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Current advances in seagrass research

Edited by
Jutta Papenbrock and Mirta Teichberg

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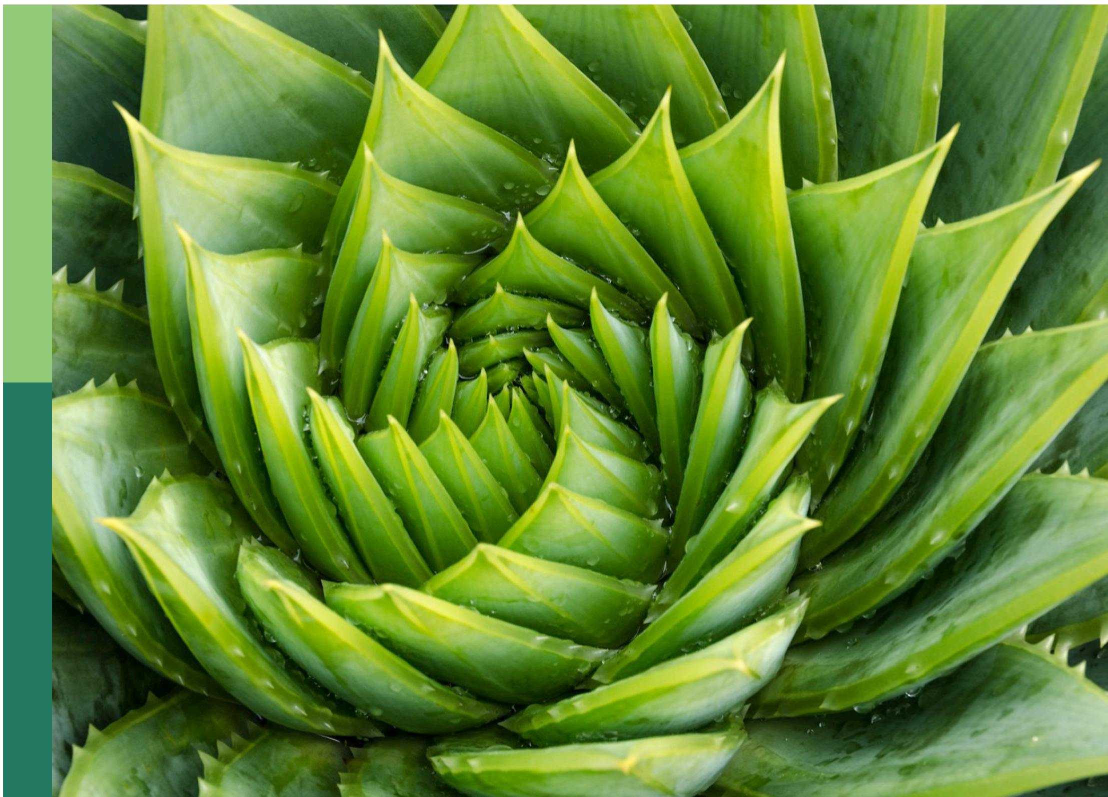

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Current advances in seagrass research

Topic editors
Jutta Papenbrock — Leibniz University Hannover, Germany
Mirta Teichberg — Ecosystems Center, Marine Biological Laboratory (MBL), United States

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EDITED AND REVIEWED BY
Eric Marechal,
UMR5168 Laboratoire de Physiologie Cellulaire Vegetale (LPCV), France

*CORRESPONDENCE
Jutta Papenbrock
✉ Jutta.Papenbrock@botanik.uni-hannover.de
Mirta Teichberg
✉ mteichberg@mbl.edu

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Editorial: Current advances in seagrass research

Jutta Papenbrock[1]* and Mirta Teichberg[2]*

[1]Institute of Botany, Faculty of Natural Sciences, Leibniz University Hannover, Hannover, Germany,
[2]The Ecosystems Center, Marine Biological Laboratory, Woods Hole, MA, United States

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Editorial on the Research Topic
Current advances in seagrass research

Seagrasses are of great ecological importance, forming large "meadows" in all continents except Antarctica and providing vital ecosystem services including primary production, carbon storage, nutrient cycling, habitat structure, and coastal protection. Seagrasses provide shelter and act as a nursery ground for commercially important small fish and invertebrates. Human activity, however, is having profound impacts on marine ecosystems, including seagrass communities. Over the last few decades anthropogenic changes, including reduced water quality, increased temperature, increased sediment loads, and higher grazing pressure, have caused global declines in seagrass populations and the area coverage of seagrass beds. Due to the valuable ecosystem services that seagrasses provide along coastlines all over the world, strategies to increase recovery of seagrass meadows are being developed; however, further research on seagrass distribution, responses to abiotic and biotic stressors and how that impacts the recovery process, acclimation or adaptation potential, and resilience to environmental change is required to support these strategies. For many regions, the exact distribution and coverage of seagrass are not well known, and simple methods to reliably detect changes in seagrass coverage need to be established for the development of locally successful conservation methods. This Research topic, therefore, aimed to advance seagrass research by bringing together different perspectives on seagrasses that highlight their ecological importance and the effects of anthropogenic pressures, as well as the potential for its recovery and restoration.

Some very basic seagrass physiological research questions have been addressed, partially with the help of genomics. It was found that the increasing degradation rate of the foundation species *Phyllospadix iwatensis* may be related to the intrinsic photosensitivity of the photosystem II oxygen-evolving complex. Ambient harsh light conditions inactivate this complex irreversibly contributing to the low resilience of this species to light stress (Wang et al.).

Knowledge of seed development in seagrasses, the only group of angiosperms to complete their life cycle submerged in marine environments, is still very limited. The regulation of the energy metabolism during the seed-to-seedling transition in *Zostera marina* was investigated by integrated metabolomic, transcriptomic, and physiological analysis (Zhu et al.). One of the main findings showed that the TCA cycle is the most effective pathway to supply energy for germination and seedling growth through the upregulation of a large number of genes and metabolites, whereas the pentose phosphate pathway plays a supplementary role during seedling establishment.

Among the most basic seagrass traits are those associated with the salt tolerance mechanism of seagrasses, essential for surviving in the marine environment. The physiological basis of salt tolerance was investigated in *Thalassia hemprichii* and substantiated with gene expression analysis (Shen et al.). The optimal salinity for *T. hemprichii* is 25 to 35 PSU. Shen et al. found that gene expression changed rapidly upon exposure to salinity stress. The differentially expressed genes regulate transport and metabolism by promoting environmental adaptation.

Another relevant adaptation factor to the saline environment is the unique composition of the cell wall of seagrasses. A study of the composition of the cell wall revealed a new combination of structural polysaccharides, such as polyanionic, low-methylated pectins, and glycoprotein elements, such as arabinogalactan proteins (AGPs) originating from both macroalgae and angiosperm land plants (Pfeifer and Classen).

As a biotic stressor, increased grazing intensity due to climate change may inhibit or prolong the recovery time of a colonizing seagrass (*Halophila ovalis*) with implications for seagrass resilience (O'Dea et al.). O'Dea et al. found that a higher grazing intensity by waterfowl on seagrasses took much longer to recover than minimal grazing intensity. Recovery times were also more variable with increased grazing intensity. Most of the recovery was due to vegetative rather than reproductive growth.

There may also be significant indirect effects of grazing within seagrass meadows. Mesograzers which often graze on epiphytic communities of seagrasses help to minimize the effects of the overgrowth of algae. However, when seagrass ecosystems are spatially connected to other macrophyte communities, such as large kelp forests, grazers preferentially choose kelp over epiphytes of seagrass (Olson et al.), indicating that allochthonous sources may be more important than previously assumed. This has implications for trophic structure and possibly could diminish the role of mesograzers in the alleviation of competition between seagrasses and their epiphytes for light.

Another example of biotic interaction that challenges seagrass ecosystems is the introduction of invasive species. The invasive salt marsh halophyte *Spartina alterniflora* was found growing further into the intertidal zone in the Yellow River Delta, China, where it threatened the habitat of the native seagrass *Zostera japonica* (Yue et al.).

However, there are also examples of the positive effects of interactions. Nested interactions between chemosynthetic lucinid bivalves and seagrass promote ecosystem functioning in contaminated sediments (Cardini et al.).

Successful approaches to restoring seagrass meadows are needed to promote recovery. Evidence was provided that the addition of nutrients to seagrass seed planting improved seedling emergence and doubled maximum shoot length, even in nutrient-rich environments (Unsworth et al.). These sources of nutrients allow for the establishment of new seedlings in their environment.

Light is one of the most important abiotic factors limiting seagrass distribution and growth. Reduction of light caused by water quality and increased sediment loads leads to seagrass decline. Xu et al. tested a novel method of suspending seagrasses in floating rafts at varying depths of the water column and measuring seagrass responses over time in Ailian Bay, Northern China, and found that over longer periods of time, seagrass highest shoot densities and reproducing shoots were found only in the shallowest water depths. This is important to determine the maximum depth at which transplants will survive, grow, and reproduce at the specific locality prior to transplantation attempts.

Reciprocal field transplantation experiments with *Zostera marina* L. revealed differences in seed germination time of two populations from different geographic regions. The analyses were supported by comparative transcriptome analysis indicating the power of combining field experiments with molecular methods (Zhang et al.).

A review paper by Nguyen et al. highlighted the current advances in seagrass research from one country, Vietnam. One goal of the review is to support decision-makers in developing science-based conservation strategies. An alarming decline in the coverage of seagrass meadows in almost all parts of Vietnam is observed. Since 1990 a decline of 46.5% or 13,549 hectares was found. Only in a few protected and/or difficult-to-reach areas was an increase observed. Conditions at those sites should be investigated in more detail to make suggestions for the conservation and recovery of seagrass meadows. Only decisions based on the interdisciplinary cooperation of scientists from all disciplines mentioned will finally lead to conserving this valuable ecosystem for humanity and biodiversity.

Another review more broadly explored the use of a trait-based approach to seagrass ecology, an approach that has been extensively applied in terrestrial plant research. Although morphological and physiological trait data have been collected for other purposes, until more recently, seagrass trait-based approaches have been minimally used. This provides an opportunity to re-explore these data with a trait-based approach. Moreira-Saporiti et al. provide a framework for exploring hypotheses linking seagrass trait responses to the environment and to ecosystem functions and services using a trait-based approach.

Many new research questions were explored by this Research Topic, yet even more questions remain open. We still need to develop non-invasive methods to better understand the spatial and temporal decline and recovery of seagrass meadows all over the world. There are still knowledge gaps in understanding the functioning and resilience of seagrass ecosystems and trophic interactions under global change. Filling these gaps would help pave the way for developing methods of seagrass restoration and have implications for their use as nature-based solutions.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

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The Cell Wall of Seagrasses: Fascinating, Peculiar and a Blank Canvas for Future Research

Lukas Pfeifer and Birgit Classen*

Department of Pharmaceutical Biology, Pharmaceutical Institute, Faculty of Mathematics and Natural Sciences, Christian-Albrechts-University of Kiel, Kiel, Germany

Seagrasses are a polyphyletic group of angiosperm plants, which evolved from early monocotyledonous land plants and returned to the marine environment around 140 million years ago. Today, seagrasses comprise the five families *Zosteraceae*, *Hydrocharitaceae*, *Posidoniaceae, Cymodoceaceae*, and *Ruppiaceae* and form important coastal ecosystems worldwide. Despite of this ecological importance, the existing literature on adaption of these angiosperms to the marine environment and especially their cell wall composition is limited up to now. A unique feature described for some seagrasses is the occurrence of polyanionic, low-methylated pectins mainly composed of galacturonic acid and apiose (apiogalacturonans). Furthermore, sulfated galactans have been detected in some species. Recently, arabinogalactan-proteins (AGPs), highly glycosylated proteins of the cell wall of land plants, have been isolated for the first time from a seagrass of the baltic sea. Obviously, seagrass cell walls are characterized by new combinations of structural polysaccharide and glycoprotein elements known from macroalgae and angiosperm land plants. In this review, current knowledge on cell walls of seagrasses is summarized and suggestions for future investigations are given.

Keywords: cell wall, seagrass, apiogalacturonan, sulfated polysaccharide, arabinogalactan-protein, lignin

Edited by:
Ugo Cenci,
Lille University of Science and Technology, France

Reviewed by:
Iker Irisarri,
University of Göttingen, Germany
Julien Le Roy,
La Cité Collégiale, Canada

***Correspondence:**
Birgit Classen
bclassen@pharmazie.uni-kiel.de

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INTRODUCTION

Around 140 million years ago, seagrasses evolved from early monocotyledonous land plants, which succeeded in conquering the marine environment. Today, they are a polyphyletic group of marine angiosperms with around 60 species in five families (*Zosteraceae*, *Hydrocharitaceae*, *Posidoniaceae, Cymodoceaceae*, and *Ruppiaceae*), which belong to the order Alismatales according to the Angiosperm Phylogeny Group IV System (APG IV, Chase et al., 2016). The genus *Ruppia*, which occurs in brackish water, is not regarded as a "real" seagrass by all authors and has been shifted to the *Cymodoceaceae* by some authors (Les and Tippery, 2013). The APG IV System and The Plant List Webpage (The Plant List, 2020) do not share this family assignment. We included *Ruppia* in this review to cover all literature with the connection to "seagrass" and used the traditional assignment to *Ruppiaceae*, resulting in five seagrass families. Seagrasses form important coastal ecosystems (Hemminga and Duarte, 2000). The worldwide endangering of these sea meadows, which provide food and habitat for many marine species, prompts the need for protection and understanding of these valuable resources. Recently, sequencing of the genomes of *Zostera marina* and

Zostera muelleri allowed better understanding angiosperm adaption to the sea (Lee et al., 2016; Olsen et al., 2016). During the evolutionary step back to the ocean, different genes have been lost (e.g., stomatal genes) or have been reduced (e.g., genes involved in the synthesis of terpenoids) and others have been regained (e.g., genes involved in sulfation; Olsen et al., 2016). Genome information further revealed that adaption to the marine habitat was accomplished by severe changes of cell wall composition (Lee et al., 2016; Olsen et al., 2016). On the other hand, the cell walls of seagrasses are poorly understood. Beside ancestral traits of land plants, one would anticipate a habitat-driven adaption process to the new environment, which is characterized by multiple abiotic (high amounts of salt) and biotic (different seagrass grazers and bacterial colonization) stressors.

Although knowledge is limited, seagrass cell walls contain polysaccharides known from angiosperm land plants, e.g., cellulose (Syed et al., 2016). On the other hand, the cell walls of some seagrasses are characterized by sulfated polysaccharides (SP) (Aquino et al., 2005; Silva et al., 2012; Kolsi et al., 2016), a common attribute of the macroalgae from the groups of red, brown and also green algae. Recently the ability to synthesize SP was proposed to be regained by marine angiosperms (Aquino et al., 2005). Another unique feature of cell walls of seagrasses is the occurrence of unusual pectic polysaccharides called apiogalacturonans. Characteristic are high amounts of low-methyl esterified galacturonic acid (GalA*p*) units substituted with the unusual monosaccharide apiose (Api*f*) (Gloaguen et al., 2010; Lv et al., 2015).

In addition to polysaccharides, glycoproteins of the hydroxyproline-rich glycoprotein family (classified in Johnson et al., 2003), are important components of cell walls of land plants. The highly glycosylated arabinogalactan-proteins (AGPs) are interesting due to their involvement in both wall architecture and cellular regulatory processes (Ellis et al., 2010; Ma et al., 2018). AGPs are ubiquitous in seed land plants (Ma et al., 2018) and have also been found in ferns, lycophytes and mosses (Classen et al., 2019). They are structurally characterized by large polysaccharide moieties comprised of arabinogalactans (AGs, normally >90% of the molecule) which are covalently linked via hydroxyproline (Hyp) to relatively small protein/peptide backbones (normally around 1–10% of the molecule). The AGs of seed plants mainly consist of type II (3,6)-galactans with 3-, 6-, and 3,6-linked β-D-galactose (Gal*p*) residues, substituted with α-L-arabinose (Ara*f*) and often minor amounts of glucuronic acid (GlcA*p*) residues (Ma et al., 2018). Distinct glycan modifications have been identified in different species and tissues and are suggested to influence both their physical properties and function. Recently, AGPs have been isolated and structurally characterized for the first time from a seagrass (Pfeifer et al., 2020). Although the common backbone structure of land plant AGPs is conserved, the glycan structures exhibit unique features, including a high degree of branching and an unusually high content of terminating 4-O-methyl-glucuronic acid (4-OMe GlcA) residues, suggesting a role of seagrass AGPs in osmoregulation (Lamport et al., 2006).

Further components of secondary walls of plants are cross-linked phenolic polymers called lignin, which are responsible for mechanical strengthening of the wall. In seagrasses, this polymer has also been detected, but often in lower amounts compared to angiosperm land plants (Opsahl and Benner, 1993; Klap et al., 2000; Martone et al., 2009; Kaal et al., 2018).

Thus, cell walls of seagrasses seem to be fascinating combinations of features known from both angiosperm land plants and marine macroalgae with new structural elements. As dried seagrass leaves might be useful for papermaking or as insulating materials, knowledge on their cell wall composition is also important from a technological point of view. This review offers a detailed summary and discussion of literature on cell wall components of seagrasses.

POLYSACCHARIDE COMPONENTS OF SEAGRASS CELL WALLS

Table 1 gives an overview on isolation and characterization processes described for cell wall polysaccharides from seagrasses. **Table 2** shows the already characterized polysaccharide structures from seagrasses.

Cellulose

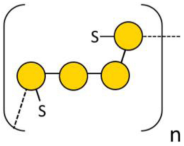
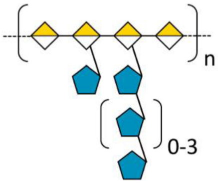
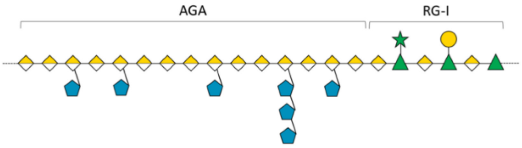
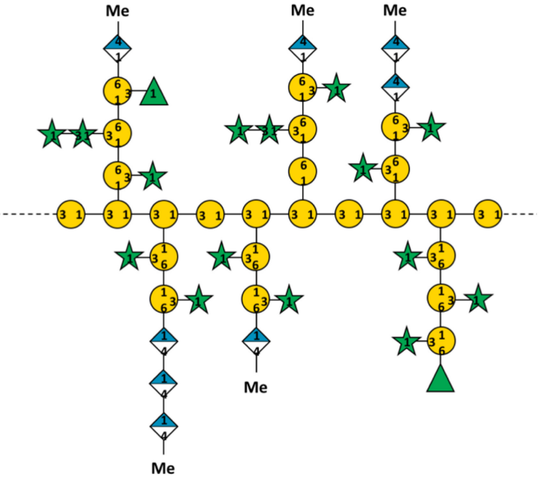
Comparable to angiosperm land plants, cellulose is a main component of seagrass cell walls. The content of cellulose has been determined in relation to dry plant material in different genera, e.g., *Halodule* (Waldron et al., 1989), *Halophila* (Baydoun and Brett, 1985; Waldron et al., 1989; Syed et al., 2016), *Posidonia* (Torbatinejad and Sabine, 2001; Torbatinejad et al., 2007; Khiari et al., 2010), *Zostera* (Davies et al., 2007), *Cymodocea*, *Enhalus* and *Thalassia* (Syed et al., 2016), and found to vary between 20% in *Posidonia australis* (Torbatinejad and Sabine, 2001) and 77% in *Enhalus acoroides* (Syed et al., 2016). A comparison between roots, leaves and rhizomes of *Halodule ovalis*, *Halodule stipulacea*, and *Halophila uninervis* revealed no obvious differences between the three species; in general, leaf tissue contained more cellulose compared to root and rhizome material (Waldron et al., 1989). It has to be taken into account, that the methods to determine the content of cellulose differ and are therefore difficult to compare (e.g., the method of Moubasher et al., 1982, is used to estimate cellulose, hemicellulose and lignin content in plant material). In general, the content of cellulose is estimated as the amount of insoluble material after hydrolysis. The methods for hydrolysis vary and furthermore, the insoluble residue is sometimes regarded as cellulose content, sometimes the carbohydrate part of the insoluble residue is determined by the photometric method of DuBois et al. (1956). Whereas in some cases only TFA (2 N) is used for hydrolysis (Waldron et al., 1989), other authors perform TFA (2 N) treatment first followed by further hydrolysis with concentrated sulfuric acid (Davies et al., 2007). In both cases, the sugar content in the insoluble residues is determined photometrically to give the cellulose content. Other authors use alkaline treatment with KOH (residue A: cellulose and lignin), followed by hydrolysis with strong sulfuric acid (residue B: lignin) and calculate the cellulose content (A–B) without determination of the carbohydrate content (Syed et al., 2016). In none of the literature reviewed, the type of cellulose

TABLE 1 | Overview on isolation and characterization processes for cell wall polysaccharides from seagrasses.

Species	Organ	Isolation	Structural analysis					References
			Monosaccharide composition	**FT-IR**	**NMR**	**MS**	**Biological activities**	
Amphibolis antarctica	Pollen grain	Enzyme containing buffer	√	–	–	–	–	Harris et al. (1994)
Cymodocea nodosa	Whole plant without root	Hot water after acetone and ethanol preextraction	√	√	√	√	√	Kolsi et al. (2016)
Halodule pinifolia	n.s.[1]	Hot water after depigmentation with acetone	–	√	–	–	√	Kannan et al. (2013)
Halodule uninervis	Leaves, rhizomes, roots	Sodium acetate buffer (10 mM), containing $CaCl_2$ (3 mM) and adjusted to pH 5.0	√	–	–	–	–	Baydoun and Brett (1985); Waldron et al. (1989)
Halodule wrightii	n.s.[1]	Sodium chloride solution (0.25 M) adjusted to pH 8.0 after depigmentation with acetone	√	√	–	–	√	Silva et al. (2012)
Halophila ovalis	Leaves, rhizomes, roots	Sodium acetate buffer (10 mM) containing $CaCl_2$ (3 mM) and adjusted to pH 5.0	√	–	–	–	–	Baydoun and Brett (1985)
Halophila stipulacea	Leaves, rhizomes, roots	Sodium acetate buffer (10 mM) containing $CaCl_2$ (3 mM) and adjusted to pH 5.0	√	–	–	–	–	Baydoun and Brett (1985)
Posidonia australis	Leaves, whole plant	Hot sulfuric acid, 0.4 N; directly from plant material	√	–	–	–	–	Bell et al. (1954); Torbatinejad et al. (2007); Torbatinejad and Sabine (2001)
Ruppia maritima	Leaves, rhizomes, roots	Papain-containing buffer at pH 6.0	√	–	√	–	–	Aquino et al. (2005)
Heterozostera tasmanica	Root hairs	Washed cell walls directly hydrolyzed in 4% (w/v) sulfuric acid	√	–	–	–	–	Webster and Stone (1994)
Phyllospadix torreyi	n.s.[1]	Hot water, 5% ammonium oxalate, 7% sodium hydroxide	√	√	–	–	–	Woolard and Jones (1978)
Zostera marina	n.s.[1]; Leaves, rhizomes, roots	1% (w/v) aqueous ammonium oxalate with following pectinase treatment; aqueous extract and Yariv-precipitation after depigmentation with acetone	√	–	√	√	√	Gloaguen et al. (2010); Pfeifer et al. (2020)
Zostera caespitosa	n.s.[1]	Ammonium oxalate, 2% (w/v)	√	√	√	√	√	Lv et al. (2015)

If more than one publication focused on one species, both are described in the "Isolation" column below each other. [1]not specified plant organ used for extraction.

TABLE 2 | Structurally described charged polysaccharides from seagrasses.

Major monosaccharide components[1]	Linkages	References	Proposed structure
🟡	1,4- and 1,3-linked Gal, 2- and 4-O sulfated	Aquino et al. (2005)	
◆ ⬟	1,4-linked GalA; 1,2-linked Api	Gloaguen et al. (2010)	
🟡 🟢 ★	1,4-linked Gal, 6-O-sulfated	Kolsi et al. (2016)	not enough data
◆ ⬟ ▲	1,4-linked GalA; 1,3-linked Api	Lv et al. (2015)	
🔵 ★ 🟡	not determined	Silva et al. (2012)	not enough data
🟡 ★ ◆	1,3-linked Gal; 1,4-linked GlcA; terminal Ara	Pfeifer et al. (2020)	

Major polysaccharide components are symbolized using the Symbol Nomenclature for Glycans (SNFG) of the NCBI-Glycan Page (National Center for Biotechnology Information (NCBI), 2020). Abbreviations: Api, apiose; Ara, arabinose; Gal, galactose; GalA, galacturonic acid; and GlcA, glucuronic acid. [1] monosaccharides are treated as "major" if they contribute more than 10% of the polysaccharide.

(α- or β-cellulose) was determined. To gain more information on that, it is recommended to perform a fractionated extraction (Galiwango et al., 2019) and use the different solubility of both types in alkaline conditions.

Hemicelluloses

Hemicelluloses are a large family of cell wall polysaccharides including different polymers. Syed et al. (2016) estimated the amount of hemicelluloses in different seagrasses using the method of Moubasher et al. (1982). The values ranged from 14 – 28% of dried seagrass material (*Thalassia hemprichii* 14%, *Halophila spinulosa* 23%, *Cymodocea serrulata* 26%, *Halophila ovalis*, and *Enhalus acoroides* 28%). For *Posidonia australis* (Torbatinejad et al., 2007; Torbatinejad and Sabine, 2001) and *Posidonia oceanica* balls (Khiari et al., 2010) the amounts were 11.7 and 21.8%, respectively. Highest values for hemicelluloses were determined for *Z. marina* (38%; Davies et al., 2007).

Xylans

Xylans are a group of plant cell wall polysaccharides with a backbone consisting of 1,4-linked β-D-xylopyranoses, which have a high degree of substitution (York and O'Neill, 2008; Hatfield et al., 2016; Peña et al., 2016; Tryfona et al., 2019). Xylans are the main hemicelluloses in the cell walls of most plant species (Tryfona et al., 2019) and play an important role in crosslinking with other structural components (cellulose, lignin). In primary cell walls of plants belonging to the *Poales* (also monocotyledonous plants), xylans are present in high amounts.

Most of the publications on seagrass xylans are based on crude monosaccharide quantification; investigations on exact structures of seagrass xylans are missing. Baydoun and Brett (1985) could show that significant quantities of xylose and arabinose were present in non-cellulosic polysaccharide fractions of *Halophila stipulacea* and *H. ovalis* and attributed this to presence of arabinoxylans. In the same study, *Halodule uninervis* showed only small amounts of xylose in the same cell wall fraction. In support of this finding, Brudecki et al. (2015) measured a low xylan content of around 5% for cell walls of *H. uninervis* with similar methods. On the other hand, *Z. marina* fibers contain 38% hemicelluloses, which are mainly xylans (Davies et al., 2007). Are they complex heteroxylans like in grasses or more similar to algal 1,4-linked- (charophytic green algae), 1,3-linked- (chlorophytic green algae/some red algae), or 1,3; 1,4-linked-homoxylans (red algae) (Hsieh and Harris, 2019)? The next step forward to answer this question would be a broader investigation: Modern methods like xylan epitope profiling (Peralta et al., 2017), capillary electrophoresis based high-throughput carbohydrate profiling (Li et al., 2013), solid-state NMR methods (e.g., Dupree et al., 2015) or HILIC-MALDI-ToF/ToF-MS/MS (Busse-Wicher et al., 2016) have to be carried out to give insights in xylan structure of seagrasses.

Mannans

Mannans are important members of the hemicellulose family, which are subdivided into linear mannans, galacto-, gluco- and galactoglucomannans (Petkowicz et al., 2001; Moreira and Filho, 2008). They have mainly structural functions, but also signaling functions are proposed (Moreira and Filho, 2008). They are present in eukaryotic algal species belonging to the divisions of Rhodophyta and Chlorophyta, where they seem to replace cellulose as the main cell wall carbohydrate (Painter, 1983). To the best of our knowledge, there are no described mannans in any seagrass species. He et al. (2015) postulated a correlation of mannan accumulation in *Dendrobium officinale* with water deficiency stress. Even though *D. officinale* is not a marine plant, this phenomenon could be also apparent in seagrass species. More studies with a focus on mannans are necessary to answer the question whether mannans are components of seagrass cell walls.

Xyloglucans

Xyloglucans are found in primary cell walls of all angiosperms, where they are responsible for the crosslinking of cellulose microfibrils (Popper and Fry, 2008; Brennan and Harris, 2011). They consist of 1,4-linked β-D-glucose residues which form a backbone substituted with α-D-xylosyl chains at *O*-6. Xyloglucans with the special side chain α-L-Fuc*p*-(1,2)-β-D-Gal*p*-(1,2)-α-D-Xyl*p*-(1,6)-β-D-Glc*p* are called fucogalactoxyloglucans and are common in primary walls of non-commelinid monocotyledons, while they are rare in commelinids. In a study with the monoclonal antibody CCRC-M1, which recognizes the epitope structure α-Fuc-(1,2)-β-Gal, *Zostera muelleri* interestingly differs in its fucogalactoxyloglucan composition from the other investigated non-commelinid monocotyledons. While most of the investigated non-commelinid species showed a widely distributed fluorescence labeling with this antibody comparable to *Arabidopsis thaliana*, *Z. muelleri* was labeled only in the phloem sieve elements (Brennan and Harris, 2011).

The low amount of fucogalactoxyloglucan in *Zostera muelleri* (Brennan and Harris, 2011) is supported by the fact that in the genome of another *Zostera* species there were only 2 GT37 genes, which encode the xyloglucan fucosyltransferases (Olsen et al., 2016). In contrast to that, *Arabidopsis* or *Oryza* have 10 or 18 of these enzyme genes, respectively.

Mixed-Linked Glucans

The so-called mixed linked glucans (MLG), consisting of β-D-glucose chains connected through 1,3- and 1,4-linkages, have been found in high amounts in the Poales order (Popper and Fry, 2004), in *Centraria islandica* (as "lichenan;" Stone and Clarke, 1992), in the genus *Equisetum* (Fry et al., 2008) and most recently in *Phaeophyceae* cell walls (Salmean et al., 2017). In the *Poales* order MLG is present at certain stages during the primary wall formation in maize (Penning et al., 2019). Baydoun and Brett (1985) as well as Waldron et al. (1989) attributed the high amounts of glucose in *Halodule uninervis* to the presence of MLG. Structure elucidation has to be performed to prove this proposal.

Pectic Polysaccharides

Pectin is a structurally complex carbohydrate family rich in galacturonic acid, including the major polysaccharides homogalacturonan, rhamnogalacturonan I, rhamnogalacturonan

II and xylogalacturonan (for an in-depth review, see Mohnen, 2008). Their characteristically high amount of acidic domains is important for the osmotic properties and can interact with ions and low-molecular-weight compounds (Willats et al., 2001).

In some seagrasses, a special pectic polysaccharide named "apiogalacturonan" is present (**Table 2**). Miroshnikov (1940) first isolated an uronic acid rich polysaccharide with gelforming properties from *Z. marina*, which he named "zosterine." Analytical characterization of zosterine was broadened by the studies of the group of Ovodova and Ovodov (e.g., Ovodova et al., 1968; Ovodov et al., 1971a,b, 1975; Popov et al., 2007), which detected D-apiose as substantial monosaccharide of this pectic fraction.

Recent studies (Gloaguen et al., 2010; Lv et al., 2015) investigated the fine structure of apiogalacturonans in two different *Zostera* species after extensive purification steps. Its structure was analyzed with modern instrumentation (enzymatic digestion + mass spectrometry of fractions; one- and two-dimensional NMR experiments). The described structures consist of an α-1,4-linked-D-galacturonan substituted at position C-2 or C-3 of GalA by single apiose residues or short oligosaccharides of apiofuranose (**Table 2**).

Apiogalacturonans of seagrasses have a very low degree of esterification around 10% (Maeda et al., 1966; Khotimchenko et al., 2012). Recent genetic studies of two *Zostera* species (Lee et al., 2016; Olsen et al., 2016) revealed an increase in unique pectin methylesterase-related domains in different proteins possibly responsible for the low degree of methyl-esterification. It is hypothesized that this represents a control mechanism for osmoregulation. Furthermore, investigations on heavy metal binding by apiogalacturonans (e.g., cerium and mercury, Khotimchenko et al., 2006, 2012; cadmium and lead, Khozhaenko et al., 2016) might help to understand biophysiological functions of apiogalacturonans in "high-charge environments" like the marine habitat.

Arabinans are composed of a α-1,5-linked arabinofuranose backbone and may occur separately in the wall or as neutral side chains of rhamnogalacturonan-I (Wefers and Bunzel, 2016). Although an "arabinan" content has been described for *Halodule uninervis* (Brudecki et al., 2015) only a very crude compositional analysis was performed in this investigation. Whether seagrasses contain arabinans is therefore unknown to date and needs to be investigated in more detail (e.g., by a fast approach with a combination of two-dimensional NMR spectroscopy with previous enzyme-assisted extraction, like established by Wefers and Bunzel, 2016).

Sulfated Polysaccharides

Sulfated polysaccharides have been found in a number of marine organisms (e.g., Pomin, 2012; Ngo and Kim, 2013) and are often described as compounds with a number of bioactivities, including antioxidant, anticancer and anticoagulant activities (Ngo and Kim, 2013). Their occurrence is mostly correlated with a saline environment (Aquino et al., 2011).

In 2005, SP were first detected in the seagrasses *Halodule wrightii* and *Halophila decipiens* as well as in the marine angiosperm *Ruppia maritima* (Aquino et al., 2005) in amounts around 1% (m/m). Structure elucidation was performed only for the galactan from *R. maritima* and revealed a 2-*O*- and 4-*O*-sulfated galactan, which consists of a repeating tetrasaccharide [3-β-D-Gal-2(OSO_3)-1,4-α-D-Gal-1,4-α-D-Gal-1,3-β-D-Gal-4(OSO_3)] (**Table 2** and **Figure 1**). In an additional study from the same working group (Aquino et al., 2011) it was shown, that the amount of this SP increased in higher salinity and disappeared in culture without salt supplement.

Silva et al. (2012) postulated the presence of a sulfated heteropolysaccharide in *H. wrightii*, consisting of glucose:xylose:galactose (1:1:0.9) with a sulfation degree of 20% and a molecular weight of ∼11 kDa. As the sample was treated with trichloroacetic acid (10%) during the isolation process (**Table 1**), which is, to the best of our knowledge, a polysaccharide-degrading and sulfate-deleting condition, the presence of this SP in *Halodule* has to be verified by further investigations.

Occurrence of a "fucoidan-like sulfated polysaccharide" has been proposed for *Halodule pinifolia* (Kannan et al., 2013). The structural data has to be regarded as preliminary as it based only on Fourier-Transformation infrared spectroscopic (FT-IR) analysis and colorimetric total sugar determination.

A sulfate containing polysaccharide fraction has been isolated from *Cymodocea nodosa* by water-extraction followed by ethanol-precipitation (Kolsi et al., 2016). Structural analytics (NMR, LC-ESI-MS) led to the proposal of a branched 1,4-linked galactosidic backbone with 6-O-sulfation and decoration with small amounts of other monosaccharides. Due to the isolation procedure (see above), the sulfated galactan is most likely accompanied by other water-soluble polysaccharides.

Callose

Callose is a homopolysaccharide composed of β(1,3)-linked glucose with some β(1,6)-branches which is typically found in sieve plate pores, in plasmodesmata, in the cell plate during cell division and also in pollen (Wu et al., 2018). It is synthesized by callose synthase (Verma and Hong, 2001) and plays an important role in biotic and abiotic stress response (Lampugnani et al., 2018). An easy method to detect callose in light microscopy is anilin blue fluorochrome staining of plant tissue. Whereas the pollen walls of *Amphibolis antarctica* showed no staining with anilin blue (Ducker et al., 1978; Harris et al., 1994), callose staining was positive between daughter cells in the pollen tetrads of *T. hemprichii* and *T. ciliatum*. Callose was also detected by this staining in pollen of *H. wrightii* and *Z. marina* (Pettit and Jermy, 1975). As reaction with this dye is not exclusive for callose (Smith and McCully, 1978), more specific methods for detection should be applied like e.g., the use of the monoclonal antibody AB 400-2. With this antibody, detection of β(1,3)- in the presence of β(1,4)- or β(1,3)-β(1,4)-linked glucan structures is possible (Meikle et al., 1991) and it was also used for immunolocalization of callose in streptophyte green algae (Herburger and Holzinger, 2015). A hint of the general ability of at least some seagrasses to synthesize callose is the presence of "Glucan synthase-like 8" genes in *Halophila* and two *Zostera* species (Lee et al., 2018). In *Arabidopsis*, these genes encode enzymes responsible for callose synthesis.

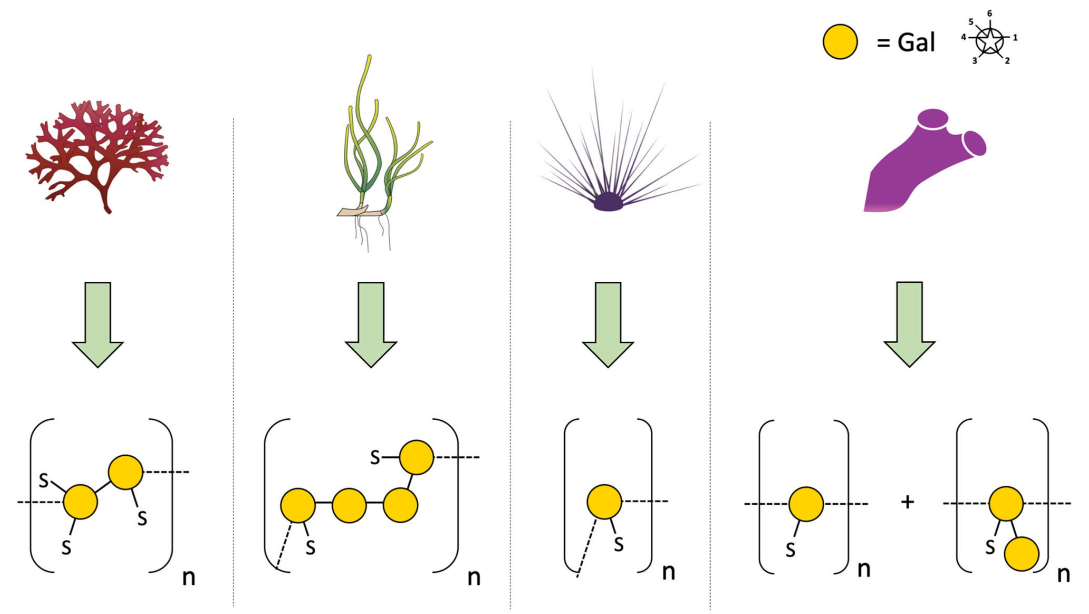


FIGURE 1 | Diverse structures of sulfated galactans from marine organisms. Sulfated polysaccharide structures from left to right: red algae: *Botryocladia occidentalis*, seagrass: *Ruppia maritima*, sea urchin: *Echinometra lucunter*, tunicate: *Styela plicata*. Illustration adapted from Aquino et al. (2005). All vector images are from Tracey Saxby, Jane Thomas, Jane Hawkey, IAN Image Library (ian.umces.edu/imagelibrary/).

WALL (GLYCO)PROTEINS

As Johnson et al. (2003) proposed, the term "wall protein" is used in this review to refer to all types of glycosylated proteins, despite their degree of glycosylation or protein-glycosylation type. The literature about this group of biomolecules from seagrasses is mainly limited to some works on pollen cell walls. Ducker and Knox (1976) first proposed the presence of specialized "glycoproteins" in pollen cell walls of *Amphibolis antarctica* as a unique adaption to be able to fulfil submarine pollination sufficiently. Based on this hypothesis Pettitt (1980) performed microscopy of *T. hemprichii* fresh-frozen pollen with β-glucosyl Yariv (βGlcY) – a reagent used for selective interaction with AGPs. AGPs, which are a group of highly glycosylated (*O*-linkage *via* hydroxyproline) proteins are involved in many plant cell wall functions and processes (Ellis et al., 2010). *T. hemprichii* pollen walls showed positive interaction with βGlcY. In discrepancy to that, Harris et al. (1994) performed extraction of *Amphibolis antarctica* pollen walls and concluded absence of hydroxyproline-rich glycoproteins – a group of wall proteins, including the extensins, AGPs and proline-rich proteins. "Small amounts of glycin-rich proteins [. . .] may be present" (Harris et al., 1994). These assumptions were based on monosaccharide and amino acid composition of acidic wall preparations. Pfeifer et al. (2020) performed the first isolation and structural characterization of a βGlcY-precipitable AGP from *Z. marina* (**Table 2**). Here whole plants, rhizomes, roots and leaves were extracted and investigated. In addition to that microscopic data and thermodynamic binding analysis showed that unique 1,4-linked- and terminal-glucuronic acids in this AGP fraction showed calcium binding properties with a K_D value in a micromolar range. This observation underlines the proposed functionality of AGPs in salt adaption (Lamport et al., 2006). A broader investigation on presence in other seagrass species could be of scientific value, especially in the light of independent adaption to the sea in minimum three seagrass lineages (Williams, 2016).

As far as *N*-linked glycosylated wall proteins are concerned, the only work on a seagrass was done by Yoshiie et al. (2012) showing that *Z. marina* contains high-mannose type *N*-glycans in high amounts. These N-linked glycans also possess a wide range of functions intensively discussed by Strasser (2014).

LIGNIN

Beside the polysaccharide components, the secondary walls of vascular land plants consist of condensed macromolecules with cross-linked phenolic monomers, called lignin. These polymers are described as containing about 30% of the organic carbon in biosphere (Boerjan et al., 2003). Due to their molecular structure, their ability to covalently complex with wall polysaccharides and their tendency to encrust cellulose microfibrils, they contribute to increasing hydrophobia in secondary plant walls which results in dehydration. An effect of this process is the gain of mechanical strength and reduction of flexibility for the tissue and for the whole plant (Doblin et al., 2010; Weng and Chapple, 2010).

