the east-west gradient described by Bricker et al. (2011). The driver of modest differences between the West and Central zones remains unclear but may be a consequence of gradient sampling.

Defense Strategies – Resistance and Tolerance

Tolerance and resistance are two basic strategies plants have when facing disease: the term "tolerance" describes a host's

ability to minimize the impact of infection on fitness without specifically reducing pathogen load in host tissue, while "resistance" characterizes the plant's ability to mount a response that actively limits pathogen reproduction (Råberg, 2014; Pagán and Garcia-Arenal, 2018). There can be trade-offs between the two strategies and they are not necessarily mutually exclusive (Pagán and Garcia-Arenal, 2018). That said, the existence of a disease tolerance strategy appears not to have been evaluated critically for any seagrass – including this study. Our data do not allow us to quantify or conclude a strategy of tolerance, but we use the concept to relate spatial patterns in our survey and to generate testable hypotheses. The composite immune metric (assimilation of all four enzyme activities) used in this study suggests that smaller (more eastern) individuals may be allocating more resources to constitutive resistance, while larger (more western) individuals appear to tolerate mostly moderate levels of infection.

Importantly, resistance and tolerance strategies yield different selective pressures (as only the former directly reduces pathogen reproduction, or infectivity), while theory predicts the two may coevolve with one another, and with pathogen virulence, in complex ways (Best et al., 2009; Carval and Ferriere, 2010; Little et al., 2010). Since tolerance can increase prevalence, it may have special relevance to disease spread in times of climate change (Kutzer and Armitage, 2016). Both strategies can be evidenced from classical reaction norm studies, relative to uninfected status (i.e., underlying vigor), where fitness is a function of pathogen loading or disease severity (Stowe et al., 2000; Baucom and de Roode, 2011). While we did not include such a study, two of our markers were consistent with an inducible response to *Labyrinthula* sp. infection, and all four markers were often associated with pathogen defenses (Duffin et al., 2020). We, therefore, interpret our immune composite measures as broadly reflecting underlying resistance. Our claim of tolerance remains conjectural, however, and we provide no alternative indirect measure aside from size differences (e.g., tolerance is often achieved through compensatory growth; Rausher, 2001; Baucom and de Roode, 2011) and consistent pathogen loading.

To explain the temporospatial relationship between immunostatus, pathogen loading, and host phenotype across the FB seascape, we introduce a simple, testable conceptual model treating resistance and tolerance as opposite (though not mutually exclusive) ends of a resource availability gradient (*y*-axis; **Figure 6A**), with the former subject to environmental perturbation. Field measures of mean immune zone responses (diamonds, colored as in **Figure 4**) are shown as a function of resources and load, but also in relation to posited defense strategies as they reflect relative directional relationships (right *y*-axis; **Figure 6A**) to resource availability. The dichotomy of load levels in the East zone (yellow) sites as either very low or very high suggests the presence of a significant but unidentified factor(s) contributing to this pattern (see below). Though other studies have shown differing relations to environmental resources (Kutzer and Armitage, 2016), recent empirical work by Zeller and Koella (2017) indicates that challenging environments can actually lead to higher levels of parasite defense, but also that higher resources can lead to the evolution of tolerance. For plants, especially longer-lived types, the expectation of high resource environments generally favoring tolerance is not new (Maschinski and Whitham, 1989), but also not universal. Although we chose leaf area as a measure of size to confirm the earlier work describing the size gradient across FB (Bricker et al., 2011), a *post-hoc* look at several fitness metrics yielded the same basic pattern, consistent with our conceptual model (**Figures 6B–D**). This relation was especially notable for below-ground biomass (**Figure 6D**), reinforcing the notion that resource availability is a key factor.

From an antagonistic coevolutionary perspective (i.e., an evolutionary arms race; Roy and Kirchner, 2000; Rausher, 2001; Kutzer and Armitage, 2016), the differential success of resistance between the inshore and offshore sites in the East zone (**Figure 4**) could reflect conditions conducive for the evolution of a more virulent strain at inshore sites. Regardless, it is clear that future work on tolerance and *Labyrinthula* sp. evolution has the potential to predict where and when more virulent strains might emerge, provide a mechanistic explanation for outbreaks, or inform conservation efforts that might target tolerance over resistance (e.g., Rohr et al., 2010; but see Carval and Ferriere, 2010).