For a long time, it was not clear whether seagrasses commonly have lignins as cell wall components, because it was hypothesized that due to the experimental methodology other phenols, being present in many seagrass tissues (Lewis and Yamamoto, 1990), could appear as "lignin." Therefore, it was stated that "evidence

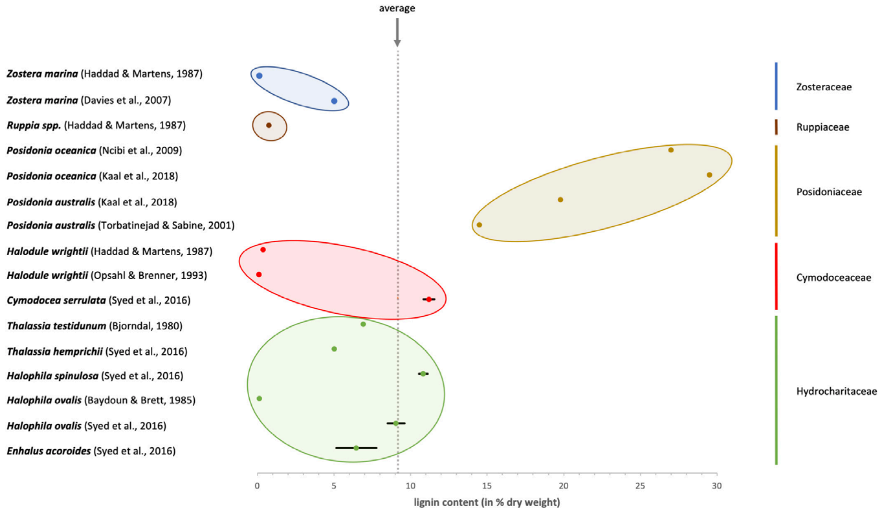


FIGURE 2 | Overview about the published values for lignin content in different seagrass species. The colors identify the membership to the five systematic families of seagrasses.

for lignin in submerged aquatic plants is not convincing. More definitive proof is necessary if these are to be considered lignin-synthesizing organisms" (Lewis and Yamamoto, 1990). Furthermore, there is no consistency in the methodologies of the existing literature. On the other hand, investigation of two seagrass species (*Posidonia oceanica* and *Z. marina*) and different tissues of these with Curie-point pyrolysis gas chromatography mass spectrometry (Py-GCMS) revealed that lignin is present in these seagrasses with variations between species and tissues (Opsahl and Benner, 1993; Klap et al., 2000). The retention of lignin during evolution from terrestrial vascular plants back to the sea might be as a useless artifact or an evolutionary advantage also in marine environment. The first idea is questionable due to the recently described occurence of lignin or lignin-like macromolecules in red algae and aquatic green algae (Martone et al., 2009). The second proposal is more conclusive. This topic is discussed intensively by Klap et al. (2000), who found that rhizomes of seagrasses are richer in lignin compared to leaves and suggested that lignification contributes to the longevity of a tissue by protecting it against microbial attack.

According to Syed et al. (2016) the amount of lignin in species of the genera *Enhalus*, *Cymodocea*, *Halophila* and *Thalassia* is low with 5–11% of dry weight. In that work, the insoluble residue after treatment with 24% potassium hydroxide followed by strong sulfuric acid was quantified as lignin.

Looking at the published data (see **Figure 2**), it seems as if the species belonging to the *Posidoniaceae* contain more lignin compared to all the other families (*Cymodoceaceae*, *Hydrocharitaceae*, *Ruppiaceae*, and *Zosteraceae*) which can be regarded as low in lignin content. It has to be taken into account that *Posidoniaceae* species are responsible for extensive detritus production. As exact definition of material used is missing in some publications, results might be influenced by degraded materials, which contain higher amounts of lignin. Therefore, results on *Posidonia* balls (e.g., Khiari et al., 2010), which are not comparable to the fresh *Posidonia* material, were excluded from **Figure 2**.

Knowledge about lignin is also of interest, because recent publications on blue carbon stocks (Barry et al., 2018; Serrano et al., 2020) showed need of lignin data for their calculations.

INFORMATION ON CELL WALL RELEVANT GENES

With the increasing field of -omics studies more and more data is accessible, which can be used for nearly each kind of comparison between marine angiosperms and other plant groups or in the polyphyletic group of seagrasses itself. Up to now two full genome sequences have been published. The authors compared the genomes of *Z. marina* (Olsen et al., 2016) or *Z. muelleri* ssp. *capricornii* (Lee et al., 2016), respectively, with the only other sequenced relative from the order of Alismatales, *Spirodela polyrhiza* and in addition to that to some angiosperm land plants. These two publications gave insight into the molecular adaptation steps to marine environment and supported that severe changes in cell wall composition were necessary.

Sablok et al. (2018) provided access to a transcriptomics database called "SeagrassDB", which allows authors to perform comparative trancriptomics on eight seagrass species and other aquatic plant species. With the same idea Wissler et al. (2009) implemented the "Dr. Zompo" database, which is focused on *Z. marina* and *Posidonia oceanica*. Both resources could enable specialists on different cell wall components to test their hypotheses, regarding for example the variety of carbohydrate active enzymes (**Table 3**).

For a detailed overview about molecular profiling and – omics techniques with a full list of all accessible data on seagrasses until the year 2016, see Davey et al. (2016).

TABLE 3 | Enzymes with activities in biosynthesis of the different cell wall components.

Cell wall component	Species	Enzymes*	References
Cellulose	*Z. marina*	12 CESA; 4 CSLG	Olsen et al. (2016)
Xylans	*Z. marina*	9 GH3; 3 GH10; 1 GH51; 39 GT8; 10 GT14; 36 GT47; 13 GT61; 1 CE6	Olsen et al. (2016)
Mannans	*Z. marina*	6 CSLA; 3 CSLD; 4 GT5_7	Olsen et al. (2016)
Xyloglucans	*Z. marina*	3 CSLC; GH16; 5 GT34; 2 GT37	Olsen et al. (2016)
	Zostera	Xyloglucan endotransglucosylase/hydrolase 5	Lee et al. (2018)
Mixed-linked glucans	*Z. marina*	absent	Olsen et al. (2016)
Pectins	*Z. marina*	39 GT8; 36 GT47; 56 GH28; 16 PL1; 63 CE8	Olsen et al. (2016)
Sulfated polysaccharides	*Z. marina*	to be further investigated	Olsen et al. (2016)
Callose	*Z. marina*	19 CBM43; 40 GH17; 10 GT48;	Olsen et al. (2016)
	Zostera	Glucan synthase-like 8	Lee et al. (2018)
Wall (glyco-)proteins	*Z. marina*	4 DUF579; 14 GT14; 25 GT31; 4 GT61; 13 GT77	Pfeifer et al. (2020)
Lignin	*Z. marina*	not investigated	

**Activities were used as stated in the respective reference.*

TECHNICAL APPLICATIONS

Large amounts of different seagrasses wash on beaches of the world each year, sometimes causing even environmental problems. Therefore, an economic use of this resource would be desirable. For *Posidonia australis*, use as foodstuff for ruminant animals has been proposed (Torbatinejad et al., 2007). Another possible application of seagrass material is use as source of fibres. Today, pulp production from non-wood material increases, sometimes due to shortage of hard-wood fiber material (Saijonkari-Pahkala, 2008). Use of seagrass material for papermaking has been evaluated for different seagrass species, e.g., *Z. marina* (Davies et al., 2007), *P. oceanica* (Khiari et al., 2010), *E. acoroides*, *C. serrulata*, *H. ovalis*, *H. spinulosa*, and *T. hemprichii* (Syed et al., 2016). The comparable low lignin content is an advantage as bleaching costs are lower. Among the species investigated by Syed et al. (2016), *Enhalus acoroides* was most preferable for papermaking because of the highest cellulose content combined with the longest fibers. Another interesting feature of dry seagrass material is its suitability as environmentally friendly, fire-resistant insulating material. Already at the beginning of the 20th century, wild gathering of the leaves of *Z. marina* formed the basis of an insulation industry in North America (Wyllie-Echeverria and Cox, 1999). Today, there are efforts to revive this industry, e.g., *Posidonia oceanica* balls are gathered and sold as insulating material. Their thermophysical behavior seems to be very promising for this purpose and only a few pre-treatment steps with sodium hydroxide could enhance the properties of *Posidonia* fibers to a level comparable to industrial insulating materials (Hamdaoui et al., 2018). Another approach for construction purposes has been registered for patent in 2018 (Pavlakis, 2018). The patent holder sells environmentally friendly panels build from *Posidonia* seagrass balls. Furthermore, the combination of a relatively high amount of cellulose and hemicelluloses together with a low lignin content makes seagrasses an interesting starting material for the production of biofuel. For this kind of use lignin usually has to be removed to enable successful chemical or enzymatic degradation in acceptable yields (Mukherjee et al., 2016). This approach was proposed for *Z. marina* detritus in Turkey (Ncibi et al., 2014).

CONCLUSION AND PERSPECTIVES

Conclusion With Regard to Existing Work

Despite the ecological importance of seagrasses, which form important coastal ecosystems worldwide, the seagrass cell wall as a whole is poorly understood. The content of cellulose, hemicelluloses and lignin is often estimated by simple procedures using different hydrolytic methods and weighing of the residual material. On the other hand, some detailed investigations of single seagrass polysaccharides in single species shed light on some aspects of seagrass cell wall adaption to the marine habitat (e.g., Aquino et al., 2005; Gloaguen et al., 2010; Lv et al., 2015; Pfeifer et al., 2020). All together, similarities and differences between the different seagrass families and species and also the comparison to cell walls of monocotyledonous land plants are still a mystery.

Especially the hemicelluloses are poorly investigated up to now. Although a content of over 30% in relation to dry plant material has sometimes been estimated for these polysaccharides, most publications comprise only rough determinations of components without in-detail structural elucidations like it is state-of-the-art in this particular field of cell wall research. For technical applications, knowledge on cellulose content and nature of hemicelluloses are necessary to evaluate the suitability of seagrass material for papermaking or production of biofuels.

With regard to pectic polysaccharides, the presence of apiogalacturonans not known from land plants has been shown for two *Zostera* species (Gloaguen et al., 2010; Lv et al., 2015). Up to now it is unknown whether apiose-rich carbohydrates are only limited to a few seagrass species or a general feature, being relevant for angiosperm life in the marine habitat.

Another unique feature of seagrass cell walls might be the presence of SP. A sulfated galactan from *Ruppia maritima* has been isolated and carefully characterized (Aquino et al., 2005). Although there are some proposals for the presence of other

SP in seagrasses (e.g., Aquino et al., 2011; Silva et al., 2012; Kolsi et al., 2016), proof of sulfate by colorimetric assays or FT-IR is not sufficient, as seagrasses contain other sulfated compounds like e.g., zosteric acid or sulfated flavonoids (Zidorn, 2016). According to Olsen et al. (2016), an expansion of arylsulfotransferases in *Zostera* compared to land plants might correlate with the ability to synthesize SP. Careful evaluation whether SP are a general feature of seagrasses is urgently needed.

In the field of wall proteins, the recent findings from our group (Pfeifer et al., 2020) could underline the hypothesis of an involvement of highly charged AGPs in calcium storage and –signaling (Lamport et al., 2006; Lamport et al., 2020). To answer the question whether AGPs are present in other seagrass species and whether they are structurally comparable to *Zostera* AGP is another challenge for the future.

Although a high number of literature on lignin in seagrasses is available, more definite proof with modern methodologies, like Py-GC-MS (e.g., procedure of van Erven et al., 2017) or qNMR (e.g., procedure of Capanema et al., 2005) is necessary with a focus on more species from all seagrass families.

Main Challenges for the Future

At first, comprehensive approaches on evaluation of seagrass cell wall composition are needed to understand the general composition of the cell walls of these angiosperms living in an extreme environment. This could be done by sequential extraction of seagrass material following published protocols (e.g., O'Rourke et al., 2015; Raimundo et al., 2016) and analytical characterization of the different cell wall fractions. To get information on similarities and differences between the cell walls of different seagrass families, so many species as possible, belonging to the different seagrass families, have to be investigated. It has to be taken into account, that the availability of seagrass material is often limited due to the strict rules for protection of seagrasses in their natural habitats.

Furthermore, it has to be investigated whether the interesting unique polysaccharides/glycoproteins which have been isolated and carefully characterized for single seagrass species are also present in other or even all seagrasses. Especially the apiogalacturonans (Gloaguen et al., 2010; Lv et al., 2015), sulfated galactans (Aquino et al., 2005) and highly charged AGPs (Pfeifer et al., 2020) are interesting candidates for adaption to the marine habitat. Comparable to marine algae, charged polysaccharides/glycoproteins of the cell wall seem to be essential to cope with salt stress. A future task will be to elucidate the adaption strategies of the different seagrass lineages that evolved to marine environment independently. Furthermore, the cellular mechanisms involved in protection against salt have to be investigated. Both sulfate groups and uronic acids are able to bind Ca^{2+} ions, which play a crucial role in both the regulation of transport and exclusion of Na^+ and other mineral ions at the plasma membrane of plant cells and are able to protect a salt-sensitive species (*Phaseolus vulgaris*) against damage caused by NaCl present in the cell culture medium (Lahaye and Epstein, 1969). For AGPs of *Z. marina*, a strong binding of Ca^{2+} has already been shown by bio-layer interferometry (Pfeifer et al., 2020).

Finally, full genome sequencing of more seagrass species is necessary to get widespread information on cell wall related genes of these fascinating organisms. Identification of seagrass genes involved in adaption of cell walls to salt water could provide information how agricultural crops might tolerate an increasingly dry and saline environment.

AUTHOR CONTRIBUTIONS

LP and BC performed literature search and evaluated the published data. LP created tables and figures. Both authors discussed the results and wrote the final manuscript. Both authors contributed to the article and approved the submitted version.

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In situ Responses of the Eelgrass *Zostera marina* L. to Water Depth and Light Availability in the Context of Increasing Coastal Water Turbidity: Implications for Conservation and Restoration

Shaochun Xu*[1,2,3,4,5], *Pengmei Wang*[1,2,3,4,5], *Feng Wang*[1,2,3,4,5], *Peng Liu*[1,2,3,4,5], *Bingjian Liu*[1,2,3,4,5], *Xiaomei Zhang*[1,2,3,4], *Shidong Yue*[1,2,3,4,5], *Yu Zhang*[1,2,3,4,5] and *Yi Zhou*[1,2,3,4,5]

[1] CAS Key Laboratory of Marine Ecology and Environmental Sciences, Institute of Oceanology, Chinese Academy of Sciences, Qingdao, China, [2] Laboratory for Marine Ecology and Environmental Science, Qingdao National Laboratory for Marine Science and Technology, Qingdao, China, [3] Center for Ocean Mega-Science, Chinese Academy of Sciences, Qingdao, China, [4] CAS Engineering Laboratory for Marine Ranching, Institute of Oceanology, Chinese Academy of Sciences, Qingdao, China, [5] College of Earth Sciences, University of Chinese Academy of Sciences, Beijing, China

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***Correspondence:**
Yi Zhou
yizhou@qdio.ac.cn

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Accelerating losses of seagrass meadows has led to efforts to restore these highly productive and beneficial ecosystems globally. Depth and light availability are critical determinants of seagrass restoration success. Eelgrass (*Zostera marina* L.) is the dominant seagrass species in the temperate northern hemisphere, but its global distribution has reduced dramatically. The main aims of this study were to determine: (1) the depth limit for *Z. marina* survival in Ailian Bay, north China, and (2) how light availability affects the growth and recruitment of *Z. marina* as a basis for identifying a suitable depth range for successful restoration. To achieve these aims, *Z. marina* shoots were transplanted from a nearby donor site, Swan Lake, to an experimental site, Ailian Bay, and the temporal responses of *Z. marina* shoots to light availability at water depths ranging from 1 to 8 m were investigated using *in situ* suspended cultures. Four suspended shoot transplantation experiments were conducted in 4 years. The results showed that the transplanted *Z. marina* shoots could survive and branch during an annual growth cycle, permanently underwater, at a depth $\leq$3 m. Due to the local turbidity of the waters in Ailian Bay, a depth of 4 m led to sufficient light deprivation (reduced to 6.48–10.08% of surface irradiance) to negatively affect seagrass shoot density and clonal reproduction. In addition, reproductive shoot density also tended to decline with water depth and light deprivation. Our results indicated that *Z. marina* population recruitment, through sexual and asexual (clonal growth) reproduction, were negatively affected by increasing water depth and light deprivation. These findings may provide a suitable depth range for the successful restoration of *Z. marina* in local coastal waters. They may also be applied to the management and restoration of *Z. marina* globally.

Keywords: seagrass meadow, depth limit, light requirement, response, methodology, reproduction, restoration, *Zostera marina*

INTRODUCTION

Seagrass meadows are among the most productive plant communities, providing habitats, food, and nurseries for a variety of marine organisms (Costanza et al., 1997; Verweij et al., 2008; Barbier et al., 2011; Liu et al., 2013; Unsworth et al., 2018b), regulating nutrients (Barbier et al., 2011), and functioning as key sites for global carbon storage in the biosphere (Fourqurean et al., 2012). Unfortunately, due to the impact of multiple stressors (environmental, biological, and climatological) (Collier and Waycott, 2009; Unsworth et al., 2018a), seagrass meadows have been declining since 1990 at a rate of 7% per annum (Waycott et al., 2009). With the development of coastal construction, the turbidity of coastal waters has been increasing, thus the light availability has been reducing (Elsahragty and Kim, 2015). Reduced light availability due to increased anthropogenic nutrient loading and sedimentation has been identified as one of the primary causes of seagrass loss (Jennifer et al., 2003; Orth et al., 2006). Furthermore, light is often considered one of the most critical factors that controls the distribution and growth of eelgrass (Lee et al., 2007; Zhang et al., 2020).

The eelgrass *Zostera marina* L. is the dominant seagrass species throughout the Atlantic and Pacific coasts of the temperate northern hemisphere (Green and Short, 2003). In China, eelgrasses are distributed in the coastal waters of Shandong, Hebei, and Liaoning provinces (Zheng et al., 2013; Xu et al., 2020a,b). Historical distribution information (1950–2013) of eelgrass specimens (Biological Museum, Chinese Academy of Sciences, Qingdao, China) and previous literature (Yang and Wu, 1984; Editorial Board of China Bay Survey, 1991; Ye and Zhao, 2002; Guo et al., 2010; Zheng et al., 2013) have revealed that a large number of eelgrass meadows having contracted sharply or disappeared (more than 80%). This is due to increased anthropogenic nutrient loading and sedimentation, according to the National Seagrass Resource Survey (2015–2020). Similarly, previously abundant *Z. marina* in Ailian Bay, north China, has declined dramatically and is currently mainly distributed in the sea cucumber ponds, according to local fishermen.

Faced with an increasing rate of eelgrass decline, eelgrass restoration throughout its distribution area in temperate northern hemisphere has become an important management tool to mitigate seagrass losses and to enhance critical ecosystem functions (Marion and Orth, 2010; Zhou et al., 2014; van Katwijk et al., 2016; Liu et al., 2019). Unfortunately, only 37% of seagrass restoration efforts have been successful in recent years (van Katwijk et al., 2016). Insufficient light availability has been identified as the one of the primary causes of eelgrass transplantation failure (e.g., Moore et al., 1997; van Katwijk et al., 2016); therefore, understanding their ability to acclimate to a range of light availability may be the key to ensuring their survival in restoration efforts (Eriander, 2017; Zhang et al., 2020).

The light requirements of seagrass have been investigated in many studies, and comprehensively reviewed by Lee et al. (2007); it has been revealed that the minimum light requirements of seagrass vary with species (Lee et al., 2007; Statton et al., 2018) and also within species depending on site-specific conditions (Lee et al., 2007). Therefore, it is important to establish the local minimum light requirement for individual species in an area of interest (Bulmer et al., 2016). The maximum depth at which seagrass grows provides an insight into the local light environment and the minimum light requirements of that seagrass. This parameter is also described as the maximum depth limit or depth of colonization. Seagrass coverage declines with water depth, and a light availability gradient exists along this natural depth distribution (Duarte, 1995). Depth has been shown to be a critical determinant of seagrass restoration success, suggesting it is imperative for identifying the most resilient areas that are most suitable for conservation and restoration (Aoki et al., 2020). Therefore, the depth of colonization of eelgrass in Ailian Bay needs to be confirmed for future restoration efforts.

To date, many studies have been performed to determine the light requirements of seagrass (Abe et al., 2003; Beck et al., 2018). Previous seagrass light requirements were estimated in field investigations (Abal et al., 1994; Abe et al., 2003; Ochieng et al., 2010; Zhang et al., 2020) and laboratory experiments (Abal et al., 1994; Moore and Wetzel, 2000; Eriander, 2017). Laboratory experimental systems remove important contextual factors of natural ecosystems (Crain et al., 2008), such as changes in water levels (due to tides and waves), episodic turbidity, and epiphyte growth which all contribute to the reduction of light availability to seagrass (Abal et al., 1994). Field investigations have been conducted in natural seagrass beds, but not necessarily in severely degraded areas. Moreover, responses of seagrass to light reduction are also dependent on site-specific conditions (Lee et al., 2007).

In the present study, an *in situ* suspended culture experiment was conducted in an area of interest, to directly examine long-term responses of transplanted *Z. marina* shoots to a depth gradient. Four suspended shoot transplantation experiments were conducted in 4 years. The aims of this study were to determine: (1) the depth limit for the survival of eelgrass (*Z. marina*) in Ailian Bay, and (2) how light availability affects the growth and recruitment of *Z. marina* as a basis for identifying a suitable depth range for successful restoration. The results from this study could provide important implications for future management and restoration of eelgrass, to avert further loss and enhance the potential for recovery. Additionally, *in situ* suspended seagrass cultures are described that may serve as a useful methodology for future field simulation experimentation.

MATERIALS AND METHODS

Study Sites

Donor Site

The donor site was located in Swan Lake, Rongcheng City, north China (**Figure 1**). Swan Lake is a 4.8-km^2 marine lagoon that is connected to Rongcheng Bay by a narrow mouth, 86 m in width. The lagoon, a national nature reserve for the whooper swan, *Cygnus cygnus,* provides food resources and the largest wintering habitat for this bird in Asia (Wang et al., 2017). *Zostera marina* is distributed in the intertidal to subtidal zone, forming a meadow of ∼2.3 km^2 (Zhou et al., 2015; Xu et al., 2019). The environmental conditions were shown in **Table 1**.

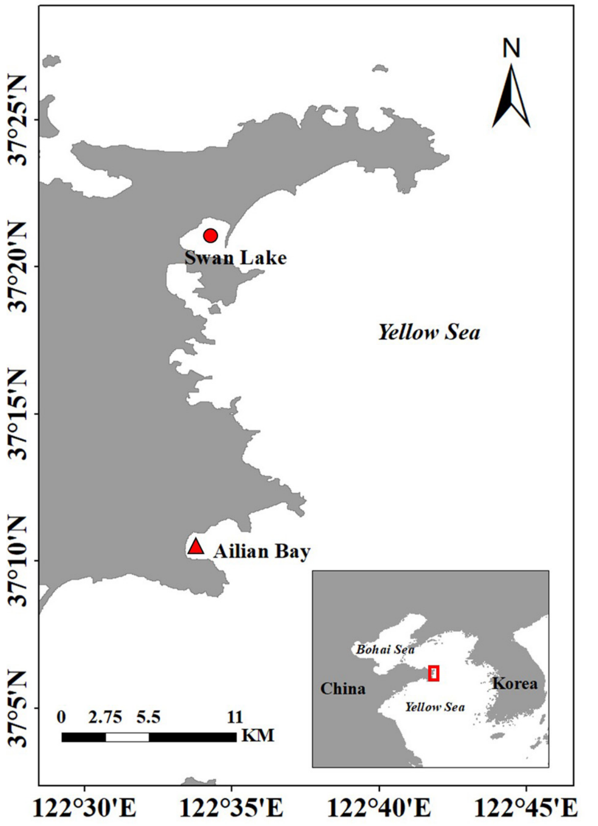

FIGURE 1 | Location of the donor site in Swan Lake (●) and the experimental site in Ailian Bay (▲), north China.

Experimental Site

The experimental site was located in Ailian Bay, Rongcheng City, ∼20 km from Swan Lake (**Figure 1**). It is a natural semi-enclosed bay. The bay is an important aquaculture area for north China (Yang et al., 2018), with more than 60% of it used for floating raft cultures of kelp or shellfish (scallops, oysters, and abalone). *Z. marina* is mainly distributed in sea cucumber ponds of this bay. The environmental conditions were shown in **Table 1**.

Collection of Adult Plants

Adult plants were collected from the intertidal area in Swan Lake at low tide. *Z. marina* materials were collected carefully with shovels, and adult shoots that had at least 1–2 cm of rhizome with roots were selected. Twenty centimeters of leaf blade and leaf sheath were retained, and the extra part was removed by scissors (Zhou et al., 2014; Liu et al., 2019). The transplantation period ran from April to September (**Table 2**).

Transplantations

Four suspended shoot transplantation experiments were performed in 4 years (2010, 2011, 2012, and 2014; see **Table 2**). Shoots were transplanted using a stone anchoring method (Zhou et al., 2014). This method involves anchoring a transplanting unit (PU) consisting of three to four shoots with rhizomes and roots to a small elongate stone 50–150 g in weight using biodegradable thread or thin rope (e.g., cotton thread or hemp string). The small stones were collected from the seashore in Swan Lake. PUs were buried in an experimental device (PE box or PVC pot; **Table 2** and **Figures 2B,C**), which contained a bottom layer of sediment taken from the bank of Swan Lake. The rhizomes were placed at a depth of 2–4 cm in sediments and on the side of the stone. The initial shoot number per device for the four experiments ranged from 10 to 20 shoots (**Table 2**). The experimental devices planted with *Z. marina* materials were then transported to Ailian Bay and tied to the rafts with polyethylene ropes (**Figure 2A**). Four transplantation experiments were conducted along a depth gradient (**Table 2**). To balance the experimental devices in the water, a one 1-kg plumb ball was tied to the bottom of each device.

Monitoring Method

The main monitored parameters included shoot number and height in all four experiments, and flowering shoot number and shoot height were also monitored in 2014. Monitoring time and frequency were shown in **Table 2**.

Environmental Parameters

Water temperature (°C), salinity (ppt), dissolved oxygen content (DO, mg L^{-1}), pH, chlorophyll content ($\mu g\ L^{-1}$), and depth (m) were measured using a multi-parameter water quality sonde (YSI 6600, United States). The parameters were measured over a 2–15 min period at each depth. The measuring sonde was moved up and down the water column while several measurements were recorded at various depths (1, 2, 3, 4, and 6 m), all within an hour. Variations in water parameters with depth were measured five times in total during April, May, June, August, and November 2014.

TABLE 1 | Environmental conditions of the donor and experimental sites.

Sites and locations		Environmental conditions
Donor site	Swan Lake 122°34′E, 37°21′N	A marine lagoon. Salinity: 29.8–31.3 psu; annual average water temperature: 14.7°C; average depth: ∼2 m; irregular semidiurnal mixed tides (tidal range of ∼0.9 m) (Liu, 2012). Annual concentrations of NH_4^+, NO_3^-, NO_2^-, and PO_4^{3-} were 2.14 ± 1.29, 1.31 ± 1.53, 0.15 ± 0.10, and 0.28 ± 0.17 $\mu mol\ L^{-1}$, respectively (Zhou et al., 2015).
Experimental site	Ailian Bay 122°34′N, 37°10′E	A natural semi-enclosed bay. Salinity: 26.3–31.1 psu; annual average water temperature: ∼15.1°C; water depth: <14 m; irregular semidiurnal mixed tides (tidal range of ∼1.8 m). Annual concentrations of NH_4^+, NO_3^-, NO_2^-, and PO_4^{3-} were 2.63 ± 0.88, 2.11 ± 1.34, 0.27 ± 0.07, and 0.31 ± 0.26 $\mu mol\ L^{-1}$, respectively (Liu, 2012).

TABLE 2 | Four suspended *Z. marina* shoot transplantation (from Swan Lake to Ailian Bay) experiments.

Transplantation time	Experiment period	Depth gradient	Number of duplicates per depth	Initial shoot number per device	Monitoring time or frequency	Monitoring index	Experimental device
Jun 2010	Jun 2010 to Jul 2011	2, 4, 6, and 8 m	4	12	Dec 2010 and Jul 2011	Shoot number and height	PE box (30 cm × 20 cm × 15 cm)
Apr 2011	Apr to Sep 2011	2, 4, 5, 6, and 7 m	4	12	Jul 2011	Shoot number and height	PE box (30 cm × 20 cm × 15 cm)
Sep 2011	Sep 2011 to Nov 2012	2, 3, 4, 5, and 6 m	4	20	Sep 2011 to Nov 2012; monitoring every 1–2 months	Shoot number and height	PE box (30 cm × 20 cm × 15 cm)
Apr 2014	Apr to Oct 2014	1, 2, 3, 4, and 6 m	5	10	Apr to Oct 2014; monitoring approximately monthly	Total and flowering shoot number, and shoot height	PVC pot (D = 20 cm, H = 12 cm)

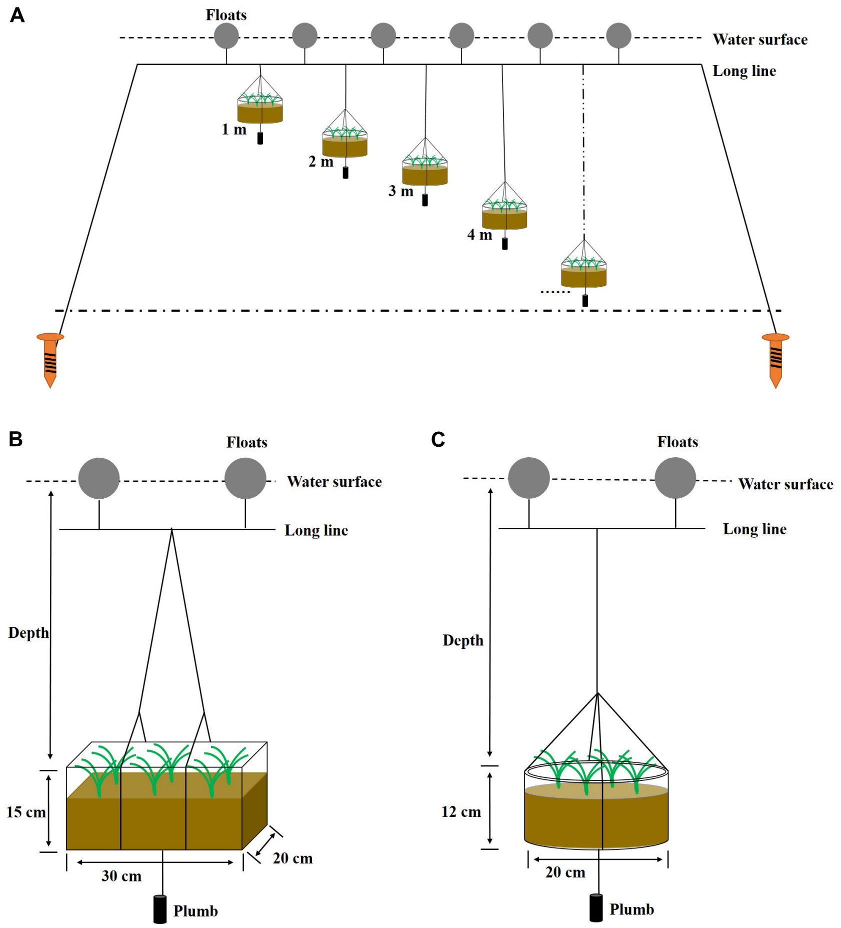

FIGURE 2 | Floating raft **(A)**, PE box **(B)**, and PVC pot **(C)** used for suspended *Z. marina* transplantation experiments. PE boxes were used in 2010–2012, and PVC pots in 2014.

Light availability (μmol photons m^{-2} s^{-1}) was measured using an ECO-PARSB sensor (Sea-Bird Scientific, United States). The light sensor was moved up and down the water column with light availability measurements recorded at various depths (0, 1, 2, 3, 4, and 6 m), all within an hour. Light intensity was obtained at four different days/times in total, in May,

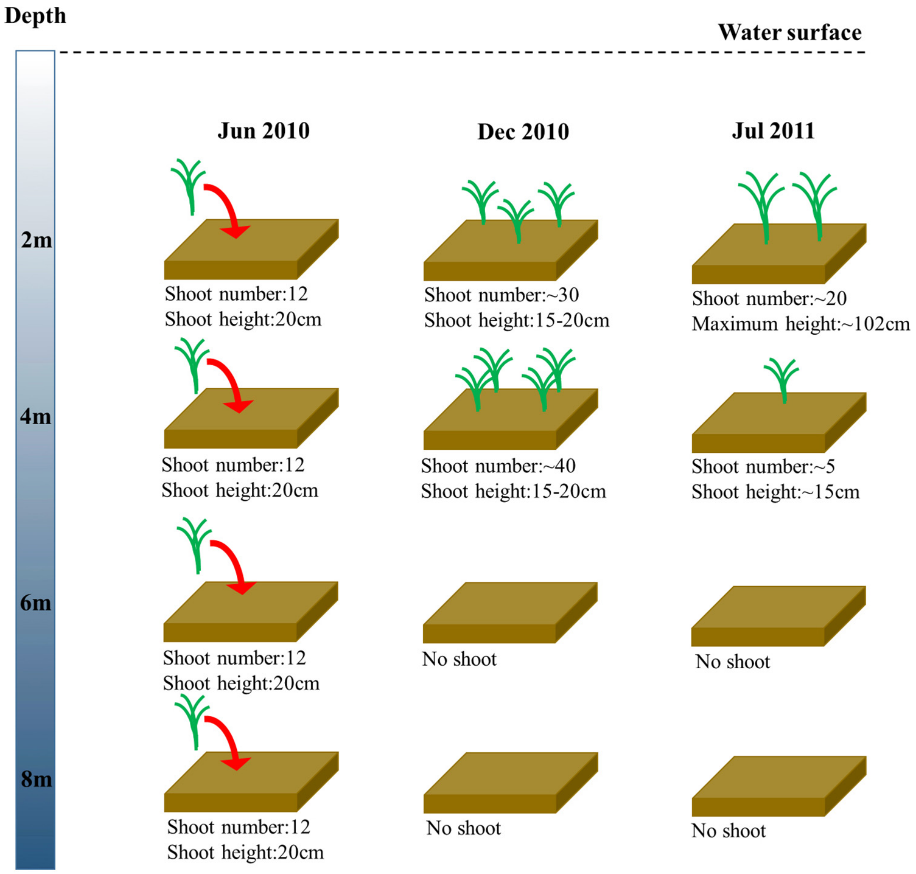

FIGURE 3 | Changes in *Z. marina* shoot number and shoot height along a depth gradient in Ailian Bay in a shoot transplantation experiment beginning in June 2010.

August, and October 2014, and the light intensity was measured every minute.

Data Analyses

For the shoot transplantation experiment beginning in April 2014, differences among seagrass variables at the various depths were tested using a one-way ANOVA, and the specific treatment differences were identified using independent *t*-test. Simple linear regression was used to test the significance of the relationships between water depth and temperature. Before the analyses, the homogeneity of variance was tested using Levene's test. Differences were considered significant at a probability level of $p < 0.05$. SPSS 20.0 was used for all data analyses. Simple linear regression analyses were considered significant at a probability level of $p < 0.05$.

Seagrass Responses to Transplantation Depth

To quantitatively analyze the responses of viable transplanted shoots to water depth and time from deployment in the 2014 experiment, the following linear model (see Zhang et al., 2020) was applied:

$$S(z) = az + b \tag{1}$$

Where S(z) is shoot density, z is depth at each time point of each year, and a and b represent equation coefficients. Here, S(z) is the vegetative shoot density, not the total shoot density, because vegetative shoot density is more suitable for the evaluation of lateral shoot branching, and reproductive shoots disappear in August every year. Any depths at which the measured shoot density overlapped with zero were excluded from linear model fitting. All linear regressions were performed using R version 3.6.3.

Such analyses were not performed for reproductive density or shoot height because quantitative relationships, such as those expressed in equation (1), could not be identified from a visual inspection of the data.

Light Variation With Depth

To explore whether water clarity varied spatially, and thus determine if the depth limits obtained from the analysis were affected by any spatial variations in water clarity, the spatial correlation of water turbidity with depth was assessed (see Zhang et al., 2020). This was achieved using non-linear regression to fit each of the four distributions for the dependence of light intensity on depth to Beer's Law (Kirk, 1985):

$$I = I_0 \, exp(-K_d^* z) \tag{2}$$

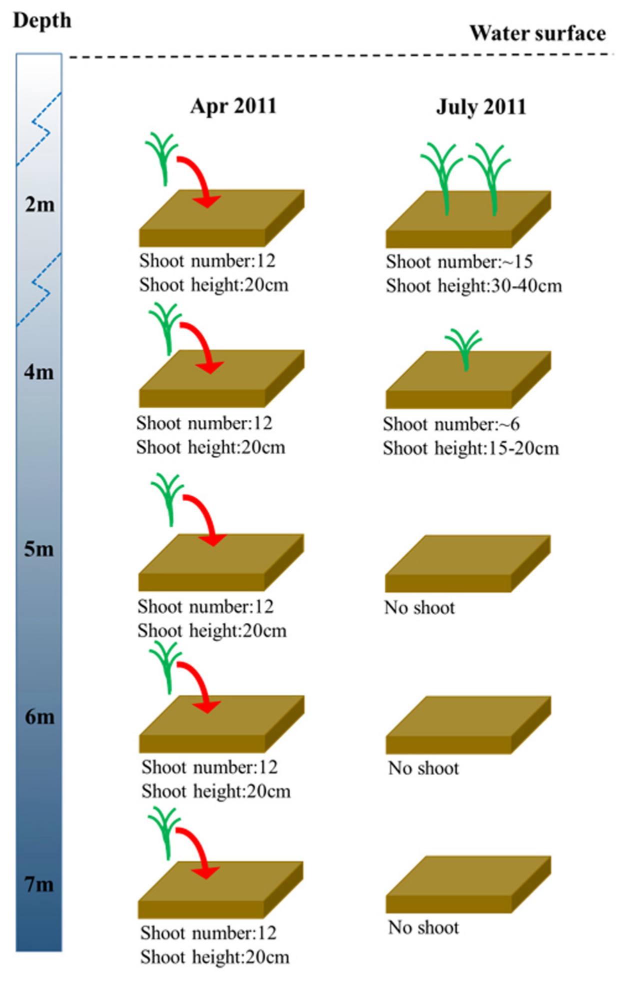

FIGURE 4 | Changes in *Z. marina* shoot density and shoot height along a depth gradient in Ailian Bay in a shoot transplantation experiment beginning in April 2011.

where, I represents the light availability at water depth z (m), I_0 represents the surface light, and $K_d{}^*$ represents the light attenuation coefficient (m^{-1}), which was not corrected for the sun's location in the sky.

Improvement of the Experimental Device

Some PE boxes were overturned by marine currents during 2010–2011; therefore the design parameters of the experimental device were altered. In order to reduce the impact of currents on the experimental device, improvements to the stability of the device in seawater were attempted by reducing the drag force. The drag force is related to fluid density, frontal area of object, drag coefficient, and the velocity of fluid, and the shape of an object has a large effect on the amount of drag (Batchelor, 1967). This can be expressed as follows:

$$F_d = 0.5\rho\mu^2 C_d A \tag{3}$$

where, F_d is the drag force, ρ is the mass density of the fluid, μ is the flow speed of the object relative to the fluid, C_d is the drag coefficient, and A is the frontal area.

In order to change the shape (reducing drag coefficient) and reduce the frontal area of the experimental device, PVC pots were alternatively used in 2014. The drag coefficient ranges of the PE box (cuboid) and PVC pot (short cylinder) were 1.05–2.05 (Aaronaes et al., 2015) and <0.64 (Mohammed et al., 2016), respectively.

RESULTS

Suspended Shoot Transplantation Experiment

In the shoot transplantation experiment beginning in June 2010, shoot densities at 2 m and 4 m increased by more than 100%, while there was no *Z. marina* remaining at 6 m and 8 m after approximately half a year of experimentation (**Figure 3**). After approximately a year of experimentation, the transplanted *Z. marina* at 2 m and 4 m successfully completed a 1-year life cycle, and reproductive shoots, with a maximum length of 102 cm, were found at 2 m. The shoot density at 2 m was four-fold greater than that at 4 m. There was also no *Z. marina* remaining at 6 m and 8 m after approximately a year of experimentation.

In the shoot transplantation experiment beginning in April 2011, in order to test whether the depth between 4 and 6 m was suitable for eelgrass survival, *Z. marina* was transplanted at 5 m. After 3 months of experimentation, *Z. marina* survived only at 2 m and 4 m, and there was no *Z. marina* found at 5 m, 6 m, or 7 m (**Figure 4**).

In the shoot transplantation experiment beginning in September 2011, shoot densities decreased gradually in autumn and winter, increased slightly in March 2012, and then maintained at a low level (**Figure 5A**). From September to November 2012, shoot densities at 2 m and 3 m increased rapidly, and shoot densities at 2 m and 3 m increased approximately three- to four-fold, indicating that lateral shoots were germinating and growing rapidly during this period. In November, there was ~90 shoots per box at 2 m and ~30 shoots per box at 3 m. Shoot density at 4 m maintained at a low level, with approximately two shoots per box in November, which was significantly lower than that at 2 m and 3 m. In December 2011, there was no *Z. marina* remaining at 5 m and 6 m; therefore, 20 shoots per box were re-transplanted at 5 m and 6 m; however, *Z. marina* had completely disappeared again in August.

Shoot height at 2 m, 3 m, and 4 m decreased in autumn and winter, and increased significantly from spring to summer (**Figure 5B**). Shoot height decreased with increasing water depth. The maximum shoot height at 2 m was ~80 cm in August 2012, and ~60 cm at 3 m and 4 m in September. Plant height began to decrease after summer, and it was ~30 cm at the above mentioned depths in November.

In the shoot transplantation experiment beginning in April 2014, total shoot densities at different depths were relatively stable in the first 3 months (**Figure 6A**). After the initial stable period, total shoot densities in shallow water (depths of 1 m and 2 m)

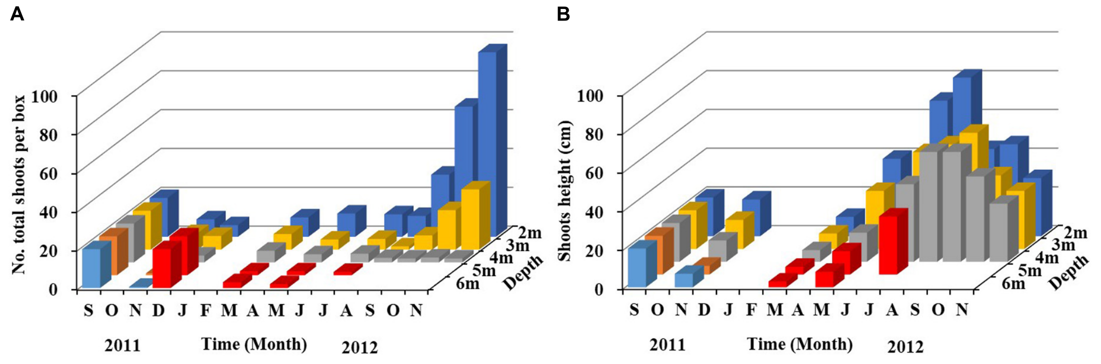

FIGURE 5 | Temporal changes in *Z. marina* shoot density **(A)** and shoot height **(B)** along a depth gradient in Ailian Bay in a shoot transplantation experiment beginning in September 2011. Since *Z. marina* was not found at 5 m and 6 m in December 2011, 20 shoots per box were re-transplanted at 5 m and 6 m (red column). The values in the figure represent the means.

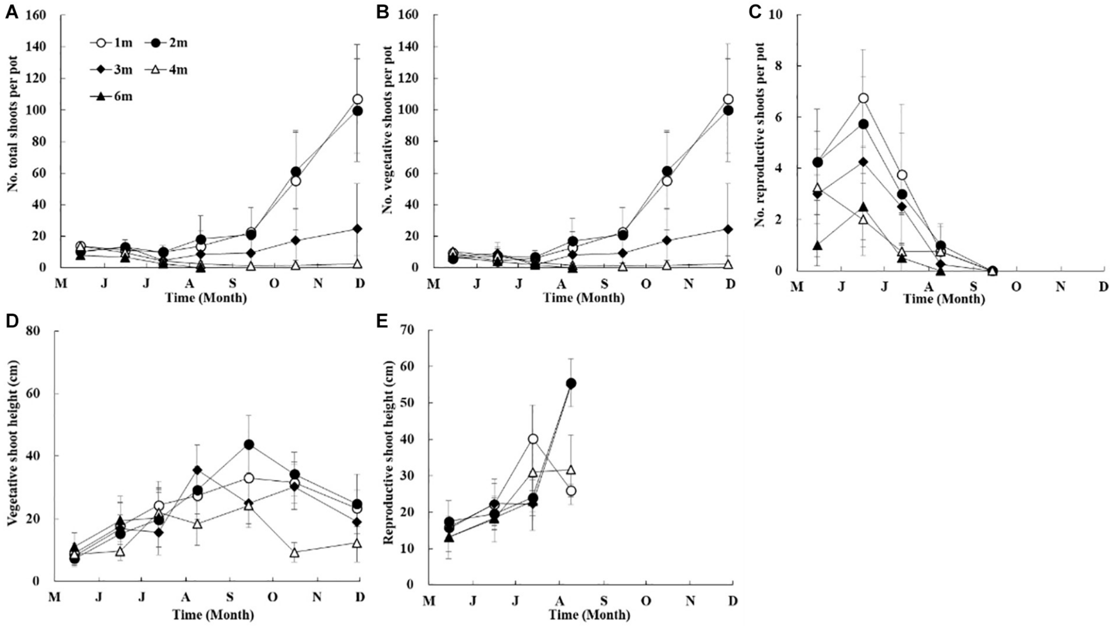

FIGURE 6 | Temporal changes in *Z. marina* total shoot density **(A)**, vegetative shoot density **(B)**, reproductive shoot density **(C)**, vegetative shoot height **(D)**, and reproductive shoot height **(E)** along a depth gradient in Ailian Bay in a shoot transplantation experiment beginning in April 2014.

increased rapidly, those at 3 m increased gradually, while those in deeper waters (depth >3 m) declined. The maximum total shoot densities at 1 m and 2 m were significantly higher than those at 3 m ($p < 0.05$), and approximately three-fold greater than those at 3 m. Very few shoots at 4 m depth survived until the end of the experiment, and shoots at 6 m depth had completely disappeared within 4 months. The temporal and spatial trends of vegetative shoots densities were consistent with those of the total shoots (**Figure 6B**). Flowering shoots were observed from May to August at 1 m, 2 m, 3 m, and 4 m, and were also sporadically observed at 6 m (**Figures 6C,E**).

Shoot height at different depths increased gradually from May to August 2014 (**Figure 6D**). The maximum shoot height at 1 m and 2 m was ~30 cm and ~40 cm in September, respectively, and that at 3 m was ~35 cm in August. Plant height began to decrease after summer, and it was ~20 cm at the above-mentioned depths in November. However, the maximum shoot height at 4 m was ~20 cm in September, and ~10 cm in November (**Figure 6D**).

The linear regression of shoot density vs. depth revealed significant differences between depths in September and November for the 2014 experiment ($p < 0.05$, **Figure 7**).

Correlations Between Water Parameters and Depth

Salinity, DO, and pH varied slightly with water depth. The chlorophyll content (Chl) changes with water depth were mostly

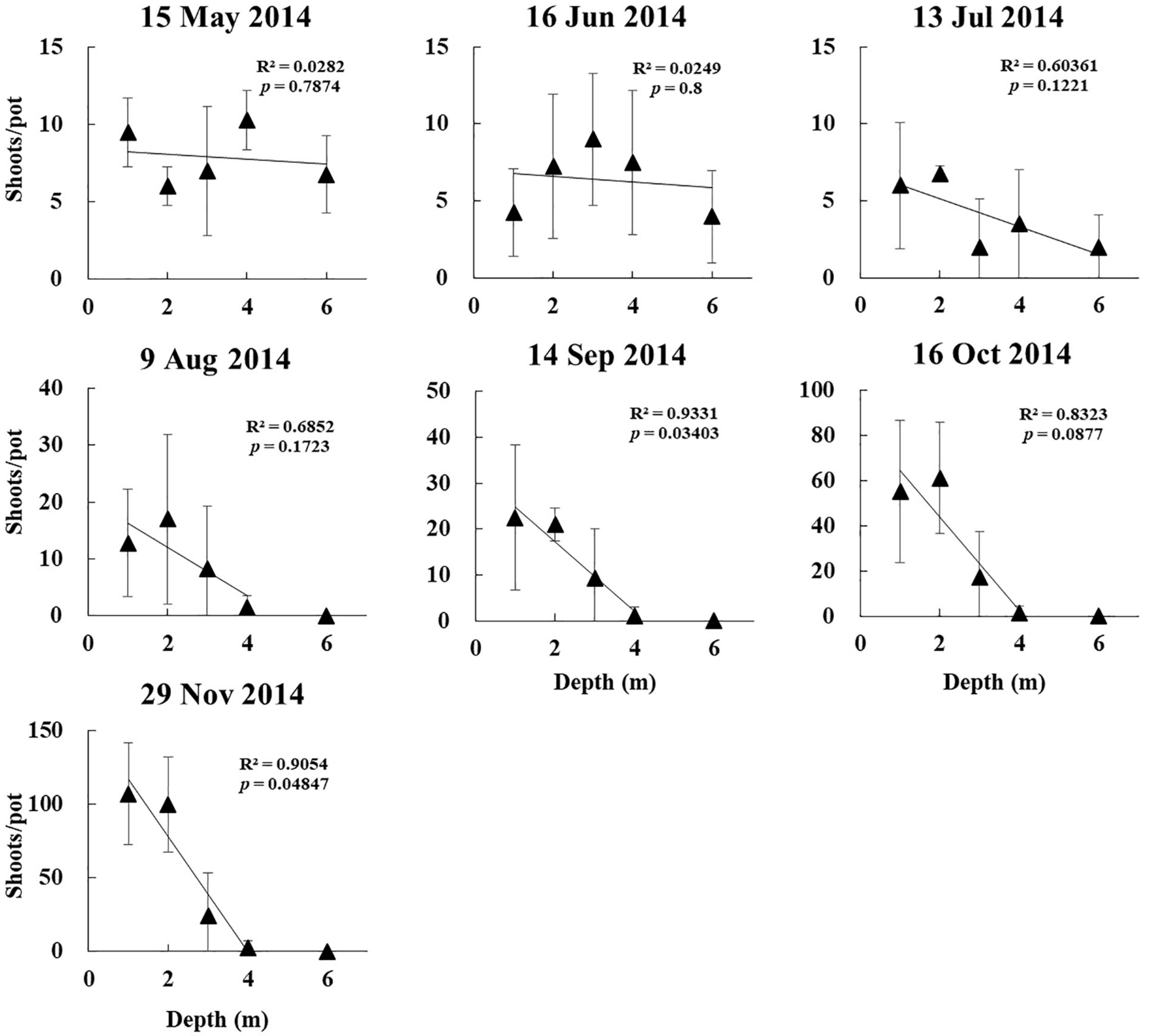

FIGURE 7 | Linear regression of *Z. marina* vegetative shoot density vs. depth in a shoot transplantation experiments beginning in April 2014.

slight. Temperature changes with water depth were relatively high in the warm season (April, May, June, and August 2014), ranging from 0.28 to 0.75°C/m (**Figure 8**). The regression of temperature vs. depth in May, June, August, and November were all significant (**Figure 9**; $p < 0.05$). Light intensity changed significantly as depth increased, and all four of the light vs. depth distributions adhered well to Beer's Law ($R^2 \geq 0.82$; **Figure 10**). These results suggest that the water clarity in Ailian Bay did not substantially vary spatially with depth.

DISCUSSION

Zostera marina is a major habitat-forming species in north China. Its distribution may be threatened by changes in water clarity through increased sedimentation and nutrient input from agricultural and urban development (Thrush et al., 2004; Orth et al., 2006; Ralph et al., 2007). The present study defined the maximum depth limit for eelgrass (*Z. marina*) and provided a suitable depth range for successful restoration in local coastal waters, north China. More broadly, this study assessed the effect of light availability on the growth and recruitment of *Z. marina* conducted by *in situ* transplanted suspended cultures.

In situ Suspended Cultures and Improvement of the Experimental Device

Site selection for potential seagrass transplantation is crucial for restoration success, and environmental parameters limit suitable habitat availability for seagrass restoration (van Katwijk et al., 2016). One essential parameter determining seagrass restoration success is depth (Aoki et al., 2020). However,

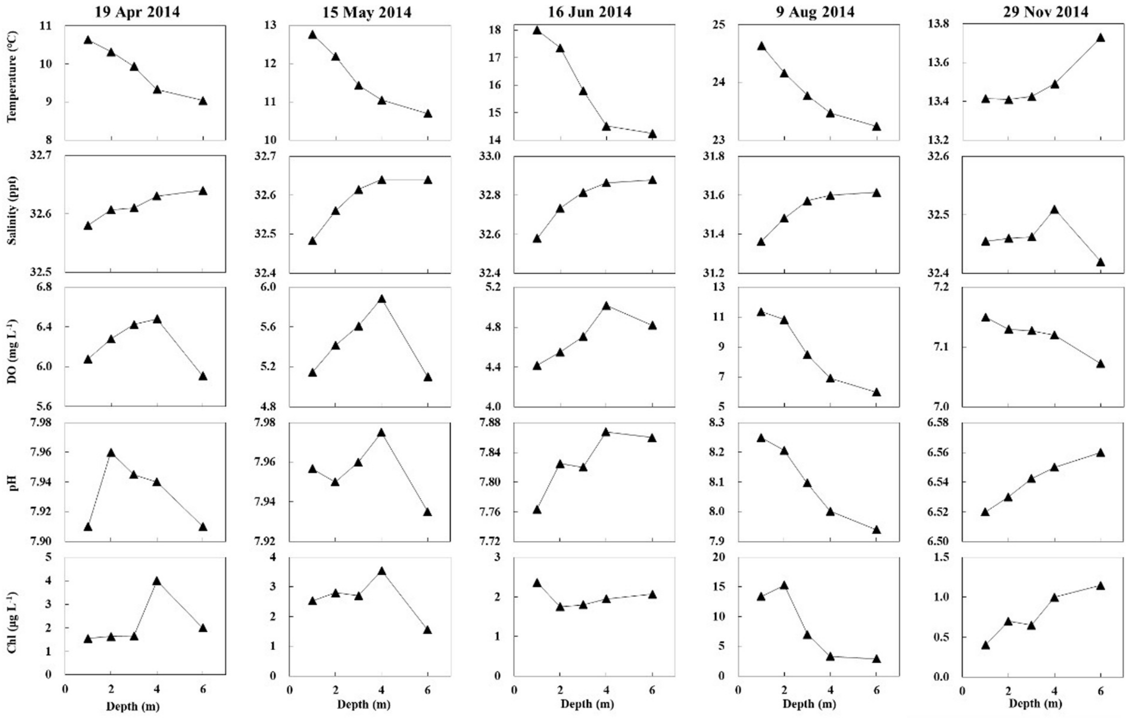

FIGURE 8 | Changes in water temperature, salinity, dissolved oxygen (DO), pH, and chlorophyll content with depth in Ailian Bay in 2014.

suggestions for site selection for seagrass restoration are mainly based on either *in situ* investigations or laboratory experiments. Laboratory experimental systems remove important contextual factors under natural conditions (e.g., waves, episodic turbidity, and epiphyte growth) (Abal et al., 1994; Crain et al., 2008), and the minimum light requirement for eelgrass in laboratory experiments has been shown to be lower than that in the field (Short, 1991). For field investigations, results might be difficult to generalize for other locations because seagrass responses to light reduction are dependent on site-specific conditions (Lee et al., 2007).

To overcome inherent weaknesses of the abovementioned experiments, an *in situ* suspended culture experiment was developed in this study to directly examine long-term responses of transplanted *Z. marina* shoots to a depth gradient, in order to determine the depth limit for eelgrass restoration. Moreover, *in situ* suspended cultures may prove to be a useful method for seagrass research, e.g., studying the effect of habitat type on eelgrass seed germination (unpublished data). Over the 4 years of *in situ* suspended experiments, the stability of the experimental device was improved by switching from PE boxes to PVC pots, based on device drag force comparisons. The drag force of the PE box was approximately double to six-fold that of the PVC pot; indicating a greater stability of the PVC pot, and consequently none of these were overturned by marine current. In addition, because it is much easier for the PE box to rotate around its vertical axis under the influence of current, the PVC pot appears to be a more stable device for suspended cultures of eelgrass. For marine areas with strong currents, a heavier plumb ball can be added at the bottom of the experimental device to improve the stability of the system.

Environmental Factors Contributing to the Depth Limit of *Z. marina*

Eelgrass shoot transplantation experiments provide evidence that eelgrass may have the ability to recolonize at an optimal depth of ≤3 m in Ailian Bay, where eelgrass has declined dramatically. The value of the *Z. marina* depth limit determined for Ailian Bay in this study was higher than that of the distribution depth (1–2 m; Xu et al., 2018, 2019) of natural populations at the donor site (Swan Lake). The depth limit in Ailian Bay is within the range reported previously, and *Z. marina* grows from the intertidal zone to depths of ∼10 m depending on water clarity (Jackson et al., 2013). However, even with sufficient light availability, seagrass can still decline if impacted by other environmental factors, such as increasing temperature (Marbà and Duarte, 2010) and high hydrogen sulfide content in sediment (Fraser and Kendrick, 2017). In China, land reclamation, clam harvesting, and mariculture have been suggested to be the main factors causing eelgrass habitat loss (Huang et al., 2006; Zheng et al., 2013).

Light availability, temperature, and inorganic nutrients are considered as major factors controlling seagrass growth (Lee et al., 2007). In the present study, light availability was identified as the most important factor determining the depth limit and

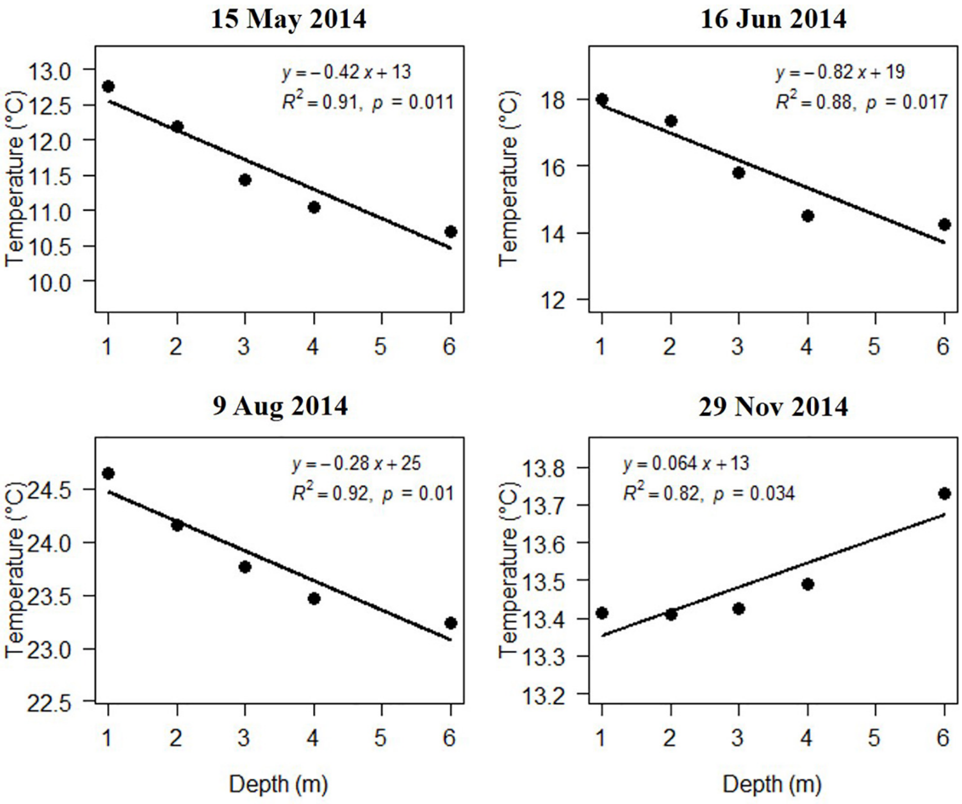

FIGURE 9 | Variation in temperature with water depth in Ailian Bay at various dates during 2014.

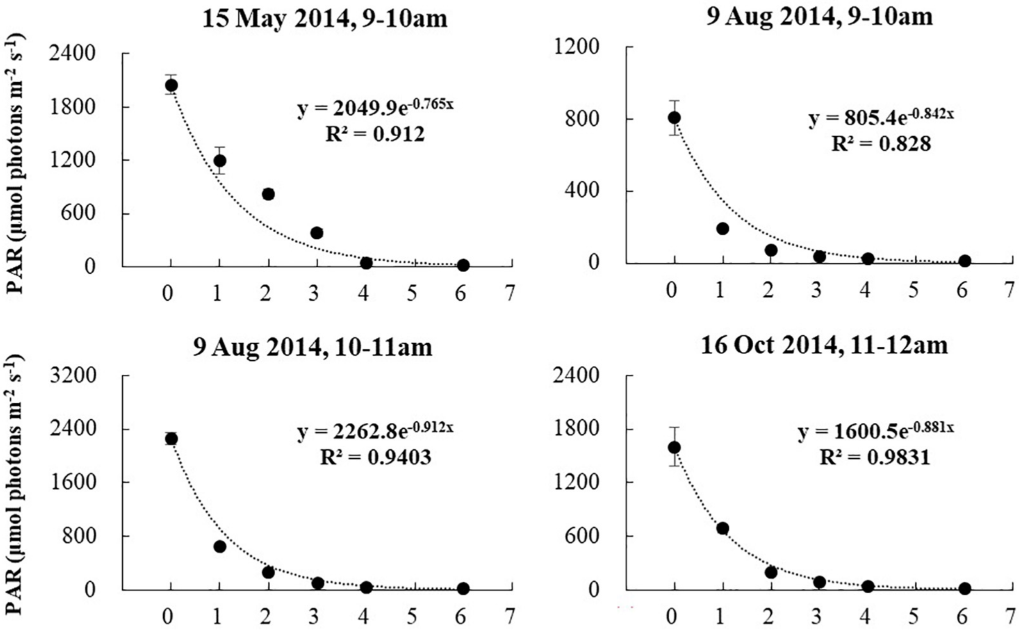

FIGURE 10 | Variation in light availability with water depth in Ailian Bay at various dates and times during 2014. The fitted line is an exponent curve representing the fit of Beer's law (equation 2) to the data.

growth of *Z. marina*, since there was little spatial variation vertically in other measured water parameters. In the present study, although *Z. marina* at 4 m depth persisted during the experimentation period, the shoot number, which was significantly lower than at shallow depths ($\leq$3 m), appeared to show a downtrend. *Z. marina* at 4 m depth might disappear over a longer time scale with 1.75–2.88% of surface irradiance, since these values are lower than the minimum light requirement of 4–36% of surface irradiance for seagrass reported previously (Ralph et al., 2007). With 4.09–18.75% of surface irradiance, within the minimum light requirement of 4–36% (Ralph et al., 2007), the shoot number at 3 m depth presents seasonal variation with asexual reproduction in autumn. Therefore, *Z. marina* may be able to survival and recolonize at an optimal depth of $\leq$3 m in Ailian Bay over a longer time scale.

Sustained elevated temperatures can cause plant mortality (Hammer et al., 2018). Several studies have reported negative temperature effects on eelgrass morphology and survival over a range of 25–30°C (Orth and Moore, 1986; Touchette et al., 2003; Moore and Jarvis, 2008; Moore et al., 2014). The highest surface water temperature in Ailian Bay from August 2010 to September 2011 was 22.4°C, recorded by Liu (2012), indicating that eelgrass are not generally exposed to stressful temperatures in this area. Moreover, temperature has a small effect on eelgrass lateral branching (Eriander, 2017). For salinity, pH, and chlorophyll, the vertical variations in Ailian Bay were minimal, and thus likely have limited effects on seagrass productivity variation with depth (Lirman and Cropper, 2003). The DO concentrations at all depths were relatively high, except in June 2015 when phytoplankton abundance in the water column was high, leading to deeper areas experiencing anoxia (1–3 mg L^{-1}) (Moore and Jarvis, 2008). Regarding nutrient concentrations, there are no significant differences between the surface water and bottom water (Li et al., 2018).

Correlating *Z. marina* Depth Limit to Minimum Light Requirements

As shown in **Figure 9**, all four of the light vs. depth distributions adhered well to Beer's Law ($R^2 \geq 0.82$), indicating that the variation in turbidity with water depth was not substantial. Since long-term measurements of subsurface photosynthetically active radiation were not recorded for the duration of the experiment, it is difficult to convert the estimated depth threshold of 3 m to a similarly precise light threshold for *Z. marina*. However, combining all estimated values of K_d* shown in **Figure 9**, the light experienced at 3 m depth was between 6.48 and 10.08% of the surface irradiance. These values agree well with the minimum light requirement of 4–36% of surface irradiance for seagrass reported previously (Ralph et al., 2007).

Shoot Density as an Indicator of Seagrass Response to Light Reduction

In situ reductions of light are known to affect seagrasses in a variety of ways. Several studies have shown stunted growth, reduced biomass, and lower densities in response to reduced light (Abal et al., 1994; Collier et al., 2009, 2016). Shoot density is determined by lateral shoot production (Olesen and Sand-Jensen, 1994a), thus branching frequency is also reduced at low light availability. The production of new lateral branching shoots is essential for restoration success, and a high lateral branching frequency would aid in the establishment and expansion of transplants, thereby reducing the risk of failure due to stochastic events (Olesen and Sand-Jensen, 1994b). Furthermore, the present experiment conducted in autumn 2011 demonstrated shoot loss of 60–82.5% at $\leq$4 m depth over the winter of 2011, increasing the risk of complete transplant mortality over winter, and indicating that high lateral shoot production in autumn of 2012 may be essential for reducing the risk. Therefore, it would be more useful, for restoration purposes, to determine the minimum light requirement for high lateral branching rather than for general growth and maintenance (Eriander, 2017).

Implications for Restoration

Depth is a critical determinant of seagrass restoration success (Aoki et al., 2020). These results suggest that the greatest eelgrass survival may occur in areas $\leq$3 m depth, which are therefore the most suitable sites for restoration in Ailian Bay. Moreover, the current transplantation experiments have demonstrated greater survival and lateral branching frequency of shoots when restoration occurs in the spring (2014) than in the autumn (2011). Considering the similar conclusions reported by previous researchers, this indicates that restoration trials occurring in the spring and early summer will result in the greatest survival of shoots (Vichkovitten et al., 2007). This is because transplants have sufficient time to acclimatize, store carbohydrates, grow, and undergo lateral shoot branching during the first season (Eriander, 2017). In addition, the present findings suggest that the technique of suspending cultures of eelgrass, can be used to improve water quality, through regulating nutrient cycles, and attracting fish through the provision of food (e.g., zooplankton) and habitats.

CONCLUSION

This study presented a novel method (*in situ* suspended cultures) to directly examine long-term responses of transplanted *Z. marina* shoots to a depth gradient, in order to determine the depth limit (light requirement) for eelgrass restoration. The findings indicate that adult *Z. marina* transplants originating from a nearby matching donor site (Swan Lake) can successfully acclimatize and be used for restoration within degraded areas (Ailian Bay) requiring eelgrass restoration. These results provide a suitable depth range for successful restoration of eelgrass in Ailian Bay, north China. More broadly, this work may provide useful knowledge for the global management and restoration of seagrass.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

SX: writing – original draft, conceptualization, methodology, investigation, and validation. PW, FW, PL, and BL: investigation, formal analysis, and writing – review and editing. XZ: data curation, formal analysis, investigation, and writing – review and editing. SY and YuZ: investigation and data curation. YiZ: funding acquisition, supervision, methodology, investigation, and writing – review and editing. All authors contributed to the article and approved the submitted version.

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Can the Non-native Salt Marsh Halophyte *Spartina alterniflora* Threaten Native Seagrass (*Zostera japonica*) Habitats? A Case Study in the Yellow River Delta, China

Shidong Yue[1,2,3,4,5,6], Yi Zhou[1,2,3,4,5,6*], Shaochun Xu[1,2,3,4,5,6], Xiaomei Zhang[1,2,3,4,5,6], Mingjie Liu[1,2,3,4,5,6], Yongliang Qiao[1,2,3,4,6,7], Ruiting Gu[1,2,3,4,5,6], Shuai Xu[1,2,3,4,5,6] and Yu Zhang[1,2,3,4,5,6]

[1] CAS Key Laboratory of Marine Ecology and Environmental Sciences, Institute of Oceanology, Chinese Academy of Sciences, Qingdao, China, [2] Laboratory for Marine Ecology and Environmental Science, Qingdao National Laboratory for Marine Science and Technology, Qingdao, China, [3] Center for Ocean Mega-Science, Chinese Academy of Sciences, Qingdao, China, [4] CAS Engineering Laboratory for Marine Ranching, Institute of Oceanology, Chinese Academy of Sciences, Qingdao, China, [5] University of Chinese Academy of Sciences, Beijing, China, [6] Shandong Province Key Laboratory of Experimental Marine Biology, Qingdao, China, [7] Qingdao University of Science and Technology, Qingdao, China

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Edited by:
Eric Marechal,
UMR 5168 Laboratoire de Physiologie
Cellulaire Vegetale (LPCV), France

Reviewed by:
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Leibniz Centre for Tropical Marine
Research (LG), Germany
Lukas Dahlin,
Biosciences Center, National
Renewable Energy Laboratory,
United States

***Correspondence:**
Yi Zhou
yizhou@qdio.ac.cn

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Seagrass meadows are critical ecosystems, and they are among the most threatened habitats on the planet. As an anthropogenic biotic invader, *Spartina alterniflora* Loisel. competes with native plants, threatens native ecosystems and coastal aquaculture, and may cause local biodiversity to decline. The distribution area of the exotic species *S. alterniflora* in the Yellow River Delta had been expanding to ca.4,000 ha from 1990 to 2018. In this study, we reported, for the first time, the competitive effects of the exotic plant (*S. alterniflora*) on seagrass (*Zostera japonica* Asch. & Graebn.) by field investigation and a transplant experiment in the Yellow River Delta. Within the first 3 months of the field experiment, *S. alterniflora* had pushed forward 14 m into the *Z. japonica* distribution region. In the study region, the area of *S. alterniflora* in 2019 increased by 516 times compared with its initial area in 2015. Inhibition of *Z. japonica* growth increased with the invasion of *S. alterniflora*. *Z. japonica* had been degrading significantly under the pressure of *S. alterniflora* invasion. *S. alterniflora* propagates sexually via seeds for long distance invasion and asexually by tillers and rhizomes for short distance invasion. Our results describe the invasion pattern of *S. alterniflora* and can be used to develop strategies for prevention and control of *S. alterniflora* invasion.

Keywords: biological invasion, anthropogenic introduction, *Spartina alterniflora*, seagrass, *Zostera japonica*, intertidal zone

INTRODUCTION

Biological invasion is a significant component of human-caused global environmental change. Biotic invaders can establish a new range in which they proliferate, spread, and persist to damage the environment (Mack et al., 2000). Animal invaders can cause extinctions of vulnerable native species. For example, the Nile perch (*Lates niloticus*) has contributed to the extinction of more than 200 endemic fish species through predation and competition for food (Lowe et al., 2000). Plant invaders can completely alter the energy budgets and nutrient cycling in a native ecosystem and can greatly diminish the diversity of native species. For example, the water hyacinth (*Eichhornia crassipes*) dramatically reduces biological diversity in aquatic ecosystems through shading and crowding of native aquatic plants (Lowe et al., 2000).

Spartina alterniflora is a perennial halophyte that is native to the Atlantic coast of the Americas from Canada to Argentina (Meng et al., 2020). *S. alterniflora* has high tolerance and adaptability to environmental stressors due to fast growth, well-developed below-ground tissues, high salt tolerance, and high reproductive capacity through both asexual and sexual reproduction, making it a good "ecosystem engineer" (Simenstad and Thom, 1995; Qin et al., 1998; Crooks, 2002; Chung et al., 2004; Zhang et al., 2004; Chung, 2006; Huang and Zhang, 2007). Due to these biological traits, *S. alterniflora* has been introduced throughout Europe, North America, Australia, and Asia in efforts to prevent shoreline erosion (Maricle and Lee, 2002). In December of 1979, *S. alterniflora* was introduced into China (Chung, 1993). In 1985, *S. alterniflora* in China covered ∼260 ha (Meng et al., 2020). In 2003, *S. alterniflora* was listed as an invasive species by the State Environment Protection Administration of China. Satellite images showed that the area covered by *S. alterniflora* had reached 34,451 ha in 2007 (Zuo et al., 2012). Today, *S. alterniflora* has expanded to around 50,000 ha of the coastal regions of China (Meng et al., 2020).

As a biotic invader, *S. alterniflora* competes with native plants, threatens native ecosystems and coastal aquaculture, and may cause local biodiversity to decline (Callaway and Josselyn, 1992; Daehler and Strong, 1996; Brusati and Grosholz, 2007; Zuo et al., 2012). The introduction of *S. alterniflora* resulted in a significant decrease of abundance, coverage, and seed and fresh corm output of *Scirpus mariqueter* which is a native species of sedge in Dongtan of Chongming Island (Chen et al., 2004; Wang et al., 2012). Xia et al. (2015) reported that *S. alterniflora* actively accumulated and stored sulfur in its tissues, leading to a high sulfide level in the invaded environment, which was harmful to some native species. Populations of native plants such as *Suaeda glauca* and *Phragmites communis* have decreased rapidly in Yancheng National Nature Reserve due to expansion of alien *S. alterniflora*, and the habitat changes are believed to be at least partially responsible for dramatic declines of wintering red-crowned crane populations (Liu C. et al., 2013). Ge et al. (2012) reported that the biodiversity of macrobenthic communities increased in the initial stage of *S. alterniflora* invasion and then decreased in the middle and final stage of invasion in Wenzhou Bay, China.

Seagrasses provide habitat, food, and nurseries for a variety of marine organisms, attenuate currents and waves, alter nutrient cycling and food web structures, and stabilize sediments (Costanza et al., 1997; Jackson et al., 2001; Barbier et al., 2011; Liu X.J. et al., 2013; Nordlund et al., 2018; Unsworth et al., 2019). Seagrass meadows are critical ecosystems, and they are among the most threatened habitats on the planet. Waycott et al. (2009) reported that 29% of the known areal extent of seagrass meadows has disappeared since seagrass areas were initially recorded in 1879. Human disturbances such as eutrophication and habitat loss due to dredging, anchoring, and coastal construction play a key role in the loss of seagrasses (Orth et al., 2006; Ralph et al., 2006; Salinas et al., 2020).

After *S. alterniflora* was first introduced to the Yellow River Delta in 1990, it grew and spread rapidly. By 2018, its distribution region had expanded to 4005.89 ha, which was 2557 times as its initial colonization area (Ren et al., 2019). Zhang X.M. et al. (2019) reported that the seagrass *Zostera japonica* Asch. & Graebn. on both sides of the mouth of the Yellow River covered about 1031.8 ha. *Z. japonica* is an intertidal seagrass species that is native to the Western Pacific Ocean from Russia to Vietnam (Miki, 1933). This species is widely threatened by climate change and human activity in its native range in Korea, Japan, and China (Lee et al., 2004; Abe et al., 2009; Hodoki et al., 2013; Zhang et al., 2014, 2015, 2020a,b; Lin et al., 2018; Yue et al., 2020). The niche of *S. alterniflora* overlaps with that of *Z. japonica* on low tidal beaches. However, competition between *S. alterniflora* and *Z. japonica* has rarely been studied experimentally in the field.

In May 2015, we found an initial invasion of *S. alterniflora* (ca.100 m^2) at *Z. japonica* meadows in our study region, and *S. alterniflora* had been occurring in small patches surrounded by *Z. japonica* meadows. In this study, we investigated the competitive effects of *S. alterniflora* on *Z. japonica* in the study region to assess the possible consequences of the introduced *S. alterniflora* on the native *Z. japonica* communities in the Yellow River Delta. We document the invasion pattern of *S. alterniflora* by *in situ* investigation and our work can be used to develop strategies for prevention and control of *S. alterniflora* invasion.

MATERIALS AND METHODS

Study Sites

The study region (37° 51′ 7″ N, 119° 5′ 47″ E) is located in the Yellow River Delta, Shandong Province, China (**Figure 1**). The climate is temperate monsoonal, with an average annual precipitation of 560 mm and average annual temperature of 12.9°C (Han et al., 2018). The tides are irregular semidiurnal, with an average tidal amplitude of 1.1–1.5 m (Hu and Cao, 2003). The broad mudflat of the Yellow River Delta displays distinct vegetation zones, producing a unique wetland landscape extending from sea to land. *Z. japonica* meadows occur in the sea, with *S. alterniflora*, *Suaeda salsa*, *Tamarix chinensis*, and reed marsh occurring in the landward direction (Zhang X.M. et al., 2019). Based on the vegetation differences, the study region can be divided into the *S. alterniflora* distribution region, the ecotone (*S. alterniflora* and *Z. japonica*) and the *Z. japonica* distribution region.

Distribution of *Spartina alterniflora* and *Zostera japonica* in the Study Region

In May 2015, we used mobile phone to record the distribution of *S. alterniflora* in the study region. In October 2016 and July 2019, the total distribution region of *S. alterniflora* was photographed by aerial vehicle (DJI Phantom 3 Advanced) during low tide.

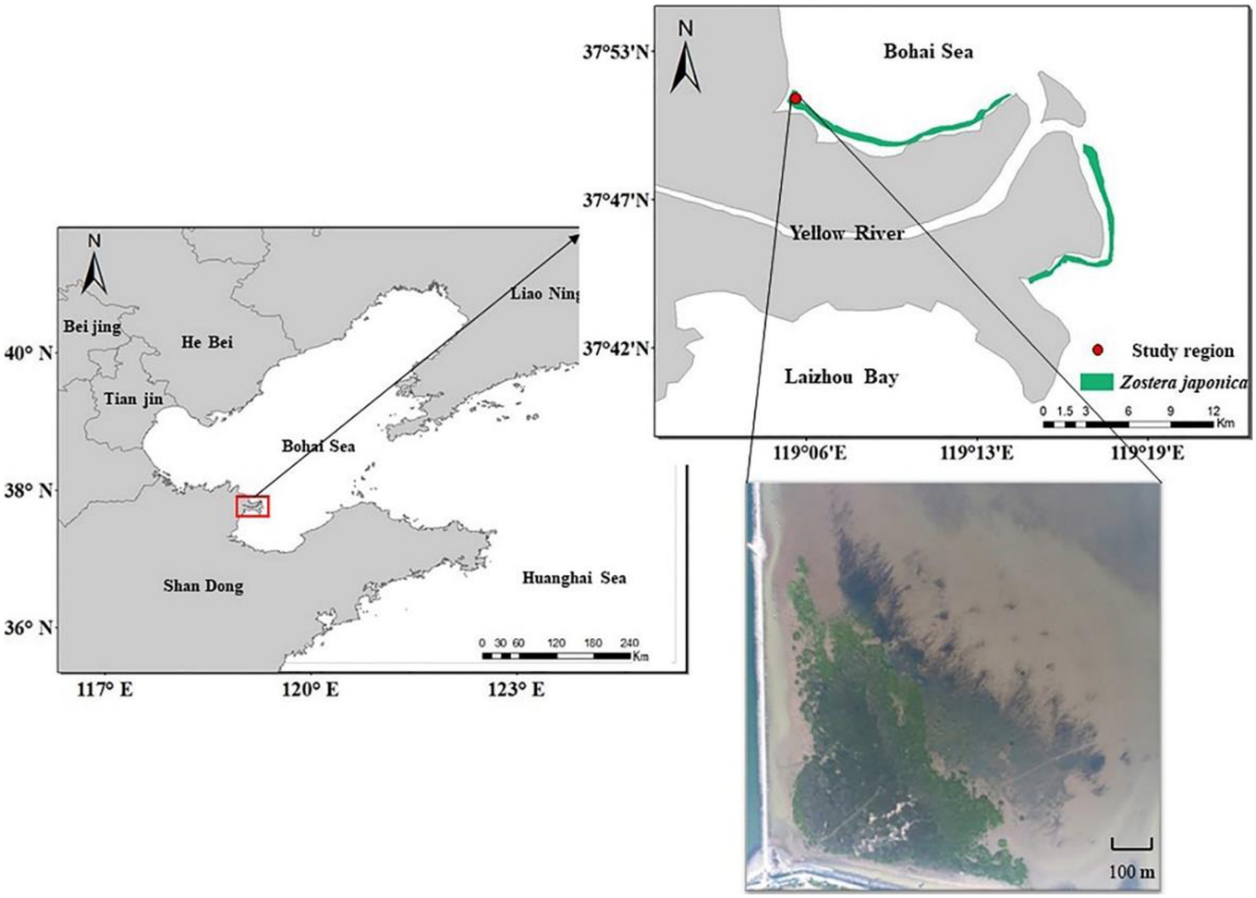

FIGURE 1 | Study region in the Yellow River Delta.

Then we estimated the distribution area of *S. alterniflora* by using road as a reference.

In July 2019, during peak biomass, the range of *Z. japonica* meadows was examined by walking during low tide or by rowing during mid to high tide, using GPS to record accessible boundary points.

To estimate the sediment accretion due to the invasion of *S. alterniflora* in the study region, we recorded the corresponding tide level from the same place (37° 51′ 6″ N, 119° 5′ 53″ E) exposed to the sea surface in May 2015 and May 2019. The tidal regime for the study region was provided by the National Marine Data Information Center of China.

Environmental Parameters

Four sediment cores (diameter = 10.5 cm, depth = 12 cm) were collected from each region to measure grain size distribution using laser diffraction analysis (Short and Coles, 2001). These sediment cores were also used to determine the concentration of sediment organic matter (OM, in %DW), which was calculated as the fractional weight loss of dry sediment in the samples after combustion at 550°C for 4 h (Heiri et al., 2001). To compare the sulfide content in sediment in the *S. alterniflora* and the *Z. japonica* distribution regions, four sediments cores (diameter = 10.5 cm, depth = 12 cm) were collected from the two regions and measured in the laboratory by using the iodometric method (National marine environmental monitoring center [NMEMC], 2007).

Field Investigation

Three parallel transect lines running perpendicular to the shoreline were marked for sampling in the study region. All transects began in the *S. alterniflora* distribution region and ended in the *Z. japonica* distribution region. Eleven sampling points were marked at an interval of 7 m along each transect line. Sampling was conducted in April, July, and October, 2019. One sediment core (diameter = 10.5 cm, depth = 12 cm) was haphazardly collected within 1 m of each sampling point along each transect.

All samples were sieved (2 mm) with seawater *in situ* to remove most of the sediment and then cleaned using tap water in the laboratory. For each sample, total number of shoots was counted to provide shoot density (shoots $\cdot$ m^{-2}). The height (cm) of 15 shoots was measured. All shoots were dried to a constant weight at 60°C to estimate total shoot biomass (g DW $\cdot$ m^{-2}). We also counted the density (shoots $\cdot$ m^{-2}), height (cm), and biomass (g WW $\cdot$ m^{-2}) of seedlings, which were only observed in April,

2019. All of these biological parameters were measured separately for *S. alterniflora* and *Z. japonica*.

Transplant Experiment

We designated three transplant sites (S1, S2, and S3) for the experiment in which we transplanted *Z. japonica*. S1 was located in the *S. alterniflora* distribution region, which represented the completed stage of invasion. S2 was located at the edge of the *S. alterniflora* distribution region, which represented the intermediate stage of invasion. S3 was located in the ecotone, which represented the initial stage of invasion.

All transplant experiments began in July, 2019. For each transplant site, four transplant plots (1 m × 1 m) were designed parallelly to the coast with an interval of 2 m. We assumed that *Z. japonica* might not grow at S1, thus we added another three transplant plots (1 m × 1 m) at S1 to ensure adequate monitoring data. In each plot, all *S. alterniflora* were removed using a sickle and hoe. Sod consisting of *Z. japonic*a and sediment was collected from the center of the *Z. japonica* distribution region using tools for pushing the plant material horizontally below the rhizome layer. Then, these sods were transplanted into the pre-dug plots from which *S. alterniflora* were removed.

After transplantation, seven sediment cores (diameter = 10.5 cm, depth = 12 cm) were collected semimonthly at S1, and four sediment cores (diameter = 10.5 cm, depth = 12 cm) were collected semimonthly at S2 and S3. All cores were sieved (2 mm) with seawater *in situ* to remove most of the sediment, and the plant materials then were taken back to the laboratory. For each sample, plant material was washed using tap water and divided into above-ground (shoots: sheath and leaves) and below-ground (rhizomes and roots) parts. The shoot height (cm) was measured and the numbers of vegetative and flowering shoots were counted to provide shoot density (shoots · m^{-2}). Total tissues were dried to a constant weight at 60°C to estimate total above-ground and below-ground biomass (g DW · m^{-2}).

Statistical Analysis

For the field investigation data, we used a one-way analysis of variance (ANOVA) to test the significance of differences in the total shoot density, biomass of *S. alterniflora*, and *Z. japonica* among time and sampling points and to test for significance of differences in seedling density and biomass among the 11 sampling points along the transect line. The significance of differences in grain sizes and OM among three regions was also tested by using a one-way ANOVA. An independent-samples *t*-test was used to test the significance of differences in the sulfide content in sediment among *S. alterniflora* and *Z. japonica* distribution regions. For the transplant experiment data, we used one-way ANOVA or independent-samples *t*-test to test the significance of differences in the reproductive shoot density, vegetative shoot density, total shoot density, below-ground biomass, above-ground biomass, total biomass, vegetative shoot height, and reproductive shoot height among time points and transplant sites. When the data did not satisfy the homogeneity of variance requirement, a Kruskal-Wallis Test was used to test the significance of differences. Multiple comparisons were performed using the Duncan method, and the level of significance was set at $p < 0.05$. Statistical analyses were conducted using SPSS 17.0. All values are reported as mean ± SD.

RESULTS

Distribution of *Spartina alterniflora* and *Zostera japonica* in the Study Region

In 2015, the area of *S. alterniflora* was ca.100 m^2. In 2016, it had extended over ca. 1.25 ha. By 2019, the distribution region had expanded to ca. 5.16 ha (**Figures 2A,B**; **Supplementary Figure 1**).