Although not designed to evaluate tolerance *per se*, studies by Brakel et al. (2014, 2019) invoke tolerance to SWD by suggesting that intra-leaf and/or younger-leaf growth compensates for lesion size, though to what extent this is adaptive is unclear. For example, in Brakel et al. (2014), it appears any benefit to increased growth could be offset by the decreased rate of emerging leaves relative to non-infected shoots. In Brakel et al. (2019), their measure of net leaf growth outpacing lesion size appears to show compensatory growth within individuals, but a measure of growth for uninfected individuals (i.e., vigor) is not provided, and possible loss of leaf function above the lesion (Steele et al., 2005) is not addressed. The effect also appears to diminish with infection intensity. Finally, Brakel et al. (2017) found that growth did not keep pace with lesion extent. In summary, it seems that eelgrass may be capable of partial compensation, but it is unclear under what conditions, or what effect this might have on below-ground reserves and thus long-term fitness.

FB Salinity Trends

Florida Bay is part of the world's largest restoration initiative with projected costs of over a billion dollars; one of the main tools under management control is freshwater flow into FB [U.S. Army Corps of Engineers and U.S. Department of the Interior (USACE-DOI), 2015]. While earlier studies do not identify specific phylotypes of *Labyrinthula* sp., it was recognized early on that eelgrass wasting disease of the 1930s was less common in lower salinity meadows (Muehlstein, 1989; Burdick et al., 1993). Similarly, both cultured isolates and controlled mesocosm observations show better growth or disease progression above about ~10–25 psu, but below ~40–50 psu for both eelgrass (Young, 1943; Pokorny, 1967; Short et al., 1987; Muehlstein et al., 1988; McKone and Tanner, 2009) and turtlegrass