The distance between the shore at the study region to the lower limits of *Z. japonica* meadows was 537 m, while the distance between the upper and lower limits of *Z. japonica* meadows was only 165 m in July 2019.

The place was exposed to the sea surface at the tidal water level of 50 cm in May 2015, while it was exposed to the sea surface at the tidal water level of 80 cm in May 2019. It shows that approximately 30 cm sediment was accumulated owing to the invasion of *S. alterniflora* in the study region (**Figure 2C**).

Environmental Parameters

The sediment particulate size composition in all the regions was mainly sand (**Figure 3**), while the percentage of sand at *S. alterniflora* distribution region was greater than at *Z. japonica* distribution region [$F_{(2,9)} = 8.887$, $p < 0.05$]. There was no significant difference in OM among region [**Table 1**; $F_{(2,9)} = 0.646$, $p > 0.05$]. The sulfide content of sediment in *S. alterniflora* distribution region (237.50 ± 88.88 mg · kg^{-1}) was greater than in *Z. japonica* distribution region (156.25 ± 58.55 mg · kg^{-1}).

Competition Between *Spartina alterniflora* and *Zostera japonica*

There were significant differences among sampling points in total shoot density, biomass of *S. alterniflora*, and *Z. japonica* in each sampling time ($p < 0.05$). The total shoot density of *S. alterniflora* and *Z. japonica* decreased and increased with moving seaward, respectively (**Figure 4**). Biomass showed the same trend as shoot density (**Figure 5**). In April, the biomass of *S. alterniflora* at points 5 and 8 was higher than at the adjacent points (**Figure 5A**). In July and October, the biomass of *S. alterniflora* at point 8 was still higher than that of the adjacent points (**Figures 5B,C**).

At point 1, the total shoot density of *S. alterniflora* in April (4,852.90 ± 808.82 shoots · m^{-2}) was higher than that in October (2,503.48 ± 657.02 shoots · m^{-2}) and July (1771.69 ± 546.05 shoots · m^{-2}) [$F_{(2,6)} = 16.852$, $p < 0.05$]. At point 3, the biomass of *S. alterniflora* in April (1,153.14 ± 1,280.93 g DW · m^{-2}) was lower than that in July (2,612.86 ± 903.89 g DW · m^{-2}) and October (3,799.51 ± 1,176.13 g DW · m^{-2}) [$F_{(2,6)} = 13.330$, $p < 0.05$]. At points 4 and 5, the total

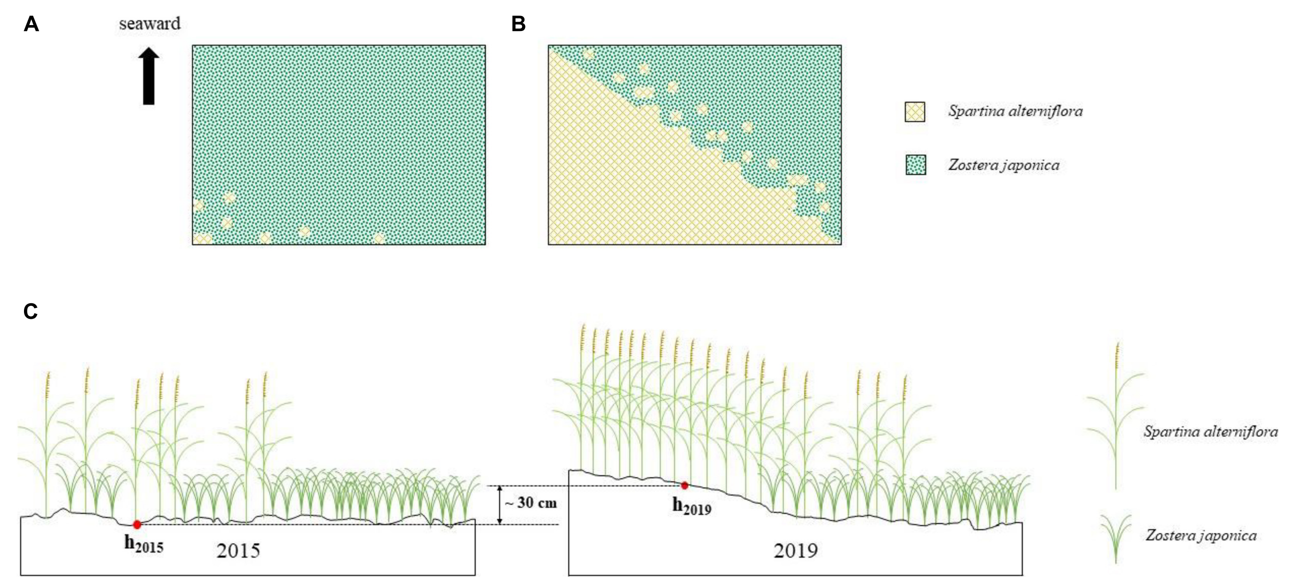

FIGURE 2 | *Spartina alterniflora* invasion of *Zostera japonica* meadows [**(A)** 2015; **(B)** 2019] and the sediment accretion in the study region **(C)**.

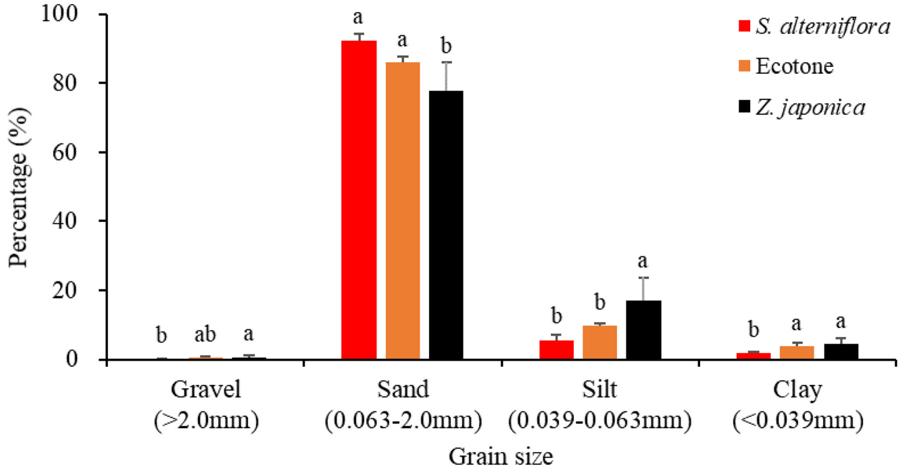

FIGURE 3 | Sediment grain sizes at the three regions. Values are mean ± SD. Different letters indicate significant difference between different regions at same size of grain.

shoot density of *Z. japonica* decreased with sampling time [$F_{(2,6)} = 9.188$, $p < 0.05$; $F_{(2,6)} = 9.046$, $p < 0.05$, respectively]. At point 9, the total shoot density of *Z. japonica* was highest in July (3,851.51 ± 1110.28 shoots · m^{-2}), followed by April (1848.72 ± 115.55 shoots · m^{-2}) and October (1,502.09 ± 200.13 shoots · m^{-2}) [$F_{(2,6)} = 11.256$, $p < 0.05$]. At points 7, 9, 10, and 11, the biomass of *Z. japonica* was highest in July, followed by April and October ($p < 0.05$).

In April, *Z. japonica* was observed from point 3 to point 11 and *S. alterniflora* was observed from point 1 to point 8 (**Figure 6A**). In July and October, *Z. japonica* was no longer observed at points 3 and 4, and *S. alterniflora* was observed at points 9 and 10 (**Figures 6B,C**). *S. alterniflora* shoots collected at sampling points 9 and 10 in July 2019 had small shoot height (<20 cm), biomass (<10 g WW · m^{-2}), and density (<50 shoots · m^{-2}); these shoots could be easily uprooted and were obviously grown from dormant seeds that germinated in May 2019. This finding shows that *S. alterniflora* had pushed forward 14 m into the *Z. japonica* distribution region.

In April, seedlings of *S. alterniflora* and *Z. japonica* were found in the samples. The seedling density and biomass of *S. alterniflora* differed significantly among sampling points [$F_{(10,22)} = 5.809$, $p < 0.05$; $F_{(10,22)} = 8.459$, $p < 0.05$, respectively]. The seedling biomass of *S. alterniflora* and *Z. japonica* decreased and increased with moving seaward, respectively (**Figure 7A**). Seedling density showed the same as biomass (**Figure 7B**). At point 8, the seedling density (115.55 ± 200.13 shoots · m^{-2}) and biomass (2.70 ± 4.67 g WW · m^{-2}) of *S. alterniflora* were higher than at adjacent points (**Figure 7**). However, seedling density and biomass of *Z. japonica* did not differ significantly among sampling points [$F_{(10,22)} = 0.741$, $p > 0.05$; $F_{(10,22)} = 0.883$, $p > 0.05$, respectively].

Effects of Different *Spartina alterniflora* Invasion Stage on *Zostera japonica*

Fifteen days after transplantation, the reproductive shoot density, vegetative shoot density, and total shoot density of *Z. japonica* at S1 were lower than those at S2 and S3 [**Figure 8**; Chi-square$_{(2)}$ = 11.343, $p < 0.05$; Chi-square$_{(2)}$ = 11.809, $p < 0.05$; $F_{(2,12)} = 36.916$, $p < 0.05$, respectively]. The aboveground biomass, total biomass, vegetative shoot height, and reproductive shoot height at S1 were lower than those at S2 and S3 [**Figures 8**, **10**; $F_{(2,12)} = 20.697$, $p < 0.05$; $F_{(2,12)} = 6.973$, $p < 0.05$; $F_{(2,12)} = 8.073$, $p < 0.05$; Chi-square$_{(2)}$ = 8.480, $p < 0.05$, respectively]. One month after transplantation, all *Z. japonica* at S1 had died. The total shoot density and biomass at S2 were lower than those at S3 [**Figures 7**, **8**; Chi-square$_{(2)}$ = 7.659, $p < 0.05$; Chi-square$_{(2)}$ = 8.199, $p < 0.05$, respectively].

No significant differences among time points for total shoot density and biomass at S3 were detected [**Figures 8**, **9**; $t_{(6)} = 2.289$, $p > 0.05$; $t_{(6)} = 0.586$, $p > 0.05$, respectively]. The vegetative shoot height and reproductive shoot height at S3 decreased over time (**Figure 10**).

DISCUSSION

In this study, we reported, for the first time, the competitive effects of the exotic plant (*S. alterniflora*) on seagrass (*Z. japonica*) by field investigation and a transplant experiment in the Yellow River Delta. Our results indicated that the seagrass meadow had been degrading significantly under the pressure of *S. alterniflora* invasion. Our results document the invasion pattern of *S. alterniflora* and can be used to develop strategies for prevention and control of *S. alterniflora* invasion.

Distribution of *Spartina alterniflora* and *Zostera japonica* in the Study Region

By 2019, *S. alterniflora* covered ca. 5.16 ha, which was 516 times greater than its initial area in 2015. In Jiangsu Province, the total planting area of *S. alterniflora* was 278.93 ha along the north coast of Sheyang in 1982–1992, but it rapidly spread and increased to 1160.69 ha by July 1, 1994 (Zuo et al., 2012). Ren et al. (2019) used remote sensing technology to monitor distributional changes of *S. alterniflora* in the Yellow River Delta. They reported that

TABLE 1 | The concentration of sediment organic matter and sulfide content at the three regions.

Region	OM (%)	Sulfide (mg · kg^{-1})
Spartina alterniflora	2.93 ± 0.30	237.50 ± 88.88
Ecotone	2.72 ± 0.38	–
Zostera japonica	2.75 ± 0.08	156.25 ± 58.55

Values are mean ± SD.

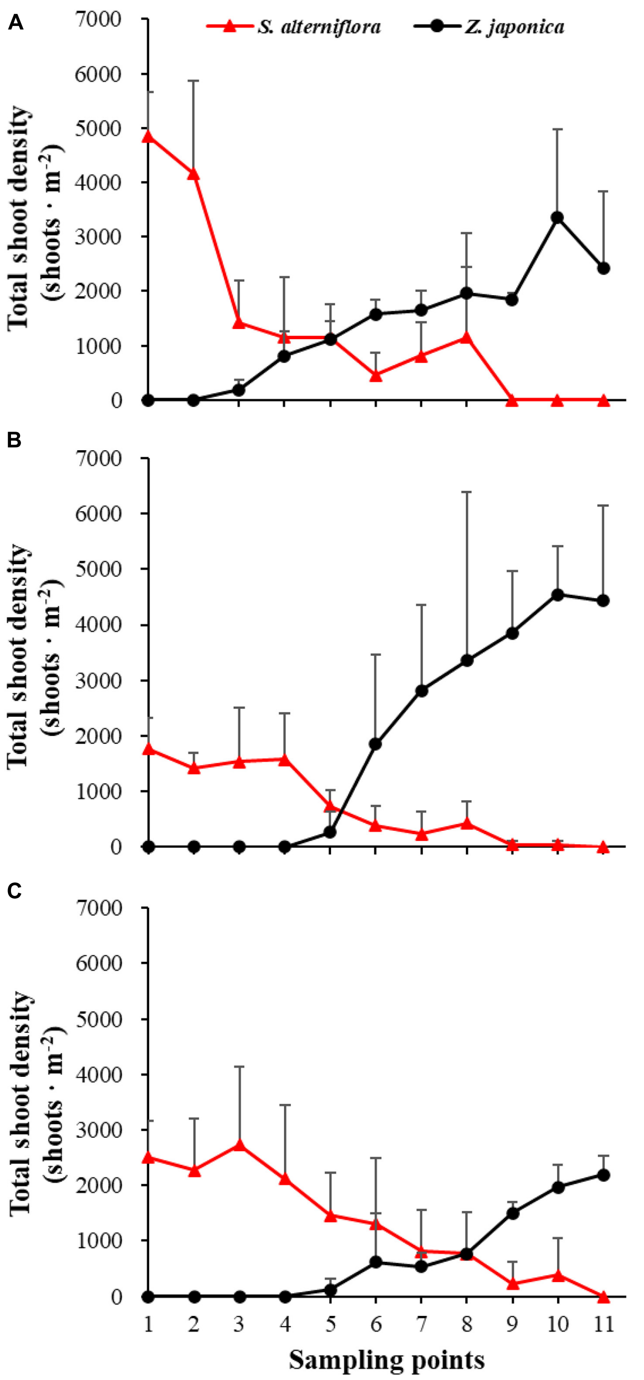

FIGURE 4 | Total shoot density of *Spartina alterniflora* and *Zostera japonica* at the 11 sampling points in April **(A)**, July **(B)**, and October **(C)** 2019. All *S. alterniflora* shoots collected at sampling points 9 and 10 in July 2019 were grown from dormant seeds that germinated in May 2019. Values are mean ± SD.

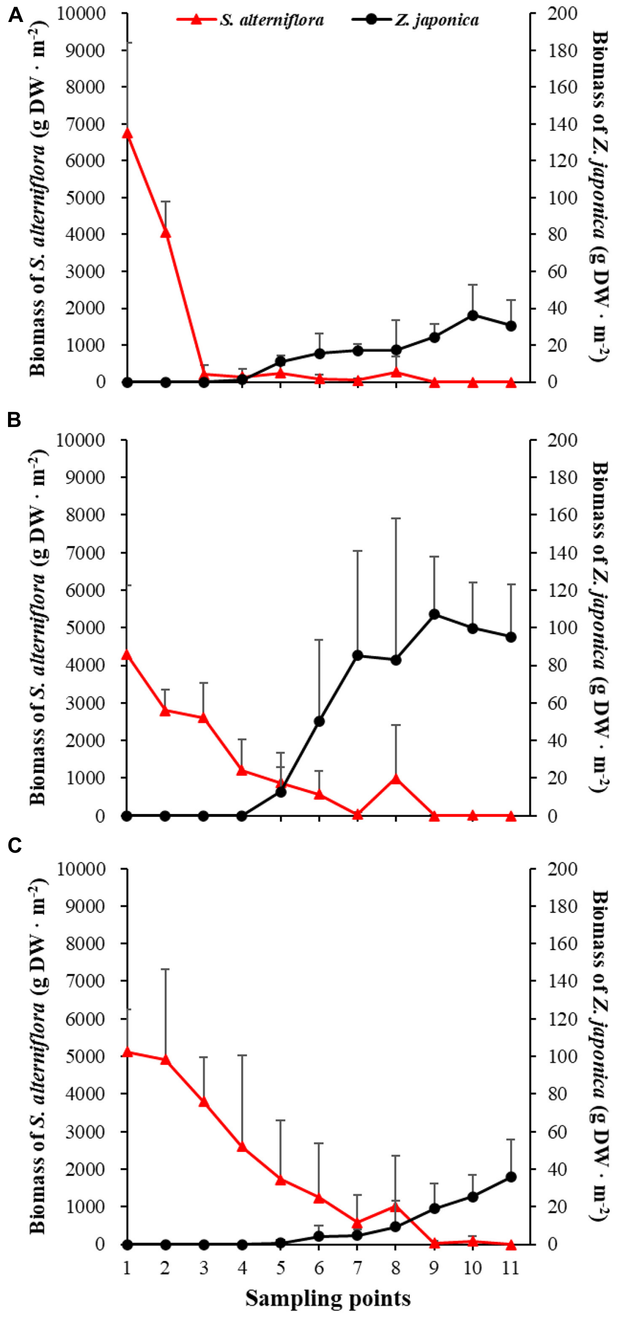

FIGURE 5 | Biomass of *Spartina alterniflora* and *Zostera japonica* at the 11 sampling points in April **(A)**, July **(B)**, and October **(C)** 2019. Values are mean ± SD.

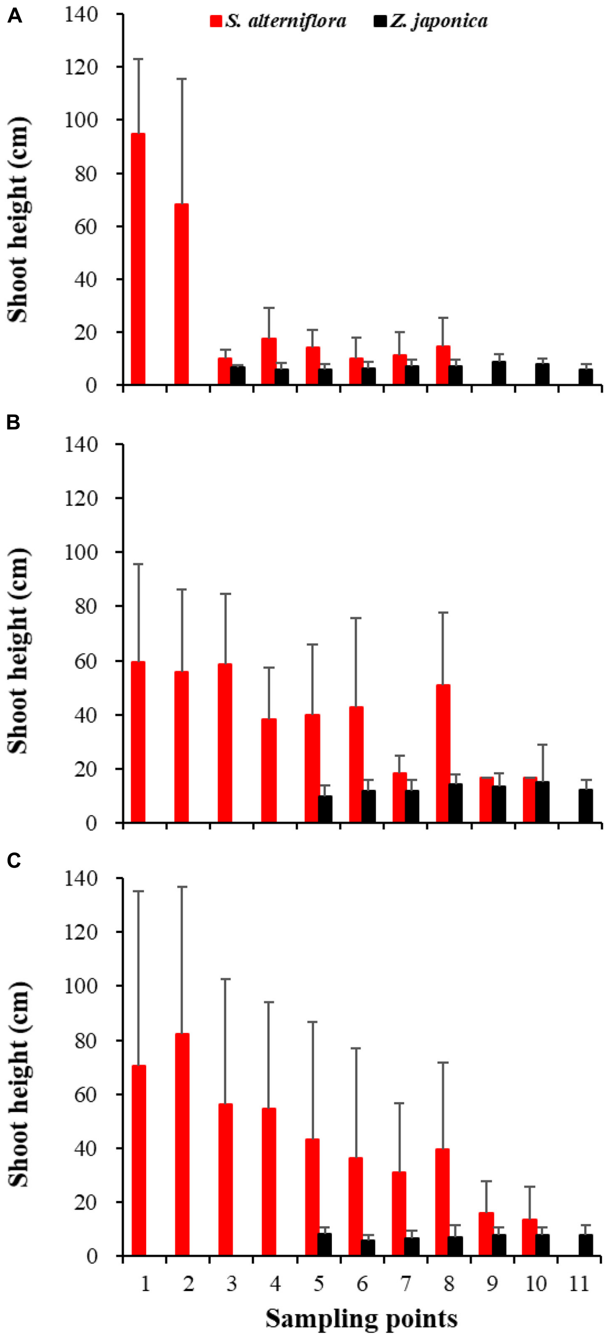

FIGURE 6 | Shoot height of *Spartina alterniflora* and *Zostera japonica* at the 11 sampling points in April **(A)**, July **(B)**, and October **(C)** 2019. Values are mean ± SD.

S. alterniflora was first identified in Landsat 5 images in 2008 on the north bank with an initial area of 0.72 ha, whereas it was first identified on the south bank in 2011 with an initial area of 1.61 ha. Since 2011, *S. alterniflora* has rapidly colonized the modern Yellow River Delta. Chen et al. (2020) reported that Fast Super-Resolution Convolutional Neural Networks (FSRCNN) were very effective at discerning and estimating the size of small *S. alterniflora* patches (<50 m^2). They found that the total patch area of *S. alterniflora* at one site increased 13 times from 11.54 ha to 157.42 ha since 2012.

The distance between the upper and lower limits of *Z. japonica* meadows was greater than 550 m in 2015 (Zhang X.M. et al., 2019), while the distance we observed was only 165 m in July 2019. The result means *Z. japonica* was seriously

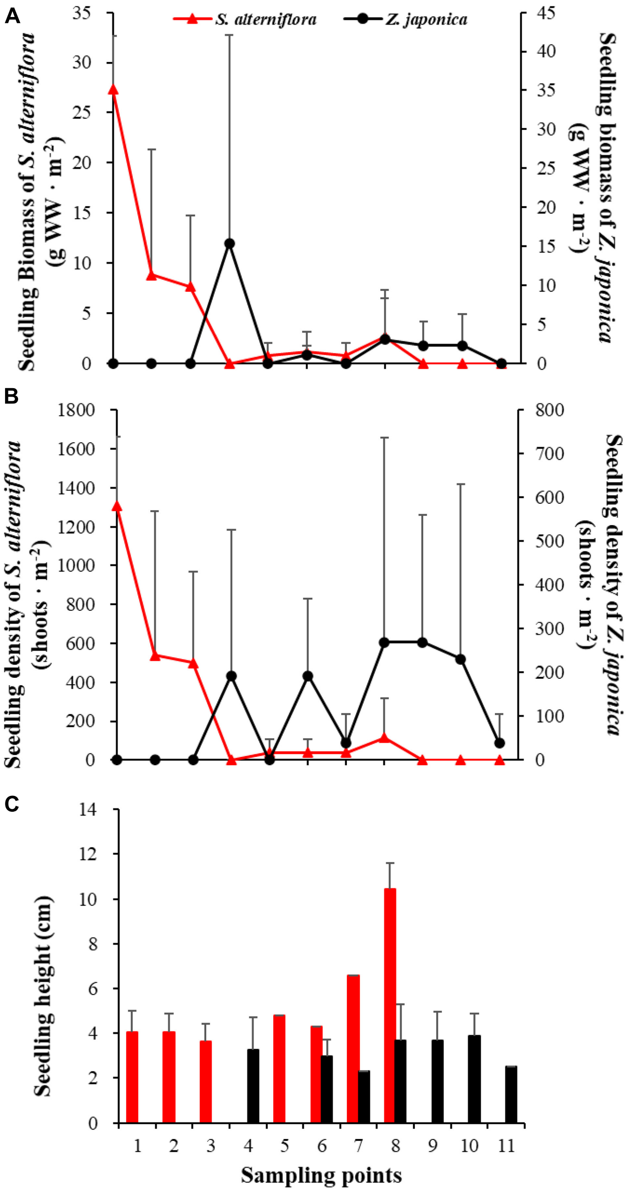

FIGURE 7 | Seedling biomass **(A)**, density **(B)**, and height **(C)** of *Spartina alterniflora* and *Zostera japonica* at the 11 sampling points in April 2019. Values are mean ± SD.

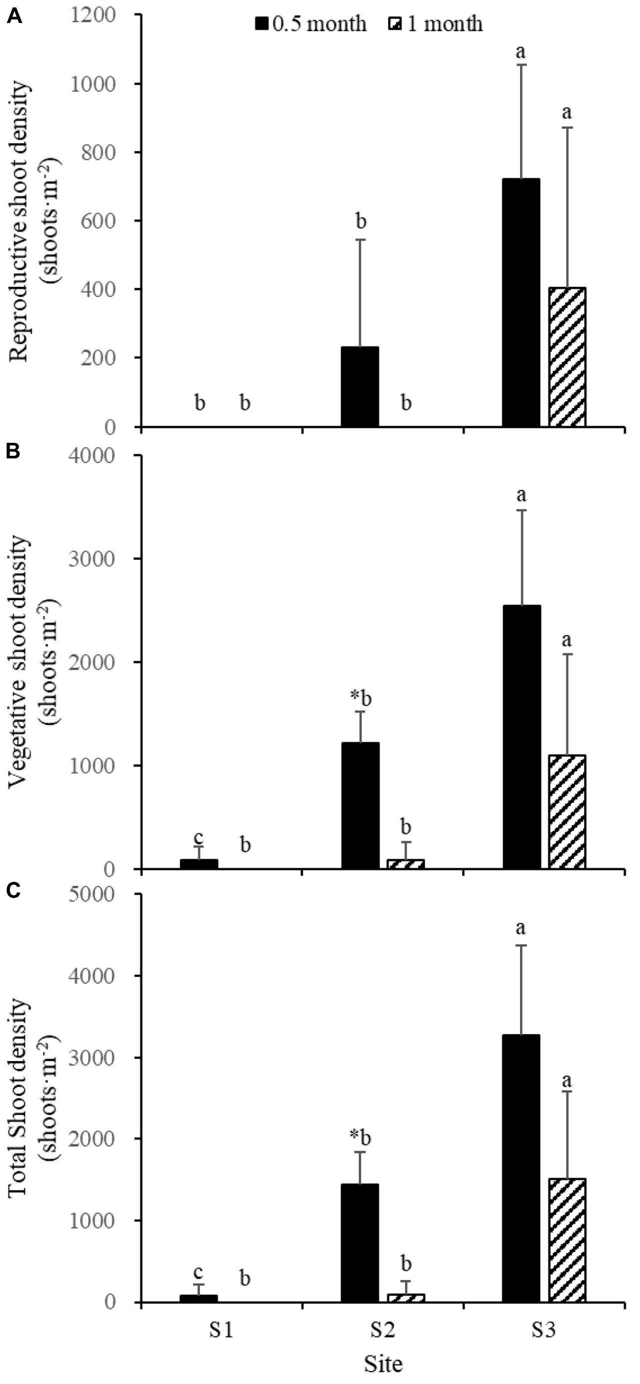

FIGURE 8 | Reproductive shoot density **(A)**, vegetative shoot density **(B)**, and total shoot density **(C)** of *Zostera japonica* in the three transplant sites at 0.5 and 1 month after transplantation. Values are mean ± SD. *Indicates significant difference between different time points in the same transplant site. Different letters indicate significant difference between different transplant sites at the same time point.

degraded and had not moving seaward under the pressure of *S. alterniflora* invasion.

In the Yellow River Delta, the invasion of *S. alterniflora* is a common phenomenon at the *Z. japonica* meadows. At another site, a limited initial invasion of *S. alterniflora* (ca.30 m^2) at *Z. japonica* meadows was observed in August, 2015; however, after 5 years, large areas of this site were also invaded by *S. alterniflora* (**Supplementary Figure 2**).

Invasion Pattern of *Spartina alterniflora*

In our field investigation, *S. alterniflora* pushed forward 14 m into the *Z. japonica* distribution region between April and July 2019, which illustrates its powerful invasion ability. Liu et al. (2020a,2020b) reported that the height, density, above-ground biomass, and reproductive ability of

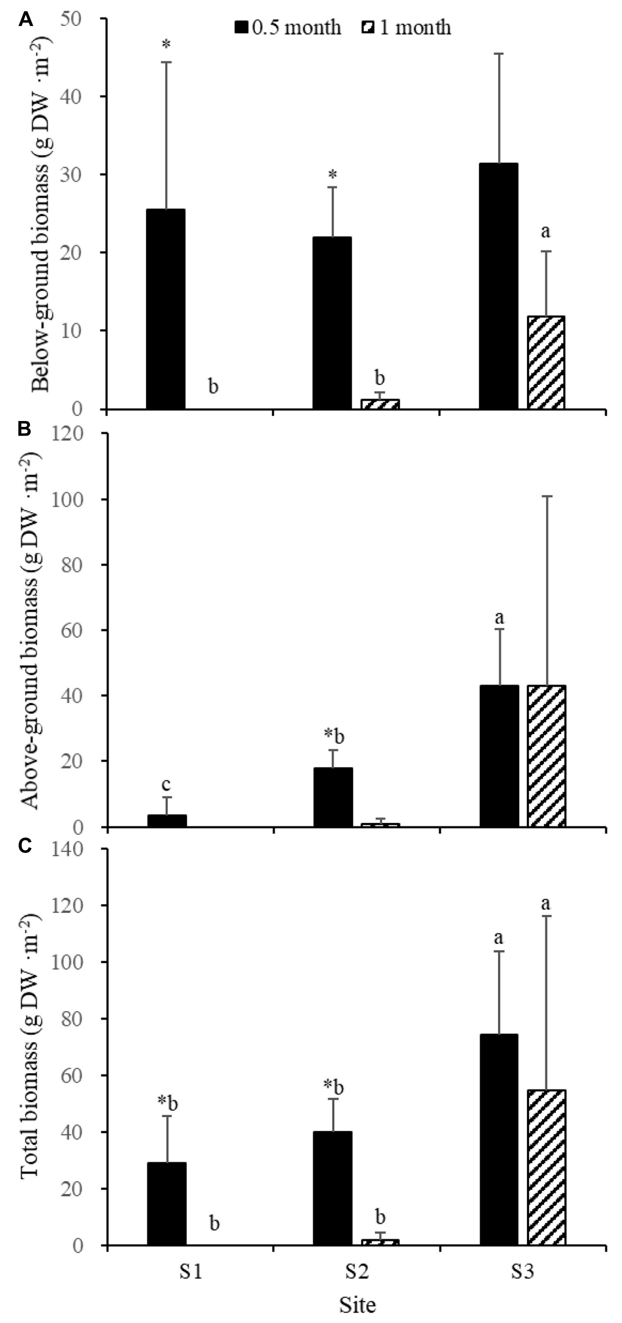

FIGURE 9 | Below-ground biomass **(A)**, above-ground biomass **(B)**, and total biomass **(C)** of *Zostera japonica* in the three transplant sites at 0.5 and 1 month after transplantation. Values are mean ± SD. *Indicates significant difference between time points in the same transplant site. Different letters indicate significant difference between different transplant sites at the same time point.

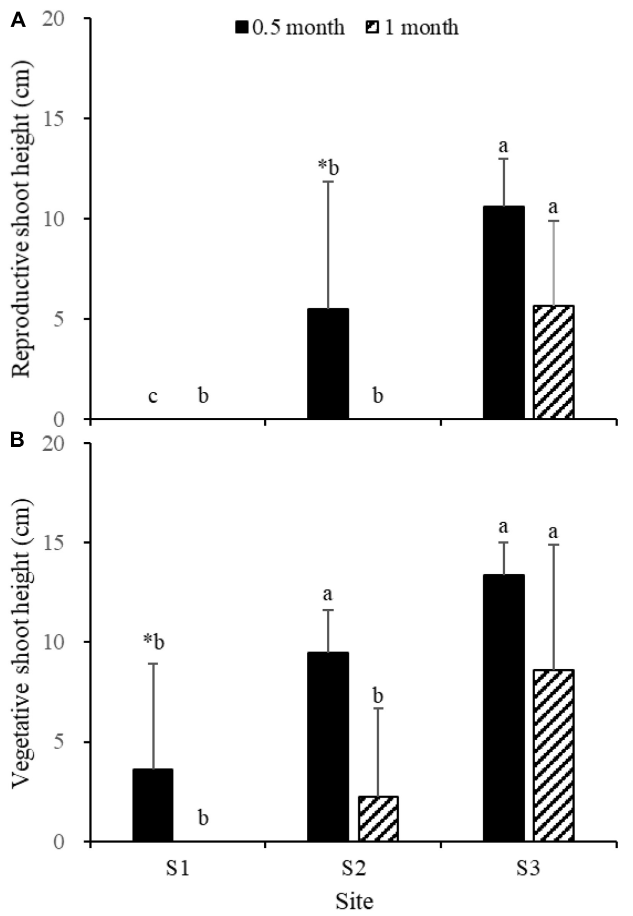

FIGURE 10 | Reproductive shoot height **(A)** and vegetative shoot height **(B)** of *Zostera japonica* in the three transplant sites at 0.5 and 1 month after transplantation. Values are mean ± SD. *Indicates significant difference between time points in the same transplant site. Different letters indicate significant difference between different transplant sites at the same time point.

S. alterniflora in the Chinese range were greater than those in the native range.

Seedlings of *S. alterniflora* are smaller than adult shoots and can reach sexual maturity within 3–4 months (Meng et al., 2020). In this study, *S. alterniflora* seedlings were recorded in spring, while seed maturation occurred in Autumn, indicating that seeds did not germinate immediately after maturity. *S. alterniflora* propagates sexually via seeds and asexually by tillers and rhizomes. *S. alterniflora* shoots collected at sampling points 9 and 10 in July 2019 could be easily uprooted and were very small; these shoots were obviously grown from dormant seeds that germinated in May 2019.

S. alterniflora has aerenchyma, making it adaptable to the anoxic environment (Meng et al., 2020). Therefore, seedlings could survival in the *Z. japonica* distribution region that was characterized by relatively deep seawater. We observed multiple peaks of biomass of *S. alterniflora* as the sampling point number increased, which represented the irregularly shaped patches of *S. alterniflora*. In the initial stages of *S. alterniflora* invasion, sparse patches arising from germinating seeds are distributed on the tidal flats (Ma et al., 2019).

S. alterniflora has a high tolerance and adaptability to environmental stressors (including lower temperature). During winter, the above-ground tissues of *S. alterniflora* turn yellow

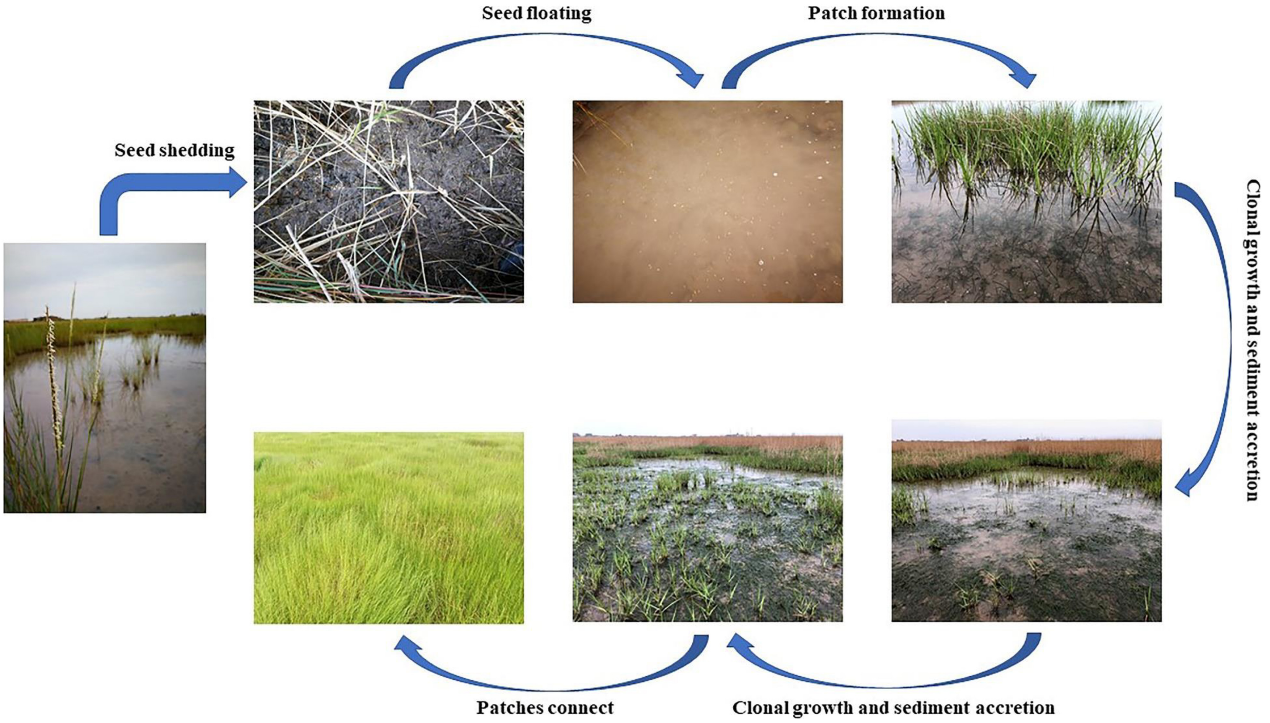

FIGURE 11 | The invasion pattern of *Spartina alterniflora* in *Zostera japonica* meadows.

but keep standing (**Supplementary Figure 3**). Therefore, *S. alterniflora* would not die off at sampling points 9 and 10 throughout the winter. In addition, we investigated the growth of *S. alterniflora* at sampling points 9 and 10 in June, 2020; the results showed that the shoot height was greater than 25 cm, and the shoot density was greater than 250 shoots · m^{-2}. These values were greater than those in October 2019 (except the shoot density at point 10). These data indicated that *S. alterniflora* at sampling points 9 and 10 had spread locally by clonal growth in 2020.

We found that the biomass of *Z. japonica* increased in July and decreased in October within the *Z. japonica* distribution region. However, the biomass of *Z. japonica* decreased over time in the front part of the ecotone. Zhang X.M. et al. (2019) previously described the seasonal changes in *Z. japonica* in the Yellow River Delta. They reported that growth increased in June and July, peaked in August, and dramatically declined by October. The higher height of *S. alterniflora* shielded the sunlight originally used by *Z. japonica* and thus inhibited growth of the seagrass. The biomass of *S. alterniflora* in October was higher than that in July, which indicated that the growth ability of *S. alterniflora* was greater than that of *Z. japonica*.

Over the past century, the coastal erosion of many deltas was triggered by the decrease of sediment loads of many rivers owing to damming and irrigation as well as improved land-use practices, such as the Yellow River Delta (Milliman, 1997; Wang et al., 2007; Syvitski et al., 2009; Bi et al., 2014; Jiang et al., 2017). Waves impacting the shoreline can suspend sediment while currents can transport these materials elsewhere, causing erosion (Fan et al., 2018). Higher growth rates of *S. alterniflora* promote greater standing biomass, which in turn slows water velocity on the marsh platform, lowers wave height, reduces erosion, and enhances mineral sediment deposition (Kirwan and Megonigal, 2013). It is suggested that the invasion of *S. alterniflora* increased the accumulation of sediment. Gao et al. (2014) reported that *S. alterniflora* can enhance the settling flux of suspended sediment and the deposition rate on the tidal flats by reducing the near-bed shear stress associated with tidal currents. Sediment accretion may increase the ecological niche of *S. alterniflora*, which is one potential explanation for the successful invasion of *S. alterniflora* into *Z. japonica* meadows.

S. alterniflora propagates sexually via seeds for long distance invasion and asexually by tillers and rhizomes for short distance invasion (**Figure 11**). The seeds fall off the plant after maturing and float with the tide into the seagrass bed, where sparse patches of the invader arise from germinating seeds. Subsequently, the deposition rate increases gradually as the density of clonal ramets increases, and over time the growth of *Z. japonica* is inhibited. Ultimately, patches of *S. alterniflora* connect and replace the seagrass bed community through vegetative reproduction.

Ecological Effects of *Spartina alterniflora* Invasion

Results of the transplant experiment showed that inhibition of *Z. japonica* growth increased with the invasion stage of *S. alterniflora*. The ecological functions of *Z. japonica* meadows may be reduced by invasion of

S. alterniflora. Zhang Y. et al. (2019) reported that the invasion of *S. alterniflora* into the Chinese coastal wetlands caused profound biotic homogenization of soil communities across latitudes. In the Chongming wetland of Shanghai, the species and number of birds in the *S. alterniflora*-vegetated community were much lower than in native reed communities (Meng et al., 2020). The Yellow River Delta National Nature Reserve is an important transit point, habitat, and breeding ground for bird migration. A high-density *S. alterniflora* population can act as an "isolation belt" between birds and their food resources, which may decrease bird diversity.

Significant quantities of carbon can be sequestered by, stored in, and released from seagrass meadows (Prentice et al., 2020). Salinas et al. (2020) reported that the loss of seagrass due to human and natural disturbances may have caused the release of 11–21 Tg CO_{2-eq} in Australia since the 1950s. Yang et al. (2013) reported that soil organic carbon was significantly increased in the upper 0–30 cm soil layer after *S. alterniflora* invaded the *S. salsa* and *Cyperus malaccensis* communities. Wang et al. (2019) found that the soil total C content was $13.3 \pm 0.3\ t \cdot ha^{-1}$ in the native mangrove regions, while the soil total C content was $10.9 \pm 0.3\ t \cdot ha^{-1}$ in the invasive *S. alterniflora* regions. Thus, the carbon buried in *Z. japonica* meadows may change after *S. alterniflora* invasion. Measuring soil organic carbon (SOC,%) by organic matter (OM,%) analyzer can significantly reduce cost per sample analysis over the long-term (Howard et al., 2014). Craft et al. (1991) reported the relationships between OM (%) and SOC (%) in tidal salt marsh [$SOC = 0.40 \times OM + 0.0025\ (OM)^2$, $r^2 = 0.99$]. Also, Fourqurean et al. (2012) reported the relationships between OM (%) and SOC (%) in seagrass meadows ($SOC = 0.43 \times OM – 0.33$, $r^2 = 0.96$). Therefore, we used the first equation to estimate the SOC content in the *S. alterniflora* distribution region and the ecotone. The SOC content in the *Z. japonica* distribution region was estimated by the second equation. The results showed that SOC content (%) increased significantly in the upper 0–12 cm soil layer after *S. alterniflora* invaded the *Z. japonica* communities (**Supplementary Table 1**; $p < 0.05$). Due to the limitation of sampling methods, the difference in SOC density was unknown; and the difference in SOC in deep sediment (12–100 cm) at different regions should be investigated in the future.

In conclusion, we found that *S. alterniflora* pushed 14 m into the *Z. japonica* distribution region within 3 months and that the invasion ability of *S. alterniflora* was greater than the growth ability of *Z. japonica*. The growth of *Z. japonica* was inhibited gradually with the invasion of *S. alterniflora*. Our results describe the invasion pattern of *S. alterniflora* and can be used to develop strategies for the prevention and control of *S. alterniflora* invasion.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

SY and YZ conceived the ideas and designed methodology and led the writing of the manuscript. SY, SCX, XZ, ML, YQ, RG, SX, and YuZ collected the data. SY analyzed the data. All authors contributed critically to the drafts and gave final approval for publication.

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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.2021.643425/full#supplementary-material

Supplementary Table 1 | The SOC content (%) at the three regions. Values are mean ± SD. Different letters indicate significant difference between different regions.