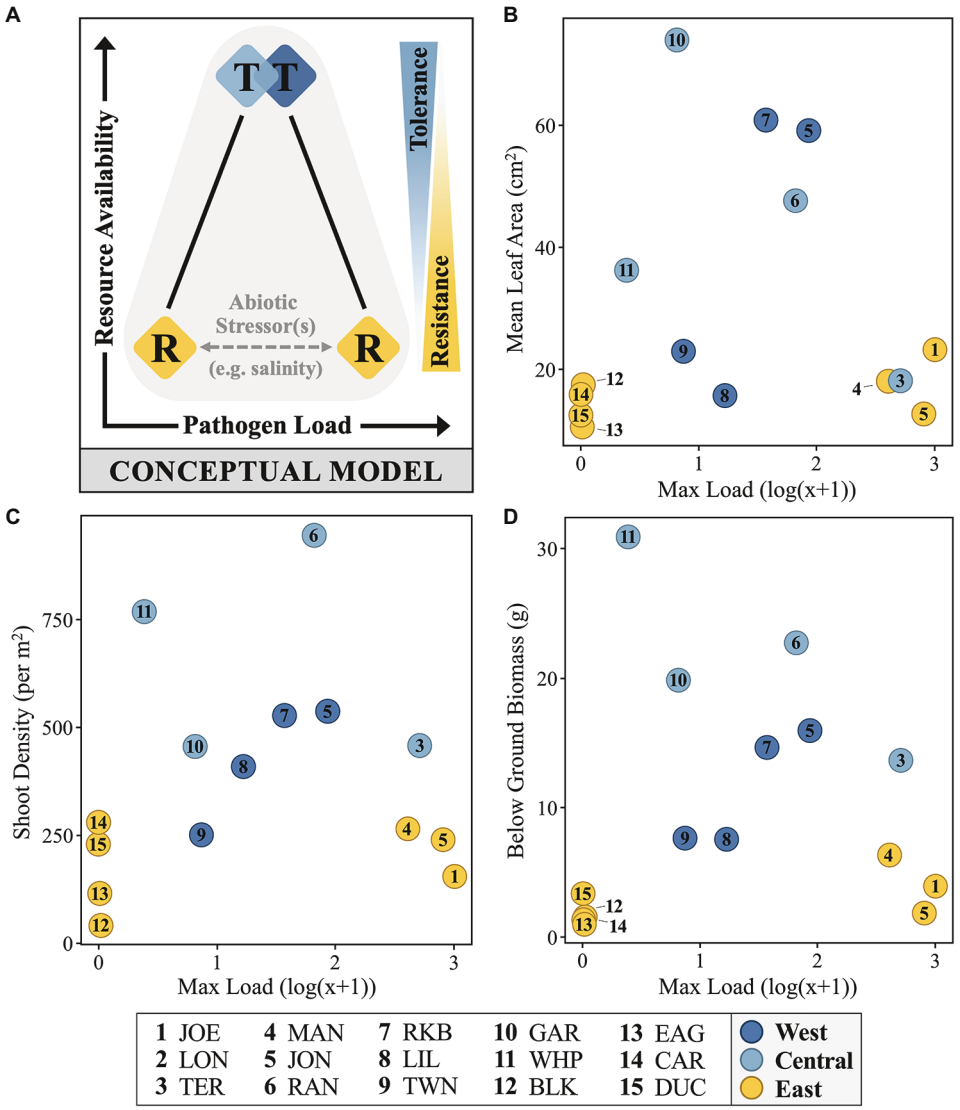

FIGURE 6 | A conceptual model **(A)** and selected fitness metrics **(B–D)** relating immunostatus, *Labyrinthula* pathogen loading and host phenotype of FB turtlegrass. In part **(A)**, colored diamonds represent field measures of mean immune zone responses adopting a strategy somewhere along the tolerance ("T") and resistance ("R") continuums (represented as blue/yellow wedge gradients, right). Measures of pathogen load and resource availability increase along the *x*- and *y*-axes, respectively. Influence of abiotic stressors (represented by gray horizontal dashed line), such as salinity stress, is suggested to play a role in determining pathogen load in hosts presenting with a strategy of resistance ("R"). In parts **(B–D)**, data are plotted along similar *x*- and *y*-axes as in the conceptual model; here, pathogen load is represented as the log(x + 1) value of the most infected individual at a given site and resource availability is substituted with three measures of morphological data that serve as reasonable proxies for plant fitness: mean leaf area (cm^2), shoot density (per m^2), and below ground biomass (g).

pathosystems (Martin et al., 2009; Trevathan et al., 2011; Bishop et al., 2017). However, to our knowledge, more detailed studies of salinity effects within the turtlegrass pathosystem are lacking.

Florida Bay is a seasonally hypersaline, reverse estuary with spatiotemporally variable salinities driven largely by direct rainfall, runoff, shallow bathymetry, and extreme climatic events (i.e., hurricanes and droughts; Kelble et al., 2007; Cole et al., 2018). Annual trends in FB salinity indicate that there are predictable, widespread oscillations between hypersaline conditions in early summer and, in the coastal basins, hyposaline conditions in the early-winter (Kelble et al., 2007), so it is not surprising that daily measurements antecedent to our early June 2015 sampling were between 35 and 55 psu, above the optimal range of turtlegrass (**Figure 5A**). One site, JOE, experienced a rapid salinity decline within 30 days of our collection, likely the result of an acute runoff event from the Everglades (Kelble et al., 2007; Sullivan et al., 2014). Interestingly, the sampling site in Joe Bay also experienced the highest pathogen loads, in both prevalence and severity of *Labyrinthula* spp. infection. Superficially, it might

be surprising that the only site where salinity fell within the host optimal range during the time of collection also experienced the highest infection. However, high salinity is equally or more likely to inhibit pathogen growth and/or transmission (e.g., Martin et al., 2009; Bishop et al., 2017), and to the extent that pathogens can respond more quickly than host resistance efforts, short-term salinity could explain the small-scale patterns we documented.