Supplementary Figure 1 | *Spartina alterniflora* invasion of *Zostera japonica* meadows [**(A)** May 2015, low tide; **(B)** October 2016, high tide; **(C)** July 2019, low tide)].

Supplementary Figure 2 | *Spartina alterniflora* invasion of *Zostera japonica* meadows in another site [**(A,B)** August 2015; **(C,D)** June 2020; the red five-pointed star was represented the location of this site]. ZJ, *Zostera japonica*; SA, *Spartina alterniflora*.

Supplementary Figure 3 | Field photo of the study region [**(A)** April 27, 2019; **(B)** July 21, 2019].

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Reciprocal Field Transplant Experiment and Comparative Transcriptome Analysis Provide Insights Into Differences in Seed Germination Time of Two Populations From Different Geographic Regions of *Zostera marina* L.

Yu Zhang[1,2,3,4,5,6†], *Shaochun Xu*[1,2,3,4,6†], *Shidong Yue*[1,2,3,4,5,6], *Xiaomei Zhang*[1,2,3,4,6], *Yongliang Qiao*[7], *Mingjie Liu*[1,2,3,4,5,6] and *Yi Zhou*[1,2,3,4,5,6*]

[1]CAS Key Laboratory of Marine Ecology and Environmental Sciences, Institute of Oceanology, Chinese Academy of Sciences, Qingdao, China, [2]Laboratory for Marine Ecology and Environmental Science, Qingdao National Laboratory for Marine Science and Technology, Qingdao, China, [3]Center for Ocean Mega-Science, Chinese Academy of Sciences, Qingdao, China, [4]CAS Engineering Laboratory for Marine Ranching, Institute of Oceanology, Chinese Academy of Sciences, Qingdao, China, [5]University of Chinese Academy of Sciences, Beijing, China, [6]Shandong Province Key Laboratory of Experimental Marine Biology, Qingdao, China, [7]Qingdao University of Science and Technology, Qingdao, China

Edited by:
Peter J. Lammers,
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United States

Reviewed by:
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Jutta Papenbrock,
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***Correspondence:**
Yi Zhou
yizhou@qdio.ac.cn

†These authors have contributed equally to this work

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Seagrasses are the only submerged marine higher plants, which can colonize the sea through sexual (*via* seeds) reproduction. The transition between seed dormancy and germination is an important ecological trait and a key stage in the life cycle of higher plants. According to our observations, the seeds of *Zostera marina* L. (eelgrass) in Swan Lake (SL) and Qingdao Bay (QB) in northern China have the same maturation time (summer) but different germination time. To investigate this phenomenon, we further carried out reciprocal transplantation experiment and transcriptome analysis. Results revealed that differences in the seed germination time between the two sites do exist and are determined by internal molecular mechanisms as opposed to environmental factors. Furthermore, we conducted comparative transcriptome analysis of seeds at the mature and early germination stages in both locations. The results that the number of genes related to energy, hormone and cell changes was higher in SL than in QB, could account for that the dormancy depth of seeds in SL was deeper than that in QB; consequently, the seeds in SL needed to mobilize more related genes to break dormancy and start germination. The results could have important practical implications for seagrass meadow restoration *via* seeds and provide in-depth and comprehensive data for understanding the molecular mechanisms related to seagrass seed germination.

Keywords: seagrass, biogeographical environment, seed germination, dormancy depth, transcriptome

INTRODUCTION

Seagrass meadows are key ecosystems, and they are among the most threatened habitats on the planet. Seagrasses are the only submerged marine higher plants with a system of underground root and rhizome (Short et al., 2007). While colonizing the sedimentary shorelines of the world's oceans, seagrasses have experienced genomic losses and gains to achieve the structural and physiological adaptations required for its marine lifestyle, arguably the most severe habitat shift ever accomplished by flowering plants (Olsen et al., 2016). As a functional group, they provide the basis for productive ecosystems along the coasts of all continents except Antarctica, and the ecosystem services they provide are on a par with tropical rainforests and coral reefs (Costanza et al., 1997; Fourqurean et al., 2012; Olsen et al., 2016). Seagrass species colonize the sea through both sexual (*via* seeds) and asexual (*via* clonal growth of rhizomes) reproduction (Phillips and Menez, 1988; Darnell et al., 2015; Zhang et al., 2020). Low pollination success, restricted dispersal of pollen and seeds, and low survival of seeds and seedlings, means that successful recruitment *via* sexual reproduction is restricted (Les, 1988; Laushman, 1993; Reusch, 2003); therefore, asexual reproduction is the main method of recruitment (Duarte and Sand-Jensen, 1990; Rasheed, 2004). Despite this, sexual reproduction is the only way to maintain the genetic diversity of the population, which improves resistance to adverse environments and resilience to disturbances (Ehlers et al., 2008; Cabaco and Santos, 2012; Xu et al., 2018). Furthermore, sexual reproduction also plays an important role in colonizing new habitats and recolonizing severely declined seagrass meadows by dispersing seeds from parental meadows (Harwell and Orth, 1999; Jarvis and Moore, 2010; Kendrick et al., 2012).

The seagrass *Zostera marina* L., or eelgrass, is the most widespread marine angiosperm species throughout the temperate northern hemisphere of the Pacific and Atlantic (Green et al., 2003; Olsen et al., 2016). Many studies have reported that sexual reproduction is crucial for the maintenance of within-species genetic diversity of eelgrass populations (Larkum et al., 2005; Kendrick et al., 2012; Ort et al., 2012). During sexual reproduction, eelgrass seeds are produced after flowering (Ort et al., 2012). No regional studies have directly documented long-distance dispersal, but genetic isolation by distance studies have suggested 150 km as an upper limit for the natural dispersal distance of *Z. marina* (Olsen et al., 2004; Muñiz-Salazar et al., 2005), which provides evidence for the colonization of new habitats with seeds. In most cases, once eelgrass seeds are released from the flowering shoots, they are deposited on or in the sediment, forming the seed bank (Fenner et al., 2005). A certain proportion of the seeds in the seed bank are maintained in a suitable physiological state conducive for germination to occur at an ecologically advantageous time. This reproductive strategy is vital for enhancing genetic diversity and avoiding the catastrophic loss of a plant population (Venable and Brown, 1988; Reynolds et al., 2013). Although much of the practical work on restoration of seagrass beds is carried out using adult seagrass plants, this approach has its limits. Apart from the huge labor and financial support required, the main problem is the potential loss of genetic diversity when adult plants are used to rebuild populations (Williams and Davis, 1996; Williams, 2001). In contrast, restoration of seagrass beds by seeding can avoid these problems, especially the loss of genetic diversity. Several restoration efforts have already shown that large areas of seagrass beds can be successfully restored by seeding (McGlathery et al., 2012; Orth et al., 2012, 2020; Orth and McGlathery, 2012), and the high genetic diversity in seagrasses restored using seeds rather than adult plants confers a greater level of ecosystem resilience to the restored seagrass meadows (Reynolds et al., 2012).

The transition between seed dormancy and germination is an important ecological trait and key stage in the life cycle of higher plants, which ensures species survival (Cadman et al., 2006; Holdsworth et al., 2008). Most seeds can survive long periods of unfavorable conditions and only germinate and grow into plants under the most favorable conditions. This ability stems, in part, from the existence of a dormancy mechanism (Cadman et al., 2006). Whether seeds are dormant or non-dormant and the type and depth of dormancy varies between species, or even between individuals of the same species (Baskin and Baskin, 2004). Seed dormancy and germination have been studied for decades in terrestrial higher plants (*Arabidopsis thaliana*, *Oryza sativa*, *Triticum aestivum*, *Hordeum vulgare*, etc.) at morphological, physiological, genetic, and molecular levels (Barrôco et al., 2005; Fait et al., 2006; Sreenivasulu et al., 2008; Howell et al., 2009; Galland et al., 2014; Han et al., 2017; He et al., 2020). As the only marine higher plant, the seed ecology of eelgrass has been studied to a certain extent (Churchill, 1992; Moore et al., 1993; Sugiura et al., 2009; Wang et al., 2016, 2017; Xu et al., 2016), but little is known about the molecular mechanisms of eelgrass seed germination.

We have observed that the seed germination time of *Z. marina* differed between Swan Lake (SL) and Qingdao Bay (QB) in northern China over a number of years. The time of seed maturation (summer time) is same in both places, but the time of germination is totally different. Seed germination in QB began in autumn of the same year as seed maturation, and most seeds germinated in October and November. In contrast, the seed germination period in SL is in spring of the year following seed maturation, from March to the end of May. Globally, a number of studies have found that the germination seasons of different geographical populations of eelgrass differ. For example, the seeds of eelgrass populations in the northeastern Pacific Ocean mainly germinate in spring and autumn (Kentula, 1982; Phillips and Backman, 1983), while populations in the northwestern Atlantic mainly germinate in autumn (Orth and Moore, 1986; Jarvis and Moore, 2010). Populations on European coasts mainly germinate in spring, but some can germinate in winter (Harrison, 1993; van Lent and Verschuure, 1994). Based on this phenomenon, we hypothesize that inherent genetic traits rather than environment determine the germination time of seeds, and the germination time does not change with the environment in which the seeds are planted. Therefore, we designed a field experiment in which seeds collected from QB and SL were reciprocally transplanted and their germination time was recorded. Comparative transcriptome analysis was then carried out to analyze the different events in eelgrass seed germination between SL and QB, which provides in-depth and comprehensive information for researchers relating to the molecular mechanism of seagrass seed germination.

MATERIALS AND METHODS

Study Sites

SL (37°21′N, 122°34′E) is a marine lagoon in Weihai City, northern China covering an area of 4.8 km^2 (**Figure 1**). SL is connected to the Yellow Sea by a narrow inlet that is 86 m wide (Zhang et al., 2020) and experiences irregular semidiurnal mixed tides, with a mean tidal range of 1.65 m. According to an investigation of environmental variables carried out by Xu et al. (2018), the water temperature shows an annual pattern ranging from −2.30 (February) to 25.60°C (August), and the annual average water temperature was 16.13 ± 7.76°C. Salinity ranges from 31.3 to 33.7 in SL. The sediment in the lake is mainly sandy and the daily photosynthetic photon flux densities increased from January and were highest in the summer months (June–July) (Xu et al., 2018). This lagoon is a suitable habitat for seagrasses with both *Zostera japonica* and *Z. marina* present (Zhou et al., 2015). *Zostera japonica* mainly occurs in the narrow mid-upper intertidal zone (Zhang et al., 2015, 2020); while *Z. marina* grows in the lower intertidal and subtidal zone (Zhou et al., 2015; Xu et al., 2018, 2019). There is a large difference in size between the two species, and the shoot height of *Z. japonica* and *Z. marina* in SL is shown in **Table 1**.

QB (36°03′N, 120°20′E) is an open bay in Qingdao City, northern China (**Figure 1A**). The tides in QB are regular semidiurnal, with a mean tidal range of 4.8 m (Zhang et al., 2020). According to research carried out by Xu et al. (2018), the water temperature exhibits an annual pattern ranging from −0.50 (February) to 28.00°C (August), and the annual average water temperature is 17.94 ± 7.80°C. The salinity in QB ranges from 31.6 to 32.7. The sediment in the bay is mainly silt and sand, and the daily photosynthetic photon flux density is similar to that in SL (Xu et al., 2018). This bay has extensive areas of seagrass meadows dominated by *Z. marina*, but there also occurs a small area of *Z. japonica*. The distribution and shoot height (**Table 1**) of the two species in QB are similar to SL. The morphological comparison between *Z. japonica* and *Z. marina* is shown in **Figure 1B**.

Experiment 1: Seed Transplant Experiment

Seed Collection

Reproductive shoots of *Z. marina* with spathes containing seeds were collected by hand from QB and SL in July 2017. Reproductive shoots were transported to the laboratory, where they were stored in a 600-μm mesh bag and placed in an aerated flow through tank (1 m × 1.2 m × 1.5 m). Mature seeds were naturally released from the flowering shoots and were then collected and sieved to remove any detritus and larger material. Following this, all seeds were kept in a running seawater tank until the initiation of seed reciprocal transplanting.

Seed Transplanting and Germination

Eelgrass seeds from SL were planted in QB on 21 August 2017. Seeds were placed in 12 polyvinyl chloride tubes (15 cm long, 1.2 cm inside diameter). Plastic mesh caps (1 mm × 1 mm) were placed at the bottom of each of the tubes, and the tubes were filled with dry sediment collected from the banks of QB. The sediment was saturated with natural seawater. Intact and full eelgrass seeds were selected, and groups of 50 seeds were planted in each tube at a burial depth of ~2–3 cm (Jarvis and Moore, 2015). Plastic mesh caps (5 mm × 5 mm) were placed at the top of each of the tubes to prevent sediment or seeds from washing out. Tubes were buried in the sediment, and the top of the tubes were level with the ground. At the same time, another 12 replicate tubes containing seeds from SL were also planted in SL for comparison. Meanwhile, 12 replicate tubes each containing 50 eelgrass seeds from QB were planted in SL on 25 August 2017. At the same time, another 12 replicate tubes containing seeds from QB were also planted in QB for comparison.

To examine the germination of seeds, three replicate tubes containing seeds from both QB and SL were sampled in October, November, December 2017, and April 2018 in both QB and SL. The tubes were sieved (0.7 mm) carefully *in situ* (Xu et al., 2018), and the retained materials were transported to the laboratory to count the number of germinated seeds. Germination of seeds was explicitly defined as not only the rupture of the seed coat but also the emergence and growth of the cotyledon (Churchill, 1983; Brenchley and Probert, 1998).

Data Analysis

All statistical analysis was carried out using R statistical software version 3.6.2 (R Core Team, 2016). Values were presented as means ± SE.

Experiment 2: Transcriptomic Profiling Analysis

Seed Collection

Reproductive shoots of *Z. marina* with spathes containing seeds were collected by hand from QB and SL in July 2019. Collection and transportation of reproductive plants and harvesting of mature seeds was the same as described in section "Seed Collection in Experiment 1". Molecular samples of mature seeds from the two sites were collected and named as QB-M and SL-M. The remaining seeds were kept in a circulating tank at room temperature in seawater. The status of seeds was observed once every 15 days until November, when the seeds collected from QB began to germinate in large numbers. By this time, molecular samples of seeds from QB (seeds in the germination stage but not yet germinated) and SL (seeds in the dormant stage and not germinated) were collected and were named as QB-G and SL-N, respectively. In March 2020, the seeds in the water tank collected from SL began to germinate in large numbers, and molecular samples of seeds from SL (seeds in the germination

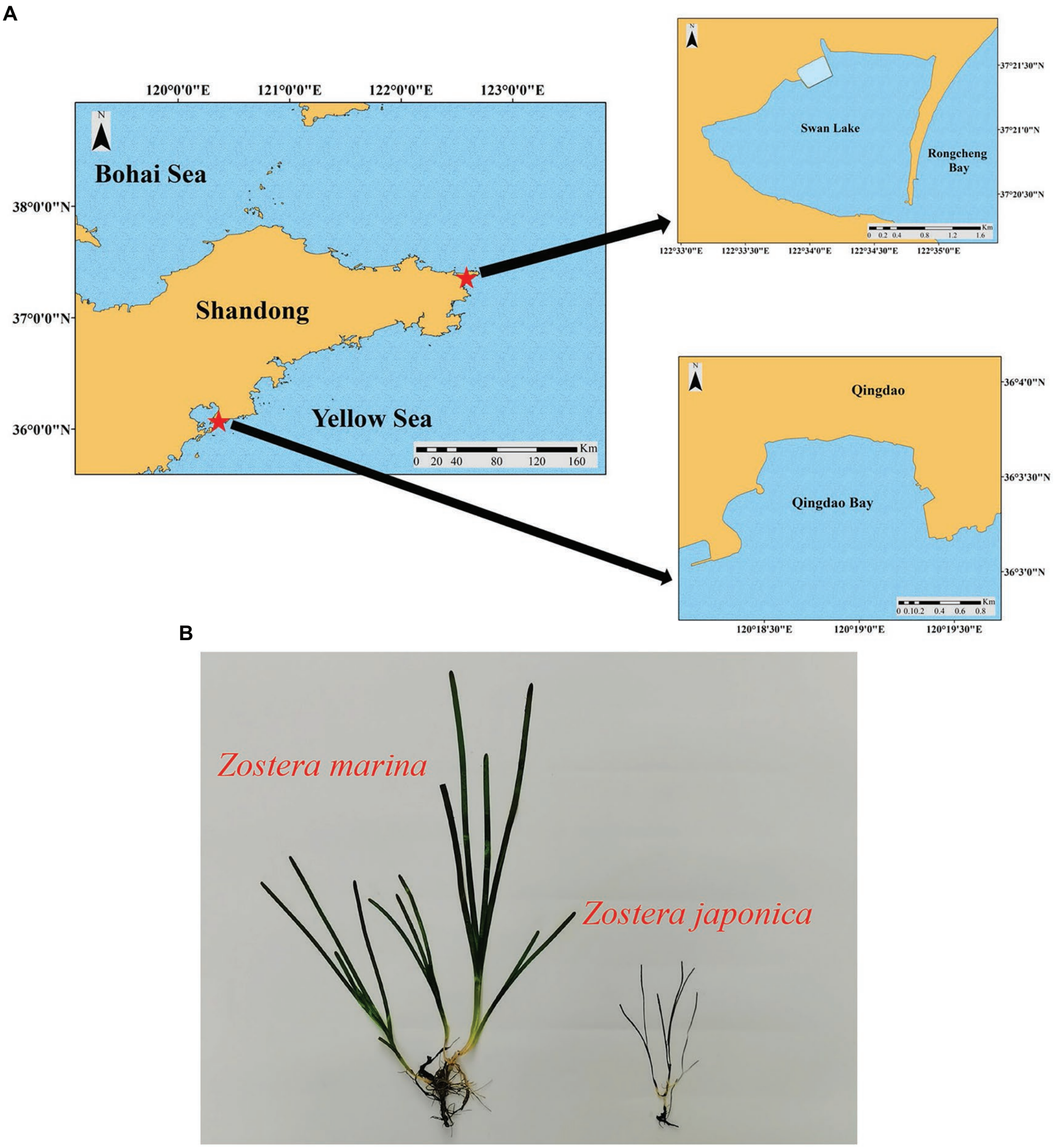

FIGURE 1 | (A) The geographical location of Swan Lake (SL) and Qingdao Bay (QB). **(B)** Morphological comparison between *Zostera marina* and *Z. japonica* (QB, in winter).

stage but not yet germinated) were collected at this time (SL-G). All molecular samples were immediately frozen in liquid nitrogen, and then stored in at −80°C. Four replicate samples, with each replicate containing approximately 10 seeds, were collected.

RNA Extraction, cDNA Library Construction, and Sequencing

Total RNA was extracted from *Z. marina* seed tissue using TRIzol® Reagent (Plant RNA Purification Reagent) according the manufacturer's instructions and genomic DNA was removed using DNase I. The RNA was tested for concentration, purity, and integrity, and only high quality RNA was used for library building OD260/280 = 1.8–2.2, OD260/230 ≥ 2.0, RIN ≥ 6.5, 28S:18S ≥ 1.0, >1 μg. The RNA-sequencing (RNA-seq) transcriptome library was prepared using a TruSeqTM RNA sample preparation kit from Illumina (San Diego, CA) using 1 μg of total RNA. After quantification with TBS380, the paired-end RNA-seq sequencing library was sequenced with the Illumina HiSeq xten/NovaSeq 6000 sequencer (2 × 150 bp read length).

Quality Control, Read Mapping, and Sequence Annotation

The raw paired end reads were trimmed and quality control was carried out with SeqPrep and Sickle software to get high

TABLE 1 | Shoot heights of *Zostera marina* and *Zostera japonica* in QB and SL.

Sites	Species	Summer	Winter	References
QB	*Zostera marina*	80.23 ± 13.83 cm	20.98 ± 10.73 cm	Xu et al., 2018
	Zostera japonica	20.53 ± 3.66 cm	13.71 ± 1.70 cm	Zhang et al., 2020
SL	*Zostera marina*	75.60 ± 20.86 cm	18.65 ± 5.29 cm	Xu et al., 2018
	Zostera japonica	20.21 ± 4.59 cm	5.77 ± 0.19 cm	Zhang et al., 2020

quality clean data. The genome of *Z. marina* was sequenced in 2016 (Olsen et al., 2016), and its annotation was updated in 2017. Therefore, *Z. marina* was used as the reference genome for subsequent sequence alignment and transcript assembly to get the mapped data. Based on the reference genome sequence, the mapped reads were assembled with Stringtie software and compared with the original genome annotation information. Finally, the functional annotations of the transcripts were summarized in the Non-Redundant Protein Sequence Database (NR), Swiss-Prot database (Swiss-Prot), the Protein families database (Pfam), Evolutionary genealogy of genes: Non-supervised Orthologous Groups (EggNOG), Gene Ontology (GO) database, and Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

Expression Analysis and Enrichment Analysis

RSEM was used to quantify gene abundance, and the expression level of each transcript was calculated according to the transcripts per million reads method. Different expression analysis was performed using DESeq2 (1.24.0), and genes with p-adjust <0.05 and $|\log2FC| > 1$ were considered to be significantly different expressed genes (DEGs). In addition, functional-enrichment analysis including GO and KEGG were performed to identify which DEGs were significantly enriched in terms of GO and metabolic pathways at Bonferroni-corrected $p \leq 0.05$ compared with the whole-transcriptome background. GO functional enrichment and KEGG pathway enrichment analysis were carried out by Goatools[1] and KOBAS.[2]

Quantitative PCR Verification

To evaluate the reliability of RNA-seq, we randomly selected eight genes (four each from SL and QB) for transcriptome validation using real-time quantitative polymerase chain reaction (RT-qPCR) and 18S rRNA was used as the internal reference gene. The RNA extracted as described in Section RNA Extraction was used as the template for reverse transcription to obtain cDNA. PCR results of the pre-experiment showed that the electrophoresis gel of each primer was a single bright band under specific conditions, indicating no specific amplification, and the primers could be used in subsequent experiments. The reaction system of RT-qPCR was as follows: 10 μl of 2× ChamQ SYBR Color qPCR Master MixPCR, 0.8 μl of both forward and reverse primers (5 uM each), 0.4 μl of 50× ROX Reference Dye, 2 μl of template (cDNA), and 6 μl of ddH_2O, made up to a total volume of 20 μl. The cycle conditions of RT-qPCR were as follows: the initial step was 95°C for 5 min, and then 40 cycles (melting at 95°C for 5 s, annealing at 50°C for 30 s, and extension at 72°C for 40 s). The target gene and internal reference gene of each sample were subjected to RT-qPCR reaction. Each treatment group had three replicates, and each replicate sample had three multiple pores. The relative expression level was calculated using the $2^{-\Delta\Delta Ct}$ method. Data were analyzed by *t*-test, and differences were considered as statistically significant if $p < 0.05$.

RESULTS

Seed Germination in the Reciprocal Seed Transplant Experiment

Seeds collected from QB had a higher seed germination rate in autumn (mainly November and December), regardless of whether they were planted in QB or SL. The highest germination rate occurred in SL in December 2017 (40.67% ± 1.76%); however, the germination rate was very low in spring (**Figure 2**). Seeds collected from SL had a very low seed germination rate in autumn, regardless of whether they were planted in QB or SL, with the germination rate close to 0; but the germination rate was relatively high in spring, with the germination rate 6.67 ± 1.33% and 36.00 ± 5.03% in QB and SL, respectively (**Figure 2**). Overall, seeds from QB mainly germinated during autumn, and seeds from SL mainly germinated during spring, which is consistent with our previous observations.

Comparison of Seed Transcriptome in Autumn (QB-G vs. SL-N)

When a large number of seeds from QB began to germinate in the circulating water tank in the laboratory in autumn, un-germinated seeds from QB and SL were collected for transcriptome analysis. At this time, the seeds from QB should have been in the germination stage (QB-G), while the seeds from SL should have been in the non-germination or dormant stage (SL-N).

After transcriptome sequencing of eight samples from QB-G and SL-N were completed, a total of 54.36 GB of clean data were obtained, the clean data of all samples were above 5.89 GB, the percentage of Q30 base was above 93.27%, and the GC content of clean reads was about 44.31%. The clean reads of each sample were aligned with the designated reference genome, and the alignment rates ranged from 89.88 to 92.91%. The Pearson correlation coefficient between biological replicates was greater than 0.97 (**Figure 3A**), indicating a good repeatability and that the data are reliable. PCA analysis showed that there was a significant difference between the SL-N and QB-G samples (**Figure 3B**). Sequencing results showed that 12,577 genes were expressed in both SL-N and QB-G, and that 821 genes were uniquely expressed in SL-N, while 370 genes were uniquely expressed in QB-G, as shown in the Venn diagram (**Figure 3C**). A total of 1,595 DEGs were identified, including 675 upregulated

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[1] https://github.com/tanghaibao/Goatools

[2] http://kobas.cbi.pku.edu.cn/home.do

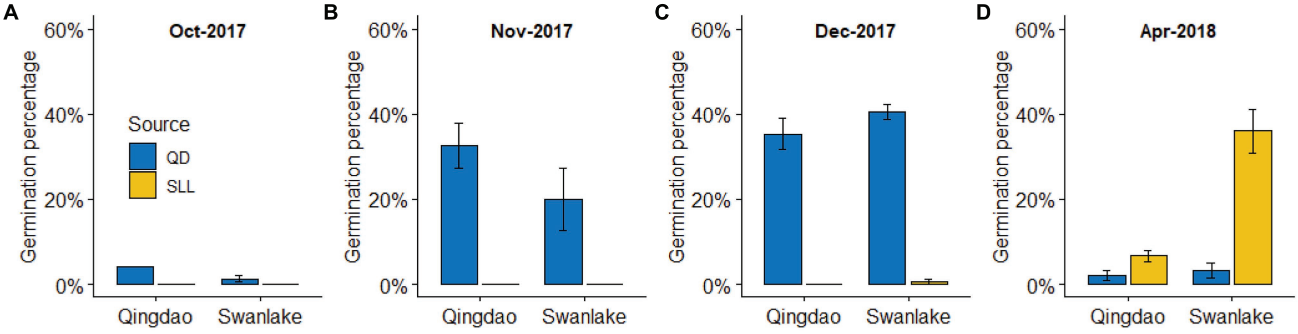

FIGURE 2 | Germination percentages (means ± SE) of eelgrass seeds in a reciprocal transplant experiment at Qingdao Bay (QB) and Swan Lake (SL). **(A-D)** represent different observation time.

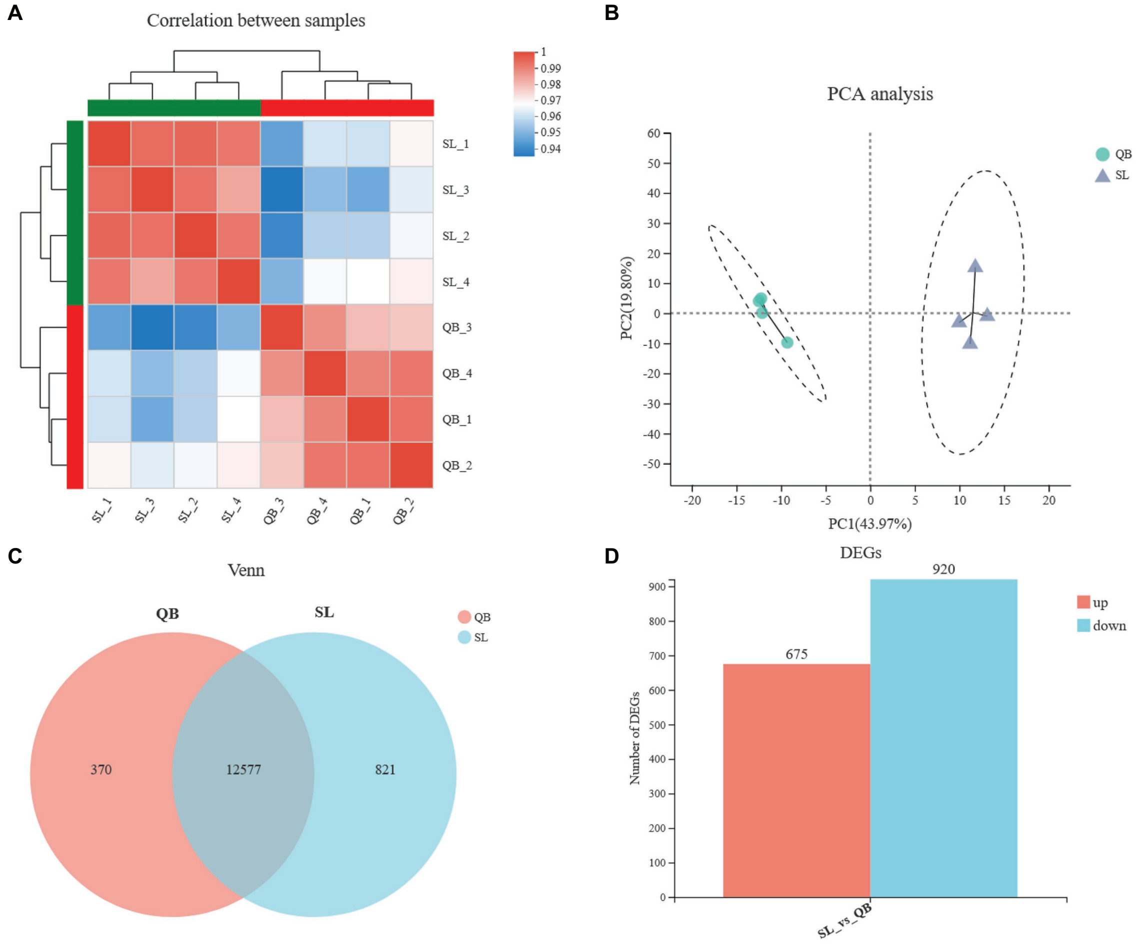

FIGURE 3 | Statistical analysis of sequencing data of QB-G and SL-N. **(A)** Pearson correlation analysis. The right and bottom side are sample names, the left and top side are sample clusters, and different colors represent the degree of correlation between two samples. **(B)** Principal component analysis based on gene expression. The distance between samples represents the degree of similarity, the horizontal and vertical axes indicate the contribution of principal component 1 (PCA1) and principal component 2 (PCA2) to the differentiated samples, respectively. **(C)** Venn analysis. Numbers represent genes that are common or unique between groups. **(D)** Statistics of differentially expressed genes (DEGs). Red group represents upregulation, blue group represents downregulation, and the vertical axis represents the number of DEGs.

genes and 920 downregulated genes (**Figure 3D**, the description of upregulation/downregulation was the result of gene changes in QB-G compared with SL-N).

The upregulated DEGs and uniquely expressed genes in QB-G were integrated into a gene set (QB_up_unique), and KEGG pathway enrichment analysis was performed (**Figure 4**). Flavonoid biosynthesis, arginine biosynthesis, phenylpropanoid biosynthesis, photosynthesis, alanine, aspartate and glutamate metabolism, glutathione metabolism, alpha-linolenic acid metabolism, nitrogen metabolism, flavone and flavonol biosynthesis, phenylalanine metabolism, and pentose phosphate pathways were significantly enriched ($p < 0.05$). On the whole, these significantly upregulated KEGG enrichment pathways are involved in energy metabolism, lipid metabolism, amino acid metabolism, and biosynthesis of secondary metabolites, which indicted that compared with the seeds from SL in the dormant stage, the seeds in the early stage of germination from QB had begun to initiate multiple metabolic pathways to mobilize the stored energy of seeds and gradually emerge from dormancy and start germination.

Comparison of Transcriptome Between Early Germination Seeds and Mature Seeds at Two Sites

Overall Comparison of Total DEGs

Transcriptome sequencing of 16 samples from SL-M, SL-G, QB-M, and QB-G was completed. A total of 107.65 GB of clean data were obtained, clean data of all samples were above 5.88 GB, the percentage of Q30 base was above 93.32%, and the GC content of clean reads was about 44.40%. The clean reads of each sample were aligned with the designated reference genome, and the alignment rates ranged from 89.91 to 94.41%. Pearson correlation coefficient between biological replicates was greater than 0.97, indicating good repeatability and that the data are reliable. PCA analysis showed that four groups of samples were separated significantly in the PC1 × PC2 dimension (**Figure 5A**). In the first dimension (X-component) of the analysis, SL-M separated from SL-G, and QB-M separated from QB-G. In the second dimension (Y-component) of the analysis, SL-M grouped with QB-M, but QB-G separated from SL-G. The RT-qPCR results are shown in **Figure 6**, and the variation trend of the expression of these genes was consistent with the results of RNA-seq detection, indicating that the gene expression data obtained by RNA-seq was reliable.

A total of 2,176 DEGs were identified by comparing QB-G and QB-M seeds, including 985 upregulated genes and 1,191 downregulated genes (**Figure 5B**). Meanwhile, a total of 4,221 DEGs were identified by comparing SL-G and SL-M seeds, including 2,135 upregulated genes and 2086 downregulated genes (**Figure 5B**). So, the number of DEGs in SL was nearly twice that in QB (the description of upregulated/downregulated was the result of gene changes in the germination stage compared with the mature stage). The DEGs identified in SL and QB were combined into a gene set, which was defined as all-germination-genes, and the cluster analysis of this gene set was performed simultaneously in two stages at two sites (**Figure 7**). Cluster analysis results showed that the gene expression patterns of SL-M and QB-M were similar, and the

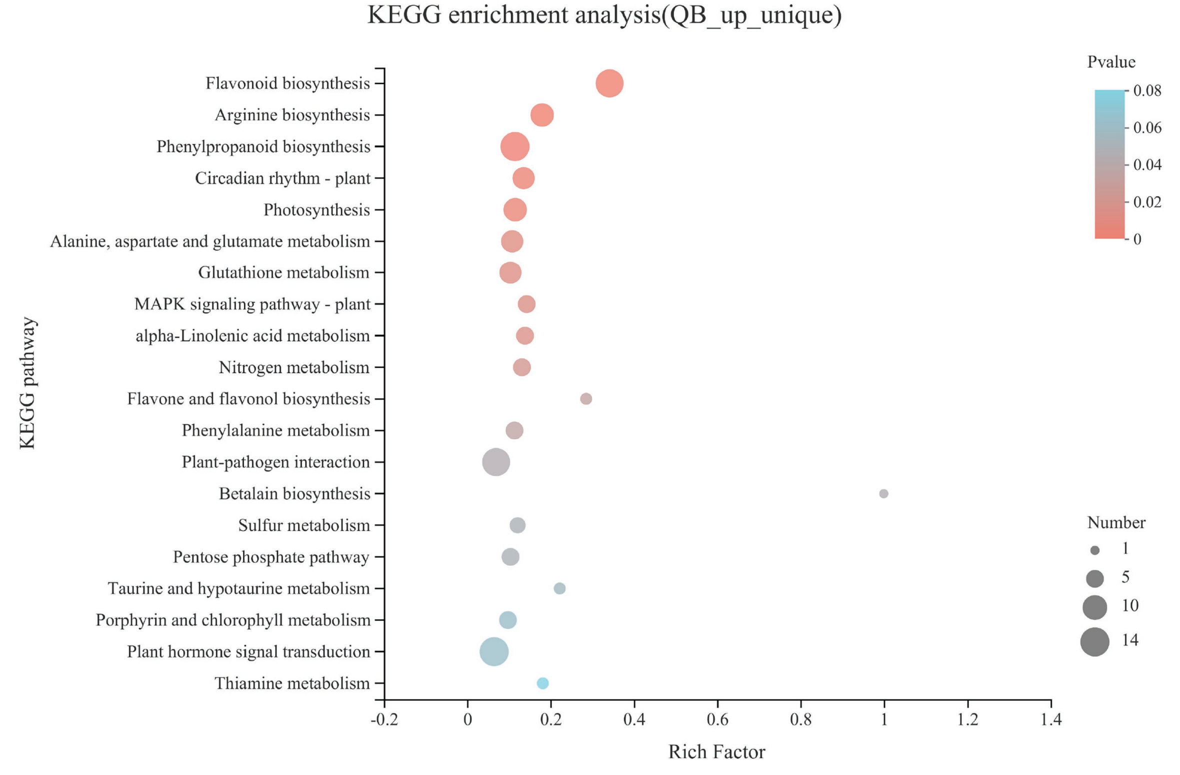

FIGURE 4 | KEGG pathway enrichment analysis of gene sets including upregulated DEGs and uniquely expressed genes in QB-G. The ordinate represents the pathway name, and the abscissa is the rich factor; the larger the rich factor is, the greater the degree of enrichment. The size of the circle indicates the number of genes enriched in pathway, the circle color represents *value of p*.

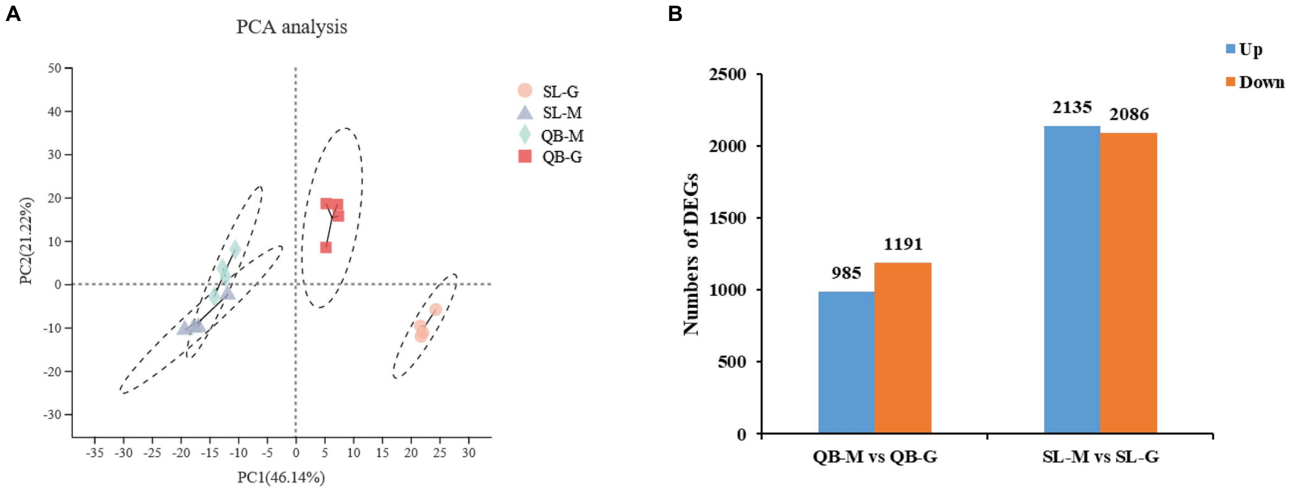

FIGURE 5 | Statistical analysis of sequencing data of SL-M, SL-G, QB-M, and QB-G. **(A)** Principal component analysis based on gene expression. See **Figure 3B** for figure interpretation notes. **(B)** Statistics of differentially expressed genes (DEGs) between the seeds at the early germination stage and mature stage. The horizontal axis represents the comparison of seed germination at different sites, blue group represents upregulation, orange group represents downregulation, and the vertical axis represents the number of DEGs.

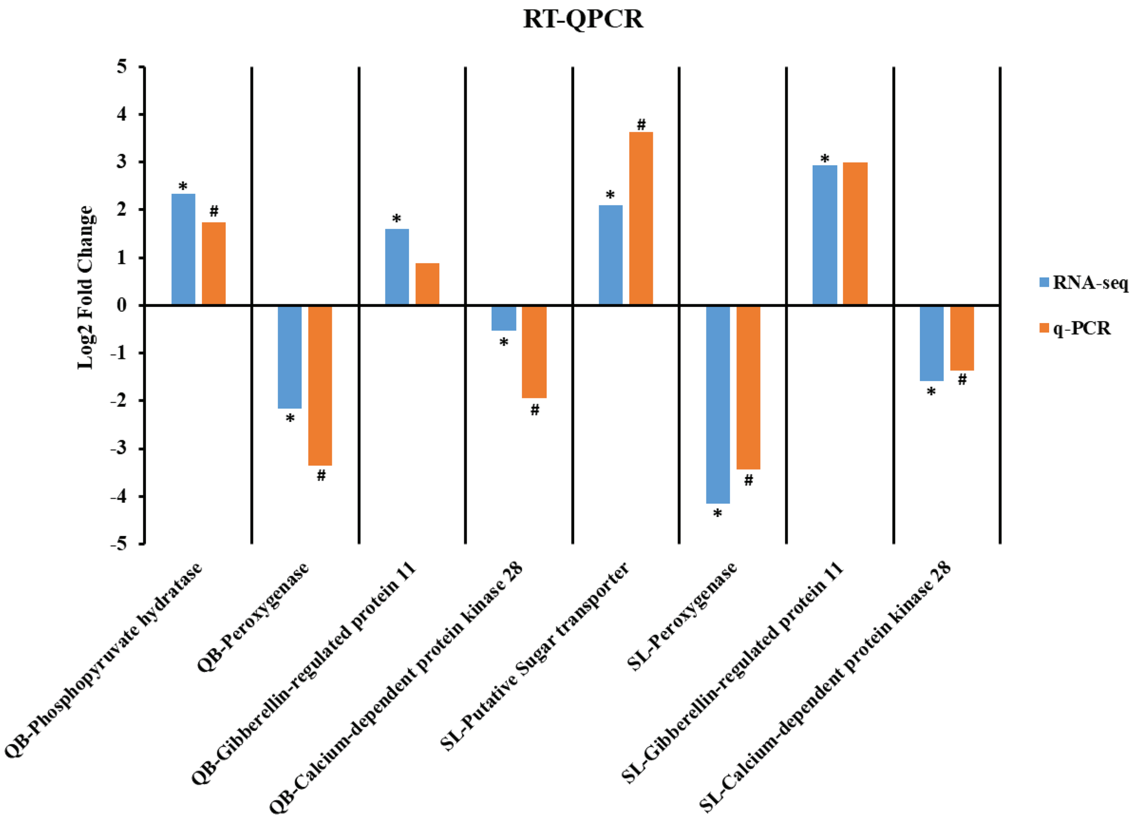

FIGURE 6 | Comparison of gene expression trend based on RNA-seq and RT-qPCR. * and # indicate a significant difference in expression between seed in the mature stage and the germination stage in the RNA-seq and RT-qPCR, respectively.

gene expression patterns of SL-G and QB-G were similar, but the expression patterns of SL-G and QB-G were significantly different in subcluster7.

KEGG pathway statistics and enrichment analysis were further analyzed for genes in subcluster7 (**Table 2** and **Figure 8**). The statistical results of the pathway are shown in **Table 2**, in which the processes, such as folding, sorting and degradation, transport and catabolism, carbohydrate metabolism and translation, contained the largest number of genes. KEGG pathway enrichment analysis showed that the process of protein processing in the endoplasmic reticulum was the most significant pathway ($p < 0.0001$).

Pathways Associated With Energy Metabolism

Upregulated DEGs in SL were analyzed by KEGG pathway enrichment (**Figure 9A**). There were 12 significant enrichment pathways ($p < 0.01$), including seven pathways related to energy metabolism and carbohydrate metabolism: pyruvate metabolism, carbon fixation in photosynthetic organisms, citrate cycle, glyoxylate and dicarboxylate metabolism, propanoate metabolism, glycolysis/gluconeogenesis, and butanoate metabolism. Similarly, upregulated DEGs in QB were also analyzed by KEGG pathway enrichment (**Figure 9B**). There were eight significantly enrichment pathways ($p < 0.01$), but only two were related to carbohydrate metabolism, which were pyruvate metabolism and glycolysis/gluconeogenesis.

Comparison of ABA/GA Related Genes

The plant hormone abscisic acid (ABA) is a positive regulator of dormancy induction and dormancy maintenance. A total of 38 genes related to ABA were found in the SL DEG set, including 17 upregulated DEGs and 21 downregulated DEGs (**Figure 10**). Further analysis revealed that nine of the 17 upregulated genes were related to ABA degradation, MYB transcription factor, and carbohydrate metabolism, and 19 of the 21 downregulated genes were related to the synthesis of ABA and response of ABA, which means that a total of 28 DEGs resulted in a decrease of ABA content. Similarly, a total of 21 genes related to ABA were found in the QB DEG set. Further analysis revealed that there were 13 DEGs resulting in the decrease of ABA content.

The plant hormone gibberellin (GA) is a positive regulator of dormancy breaking and germination induction. A total

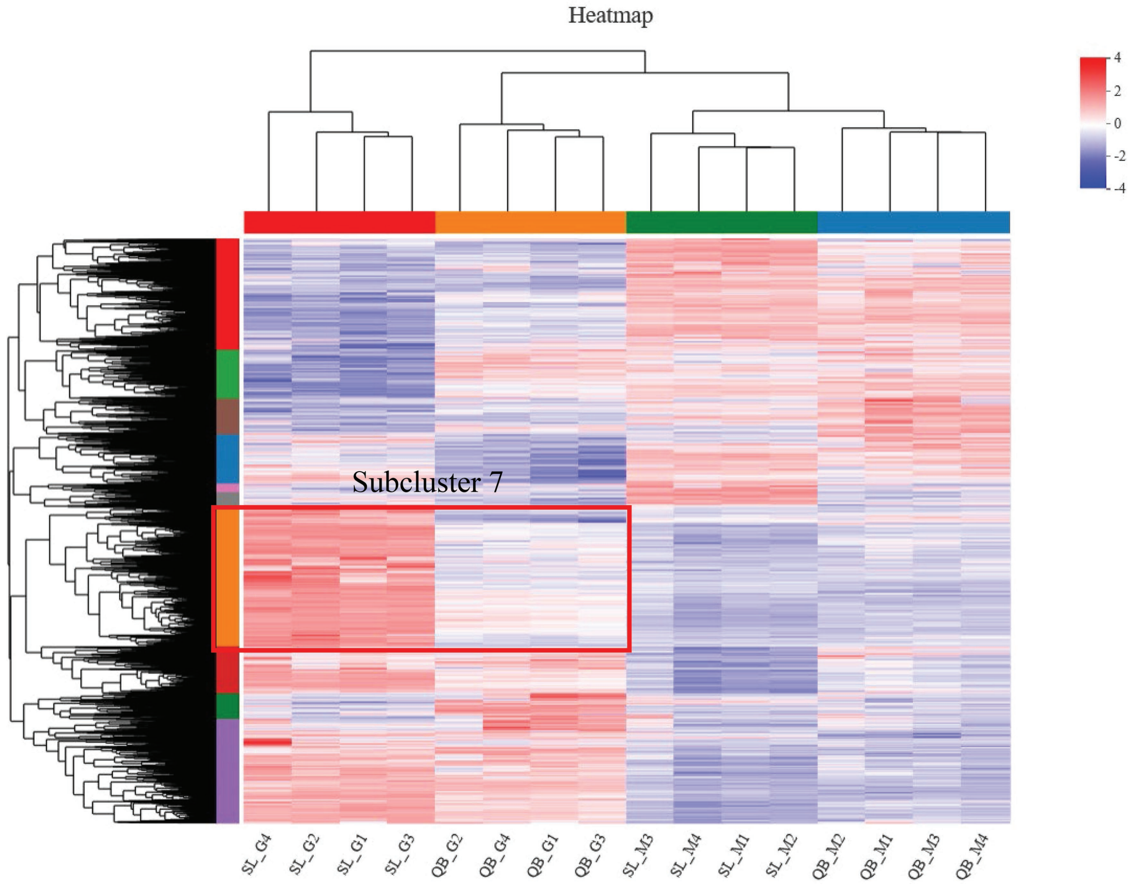

FIGURE 7 | Cluster analysis of all-germination-genes set in two stages at two sites. Each column represents a sample and each row represents a gene. The color indicates the expression value of the gene after normalization in each sample. Red means the gene is highly expressed, blue means the gene is lowly expressed, and the number next to the color bar on the top right indicates the specific trend. The tree diagram of gene cluster and the module diagram of subcluster are shown on the left side, and the closer the two gene branches are, the closer their expression levels are. The upper part is the tree diagram of sample cluster, and the lower part is the name of the samples, and the closer the two sample branches are, the closer the expression patterns of all genes in these two samples are, that is, the closer the variation trends of gene expression levels are.

TABLE 2 | KEGG pathway statistics of genes in subcluster7.

First category	Second category	Gene number
Genetic information processing	Folding, sorting, and degradation	56
Cellular processes	Transport and catabolism	50
Metabolism	Carbohydrate metabolism	44
Genetic information processing	Translation	33
Environmental information processing	Signal transduction	28
Metabolism	Amino acid metabolism	24
Metabolism	Energy metabolism	23
Metabolism	Lipid metabolism	22
Genetic information processing	Replication and repair	20
Metabolism	Metabolism of cofactors and vitamins	18
Genetic information processing	Transcription	18
Metabolism	Glycan biosynthesis and metabolism	16
Organismal systems	Environmental adaptation	13
Metabolism	Metabolism of other amino acids	9
Metabolism	Biosynthesis of other secondary metabolites	8
Metabolism	Nucleotide metabolism	8
Metabolism	Metabolism of terpenoids and polyketides	6
Environmental information processing	Membrane transport	3

of 15 genes related to GA were found in the SL DEG set, including nine upregulated DEGs and six downregulated DEGs (**Figure 10**). Further analysis revealed that all the nine upregulated genes were related to the synthesis, perception, and regulation of GA, and three of the six downregulated genes were associated with the negative regulation of GA decomposition and synthesis, which means that a total of 12 DEGs resulted in the increase of GA content. Similarly, a total of seven genes related to GA were found in the QB DEG set. Further analysis revealed that there were six DEGs resulting in an increase of GA content.

Comparison of Genes Associated With Cell Changes

Genes related to cell changes were identified in all genes from SL and QB (**Figure 11**), including genes related to cell growth, cell division, and cell proliferation. In addition, two plant hormones, auxin and cytokinin, were also included. The identification results were as follows: a total of 368 related genes were identified in SL, including 87 upregulated DEGs and 46 downregulated DEGs. A total of 365 related genes were identified in QB, including 34 upregulated DEGs and 35 downregulated DEGs. There were approximately 2.5 times more upregulated DEGs in SL than in QB. And, the number of downregulated DEGs in SL was slightly higher than that in QB.

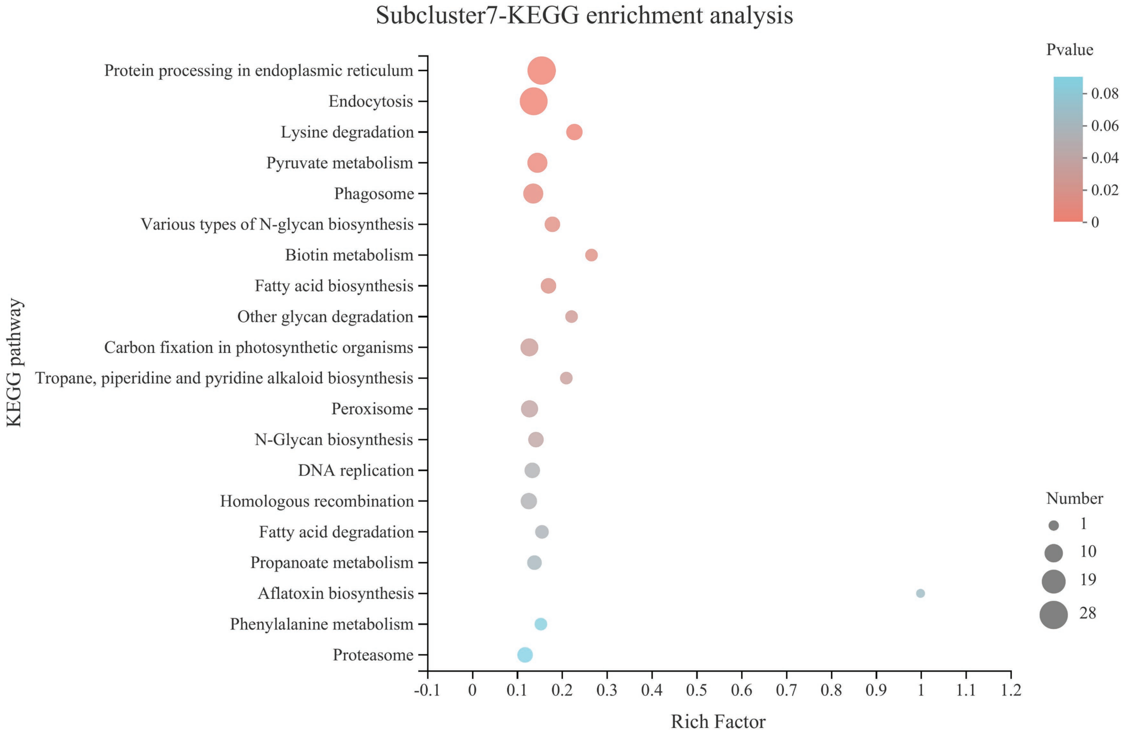

FIGURE 8 | KEGG pathway enrichment analysis of genes in subcluster7. See **Figure 4** for figure interpretation notes.

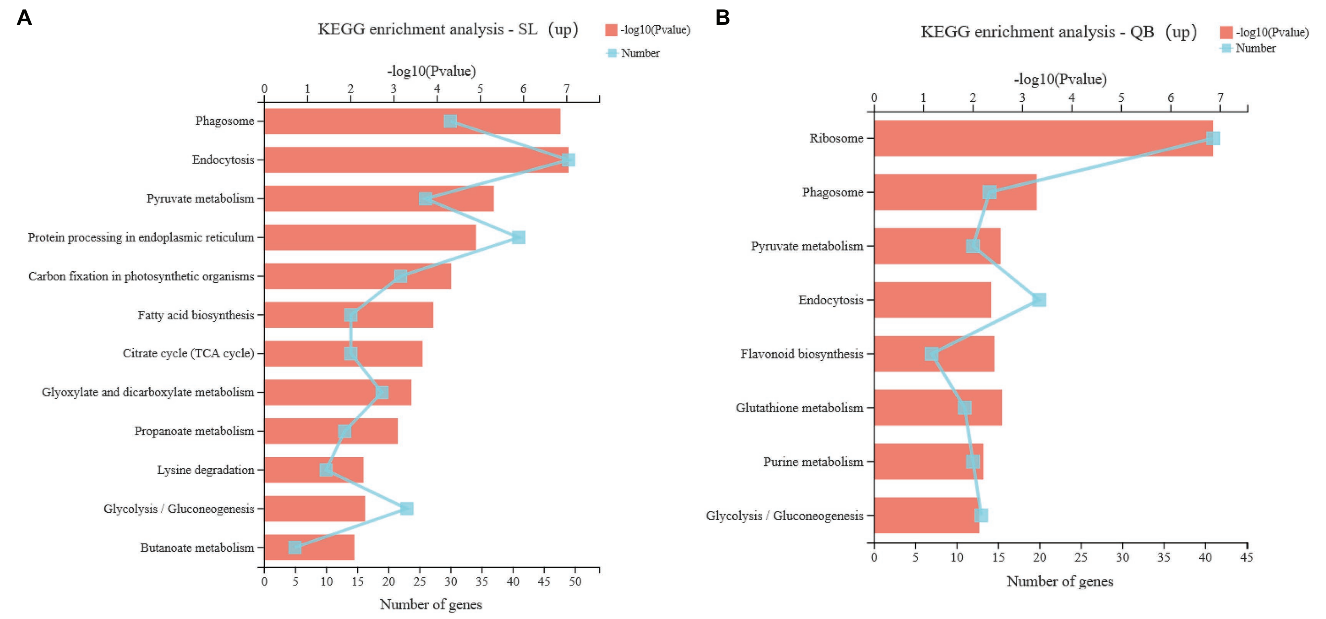

FIGURE 9 | KEGG pathway enrichment of upregulated gene sets in Swan Lake (SL; **A**) and Qingdao Bay (QB; **B**). The vertical axis represents the name of KEGG Pathway; the lower abscissa represents the number of genes in this pathway, corresponding to different points on the broken line, the upper abscissa represents the significance level of enrichment, corresponding to the height of the column; the larger the value of – log10 (value of *p*) is, the more significantly enriched the KEGG pathway is.

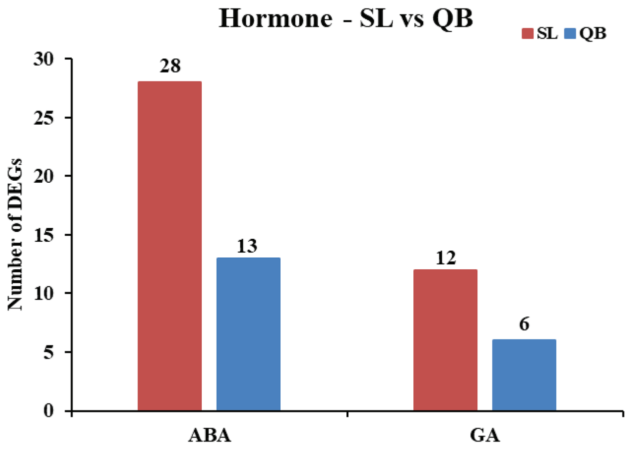

FIGURE 10 | Statistics of DEGs related to negatively regulating abscisic acid (ABA) and positively regulating gibberellin (GA) in Swan Lake (SL) and Qingdao Bay (QB).

DISCUSSION

According to the results of the seed germination time of the natural population of *Z. marina* observed for many years, the seeds from QB mainly germinate in autumn of the same year as seed maturation, and the seeds from SL mainly germinated in spring of the following year. In this study, we carried out a field experiment transplanting seeds from QB to SL and from SL to QB. The results showed that seeds from QB still germinated in autumn regardless of whether they were planted in QB or SL, and seeds from SL, whether planted in QB or SL, still germinated in the following spring. We also conducted comparative transcriptome analysis in autumn (germination stage of seeds in QB and dormant stage of seeds in SL). The results showed that genes involved in some metabolic pathways, such as energy metabolism, lipid metabolism, amino acid metabolism, and biosynthesis of secondary metabolites, were significantly upregulated in the seeds of QB compared with those of SL, which indicated that the seeds in QB were gradually emerging from dormancy and starting the germination process, while the seeds in SL were still dormant. Therefore, we believe that the factors that determine the seed germination time at the two sites are not environmental factors, but their different internal molecular mechanisms.

Comparative transcriptome analysis on seeds at the early germination stage in both places were conducted, mainly from four aspects: the overall comparison of DEGs in germination, the comparison of energy metabolism-related pathways, the comparison of major hormone-related genes controlling germination and the comparison of genes related to cell changes. Seed dormancy is a common phenomenon in the plant kingdom, which allows seeds to survive unfavorable conditions for a long time before germination and to establish plants under the most favorable conditions (Cadman et al., 2006). In many cases, seeds acquire primary dormancy during seed maturation and may enter a state of secondary dormancy if seeds are exposed to unsuitable temperature or lack adequate light (Finkelstein et al., 2008). Presumably, each seed is in a state somewhere along the continuum from deeply dormant to non-dormant, and the transition between different dormancy states is an active process

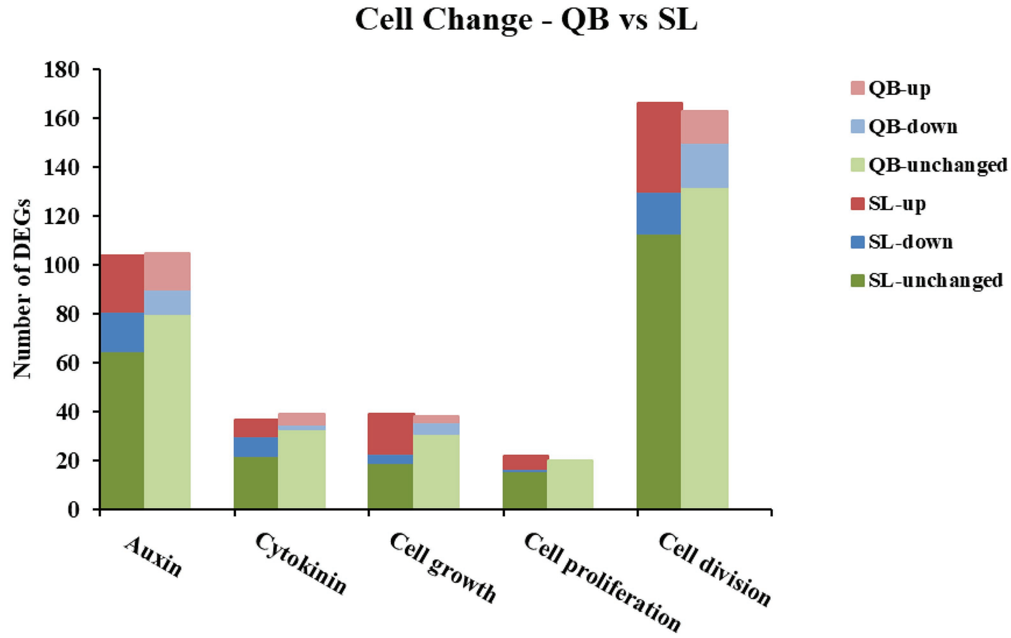

FIGURE 11 | Statistics of genes associated with cell changes in Swan Lake (SL) and Qingdao Bay (QB).

involving changes in gene expression (Finkelstein et al., 2008). To investigate whether the dormancy states of the seeds in the two regions were the same, transcriptome analysis was performed to characterize gene expression in different physiological states. Principal component analysis was applied to the expression of all RNA-seq genes between two different physiological states at two sites. The analysis found that the first dimension grouped the mature seeds of the two sites together and separated them from the seeds of the germination stage of the two sites. The second dimension showed that the seeds in the same location had a certain similarity, and the seeds in different locations had certain differences, especially the seeds in the germination stage, SL and QB had great separation. Cluster analysis (heat map) also showed that the gene expression patterns of SL-G and QB-G were similar on the whole, but the number of upregulated genes of early germination seeds in SL was much higher than that in QB. In a review of molecular studies on seed dormancy, Finkelstein concluded that the expression level of dormancy related genes was to some extent correlated with dormancy depth (Finkelstein et al., 2008). Cadman, in his study of *Arabidopsis* seed germination, found that the number of genes involved in breaking dormancy or inducing germination was much higher at the post-ripening stage than at the dormant stage (Cadman et al., 2006). Based on this, we speculated that the dormancy depth of the seeds in SL was deeper than that in QB.

Embryos must mobilize carbon and energy sources to germinate and grow (Finkelstein et al., 2008). KEGG enrichment analysis of upregulated DEGs sets in early germination seeds from SL and QB revealed that pyruvate metabolism and glycolysis were both common and significantly enriched pathways, indicating that seeds in this period had started to mobilize reserves to prepare energy supply for the subsequent germination processes. Previous studies have shown that the photosynthesis and mineral uptake systems are not active during seed germination, and the energy for seed physiological activities is provided by reserve mobilization (Yu et al., 2014). In the early stages of germination, the energy generated by molecule degradation (e.g., glycolysis, glyoxylate cycle, and tricarboxylic acid cycle) is presumed to be a key determinant of germination vigor (Galland et al., 2014). Early activation of transcripts of starch and lipid reserve mobilization pathways can provide sucrose and hexose, which provide energy until the cotyledons become photoautotrophic plants (Sreenivasulu et al., 2008). Because of the limited penetration of oxygen into dense seed tissue, the large amount of energy required for seed development seems to depend primarily on glycolysis (Yang et al., 2007). Han et al. (2017) also hypothesized that glycolysis provides the embryo with a large amount of energy for the biosynthesis of new compounds and other metabolic requirements. In addition, our KEGG enrichment results also revealed that five other pathways related to energy metabolism were significantly enriched in seeds from SL, while only two pathways, pyruvate metabolism and glycolysis, were significantly enriched in seeds from QB. This demonstrated that the energy pathway mobilized in the early germination stage of *Z. marina* seeds in SL was more active than that in QB. We hypothesized that the amount of energy required to break dormancy to initiate germination was also different, because of the different depths of seed dormancy at different sites.

A large number of studies have shown that plant hormones are key regulators of seed dormancy and germination in terrestrial plants, including ABA, GA, ethylene, auxin, and brassinosteriods, with ABA and GA being the most important regulatory factors that play antagonistic roles in seed germination (Holdsworth et al., 2008; Nambara et al., 2010; Han and Yang, 2015). ABA is a positive regulator of induction and maintenance of dormancy, and a negative regulator of germination, while GA can counteract the effect of ABA, releasing dormancy and promoting seed germination (Kucera et al., 2005). ABA deficiency is associated with the loss of primary dormancy in mature seeds during seed development in many plants, and the overexpression of ABA biosynthetic genes can increase ABA content, enhance seed dormancy, or delay seed germination (Finkelstein et al., 2002; Nambara and Marion-Poll, 2003; Kushiro et al., 2004). Studies have shown that ABA restricts embryo growth potential by inhibiting water absorption (imbibition) and cell wall loosening, which are critical steps in the initiation of germination (Schopfer and Plachy, 1984; Gimeno-Gilles et al., 2009). GA, on the other hand, weakens the restriction of barrier tissues such as the endosperm or seed coat by inducing hydrolase, induces the mobilization of seed storage materials, and stimulates the expansion of the embryo to stimulate seed germination (Bewley, 1997). Studies have found that mutants with defects in the gene encoding GA biosynthetase in some species cannot germinate (Mitchum et al., 2006; Steber, 2008). In this study, it was found that the number of genes leading to the decrease of ABA content in SL during the early germination stage was higher than that in QB, with the number in SL being twice that in QB (SL:28 vs. QB:13). Among these DEGs, those related to ABA degradation, MYB transcription factors, and carbohydrate metabolism were significantly upregulated, while those related to ABA response or ABA signal transduction were significantly downregulated. Similarly, the number of genes responsible for the increase of GA content in SL was twice as high as that in QB (SL:12 vs. QB:6). In these DEGs, those related to the synthesis, perception, and regulation of GA were significantly upregulated, while those related to the decomposition of GA were significantly downregulated. As previously mentioned, ABA and GA are positive regulators of dormancy maintenance and dormancy breaking, respectively. Our results demonstrated that there were more genes related to emergence from dormancy when the seeds were about to germinate in SL seeds compared with those from QB. Therefore, it was speculated that the dormancy depth of the seeds of SL was deeper than QB.

Seed germination is a series of physiological and morphogenetic processes, where the plant embryo resumes growth after a period of quiescence (Barrôco et al., 2005). Considerable experimental evidence compels people to believe that the resumption of cell cycle activity is a specific feature of early germination (de Castro et al., 2000; Gallardo et al., 2002; Vázquez-Ramos and De la Paz Sánchez, 2003). In terrestrial dicotyledon seeds, the embryonic root first emerges from the seed, and then the embryonic shoot emerges from the seed after absorbing water. In terrestrial monocotyledon seeds, the cotyledon and radicle are covered by a coleoptile and coleorhiza, respectively, and the coleorhiza and radicle grow out of the seed in sequence. Following this, the coleoptile is pushed up until it reaches the surface (Nonogaki et al., 2010; Rajjou et al., 2012; Xue et al., 2021). However, eelgrass is a marine monocotyledon, whose seed germination process is slightly different to that of terrestrial seeds (Taylor, 1957). When the seeds germinate, the cotyledon sheath elongates first, then the first true leaf elongates from inside of the cotyledon sheath. Following this, the second true leaf and the adventitious roots emerge from inside of the cotyledon sheath (Sugiura et al., 2009). The radicle of eelgrass seeds never develops, so no primary root is formed and the adventitious root is the only true root of eelgrass seeds (Taylor, 1957). The rapid growth of the embryos, which eventually leads to the rupture of the covering layers and the emergence of the radicle, is considered to be the completion of seed germination in terrestrial plants (Barrôco et al., 2005). At this stage, the decision of individual embryo cells reenter the cell cycle or continue to be arrested is crucial for seedling formation. The establishment of plant shape and function depends on the ability of embryo cells to resume division and differentiation (Barrôco et al., 2005). In this study, we identified genes related to cell cycle and cell changes in SL and QB gene sets, which revealed that the number of upregulated DEGs in SL was 2.5 times that in QB at the early stage of germination. Therefore, we speculate that the dormancy depth of SL seeds is deeper, so more genes related to the cell cycle and cell changes need to be mobilized in the early stages of germination to initiate the subsequent germination process.

Germination is usually controlled by both internal factors, such as genetic control, and external factors, such as water temperature, which was very important in the field (Orth et al., 2000). According to Xu et al. (2018) and our observations over the years, although the latitude of SL is only 1°higher than that of QB, the temperature of SL is much lower than that of QB in winter. The intertidal zone of the QB have little chance to be covered by ice, while most of the intertidal zone in SL is covered by ice in winter, even the entire lagoon is covered by ice in some years. Phillips et al. (1983) reported that ice scouring in winter is an important source of eelgrass mortality at the northern margin of the range in the Bering Sea. Thus, eelgrass seeds from the SL population might be not acclimated to an autumn germination, because their seedlings cannot survive the severe winter. By contrast, seeds from the QB population can germinate in autumn and their seedlings can survive the relatively warm winter. Therefore, in autumn, seeds from SL would choose to go into a deeper level of dormancy to survive the cold winter, while seeds from QB would choose to germinate, which suggests that long-term differences in winter temperatures may be an important factor for the genetic differences.

CONCLUSION

The results of field observations, the reciprocal transplant experiment, and molecular analysis were consistent, which supports our hypothesis that differences do exist in the seed germination period at two sites, and that these differences are not controlled by environmental factors but are determined by internal molecular mechanisms. Therefore, when using seed for seagrass bed restoration, it is necessary to take into account the germination characteristics of the donor seeds and choose the correct time for sowing. In addition, the comparative transcriptome analysis of seeds at the early germination stage at both sites from the aspects of overall germination DEGs, energy metabolism related pathways, major hormone genes controlling germination and genes related to cell changes, revealed that the dormancy depth of the seeds in SL was deeper than that in QB, and the long-term differences in winter temperature between the two sites might be the important factor for the genetic differences in seed germination time or dormancy depth between the two populations from different geographic regions.

DATA AVAILABILITY STATEMENT

All sequencing data are available through the NCBI Sequence Read Archive under the accession number PRJNA770030, and other data that support the findings of this study are available from the corresponding author, without undue reservation.

AUTHOR CONTRIBUTIONS

YZa: writing – original draft, conceptualization, methodology, and investigation. SX: writing – review and editing, conceptualization, and investigation (especially reciprocal seed transplant experiment). SY, XZ, YQ, and ML: investigation. YZo: funding acquisition, supervision, methodology, investigation, and writing – review and editing. All authors contributed to the article and approved the submitted version.

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Intrinsic Photosensitivity of the Vulnerable Seagrass *Phyllospadix iwatensis*: Photosystem II Oxygen-Evolving Complex Is Prone to Photoinactivation

Mengxin Wang†, Wei Zhao†, Mingyu Ma†, Di Zhang, Yun Wen, Mingyu Zhong, Chengying Luo, Zimin Hu and Quansheng Zhang*

Ocean School, Yantai University, Yantai, China

Phyllospadix iwatensis, a foundation species of the angiosperm-dominated marine blue carbon ecosystems, has been recognized to be a vulnerable seagrass. Its degradation has previously been reported to be associated with environmental changes and human activities, while there has been a limited number of studies on its inherent characteristics. In this study, both the physiological and molecular biological data indicated that the oxygen-evolving complex (OEC) of *P. iwatensis* is prone to photoinactivation, which exhibits the light-dependent trait. When exposed to laboratory light intensities similar to typical midday conditions, <10% of the OEC was photoinactivated, and the remaining active OEC was sufficient to maintain normal photosynthetic activity. Moreover, the photoinactivated OEC could fully recover within the same day. However, under harsh light conditions, e.g., light intensities that simulate cloudless sunny neap tide days and continual sunny days, the OEC suffered irreversible photoinactivation, which subsequently resulted in damage to the photosystem II reaction centers and a reduction in the rate of O_2 evolution. Furthermore, *in situ* measurements on a cloudless sunny neap tide day revealed both poor resilience and irreversible photoinactivation of the OEC. Based on these findings, we postulated that the OEC dysfunction induced by ambient harsh light conditions could be an important inherent reason for the degradation of *P. iwatensis.*

Keywords: degeneration, oxygen-evolving complex, *Phyllospadix iwatensis*, photoinactivation, seagrass

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Xenie Johnson,
Commissariat à l'Energie Atomique et aux Energies Alternatives (CEA), France

Reviewed by:
Alonso Zavafer,
The Australian National University, Australia
Kohtaro Iseki,
Japan International Research Center for Agricultural Sciences (JIRCAS), Japan

***Correspondence:**
Quansheng Zhang
ytuqsz@hotmail.com

† These authors have contributed equally to this work and share first authorship

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INTRODUCTION

The seagrass *Phyllospadix iwatensis* (Zosteraceae), characterized by a well-developed root system and reddish-brown hairy fibers on the rhizomes, is naturally distributed in the rocky intertidal zone of the Northern Hemisphere (Cao et al., 2015; Li et al., 2020). As an important constituent species of angiosperm-dominated marine blue carbon ecosystems, *P. iwatensis* can form vast "underwater meadows," which not only have an enormous ability to store carbon (Mcleod et al., 2011; Fourqurean et al., 2012) but also provide important habitat, spawning grounds, and food sources for various organisms in coastal areas (Cullen-Unsworth et al., 2018; Costa et al., 2020; Jiang et al., 2020; Rodriguez and Heck, 2020).

However, increasing amounts of research suggest that seagrasses are currently experiencing a global decline (Orth et al., 2006). *P. iwatensis* was assigned to the "vulnerable" category of the International Union for the Conservation of Nature red list in 2011 (Short et al., 2011). In China, the seaweed house, whose roof is primarily built by *P. iwatensis*, is a provincial intangible cultural heritage in Rongcheng, Shandong Province, with a history of >400 years (Yajing, 2021). However, seagrass beds in Shandong Province have shrunk by 90% during the past 20 years. At this point in time, *P. iwatensis* is scattered only in this coastal area to the extent that it is unable to meet the needs of housing restoration (Zheng et al., 2013).

Recently, with the extensive research on the degradation of seagrasses, most investigators believe that global climate change and human activities underlie the demise of their populations (Short and Wyllie-Echeverria, 1996; Short and Neckles, 1999). Since they inhabit shallow seas, seagrasses are exceptionally sensitive to changes in temperature and light. Therefore, an increase in the temperature of seawater owing to global climate change is an important cause of their degradation that cannot be ignored (Valle et al., 2014; Repolho et al., 2017; Duarte et al., 2018; Nguyen et al., 2021). Furthermore, eutrophication caused by human activities will induce the development of additional phytoplankton blooms, which can inhibit the photosynthesis performance of seagrass by reducing the light available (Hauxwell et al., 2003; Burkholder et al., 2007). Moreover, in many coastal areas, the fragmentation of habitat, pollution, overfishing, and biological invasion that can be caused directly by human activities also seriously threaten the survival of seagrasses (Short and Wyllie-Echeverria, 1996; Herrera-Silveira et al., 2010; Hall-Spencer and Harvey, 2019). Although the current causes of degradation have been studied extensively, less attention has been paid to the intrinsic vulnerability of seagrasses.

Oxidation of water molecules to electrons and molecular oxygen occurs in the oxygen-evolving complex (OEC) of photosystem II (PSII) (Cady et al., 2008; Gupta, 2020). The OEC is composed of manganese (Mn) ions, calcium (Ca) ions, peripheral proteins, and cofactors, and damage to each of these components results in the inactivation of the OEC (Nishimura et al., 2016). When the OEC is impaired, the lifetime of $P680^+$ is prolonged, since the OEC cannot transfer electrons to the PSII reaction center quickly enough. $P680^+$ is a strong oxidant that can oxidatively destroy the D1 protein in the PSII reaction center and then damage pigments such as carotenoids and chlorophyll (Tyystjärvi, 2008). We have recently observed that the OEC of the *Zostera marina* is preferentially inactivated under visible light (Tan et al., 2020; Zhao et al., 2021). Based on this, we chose to study the photosynthetic regulatory mechanisms induced by OEC photoinactivation under controlled laboratory conditions (Tan et al., 2020; Zhao et al., 2021). However, whether the dysfunction in OEC owing to photoinactivation can cause a decline in the productivity of seagrass, and even worse, in the degradation of population, is an extremely interesting topic that merits more attention.

In this article, the vulnerable seagrass *P. iwatensis* was utilized as the research object, and the OEC of *P. iwatensis* was confirmed to be prone to photoinactivation by the use of a mutually corroborative strategy of physiology and molecular biology. Furthermore, based on the fact that the photoinactivation of OEC inhibits its photosynthetic performance and even leads to irreversible damage in the photosynthetic apparatus, we suggest that the photoinactivation of OEC may be an important inherent reason for the degradation of *P. iwatensis.*

MATERIALS AND METHODS

Plant Materials

Healthy specimens of *P. iwatensis*, characterized by intact rhizome systems and fresh leaves that lacked mechanical injury (**Figure 1**), were collected from the rocky intertidal zone of Rongcheng (37° 16′N, 122° 41′E), Weihai, Shandong Province, China, throughout May 2021. The samples were precultured in aquaria with filtered seawater for 3 days under conditions of 15°C and a 10:14 h light: dark cycle with a minimum saturating irradiance of 40 μmol photons/m^2 s.

Experimental Treatments

Precultured *P. iwatensis* samples were acclimatized in the dark overnight prior to experimentation. To evaluate the dynamics of the OEC activity, dark-adapted samples were exposed to a light intensity of 400 μmol photons/m^2 s, which was the usual underwater light conditions measured in *P. iwatensis* habitats during midday. The samples were exposed for 6 h, with measurements taken every 1 h during this period. Furthermore, to investigate the ability of OEC to recover, two light environments were simulated to treat the samples. Pretreated samples were exposed to 400 μmol photons/m^2 s for 3 h to simulate the high light environment at midday under usual conditions. Following 1, 2, and 3 h of light exposure, a portion of the samples was taken for recovery under dark conditions in 15°C seawater. Measurements were taken every 1 and 3 h during exposure to light and recovery, respectively. In addition, based on the light environments of cloudless sunny neap tide days and continual sunny days, the times of exposure were extended to 6 h per day for 3 continuous days, which was established as the harsh light stress. Following light exposure, samples were recovered in the dark at 15°C. Measurements were taken at the beginning and end of each light exposure. All the experiments were conducted in aquaria. The light source was provided by an LED lamp with a color temperature range of 6,000 K (LI-COR Inc., Lincoln, NE, United States). The leaf segments were randomly collected from 2 cm above the leaf sheath before the parameters were measured, with a portion of the samples used for fluorescence measurements after 15 min of dark adaptation, while the other portions were immediately frozen in liquid nitrogen for subsequent Western blot analyses. The subscript "control" indicated that the parameter was derived from dark-adapted samples under controlled laboratory conditions. The subscript "treatment" indicated that the parameter was derived from samples treated with light stress in the laboratory. Each measurement was conducted in triplicate.

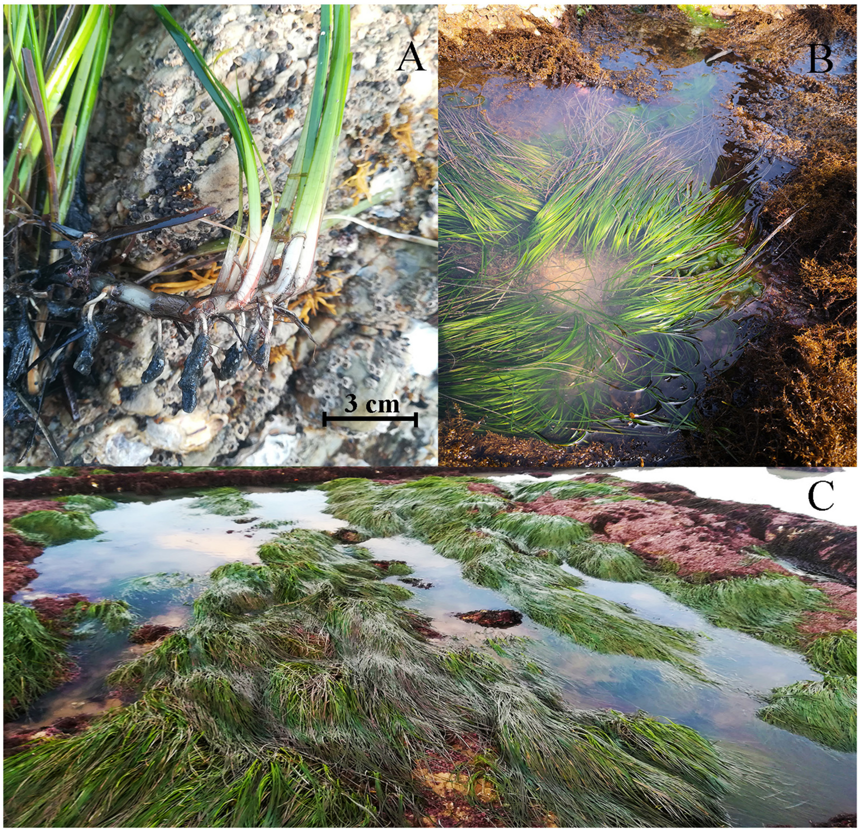

FIGURE 1 | *Phyllospadix iwatensis*, characterized by reddish-brown hairy fibers at the base of the plant and a well-developed root system **(A)**, is naturally distributed in the rocky intertidal zone and can form vast "underwater meadows" **(B,C)**.

Field *in situ* Measurements

To examine the *in situ* inactivation of the OEC, *in situ* measurements were conducted on a cloudless sunny neap tide day in May 2021 at the intertidal zone of a rocky shore with high seawater transparency in North Rongcheng (37°16'N, 122°41'E). Apparently healthy *P. iwatensis* plants were collected by diving every 2 h from 6:00 to 18:00 and at 6:00 and 8:00 on the following morning. Leaf segments that had been collected from ∼2 cm above the leaf sheath were partially used for chlorophyll fluorescence measurements, while the remainder were immediately frozen in liquid nitrogen for subsequent Western blot analyses. The light intensity of *P. iwatensis* habitats was measured using QSPL2100 (Biospherical Instruments Inc., San Diego, CA, United States). The subscript "control" indicated that the parameter was derived from samples collected at 6:00 on the first day of measurement under natural conditions in the field. The subscript "treatment" indicated that the parameter was derived from samples at different time points under natural conditions. Each measurement was conducted in triplicate.

Chlorophyll a Fluorescence Measurements

To analyze the photosynthetic physiological parameters, M-PEA-2 (Hansatech, Norfolk, United Kingdom) was used to monitor the rapid chlorophyll fluorescence induction kinetic curve (OJIP curve). The OJIP curve was induced by 5,000 μmol photons/m^2 s red light at a measurement time of 2 s. Chlorophyll fluorescence parameters were calculated as previously described (Strasser et al., 2010; Guo et al., 2020). The normalized OJIP fluorescence rise kinetics could be calculated using the formula $\Delta Vt = \Delta[(F_t - F_O)/(F_m - F_O)]$; $F_v/F_m = (F_m - F_O)/F_m$, which represented the maximal quantum yield of the PSII; $W_K = (F_K - F_O)/(F_J - F_O)$ and $\Delta W_K = [(F_K - F_O)/(F_J - F_O)]_{\text{treatment}} - [(F_K - F_O)/(F_J - F_O)]_{\text{control}}$, which reflected the extent of damage on the donor side of PSII; the active fraction of OEC centers, characterized by $\text{OEC}_{\text{centers}}$, could be calculated as $\text{OEC}_{\text{centers}} = [1 - (V_K/V_J)]_{\text{treatment}}/[1 - (V_K/V_J)]_{\text{control}}$. A G-band observed between the I-step and P-step was supposed to relate to the redox state of the end PSI acceptor pool (Zagorchev et al., 2021).