Salinity stress might also play a role in the relative success of resistance vs. tolerance strategies adopted by different turtlegrass populations of FB over longer temporal scales. The 5-year view of monthly salinity trends among sites of the East immune response zone revealed that locations with highest pathogen loads also experienced dramatic fluctuations between moderate saline conditions (i.e., within turtlegrass and *Labyrinthula* sp. optimal range) and periods of extreme hyposalinity (**Figure 5B**). Thus, chronic environmental stress may impair the immune response of the host. Specifically, we note that the relatively enhanced immune response of East zone individuals appears to have been successful at sites where longer-term hyposalinity stress events were minimal (more off-shore sites), but small-bodied high-immune activity individuals that routinely experienced hyposaline stress (i.e., more coastal basins) succumbed to *Labyrinthula* spp. infection (**Figure 5B**). From this perspective, the results generally suggest that plant morphology, immune status, and hyposalinity events interact to drive differences in the host susceptibility to pathogen loading across FB. Finally, despite our focus on salinity (due to its historic influences in the study system and compelling fit with our data), we further emphasize that this stressor represents just one of many potential abiotic drivers modulating defense strategies and wasting disease in FB turtlegrass.

Conclusion

It has been nearly a century since the massive 1930's north Atlantic eelgrass wasting events attributed to *Labyrinthula* sp., yet, we struggle to predict or describe such outbreaks (Sullivan et al., 2013, 2018). This can be attributed in part to the opportunistic nature of some *Labyrinthula* spp. – a term that may reflect more about our lack of knowledge regarding the specifics and/or variety of mechanisms affecting hosts and pathogens (e.g., emergent properties: Bull et al., 2012; Egan and Gardiner, 2016), with the latter serving only as an operational definition (Pirofski and Casadevall, 2012). Nonetheless, it is important to move forward by uncovering factors affecting disease dynamics in foundational species such as turtlegrass. Toward this end, our new tools for qPCR and resistance metrics show promise for seagrass-pathosystem studies. In addition to the strong morphological plasticity documented across the complex environmental gradient of FB, turtlegrass itself appears to possess significant physiological plasticity in immunostatus and pathogen defense strategies. Our data also hint at the role of environmental quality on both host defenses and shorter-term factors that appear to drive pathogen transmission/infection dynamics capable of overrunning host defenses. Importantly, our results were inconsistent with classic density-dependent explanations for disease prevalence, indicating that these host-pathogen dynamics are far more complex than just conditions that affect transmission. While resistance is often the metric of choice in defense studies, we argue that investigations of tolerance could provide needed perspective for disease dynamics in terms of pathogen prevalence and evolution (e.g., virulence) in a changing climate.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

CR initiated and acquired funding for this project. PD, DM, and CR conceptualized the ideas that drove the manuscript. PD and DM led methodological advancements in the qPCR assay. Pure *Labyrinthula* culture collections were carried out by DM. PD acquired samples, curated sample data, and led the formal analysis and investigation, with help from DM, BF, and CR. Environmental data acquisition and multivariate analyses relied on the expertise of BF. Visualization of data was primarily achieved by PD and BF, with input from DM and CR. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.612947/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CORRECTION
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Corrigendum: Spatial Patterns of *Thalassia testudinum* Immune Status and *Labyrinthula* spp. Load Implicate Environmental Quality and History as Modulators of Defense Strategies and Wasting Disease in Florida Bay, United States

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Keywords: immunocompetence, host-pathogen interactions, hyposalinity stress, opportunistic pathogens, environmental fluctuation, anthropogenic influences, resistance, tolerance

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A Corrigendum on

Spatial Patterns of *Thalassia testudinum* Immune Status and *Labyrinthula* spp. Load Implicate Environmental Quality and History as Modulators of Defense Strategies and Wasting Disease in Florida Bay, United States

by Duffin, P., Martin, D. L., Furman, B. T., and Ross, C. (2021). Front. Plant Sci. 12:612947. doi: 10.3389/fpls.2021.612947

In the original article, there was an error. The primer set sequences we originally provided did not accurately reflect the primer set sequences we used to conduct the study. The original study referenced, however, does contain the correct sequence (Duffin et al., 2020).

A correction has been made to *Materials and Methods* > Labyrinthula *spp. Load* > *Quantitative Real-Time PCR Procedure* > *qPCR Protocol*, paragraph one:

Primers (LabPathITS1-3F: 5′-CAA CTC AAT GAA TAT CTT GGT TTC C-3′, and LabPathITS1-3R: 5′-CCG CTT ATT GAT ATG CTT AAA TTC-3′) targeted the ITS region of the ribosomal RNA gene complex (Duffin et al., 2020). Quantitative PCR (qPCR) reactions were prepared with the following final concentrations: 1 ng μl^{-1} of DNA template, 0.025 μM of each primer, 2.7 ng μl^{-1} of BSA, 1X of iTaq SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), and nuclease free water up to 20 μl. Reactions were run in triplicate on a CFX Connect thermal cycler (Bio-Rad) with the following cycle parameters: 5 min at 95°C, followed by 45 rounds of 30 s at 95°C and 60 s at 63°C. Reactions were terminated with a melting curve analysis (65–95°C, at 0.5°C increments). Results are reported as the number of *Labyrinthula* spp. cells per mg starting seagrass tissue (dry weight, ∼5 mg). See Duffin et al. (2020) for additional details.

In the original article, there were a few errors in reporting concentrations of *Labyrinthula* strains used in the qPCR specificity assays. Specifically, there were three locations within a single sentence where "ml" was inadvertently used instead of "μl."

A correction has been made to *Materials and Methods* > Labyrinthula *spp. Load* > *qPCR Strain Specificity*, paragraph two:

First, summary information was compiled for the average cycle quantification value (Cq, also referred to as Ct) of amplified strains across previous qPCR runs (conducted before and after publication of the Duffin et al., 2020 pilot study) with varying DNA template concentrations, to assess whether the qPCR assay amplified putatively pathogenic isolates more readily (i.e., at a lower Cq value, on average) than non-pathogenic isolates. In the pilot study, we tested strains using very high concentrations of DNA isolated from pure *Labyrinthula* sp. cultures. Thus, secondly, we adjusted the concentration of starting *Labyrinthula* sp. template DNA to 25 cells per reaction (converted from *Labyrinthula* sp. cells per mg dry seagrass tissue), to better match realistic concentrations in the field. This reflects a cell count greater than the load present in >95% (and within 1 SD of the highest load detected) of our FB-collected *T. testudinum* samples with detectable levels of the pathogen (this study). Third, we accounted for the possibility that non-specific binding may occur when using pure non-pathogenic *Labyrinthula* sp. cultures as the only template, so we introduced UltraPure™ Salmon Sperm DNA Solution (Invitrogen™) as background DNA in our diagnostic qPCR assays (i.e., to mimic host "background" DNA). Fourth, we evaluated bias for the case of pathogenic types being outnumbered by non-pathogenic. Specifically, we tested pathogenic isolate "8b" and non-pathogenic isolate "98b," both of which reliably amplified in Duffin et al. (2020), but with notably different melt curve peaks at 76°C and 78.5°C, respectively, under several template DNA conditions: (1) pure *Labyrinthula* sp. culture at 1x concentration (2 μl 8b at 1x ≈ 646.8 cells per reaction; 2 μl 98b at 1x ≈ 1522.5 cells per reaction); (2) *Labyrinthula* sp. DNA equivalent of 25 cells per reaction with salmon sperm DNA to bring template volume to 20 ng total (i.e., 1 ng/μl reaction volume); and (3) both *Labyrinthula* sp. DNA loaded in a single reaction at a 1:70 ratio (∼5.36 cells of 8b; ∼375.2 cells of 98b per reaction), brought to 20 ng total with salmon sperm DNA. Each reaction was run in duplicate.

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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Duffin, P., Martin, D. L., Pagenkopp Lohan, K. M., and Ross, C. (2020). Integrating host immune status, Labyrinthula spp. load and environmental stress in a seagrass pathosystem: assessing immune markers and scope of a new qPCR primer set. PLoS One 15:e0230108. doi: 10.1371/journal.pone.0230108

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