Western Blotting Analysis

The levels of expression of the OEC peripheral proteins PsbO, PsbP, PsbQ, and PSII core protein D1 were used to verify the activities of the OEC and PSII reaction center. The chloroplasts from leaves were separated using a Plants Leaf Chloroplast Rude Divide Kit (GenMED Scientifics Inc., Arlington, MA, United States). The chlorophyll content of the separated chloroplasts was measured as described earlier

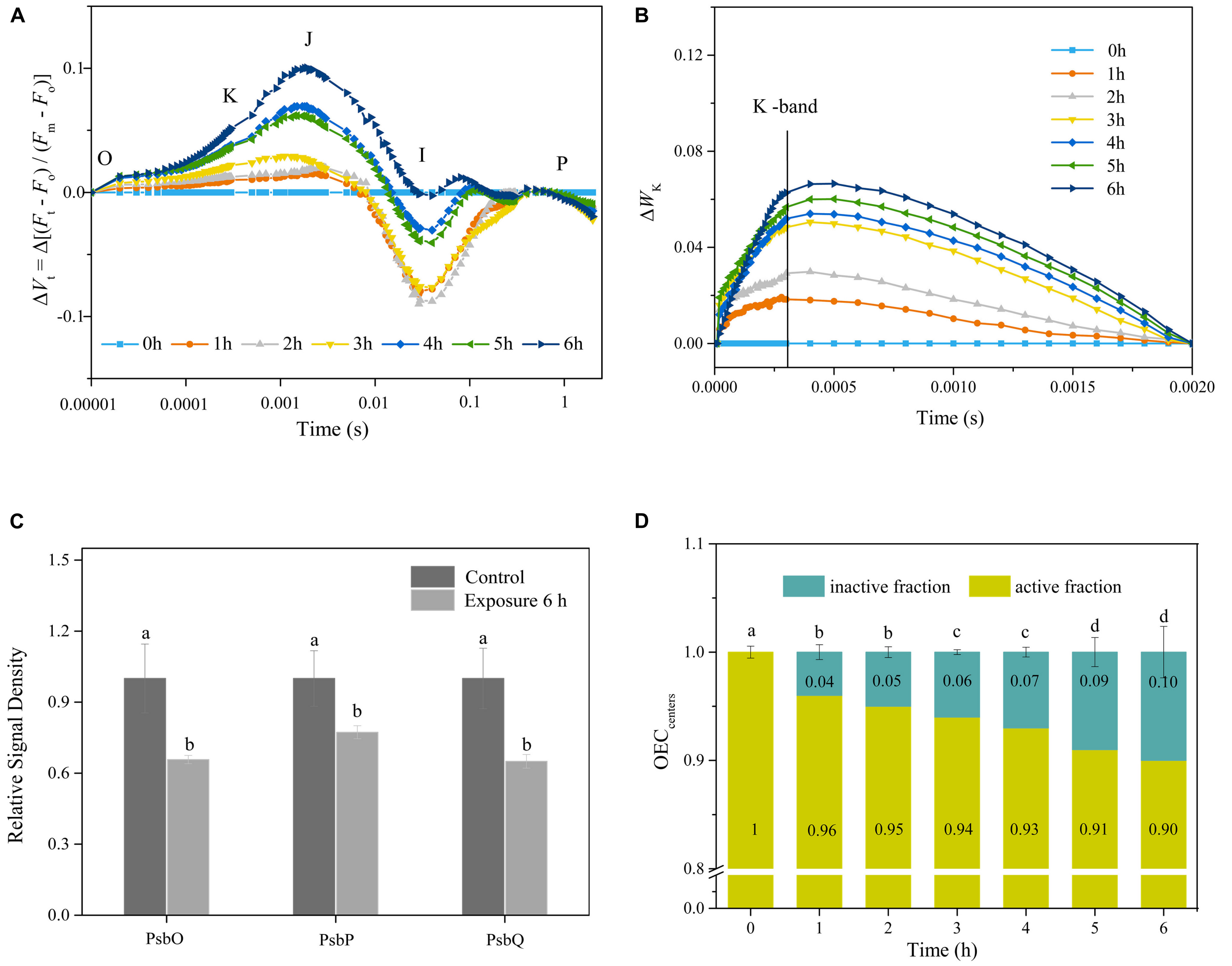

FIGURE 2 | (A,B) Time courses of normalized chlorophyll a fluorescence intensity of ΔV_t and ΔW_K curves in response to HL (400 μmol photons/m^2 s) exposure. Each curve represents the average of three replicates. **(C)** The relative expression levels of OEC peripheral proteins after 6 h HL exposure. The means ± SD were calculated from three independent samples. Different letters indicate a significant difference (Tukey's tests, $p < 0.05$). **(D)** Variations in OEC$_{centers}$ in response to HL exposure. The means ± SD were calculated from three independent samples. Different letters indicate a significant difference (Tukey's tests, $p < 0.05$).

(Porra et al., 1989). To compare quantitative differences, serial dilutions (1.25, 2.5, and 5 μg of chlorophyll corresponding to 25, 50, and 100% of the control samples) of the control samples were loaded onto the first three lanes of a 12% SDS-triglycine-PAGE gel, followed by loading equal contents of chlorophyll (5 μg) of the solubilized materials of treated samples onto the same gel for separation. They were then transferred to PVDF membranes (0.22 μm; Sartorius Stedim, Göttingen, Germany). Western blot assays with antibodies against PsbO, PsbP, PsbQ, and D1 (Agrisera, Vännäa, Sweden) were performed as described earlier (Fristedt et al., 2009). RuBisCo large subunits (RbcL) with the same loading volume (5 μg of chlorophyll) as the proteins above were used as equal loading controls. The chemiluminescent bands were quantified on a Gel Doc XR + system (Bio-Rad, Hercules, CA, United States) using Image Lab software (Bio-Rad). The total sample density in each case was normalized based on the RbcL density. Each measurement was conducted in triplicate.

Oxygen Evolution Rate Measurements

The rate of photosynthetic O_2 evolution was measured to evaluate the overall photosynthetic performance. This rate was determined using a liquid-phase oxygen electrode system (Chlorolab2 + ; Hansatech, Norfolk, United Kingdom) at 15°C. Leaf fragments (∼25 mg) were placed in the reaction chamber with 2 ml of seawater. A white light of 40 μmol photons/m^2 s was used to measure the evolution of O_2. The net photosynthetic rate (Pn) of the leaves was measured within 3 min of light irradiation, and the respiration rate (R) was measured within 2 min of the dark state. The rate of O_2 evolution (P) was calculated as $P = R + Pn$ and expressed as nmol O_2/min g fresh mass. Each measurement was conducted in triplicate.

Data Analysis

A statistical analysis of the collected parameters was performed using a one-way ANOVA in SPSS 22.0 (IBM Inc., Armonk, NY,

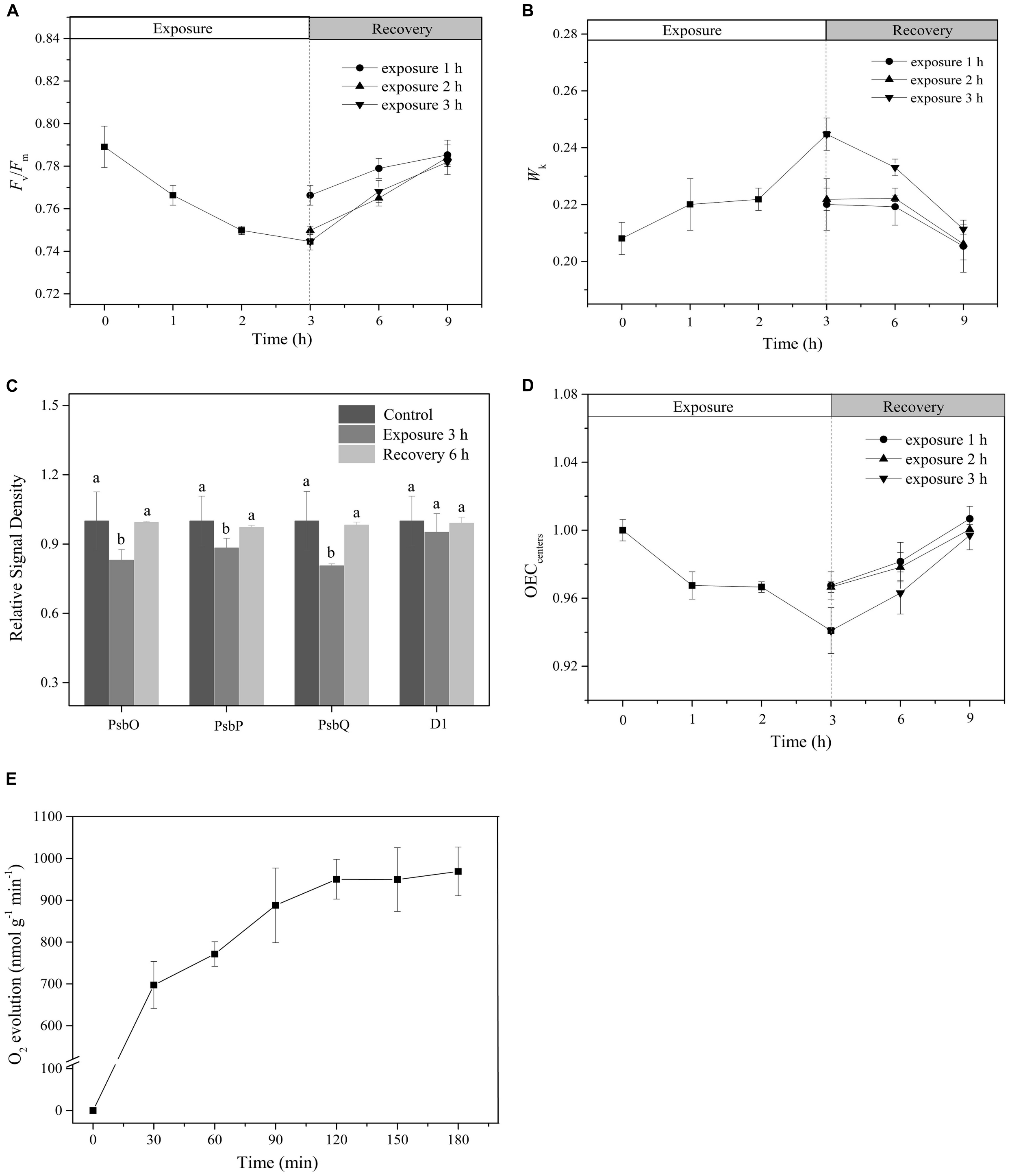

FIGURE 3 | The recovery of photoinactivated OEC. **(A,B)** Time course of changes in F_v/F_m and W_K. White and light gray rectangles represent HL (400 μmol photons/m^2 s) exposure and dark recovery period, respectively. Circle, triangle, and inverted triangle represent the different recovery start times: after HL exposure for 1, 2, and 3 h, respectively. **(C)** The relative expression levels of OEC peripheral proteins and PSII core D1 protein after 3 h HL exposure and subsequent 6 h recovery. The means ± SD were calculated from three independent samples. Different letters indicate a significant difference (Tukey's tests, $p < 0.05$). **(D)** Time course of changes in $OEC_{centers}$. White and light gray rectangles represent HL exposure and dark recovery period, respectively. Circle, triangle, and inverted triangle represent the different recovery start time: after HL exposure for 1, 2, and 3 h, respectively. **(E)** Changes in O_2 evolution rate. The means ± SD were calculated from three independent samples.

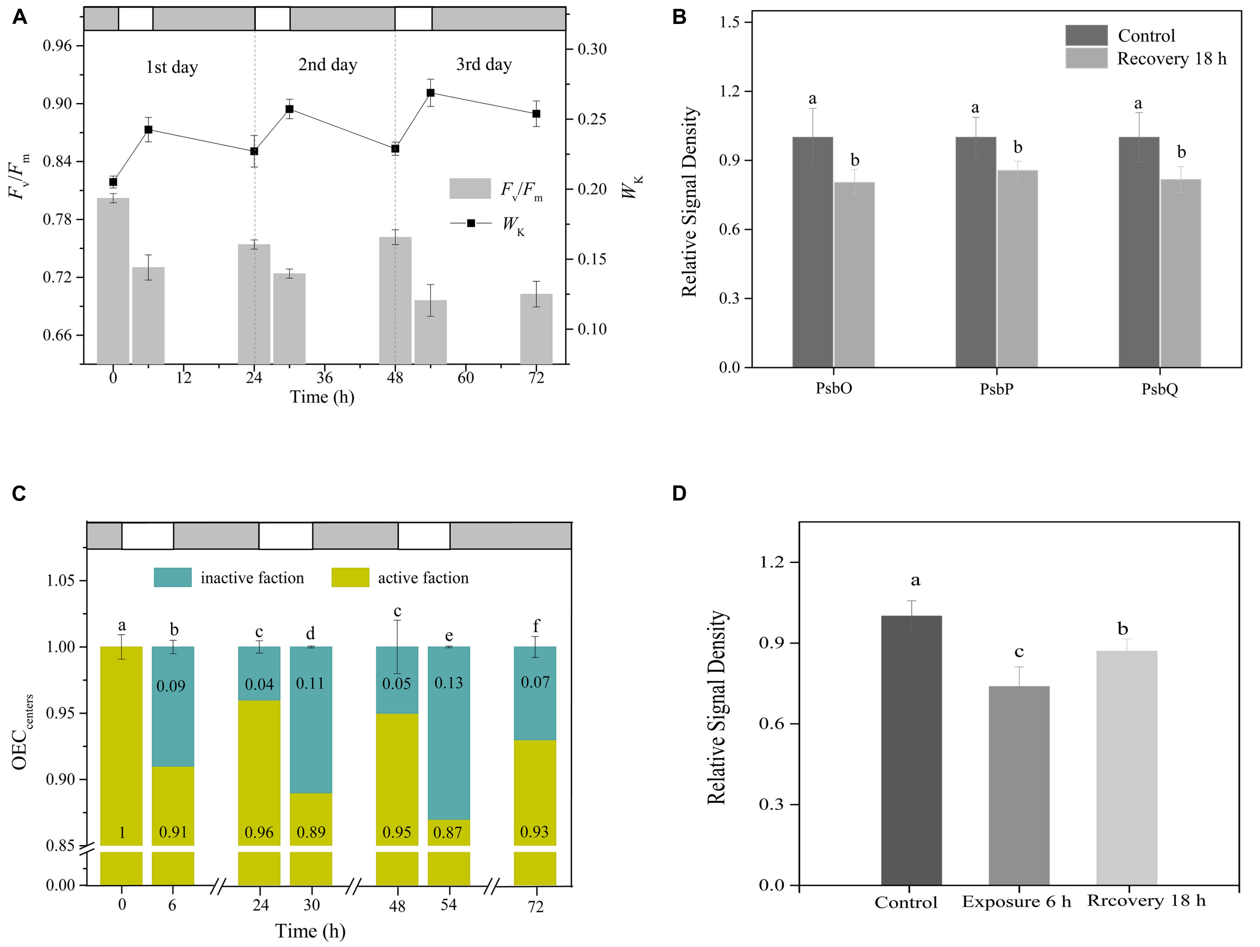

FIGURE 4 | Irreversible photoinactivation of OEC. **(A)** Time courses of changes in F_V/F_m and W_K in response to 6 h of HL (400 μmol photons/m^2 s) exposure per day for 3 continuous days. White and light gray rectangles represent HL exposure and dark recovery period, respectively. **(B)** The relative expression levels of OEC peripheral proteins after recovery from 6 h HL exposure. The means ± SD were calculated from three independent samples. Different letters indicate a significant difference (Tukey's tests, $p < 0.05$). **(C)** The changes in $OEC_{centers}$. White and light gray rectangles represent HL exposure and dark recovery period, respectively. Different letters indicate a significant difference (Tukey's tests, $p < 0.05$). **(D)** The relative expression levels of PSII core protein D1 in response to 6 h HL and the subsequent recovery for 18 h. The means ± SD were calculated from three independent samples. Different letters indicate a significant difference (Tukey's tests, $p < 0.05$).

United States). *Post hoc* comparisons were made using Tukey's trend test. Statistical significance was assessed at a threshold of $p < 0.05$.

RESULTS

Photoinactivation of Oxygen-Evolving Complex

The ΔVt curves of fluorescence rise kinetics normalized by OJIP changed with an increase in the time of exposure, suggesting that the performance of PSII was significantly affected by the duration of exposure (**Figure 2A**). The intensity of K-band at 0.3 ms in the curve of ΔW_k increased gradually with the duration of exposure, indicating that the OEC was continuously inactivated (**Figure 2B**). Furthermore, the activity of OEC, characterized by the peripheral protective protein contents of PsbO, PsbP, and PsbQ, decreased significantly during light exposure (**Figure 2C** and **Supplementary Figure 1**). Indeed, high light (HL) induced the partial inactivation of active OEC, as demonstrated by the variation in the physiological parameter $OEC_{centers}$ (**Figure 2D**). The percentage of OEC inactivation continuously increased with the duration of exposure, with 6 h of exposure resulting in about 10% of inactivation (**Figure 2D**).

Reversible Photoinactivation of Oxygen-Evolving Complex

Following a single HL exposure for 3 h, both the F_v/F_m and W_k gradually recovered during darkness (**Figures 3A,B**). Furthermore, the Western blot showed that the protein contents of PsbO, PsbP, and PsbQ increased during the recovery period (**Figure 3C** and **Supplementary Figure 1**). All these parameters

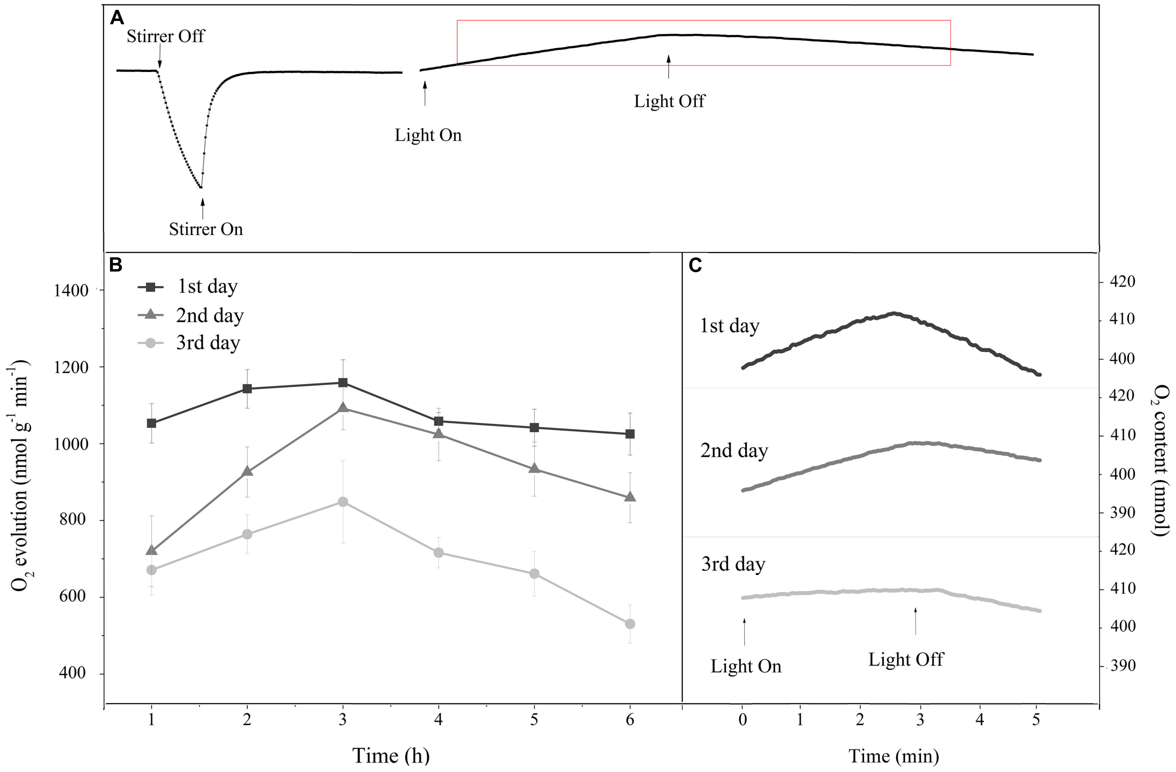

FIGURE 5 | (A) A typical oxygen content change curve in the reaction chamber of the Liquid-Phase Oxygen Electrode System for monitoring the O_2 evolution rate. **(B)** Time course of the O_2 evolution rate in response to 6 h of HL (400 μmol photons/m^2 s) exposure per day for 3 continuous days. Square, triangle, and circle represent the continuous exposure for 1st, 2nd, and 3rd day, respectively. The means ± SD were calculated from three independent samples. **(C)** Changes in the oxygen content curve during the O_2 evolution rate monitoring after 6 h of continuous HL exposure per day. The rising slope of the curve under light represents the net photosynthetic rate, and the falling slope of the curve in darkness represents the respiration rate. Each curve is based on the average of three replicates.

reached their initial levels after 6 h of recovery, which was also confirmed by the full recovery of $OEC_{centers}$ (Tukey's test, $p = 0.171$, $p = 0.13$, $p = 0.055$, $p = 0.073$, $p = 0.068$, and $p = 0.54$, respectively; **Figures 3A–D**). No net loss of the D1 protein was observed during illumination (Tukey's test, $p = 0.86$; **Figure 3C** and **Supplementary Figure 1**), indicating that the PSII reaction centers did not suffer net damage.

To further verify the effect of light exposure on the photosynthetic performance of *P. iwatensis*, the rate of O_2 evolution was measured. Within a single 3-h period of exposure to HL, the rate of O_2 evolution gradually increased, exhibiting a typical photoinduction process (**Figure 3E**), which represents a normal photosynthetic performance.

Irreversible Photoinactivation of Oxygen-Evolving Complex

Following a single 6-h exposure, F_v/F_m and W_k failed to reach their initial levels until the following day (Tukey's test, $p < 0.05$, $p < 0.05$, respectively; **Figure 4A**). This was also true for the changes in both the OEC peripheral protein contents and $OEC_{centers}$ (Tukey's test, $p < 0.05$, for both OEC peripheral proteins and $OEC_{centers}$; **Figures 4B,C** and **Supplementary Figure 1**). These observations indicated the occurrence of irreversible photoinactivation of OEC. Furthermore, irreversible oxidative damage to the PSII reaction centers was observed, as indicated by the D1 protein, which suffered a net loss during illumination and did not return to its original state by the morning of the next day (Tukey's test, $p < 0.05$; **Figure 4D** and **Supplementary Figure 1**).

During 6 h of HL exposure per day for 3 continuous days, F_v/F_m and W_k exhibited rhythmic changes of the light-dark cycle, with gradual decreases and increases, respectively, during the day and incomplete recovery at night, which made F_v/F_m and W_k keep decreasing and increasing as the days progressed (**Figure 4A**). Moreover, aside from the decrease in $OEC_{centers}$ with the extended days of exposure, the proportion of OEC that did not regain activity after dark adaptation overnight also increased daily (**Figure 4C**). These results indicated the occurrence of continuous irreversible photoinactivation of OEC.

The complete measurement process of the O_2 evolution rate is shown in **Figure 5A**. When light exposure exceeded 3 h, the rate of O_2 evolution decreased gradually, indicating that the photosynthetic performance was inhibited following long light exposure (**Figure 5B**). Additionally, under daily light stress, both the net photosynthetic rate in the light and the respiration rate in the dark decreased daily (**Figure 5C**). The O_2 evolution rate calculated from them exhibited a similar trend (**Figure 5B**).

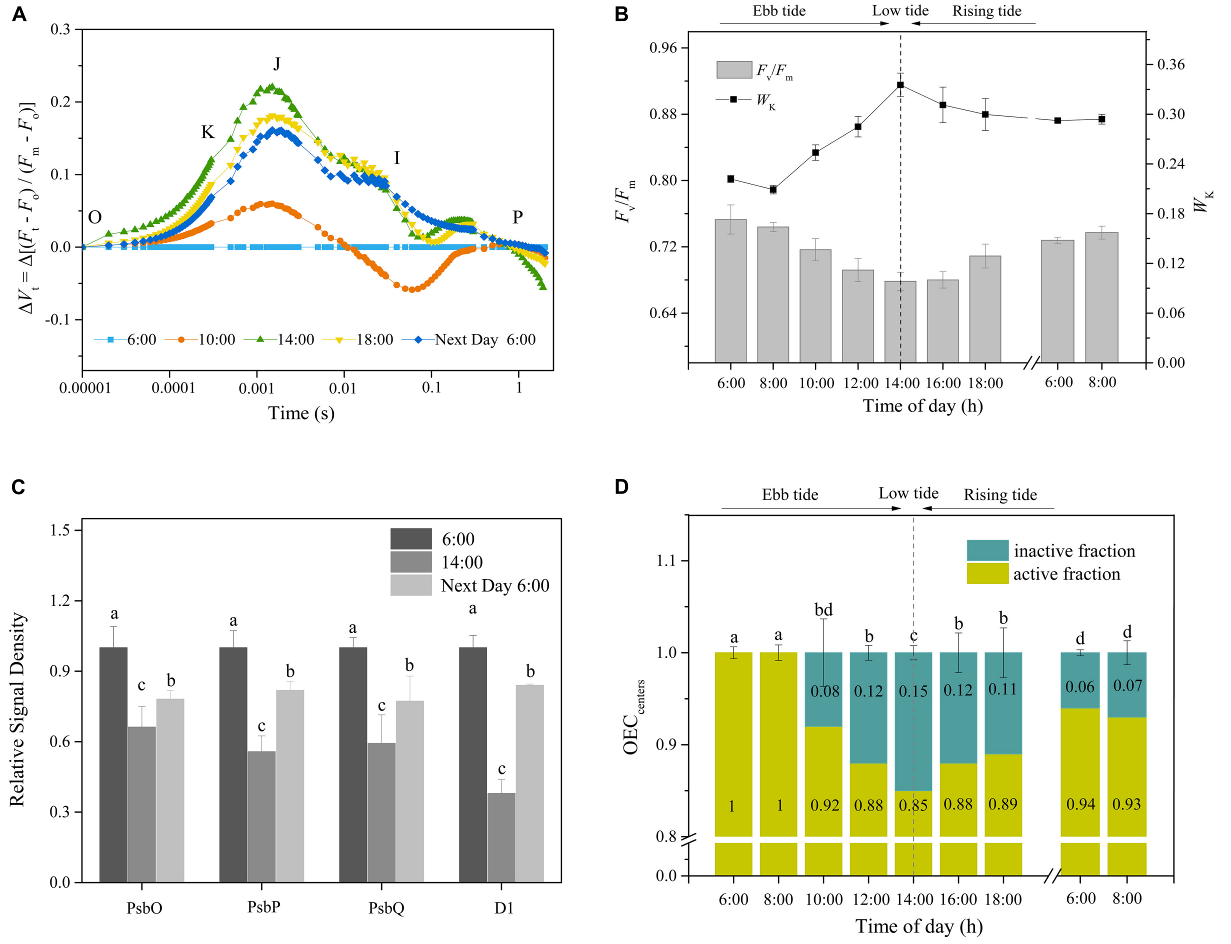

FIGURE 6 | The *in situ* occurrences of OEC photoinactivation. **(A,B)** Changes in the ΔV_t curves, F_v/F_m, and W_K in response to the tidal cycle. **(C)** The relative protein expression levels of PsbO, PsbP, PsbQ, and D1 in the early morning, lowest tide, and after dark recovery overnight. The means ± SD were calculated from three independent samples. Different letters indicate a significant difference (Tukey's tests, $p < 0.05$). **(D)** Time courses of $OEC_{centers}$ in response to the tidal cycle. Different letters indicate a significant difference (Tukey's tests, $p < 0.05$). The photosynthetic photon flux density (PPFD) at 8:00, 10:00, 12:00, 14:00, and 16:00 was around 100, 250, 350, 400, and 300 μmol photons/m^2 s, respectively. The means ± SD were calculated from three independent samples.

In situ Measurements of Oxygen-Evolving Complex Activity

To gain insight into the OEC photoinactivation of *P. iwatensis* in its natural environment, the chlorophyll fluorescence and protein contents were measured *in situ* on seagrass beds. As shown in **Figure 6A**, the ΔVt curves significantly fluctuated in a diurnal manner. In the morning, the decrease in F_v/F_m was accompanied by an increase in W_k, reaching their extreme values at 14:00. In the afternoon, F_v/F_m and W_k gradually recovered as the light intensity diminished, but their full recoveries were not observed by the next morning (Tukey's test, $p < 0.05$, $p < 0.05$, respectively; **Figure 6B**). Similarly, an incomplete recovery was also demonstrated in the OEC peripheral proteins and PSII core D1 protein contents (Tukey's test, $p < 0.05$, for both OEC and D1 proteins; **Figure 6C** and **Supplementary Figure 2**). The $OEC_{centers}$, as shown in **Figure 6D**, changed with the process of diurnal light fluctuations, with the highest rate of inactivation of approximately 15% at 14:00, which remained at 6% inactivation following an overnight recovery (Tukey's test, $p < 0.05$). Those results confirmed that *P. iwatensis* suffers irreversible OEC photoinactivation in a natural harsh light environment.

DISCUSSION

The manganese mechanism that is derived from the light-induced inactivation of the Mn cluster is an important component of the hypothesis of photo-inhibition (Ohnishi et al., 2005). This hypothesis considers that the light absorption by the Mn cluster in PSII leads to the release of Mn and the inactivation of OEC. This initial event precedes the damage to the PSII reaction center by light absorption by chlorophyll. A seminal work in pumpkin (*Cucurbita pepo*) showed that the release of an Mn ion into the thylakoid lumen was the earliest detectable step of photo-inhibition and that the action spectrum of photo-inhibition resembled the absorption spectra of Mn (III) and Mn

(IV) compounds (Hakala et al., 2005). Subsequently, Mn photoinhibition was observed by Sarvikas et al. (2006) in *Arabidopsis*, Oguchi et al. (2009) in capsicum (*Capsicum annuum*), He et al. (2015) in barley (*Hordeum vulgare*), and Oguchi et al. (2011a) and Iermak et al. (2020) in spinach (*Spinacia oleracea*), among others. Moreover, Oguchi et al. (2009, 2011a,b) suggested that both the energy excess mechanism, which states that excess energy absorbed by chlorophyll leads to PSII photoinactivation, and the manganese mechanism, operate during the process of photoinhibition, with the relative contribution of each mechanism depending on the plant species or growth conditions. All the studies described are fundamental for the corpus of manganese mechanism. In this mechanism, the OEC is inactivated by light absorbed by the Mn cluster, and a positive band in the OJIP curve at 0.3 ms, designated the K-step, may serve as a specific marker of OEC damage (Iermak et al., 2020).

In our study, both the data of relative variable fluorescence at the K point and abundance in the OEC peripheral stabilizing proteins PsbO, PsbP, and PsbQ confirmed the fact that the OEC of *P. iwatensis* was prone to photoinactivation, which was consistent with the characteristics of manganese mechanism. *P. iwatensis* primarily naturally inhabits intertidal areas where the light fluctuation depends on both daily variations and tidal changes. In our simulated experiments, the short light exposure (3 h) induced the increase in W_k and the decrease in OEC peripheral protein contents and $\text{OEC}_{\text{centers}}$ (<10%), which recovered rapidly as samples were transferred to the dark, suggesting an occurrence of the reversible photoinactivation of OEC. Moreover, the data for D1 protein content and the evolution of photosynthetic O_2 showed that photosynthesis was not significantly affected. As observed in *Z. marina*, when the OEC is partially inactivated, PSII-CEF and ascorbic acid (AsA) can supply electrons to the reaction centers, while the antioxidant system and controlled selective electron flow is not significantly activated, thereby allowing the photosynthetic system to efficiently use the limited electrons to maintain normal levels of carbon assimilation (Zhao et al., 2021). PSI-CEF operates efficiently to establish ΔpH, contributing to the maintenance of OEC stability (Tan et al., 2020). In contrast, after 6 h of light exposure, the F_v/F_m, W_k, OEC peripheral protein contents, and the $\text{OEC}_{\text{centers}}$ varied significantly and could not recover completely after spending overnight in the dark, indicating an occurrence of irreversible photoinactivation on both the OEC and PSII. Furthermore, the net loss of D1 protein and the reduced rate of O_2 evolution further suggested that a net damage to the photosynthetic system occurred. In terms of the continuous daily exposure, both the chlorophyll fluorescence and protein data showed the accumulation of inactivated OEC. The gradual decrease in the rate of O_2 evolution also indicated a continuous impairment of the photosynthetic performance. Based on these results, we postulated that the natural environments of cloudless sunny neap tide days and continuous sunny days under high seawater transparency could lead to the irreversible photoinactivation of OEC in *P. iwatensis.* To verify this hypothesis, the OEC activity of *P. iwatensis* was determined by *in situ* measurements on a cloudless sunny neap tide day. The lowest tide is at approximately midday, during neap tides, and therefore coincides with the most serious light exposure. As expected, both chlorophyll fluorescence and the level of expression of key proteins exhibited similar trends with the observations in the laboratory experiments, with the changes of W_k, F_v/F_m, OEC peripheral proteins, and $\text{OEC}_{\text{centers}}$ induced by light exposure had not fully recovered by the next morning, revealing the poor resilience of OEC. The 15 and 6% inactivation of OEC at the lowest tide and the following morning, respectively, indicated that the OEC had suffered irreversible damage under field environments. The irreversible photoinactivation of the OEC will undoubtedly affect the photosynthetic performance and may subsequently lead to plant mortality and population degradation. Therefore, we propose that the dysfunction of OEC induced by natural harsh light environments is probably an important inherent reason for the degradation of *P. iwatensis.*

The OEC of seagrasses prone to photoinactivation may be a result of the lack of protection by substances that function to shield the light owing to the absence of photoreceptors (Olsen et al., 2016; Ma et al., 2021). Additionally, chloroplasts are located on the external epidermis of leaves, which facilitate the transport and diffusion of inorganic carbon and also render the OEC prone to photoinactivation owing to the maximum light received by the chloroplasts (Beer et al., 2014).

CONCLUSION

The OEC of *P. iwatensis* is prone to photoinactivation, and it occurs in natural environments. Generally, the photoinactivation of OEC in *P. iwatensis* can be fully recovered on the same day without affecting the photosynthetic performance. However, the resilience of OEC is poor under harsh light conditions. In this case, the reduced photosynthetic performance and even the damaged photosynthetic apparatus caused by the irreversible photoinactivation of OEC will threaten the survival of seagrasses. Therefore, we suggest that OEC dysfunction induced by harsh light conditions such as cloudless sunny neap tide days and continuous sunny days under high transparency seawater may trigger destructive chronic impacts on *P. iwatensis* that can result in its degradation.

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

QZ got the project funding. QZ, WZ, MM, MZ, CL, and ZH designed the experiment. MW, WZ, MM, and YW carried out the experiment and analyzed the data. MW wrote the first version of the manuscript. DZ and QZ 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

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.792059/full#supplementary-material

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Jutta Papenbrock,
Leibniz University Hannover,
Germany

REVIEWED BY
Zhijian Jiang,
South China Sea Institute of Oceanology
(CAS), China
Kun-Seop Lee,
Pusan National University, South Korea

*CORRESPONDENCE
Ulisse Cardini
ulisse.cardini@szn.it
Gabriele Procaccini
gabriele.procaccini@szn.it

†These authors have contributed equally to this work

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Nested interactions between chemosynthetic lucinid bivalves and seagrass promote ecosystem functioning in contaminated sediments

Ulisse Cardini[1*†], Lazaro Marín-Guirao[1,2†], Luis M. Montilla[1], Ugo Marzocchi[1,3], Salvatore Chiavarini[4], Juri Rimauro[4], Grazia Marina Quero[1,5], Jillian M. Petersen[6] and Gabriele Procaccini[1*]

[1]Department of Integrative Marine Ecology, Stazione Zoologica Anton Dohrn - National Institute of Marine Biology, Ecology and Biotechnology, Naples, Italy, [2]Centro Oceanográfico de Murcia, Instituto Español de Oceanografia (IEO-CSIC), Murcia, Spain, [3]Department of Biology, Center for Water Technology (WATEC), Aarhus University, Aarhus, Denmark, [4]Division Protection and Enhancement of the Natural Capital - Italian National Agency for New Technologies, Energy and Sustainable Economic Development (ENEA), Rome, Italy, [5]Institute for Biological Resources and Marine Biotechnology, National Research Council (IRBIM-CNR), Ancona, Italy, [6]Division of Microbial Ecology, Centre for Microbiology and Environmental Systems Science, University of Vienna, Vienna, Austria

In seagrass sediments, lucinid bivalves and their chemoautotrophic bacterial symbionts consume H_2S, relying indirectly on the plant productivity for the presence of the reduced chemical. Additionally, the role of lucinid bivalves in N provisioning to the plant (through N_2 fixation by the symbionts) was hypothesized. Thus, lucinids may contribute to sediment detoxification and plant fitness. Seagrasses are subject to ever-increasing human pressure in coastal environments. Here, disentangling nested interactions between chemosynthetic lucinid bivalves and seagrass exposed to pollution may help to understand seagrass ecosystem dynamics and to develop successful seagrass restoration programs that consider the roles of animal-microbe symbioses. We evaluated the capacity of lucinid bivalves (*Loripes orbiculatus*) to promote nutrient cycling and seagrass (*Cymodocea nodosa*) growth during a 6-week mesocosm experiment. A fully crossed design was used to test for the effect of sediment contamination (metals, nutrients, and hydrocarbons) on plant and bivalve (alone or interacting) fitness, assessed by mortality, growth, and photosynthetic efficiency, and for the effect of their nested interaction on sediment biogeochemistry. Plants performed better in the contaminated sediment, where a larger pool of dissolved nitrogen combined with the presence of other trace elements allowed for an improved photosynthetic efficiency. In fact, pore water nitrogen accumulated during the experiment in the controls, while it was consumed in the contaminated sediment. This trend was accentuated when lucinids were present. Concurrently, the interaction between clams and plants benefitted both organisms and promoted plant growth irrespective of the sediment type. In particular, the interaction with lucinid clams resulted in higher aboveground biomass of *C. nodosa* in terms of leaf growth, leaf surface, and leaf biomass. Our results consolidate the notion that nested interactions involving animal-microbe associations promote ecosystem functioning, and potentially help designing unconventional seagrass restoration strategies that exploit chemosynthetic symbioses.

KEYWORDS

ecological facilitation, ecosystem restoration, nature-based solutions, chemosynthetic symbioses, *Loripes orbiculatus*, *Cymodocea nodosa*, Bagnoli-Coroglio, sediment contamination

Introduction

Seagrasses are habitat-forming marine plants that build the foundation of biodiversity hotspots in coastal marine environments (Heck et al., 2008). However, seagrass ecosystems are under threat due to a variety of human activities, such as coastal exploitation, eutrophication, and climate change (Orth et al., 2006). In many locations, seagrass meadows are becoming fragmented or have already completely disappeared, and have been replaced by bare sediments or by opportunistic macrophytes (Montefalcone et al., 2015). Thus, the restoration of seagrass habitats is often an environmental and economic imperative given that seagrasses provide key ecosystem functions and services (Reynolds et al., 2016), and has recently been recognized as a key action to address the causes of climate change and to mitigate associated effects (Gattuso et al., 2018). However, over the years, many seagrass restoration and/or transplantation programs have been costly and unsuccessful (Cunha et al., 2012; van Katwijk et al., 2016). Possibly, this is because these programs did not take into account factors such as the genetic features of donor populations (Pazzaglia et al., 2021) or the important role of positive species interactions in effectively contributing to ecosystem functioning (Cardinale et al., 2002; Bulleri et al., 2018; Valdez et al., 2020; Gagnon et al., 2021; Malkin and Cardini, 2021; Zhang et al., 2021).

Increasing evidence supports the notion that nested interactions involving animal-microbe associations (also called holobionts) fundamentally contribute to the functioning of diverse marine ecosystems (Pita et al., 2018). The most iconic example is that of coral reefs, where a symbiosis between an animal and a microalgal symbiont forms the basis of some of the most diverse ecosystems on Earth (Muscatine and Porter, 1977). Seagrasses are themselves holobionts associating with a diverse community of microbes which grow on their leaves as epiphytes or inhabit their rhizosphere (Tarquinio et al., 2019). These microbes play fundamental roles in the overall ecosystem functioning. For example, leaf epiphytes were shown to contribute significantly to the plant N needs, by fixing atmospheric N_2 or converting dissolved organic nitrogen (DON) compounds into bioavailable inorganic forms (DIN) (Agawin et al., 2016; Cardini et al., 2018; Tarquinio et al., 2018). Similarly, sulfate-reducing bacteria (SRB) and other microorganisms in the seagrass rhizosphere significantly contribute to the mineralization of organic N and phosphorus (P), and to anaerobic N_2 fixation (Welsh, 2000). Recently, Scholz et al. (2021) demonstrated the widespread relationship of cable bacteria growing in association with the root rhizosphere of aquatic plants and seagrasses. Critically, these bacteria can efficiently remove sulfide from sediments and are likely beneficial for the plant (Malkin and Cardini, 2021; Scholz et al., 2021). Other significant positive effects of microorganisms on seagrasses are for example the production of phytohormones, or defense against pathogens or toxic compounds (see Tarquinio et al. (2019) for a review).

Symbioses between macro- or meiofauna and microbes are ubiquitous and highly diverse in seagrass sediments. Sediment microorganisms can benefit a great deal by associating with invertebrates inhabiting this environment. The invertebrate host can provide the microbial symbionts with access to resources that may be unavailable, such as nutrients, or electron donors and acceptors that may not be available simultaneously in the sediment environment (Beinart, 2019). One prominent example of symbioses inhabiting seagrass sediments is the lucinid clams, where a bivalve host associates with sulfur-oxidizing bacteria that are hosted in the animal gills (Taylor and Glover, 2006). This holobiont was suggested to form a positive nested interaction with seagrasses (van der Heide et al., 2012). In this example of a nested ecosystem, the clam and its microbial symbionts are suggested to contribute to the removal of sulfide (toxic to the plant) from the sediments and thus to enhance seagrass growth (Chin et al., 2021). Additionally, a role of the lucinid clam *Loripes orbiculatus* in N provisioning to the seagrass ecosystem was recently proposed, given the ability of the symbionts to also fix atmospheric N_2 (Petersen et al., 2017; Cardini et al., 2019).

Seagrasses create the conditions for biodiversity hotspots through their role as habitat-forming species; at the same time, efforts that incorporate biodiversity as a means for the restoration of this important ecosystem may be more successful (Williams et al., 2017). Therefore, in this study, we aimed to test the importance of nested interactions between the plant (*Cymodocea nodosa*), the lucinid clams (*Loripes orbiculatus*), and their symbionts, in enhancing seagrass performance and growth in natural vs. contaminated sediments. By means of a mesocosm experiment, we explored the possibility of exploiting nested interactions for successful seagrass restoration strategies. We used a fully crossed design to examine the effect of sediment contamination (metals, nutrients, and hydrocarbons) on plant and bivalve (alone or interacting) fitness, assessed by mortality, growth, and photosynthetic efficiency, and for the effect of their nested interaction on sediment biogeochemistry. We hypothesized that the interaction between *Cymodocea nodosa* and *Loripes orbiculatus* may benefit both organisms in colonizing contaminated sediments and may provide a potential restoration strategy that exploit nested interactions as a nature-based solution in coastal polluted areas.

Materials and methods

Collection of sediments, plants, and lucinids

Collection of sediments, plants, and lucinids was carried out at the end of May 2018. Control sediment was collected north of the Gulf of Napoli, at Cape Miseno (40°47′5.75″N–14°4′36.79″E), while polluted sediments were collected within the bay of Bagnoli-Coroglio (40°48′22.10″N–14°9′44.59″E), a coastal area impacted by industrial contamination of hydrocarbons and heavy metals (Morroni et al., 2020). At each site, 150 L of surface sediment (max depth 10 cm) was collected between 5 and 10 m depth by divers using a hand-drag, and immediately transported to the laboratories of the Stazione Zoologica Anton Dohrn (SZN) in Napoli, Italy. *Cymodocea nodosa* plants were collected at Cape Miseno at ~8 m depth. Large fragments of the species were gently uprooted by divers and transported in coolers to the SZN facilities (within 2 h) to be subsequently introduced into the aquaria for plant acclimation. Specimens of *Loripes orbiculatus* were collected by scuba diving in the bay of Fetovaia, Livorno (Italy) from sediments adjacent to a *Posidonia oceanica* meadow (42°43′48″N 10°9′23″E) at ~7 m depth. The bivalves were moved to the HYDRA Institute for Marine Sciences in Fetovaia, prepared for transport in water-tight containers with a good quantity of their surrounding sediment, seawater, and a headspace for gas exchange, and transported to the SZN within 24 h from sampling.

Experimental setup

Cymodocea nodosa fragments of similar size, composed of 1 apical shoot and 8–10 connected vertical shoots, were selected for the experiment. Fragments were fixed to a plastic square mesh (mesh size: 4 cm) with cable ties to be transplanted into 61 plastic pots (20 × 30 × 15 cm). Three to four *C. nodosa* fragments were fixed to each plastic square mesh to reproduce the plant density of the meadow at the collection site (513 ± 14 shoots m^{-2}). The plastic square mesh was fixed to the top of the pots, and thereafter, sediment was carefully poured to allow roots to maintain their vertical position within the sediment. Half of the pots were filled with control sediment and the other half with polluted sediment. Thereafter, 50 lucinid bivalves of similar size (13.2 ± 1.3 mm shell length), equivalent to a realistic density of ~830 individuals m^{-2} (see van der Geest et al., 2020), were transferred onto the sediment of half of the pots to obtain a crossed design. Lucinid clams were left undisturbed and burrowed in the sediment within 8 h. The pots, filled with either polluted or control "Sediment" (factor 1; 2 levels), were thus reconstructed to recreate four types of "Community" (factor 2; 4 levels): only sediment (S), sediment + plant (P), sediment + lucinids (L), and sediment + plant + lucinids (PL). See Supplementary Figure S1 for a graphical representation of the experimental design. Four pots, one for each level of the factor Community, were allocated inside each of the six 500-L experimental tanks ($n = 3$ for each sediment type). See Ruocco et al. (2019) for a description of the aquarium system. The resulting experimental setup was let to acclimate for 1 week under the environmental conditions present *in situ* during sampling (temperature: 24.5°C; salinity: 37.5 psu; maximum noon irradiance: 275 ± 15 µmol m^{-2} s^{-1}; 12:12 h light:dark photoperiod). The same conditions were kept during the entire duration of the experiment, which lasted 6 weeks (42 days) in total.

Chemical characterization of sediments

Sediments chemical characterization was performed as already reported (Armiento et al., 2020; Morroni et al., 2020). Briefly, total organic carbon (TOC) was determined by a Leco CNS 2000 elemental analysis apparatus. Granulometric size distribution determinations were performed on a Micromeritics SediGraph 5100 X-ray particle size analyzer. Major and trace elements were determined by a PerkinElmer Optima 2000DV ICP-OES and an Agilent 7800 ICP-MS, after mineralization by a microwave-assisted acid digestion (Ethos Easy, Milestone). Hg was determined by Automatic solid/liquid Mercury Analyzer (FKV AMA-254). PAHs were analyzed according to EPA 8270D method with an Agilent 7890A-5975C GC–MS system, after extraction according to EPA 3545a method by an Accelerated Solvent Extractor (Dionex ASE 200) and silica gel cleanup (EPA 3630). Hydrocarbons in the C12–C40 range were determined by GC-FID on an Agilent 7820a system after Dionex ASE 200 extraction and Florisil cleanup. Sediment redox potential was characterized in each experimental pot at 5 and 10 cm below the sediment–water interface at the end of the experiment. Five measurements were taken in each pot at each of the selected sediment depths, by inserting a Crison Pt electrode, connected to a portable pH meter (Crison model 507), into the sediments. The electrode was calibrated with a redox standard solution (Crison 468 mV at 25°C) and redox measurements were referred to the standard hydrogen electrode (207 mV) as described in APHA (1992).

Pore water nutrients

Pore water was collected using metered stainless steel lances (Cardini et al., 2019) at the start and at the end of the experiment.

Seawater was retrieved from above the sediment (seawater control), and at 2 and 10 cm below the sediment–water interface. Two 30 ml seawater samples were retrieved from each depth and experimental pot. One sample was filtered onto 0.22 μm PES membrane filters (Merck Millipore), preserved frozen at −20°C, and analyzed for nitrogen oxides (NO_x) as the sum of nitrate (NO_3^-) and nitrite (NO_2^-), ammonium (NH_4^+), and orthophosphate (PO_4^{3-}) concentrations on a Continuous Flow Autoanalyzer (Flowsys, Systea) at the SZN laboratories. The other sample was filtered using an acid-washed 50 ml polycarbonate syringe through a pre-combusted 0.7 μm GF/F filter directly into acid-washed 30 ml HDPE sample bottles (Cardini et al., 2015). The sample was then immediately acidified with 80 μl of 18.5% HCl and stored in the dark at 4°C until analysis at the SZN by the high-temperature catalytic oxidation method on a TOC-L Analyzer with a total nitrogen (TN) unit (Shimadzu) for DOC and DON (as the difference of TN and dissolved inorganic nitrogen) quantification. No differences between "Community" levels were detected at the start of the experiment, and t0 data were thus pooled in one group and compared against the "Community" levels at the end of the experiment. Further, no differences were detected between sediment depths, and samples were thus pooled within the respective "Sediment" and "Community" level.

Plant photophysiology

A diving-PAM fluorometer (Walz, Germany) was used to characterize the functioning of the photosynthetic apparatus at the level of photosystem II (PSII). Chlorophyll fluorescence measurements were taken in two randomly selected *C. nodosa* shoots per experimental pot following Marín-Guirao et al. (2013b). Briefly, basal (F_0) and maximum fluorescence (F_m) were measured on whole-night adapted plants by the saturation pulse method to calculate the maximum quantum yield of PSII [$(F_v/F_m = (F_m - F_0)/F_m$]. Subsequently, rapid light curves (RLC) were generated on the same shoots after 4 h of illumination in experimental tanks to estimate maximum relative electron transport rates (rel-ETR_{max}). Each RLC was composed of 20 s exposure to 9 incremental irradiances. The curve fitting method developed by Jassby and Platt (1976) was used for calculating rel-ETR_{max}. Non-photochemical quenching was calculated as $NPQ = (F_m - F_m')/F_m'$; where F_m' is the maximum fluorescence of light-adapted leaves obtained from the RLCs. Measurements taken within each pot were averaged to be used as independent replicates ($n = 3$).

Plant morphology, growth, and mortality

Seagrass growth was measured as leaf elongation and rhizome growth. Leaf elongation was determined by marking the leaves of five randomly selected shoots with a needle 3 weeks after the beginning of the experiment (Zieman, 1974). Marked shoots were collected at the end of the experiment to measure the surface area of newly-formed leaf tissues ($cm^2\ shoot^{-1}\ day^{-1}$). Total leaf biomass, the number of leaves, and the percentage of the necrotic leaf surface were also determined on marked shoots. Rhizome growth was determined by marking the apical shoot of each plant fragment with plastic ties at the beginning of the experiment. Plant fragments were harvested at the end of the experiment and the newly produced tissues divided into leaves, rhizomes, and roots before being dried and weighed to estimate their biomass. Measurements taken within each pot were averaged to be used as independent replicates ($n = 3$). Finally, all shoots in each experimental pot were counted at the beginning and at the end of the experiment, and the differences normalized to the initial shoot number and expressed as a percentage of net shoot change.

Lucinid clam mortality and tissue analyses

All *L. orbiculatus* clams were counted at the end of the experiment for determining their mortality rate in the different experimental pots. Additionally, 10 clams at T0 and four clams from each pot at the end of the experiment were selected randomly, measured for their shell length, and dissected for tissue analyses. Symbiont-bearing (gill) tissue and non-symbiotic (host) tissue (i.e., the remaining tissue after removal of the gills) were separated and stored at −20°C to determine the natural $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios of the gill and host tissues as in Cardini et al. (2019). Frozen tissues were freeze-dried for 48 h, ground to fine powder, and weighed into tin capsules. Samples were analyzed for C% and N% and for $\delta^{13}C$ and $\delta^{15}N$ by continuous-flow isotope ratio mass spectrometry (IRMS, Isoprime, GV Instruments Ltd) coupled with an elemental analyzer (Costech Instruments).

Data analysis

Differences in sediment inorganic and organic nutrient concentrations, plant (photochemistry, morphology, and growth), and lucinid clam (mortality) were tested using PERMANOVA tests (Anderson, 2008) with "Sediment" and "Community" as fixed factors. The test for Redox potential additionally included the factor "Depth." The analysis was conducted using the Euclidean distance as a coefficient of dissimilarity on previously normalized data. Type 3 (partial) sum of squares was used with the unrestricted permutation of raw data (9,999 permutations). These analyses were run using the PERMANOVA tool included in the PRIMER 6+ package. A principal component analysis (PCA) was also performed to explore overall plant responses (photochemistry, morphology and growth) to "Sediment" and "Community" experimental treatments. The isotopic niche spaces of symbionts and hosts were compared among experimental pots analyzing the Bayesian standard ellipse areas (SEA_B) with the SIBER R package (Jackson et al., 2011; R Core Team, 2021).

Results

Sediment geochemistry and pore water nutrients

Both sediments had a similar grain size distribution, characteristic of sandy sediments (Table 1). However, the organic content of the two sediment types differed significantly in both their TOC and DON content, as well as for their DOC:DON ratios (Table 1).

Abundant elements showed similar concentrations in sediments from both sites (Supplementary Table S1). However, P and Fe were significantly more concentrated in polluted sediments (Table 2). Further, heavy metals and metalloids were significantly more concentrated in polluted sediments (Table 2), with some vastly exceeding environmental quality standards, such as arsenic (As), cadmium (Cd), and lead (Pb). Similarly, hydrocarbon concentrations were high in polluted sediment, with the EPA's 16 priority pollutant polycyclic aromatic hydrocarbons exceeding by 5-fold the environmental quality standard (Table 2).

Redox potential (Supplementary Figure S2; Supplementary Table S3) significantly differed between the two analyzed sediment depths ($p < 0.001$) and among communities ($p < 0.01$) but was similar between control and polluted sediments. Sediment redox potential at 5 cm depth was significantly higher in all treatments, almost double that at 10 cm depth. Regardless of depth, the redox potential in the PL community was significantly higher than that in the S community, both in the control ($p < 0.05$) and in the polluted sediment ($p < 0.01$). In the control sediment, lower redox values were found associated with the S community, with values significantly different from the L community ($p < 0.05$). In the polluted sediment, lower redox potentials were associated with the L community, which significantly differed from the redox conditions of the PL community ($p < 0.05$). Across all treatments, the highest mean redox potential was found in PL communities of polluted sediments (102.7 ± 13.7 mV), while the lowest was found in L communities of polluted sediments (26.5 ± 27.3 mV).

Inorganic and organic nutrient concentrations were stable in the aquaria seawater during the experiment (Supplementary Table S2), while they generally increased in the control sediment pore water, regardless of the "Community" level (Supplementary Figure S3; Supplementary Table S3). The same trend was observed in the polluted sediment pore water for PO_4^{3-} and DOC, although DOC increased significantly more in the P and PL community compared to the other treatments (Supplementary Figure S3). Conversely, we detected a significant decrease in NH_4^+ and DON concentrations in the polluted sediment, particularly in the PL community (Supplementary Figure S3).

TABLE 1 Sediment organic content and grain size distribution (n=6).

Parameter	Control	Polluted	*p* value
TOC (%)	0.02 ± 0.01	0.36 ± 0.06	<0.01
DOC (μM)	174.57 ± 22.02	166.39 ± 15.99	
DON (μM)	9.65 ± 2.18	35.28 ± 6.53	<0.01
DOC:DON	18.51 ± 2.53	4.82 ± 0.79	<0.01
Gravel: >2 mm (%)	0.30	0.30	
Sand: 2 > 0.063 mm (%)	99.60	99.40	
Silt: <0.063 mm (%)	0.10	0.30	

TABLE 2 Concentration of key elements and hydrocarbons in the sediment, and environmental quality standard expressed as an annual average values (EQS-AA) according to the IT law 260/2010.

Element (ppm)	Control	Polluted	EQS-AA
Fe	20,458 ± 155	102,466 ± 2,794	
P	504 ± 10	4,336 ± 68	
As	21.3 ± 0.6	**75.2 ± 2.9**	12 ± 20%
Cd	0.148 ± 0.011	**0.76 ± 0.04**	0.3 ± 20%
Cr	13.8 ± 2.4	30.9 ± 1.1	50 ± 20%
Cu	6.34 ± 0.40	157 ± 9	
Hg	0.009 ± 0.001	0.220 ± 0.019	0.3 ± 20%
Ni	7.84 ± 1.30	13.6 ± 1.4	30 ± 20%
Pb	31 ± 1	**281 ± 15**	30 ± 20%
Hydrocarbons (ppb)	**Control**	**Polluted**	
Σ PAHs (16 priority pollutants EPA)	40.5	**4,450.4**	800 ± 20%
Heavy hydrocarbons (C > 12)	18.5 ± 3.7	155.5 ± 28	

Numbers in bold indicate values exceeding the EQS-AA.

Plant photophysiology

At the end of the experiment, the photochemical efficiency of plants growing in polluted sediments was significantly higher than the efficiency of plants in control sediments (Figure 1; Supplementary Table S4). These plants also showed significantly higher values of electron transport rate (rel-ETR) but lower values of non-photochemical quenching (NPQ) (Figure 1; Supplementary Table S4). There was no indication of an effect of the community type on the photophysiology of *C. nodosa*.

Plant morphology, growth, and mortality

The total leaf surface area of *C. nodosa* interacting with *L. orbiculatus* was significantly higher irrespective of sediment pollution (Figure 2; Supplementary Table S5). These plants also showed a trend of higher leaf elongation and leaf biomass compared to plants growing in the absence of lucinid clams, but differences were deemed not significant by the statistical test (Figure 2; Supplementary Figure S4; Supplementary Table S5). New apical growth was measured for the root, rhizome, and leaf portion of the plant. Neither rhizome nor leaf apical growth showed significant differences among the experimental pots (Supplementary Figure S5; Supplementary Table S6). However, there was a significant effect of

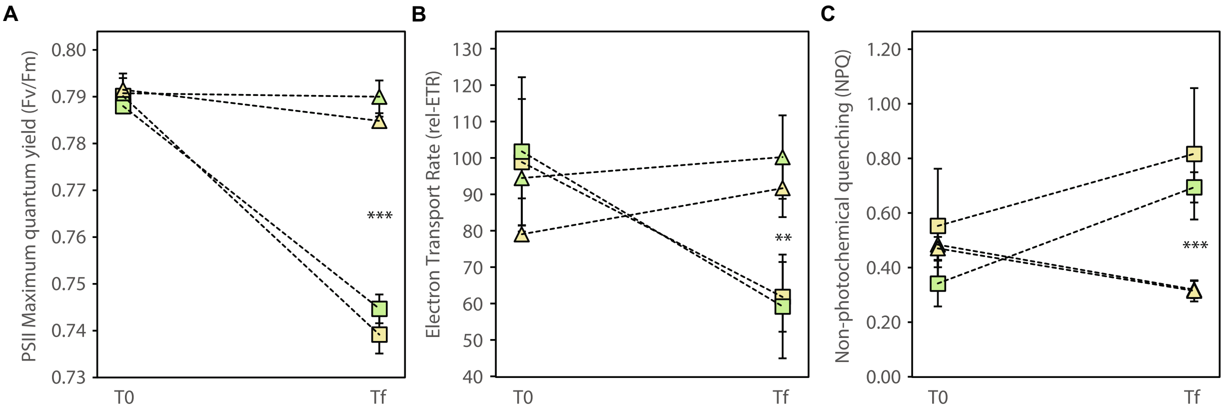

FIGURE 1
Evolution of chlorophyll a fluorescence parameters. **(A)** Maximum quantum yield of PSII (F_v/F_m), **(B)** electron transport rate (rel-ETR), **(C)** non-photochemical quenching (NPQ) at the beginning (T0) and end (Tf) of the experiment (±SE, *n*=3). Colors indicate absence (yellow) or presence (green) of the interaction with lucinid clams. Symbols are used to indicate control (quadrats) or polluted (triangles) sediment. Asterisks indicate significant differences in the factor "Sediment" at Tf (***p*<0.01; ****p*<0,001); see Supplementary Table S4 for the statistics.

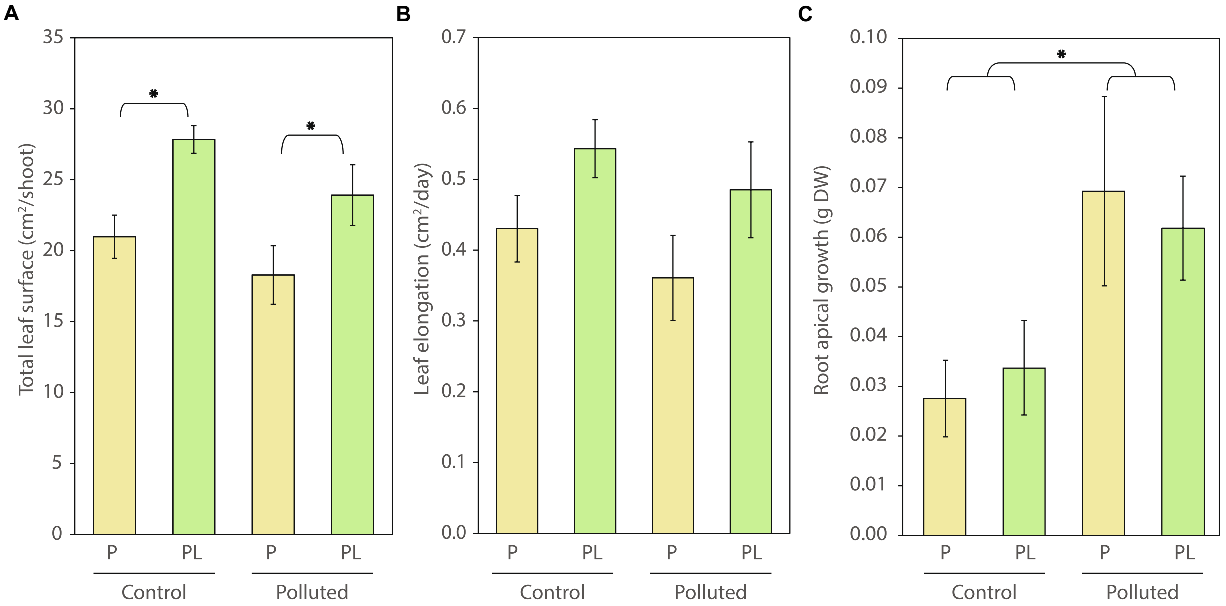

FIGURE 2
Plant morphology and growth. **(A)** Total leaf surface, **(B)** leaf elongation, **(C)** root apical growth of *C. nodosa* at the end of the experiment (±SE, *n*=3). Levels of the factor "Community" are identified with letters as indicated in the methods. Colors indicate absence (yellow) or presence (green) of the interaction with lucinid clams. Asterisks (**p*<0.05) indicate significant differences; see Supplementary Tables S5, S6 for the statistics.

the sediment type, regardless of the interaction with lucinids, on the growth of the apical roots (Figure 2; Supplementary Table S6). At the same time, there was a significant increase of necrotic tissue in *C. nodosa* from the polluted compared to the control sediment, regardless of the presence of the clams, while all plots showed a positive net shoot change during the course of the experiment regardless of the experimental treatment (Supplementary Figure S4; Supplementary Table S5).

In a principal component analysis (Figure 3), sediment type (control vs. polluted) segregated samples along axis 1 (37.9% of total variance), which was mainly correlated with photochemical parameters and the newly produced tissues by apical growth. Conversely, the community type (P vs. PL) segregated samples along axis 2 (29.9% of total variance). This axis was mainly correlated with leaf growth and shoot size (in terms of both leaf surface and leaf biomass).

Lucinid clams

Loripes orbiculatus showed significantly more mortality in the polluted sediment (Figure 4; Supplementary Table S6) compared to the control sediment ($p < 0.05$), approaching a value of 10%

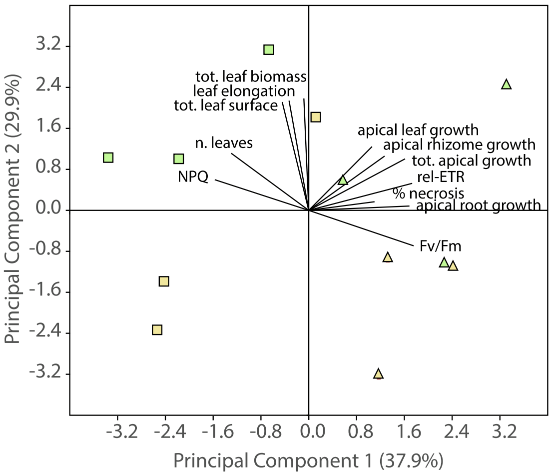

FIGURE 3
Principal component analysis testing for multivariate changes in plant variables among treatment levels. Colors indicate absence (yellow) or presence (green) of the interaction with lucinid clams. Symbols are used to indicate control (quadrats) or polluted (triangles) sediment. Note that the per cent variation explained by the principal components is indicated in the graph and refers to the fraction of the total variance explained by each axis or principal component (i.e., PC1 and PC2).

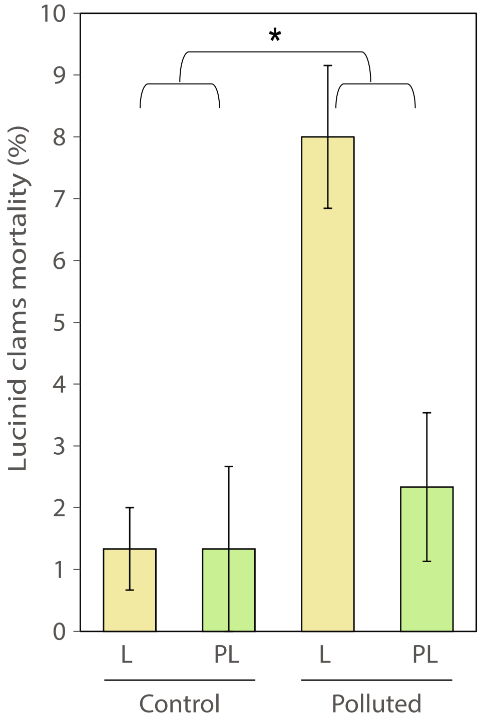

FIGURE 4
Lucinid clams mortality (±SE, n=3). Levels of the factor "Community" are identified with letters as indicated in the methods. Colors indicate absence (yellow) or presence (green) of the interaction with the plant. Asterisks (*p<0.05) indicate significant differences; see Supplementary Table S6 for the statistics.

where the plant was not present. The isotopic niche of *L. orbiculatus* sampled at the beginning of the experiment showed a differentiation between symbiont-free (rest) and symbiont-hosting (gill) lucinid clam tissues, with the latter having more negative $\delta^{13}C$ and $\delta^{15}N$ values (Figure 5) and in SEA_B (Supplementary Figure S6). At the end of the experiment, the same pattern was generally maintained in all treatments. SEA_B of *L. orbiculatus* tissues overlapped significantly between the beginning and the end of the experiment, except for symbiont-free (rest) tissues in the L treatment of the polluted sediment (Figure 5). These samples also showed the largest SEA_B (Supplementary Figure S6). The interaction of *L. orbiculatus* with the plant also caused larger SEA_B, regardless of the sediment type (Supplementary Figure S6).

Discussion

Here, we found evidence that nested interactions between chemosynthetic lucinid bivalves and seagrass promote ecosystem functioning, and that these interactions can play a role in the capacity of the mutualistic consortium to resist stress and grow in polluted sediments.

Cymodocea nodosa is tolerant to pollution

Our study confirms that *C. nodosa* is highly plastic at the morphological level. This species can in fact rapidly change the plant architecture modifying the ratio of above- vs. below-ground biomass depending on environmental conditions and resource availability (Perez et al., 1994; Marín-Guirao et al., 2018). Our study demonstrates that this plant is resistant to high doses of pollution deriving from the massive industrial contamination by trace metals and hydrocarbons found in the Bagnoli area (Armiento et al., 2020; Morroni et al., 2020).

In our experiment, *C. nodosa* plants exposed to pollution showed increased photosynthetic efficiency and apical growth. Nutrients in *C. nodosa* are mainly taken up through the root system, with leaf uptake dominant only when seawater concentrations suddenly increase after a nutrient pulse (Alexandre and Santos, 2020). In particular, ammonium is the preferential nitrogen source for *C nodosa*, while amino acids can also represent a large fraction of this species' N demand (Alexandre and Santos, 2020). In our study, the higher availability of nutrients (and particularly of ammonium and dissolved organic nitrogen) in polluted sediments likely contributed to increased uptake rates and stimulation of plant roots growth.

Other important macronutrients (P) and as well as micronutrients/trace metals (e.g., Fe, Cu, Mn, and Zn) that are used as co-factors in photosynthesis were significantly more abundant in polluted sediments, possibly explaining the more efficient use of light for *C. nodosa* plants. At the same time, our lower NPQ in plants

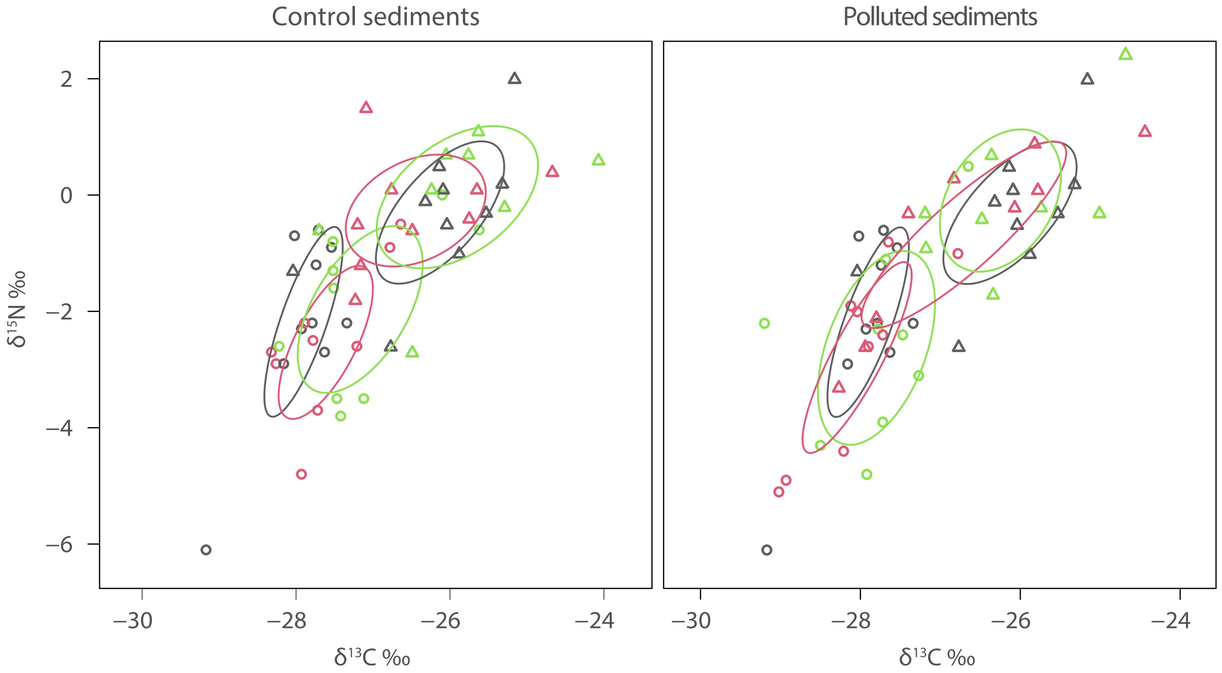

FIGURE 5
Isotopic niche of lucinid tissues exposed to the different experimental conditions. The lines enclose the Bayesian standard ellipse area (SEA_B). Initial (T0) samples are represented in grey, samples from the L treatment are in red, while those from the PL treatment are shown in green. Triangles are used for symbiont-free (rest) and circles for symbiont-hosting (gill) lucinid clam tissues. See Supplementary Figure S6 for the density plot of SEA_B.

exposed to pollution suggests that *C. nodosa* activated responses at the physiological level that enhance acclimation and plant tolerance to stress (Marín-Guirao et al., 2013a). Indeed, a recent study showed for NPQ a biphasic dose–response pattern typical of hormesis in *C. nodosa* exposed to ZnO nanoparticles (Malea et al., 2019).

In a different study, *C. nodosa* meadows growing on mining-impacted sediments were more dense and lush than meadows on control sediments (Marín-Guirao et al., 2005), further demonstrating the capacity of *C. nodosa* to tolerate stress from heavy metals. Many of these metals are accumulated by the plant if bioavailable and not sulfide-bound. Unfortunately, neither metal accumulation in plant tissues nor sulfide concentrations were quantified in this study, making it impossible to speculate whether the plant was able to cope with pollution because of their low bioavailability or despite their accumulation. Notwithstanding, it appears clear that *C. nodosa* is resistant to stress deriving from heavy metal and hydrocarbon contamination, at least when this is accompanied by a significant increase in macro and micronutrient availability that boost plant growth.

Loripes orbiculatus response depends on seagrass presence

The lucinid bivalve *L. orbiculatus* was susceptible to pollution, as indicated by the significantly higher mortality rate of clams burrowing in polluted sediment, particularly for those animals maintained in the absence of the plant. Further, in this study, we explored the response of the *L. orbiculatus* chemosynthetic symbiosis to pollution and interaction with *C. nodosa* by quantifying the isotopic niche width of the symbiotic vs. non-symbiotic clam tissues.

The isotopic niche has become an established concept in ecology because stable isotope ratios in consumer tissues are tightly linked to those in their diet (Jackson et al., 2011), offering a potentially powerful way to investigate ecological niches and trophic interactions (Yeakel et al., 2016). Recently, the method was used to also look at trophic interactions in chemosynthetic symbioses (Cardini et al., 2019). Stress-induced variability in physiological status can induce changes in isotopic niche width. For example, greater isotopic niche estimates were derived for the deposit-feeding amphipod *Monoporeia affinis* exposed to sediments contaminated with polychlorinated biphenyls, heavy metals, chlorophenols, and polycyclic aromatic hydrocarbons (Karlson et al., 2018).

Similarly, in our study, greater isotopic niche estimates were derived for non-symbiotic tissues of *L. orbiculatus* exposed to the polluted sediments, a result which is consistent with the increase in stress-induced mortality for these clams and the concomitant decrease in redox potential as a likely result of clam mortality and decay. The symbiotic (gill) tissues of clams exposed to polluted sediments showed comparable isotopic niche widths compared to the control group, suggesting that the microbial partners (hosted in the bivalve gills) may remain little affected in those individuals that overcome the stressful conditions.

Nested interactions promote ecosystem functioning

The association between lucinid bivalves and seagrass was suggested to function as a tripartite mutualism (van der Heide et al., 2012). In this system, the plant provides organic matter, which is respired by sulfate-reducing bacteria in anoxic sediments leading to formation of hydrogen sulfide, the energy source needed by the clam's chemosynthetic bacteria. At the same time, radial oxygen loss by the seagrass roots facilitates the clam's respiration. In return, the clam symbionts oxidize hydrogen sulfide back to sulfate, preventing a potential build-up of the powerful phytotoxin in sediments (Lamers et al., 2013) and thus, sulfide intrusion into the seagrass with potential to induce plant starvation and mortality (Holmer and Hasler-Sheetal, 2014). This mutualism was recently also verified in a field survey in a temperate lagoon system (van der Geest et al., 2020).

Our experiment seems to confirm these studies and the presence of ecological facilitation, irrespective of sediment type. While a longer duration of the experiment and higher replication would have likely resulted in lower variability and clearer differences among the treatments, the experiment clearly showed an effect of both pollution and the interaction between *L. orbiculatus* and *C. nodosa* on some of the investigated variables (Figure 6). The interaction of plants and lucinids significantly improved sediment oxic conditions as shown by the increase of redox potential. At the same time, *C. nodosa* enriched sediment pore water in dissolved organic carbon, a potential food source for sediment sulfate-reducers. The interaction with lucinid clams further resulted in higher aboveground biomass of *C. nodosa* in

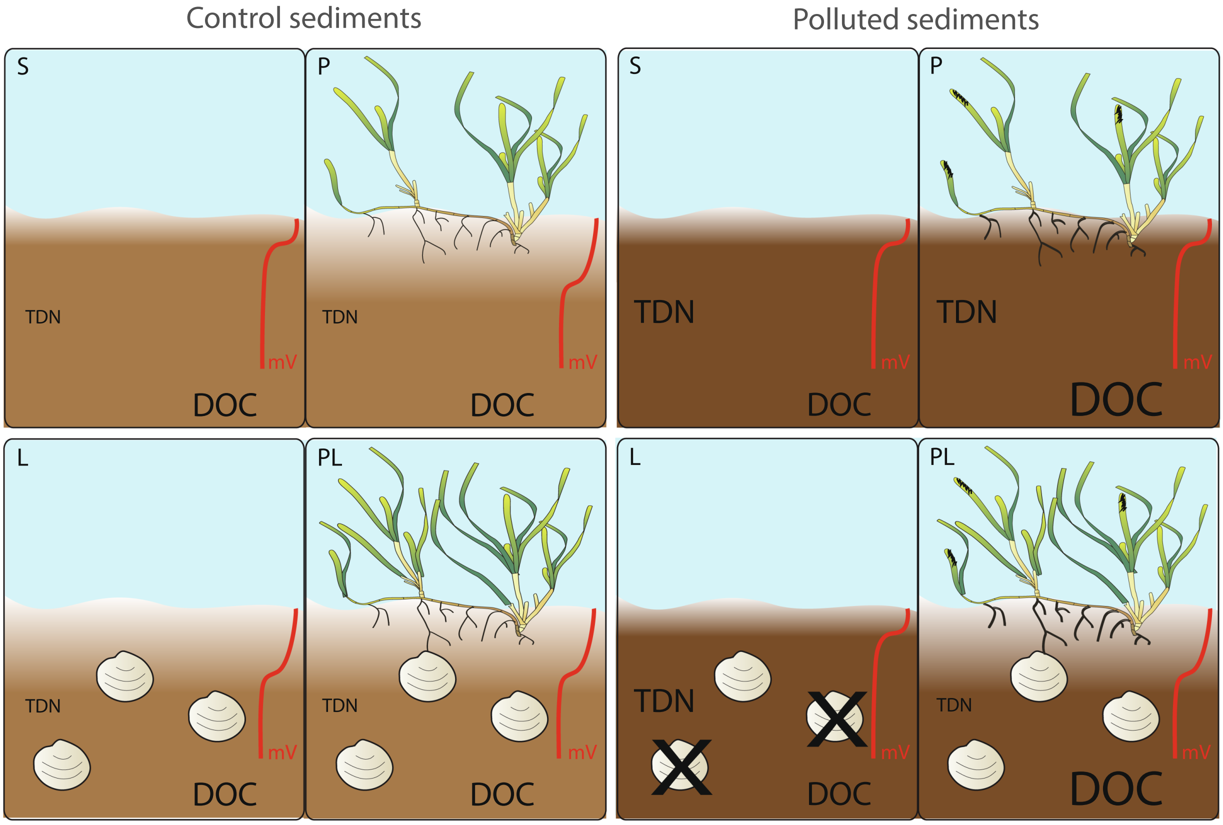

FIGURE 6
Conceptual model of facilitation of the seagrass *Cymodocea nodosa* by the lucinid clam *Loripes orbiculatus*, in control (left) vs. polluted (right) sediments. (S) represents pots with only sediment; (P) are pots with seagrass; (L) are pots with lucinids; (PL) represents pots with the plant and lucinids. Presence of lucinids resulted in increased above-ground biomass (more leaves) irrespective of the sediment type. Conversely, root apical growth (thicker roots) increased in polluted sediments, regardless of lucinid clams. Lucinid mortality was high in the polluted sediment (black crosses), but only when the plant was absent, while the percentage of plant necrotic tissue (black markings on leaves) were higher in the polluted sediment regardless of lucinids. The interaction of plants and lucinids significantly improved sediment conditions as shown by the increase in redox potential (mV) and decrease (consumption) of total dissolved nitrogen (TDN) in the polluted sediment, where the plant released large quantities of dissolved organic carbon (DOC). Seagrass symbol credit: Integration and Application Network (https://ian.umces.edu/media-library).

terms of leaf growth, leaf surface and leaf biomass, similar to the findings of van der Geest et al. (2020) for *L. orbiculatus* and *Zostera noltii* in the Thau lagoon, France.

Our study further demonstrates that the interaction between the plant and lucinid clams facilitates the consortium, especially in heavily contaminated sediments. In these sediments, the interaction with lucinids turned the plant treatment into a sink for nitrogen (both ammonium and dissolved organic nitrogen), suggesting a more efficient uptake of this nutrient when the plant and the clam are associated. While the specific mechanisms involved are difficult to pinpoint, our results seem to support the notion of a role in nitrogen cycling for *L. orbiculatus* as indicated by Cardini et al. (2019).

Further, we found that *L. orbiculatus* isotopic niche is larger (both for the animal host and for the symbiont) when associated with the plant, regardless of the type of sediment. This was previously linked to the flexible nutritional mutualism of *L. orbiculatus,* in which the clam host and its symbionts cycle between a looser trophic association and a tight chemoautotrophic partnership, changing nutritional strategy according to the environmental conditions (Cardini et al., 2019). Importantly, the present study further shows how the remarkable flexibility of this chemosynthetic symbiosis allows it to withstand heavy pollution if associated with a seagrass partner, the plant in turn benefitting and building more aboveground biomass in the presence of the clam.

Conclusion

Harnessing positive species interactions as a tool for restoration of degraded systems, or to counteract climate-driven loss of coastal biodiversity, is urgently needed (Bulleri et al., 2018). In particular, plant–bivalve interactions have been suggested to facilitate foundation species such as seagrasses, possibly helping to increase the success of restoration efforts (Gagnon et al., 2020). However, studies that mechanistically test specific interactions for their capacity to improve resistance of the whole consortium of organisms to anthropogenic stress are lacking. In this study, we showed that the interaction between *C. nodosa* and *L. orbiculatus* favors both organisms in colonizing highly polluted sediments from the Bagnoli-Coroglio area, promoting growth and resilience of the foundation species. Thus, co-restoration of *C. nodosa* and *L. orbiculatus* may be used in heavily impacted sites where other options are prone to failure and may improve restoration success leading to recovery of associated biodiversity, functioning and ecosystem services.

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 authors.

Author contributions

UC, LM-G, and GP conceived the ideas and designed methodology. UC, LM-G, JP, and GP contributed to the sampling of biological material. UC, LM-G, GP, and GQ performed the experiment and collected the data. UC, LM-G, LMM, UM, SC, and JR analyzed the data. UC and LM-G led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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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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Supplementary material

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Increased extent of waterfowl grazing lengthens the recovery time of a colonizing seagrass (*Halophila ovalis)* with implications for seagrass resilience

Caitlyn M. O'Dea[1,2]*, Paul S. Lavery[1], Chanelle L. Webster[1] and Kathryn M. McMahon[1]

[1]Centre for Marine Ecosystems Research, School of Science, Edith Cowan University, Joondalup, WA, Australia, [2]Department of Water and Environmental Regulation, Aquatic Science Branch, Joondalup, WA, Australia

Herbivore distributions and abundance are shifting because of climate change, leading to intensified grazing pressure on foundation species such as seagrasses. This, combined with rapidly increasing magnitudes of change in estuarine ecosystems, may affect seagrass resilience. While the overall resilience of seagrasses is generally well-studied, the timeframes of recovery has received comparatively little attention, particularly in temperate estuaries. We investigated how the recovery time (RT) of seagrass is affected by simulated grazing in a southwestern Australian estuary. Whilst excluding swans, we simulated different grazing intensities (25, 50, 75, and 100% removal from 1 m^2 plots) at four locations in the Swan-Canning Estuary, Western Australia during summer and tracked the recovery of seagrass over 3 months, using seagrass cover as the main measure of recovery. We found that seagrass recovered within 4–6 weeks from the lower grazing intensities (25 and 50%) and 7–19 weeks from the higher grazing intensities (75 and 100%) across the estuary. Increased grazing intensity led to not only longer recovery times (RTs), but also greater variability in the RT among experimental locations. The RT from the higher grazing intensities at one location in particular was more than double other locations. Seagrass recovery was through vegetative mechanisms and not through sexual reproduction. There was a significant grazing treatment effect on seagrass meadow characteristics, particularly belowground biomass which had not recovered 3 months following grazing. As the pressure of climate change on estuarine environments increases,

EDITED BY
Mirta Teichberg,
Ecosystems Center, Marine Biological Laboratory (MBL), United States

REVIEWED BY
Xiaomei Zhang,
Institute of Oceanology (CAS), China
Hung Manh Nguyen,
Ben-Gurion University of the Negev, Israel

*CORRESPONDENCE
Caitlyn M. O'Dea
caitlyn.odea@ecu.edu.au

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these quantified RTs for seagrass provide a baseline for understanding grazing pressure as a singular disturbance. Future work can now examine how grazing and other potentially interacting pressures in our changing climate could impact seagrass recovery even further.

KEYWORDS

recovery time, resilience, grazing simulation, trophic interactions, seagrass, herbivory, Swan-Canning Estuary, swan

Introduction

While trophic interactions between species have evolved over millennia (Durant et al., 2019), they can, nonetheless, fall out of balance as in the case of overgrazing. Overgrazing is a mismatch between the food requirements and food availability in an ecosystem (Durant et al., 2005), and has been observed in aquatic environments through waterfowl (Kollars et al., 2017), sea urchins (Eklöf et al., 2008), and turtle grazing (Fourqurean et al., 2019). This can be the result of increased herbivore abundance and, in extreme cases, has caused the functional extinction of submerged aquatic vegetation (SAV) within an ecosystem (Gangal et al., 2021). In many regions, predicted changes in hydrology have the potential to lead to increased herbivore densities in some aquatic ecosystems, with implications for the plants they depend on. For example, in regions with Mediterranean climates, wetlands can dry, partially or completely, over summer (Brinson and Malvárez, 2002). With a drying climate, these wetlands will become drier for longer periods (Semeniuk and Semeniuk, 2013; Hope et al., 2015) and nearby ecosystems, such as estuaries, are likely to become refuges for waterfowl, potentially increasing herbivore density grazing pressure on SAV.

Seagrasses are a foundation species of SAV in many estuaries (Dayton, 1972) as they create habitat, stabilize sediments, cycle nutrients, and form the base of the trophic web (Hemminga and Duarte, 2000; Unsworth et al., 2015). Like many ecosystems, seagrasses have been under threat from human impact and are declining at alarming rates globally (Waycott et al., 2009; Dunic et al., 2021). Future climate-driven changes in grazer abundance would represent an additional pressure on seagrass ecosystems. The extent of degradation from grazing pressure is often dependent on the abundance and distribution of herbivores (Choney et al., 2014), the scale of impact (O'Brien et al., 2018), and how the plants can respond (Pérez et al., 2012; Sanmartí et al., 2014). Seagrasses are commonly classified into three types based on their ability to resist or recover from disturbance: persistent, opportunistic, or colonizing species (Kilminster et al., 2015). In estuaries, the naturally variable conditions often favor colonizing seagrass species, which are small, fast-growing, and with low resistance to disturbance but the ability to recover rapidly (Kilminster et al., 2015). These seagrasses would be expected to have a relatively high potential for recovery. However, as with recovery following other forms of disturbance, the timeframes of recovery following grazing are not well understood (York et al., 2016).

Understanding recovery time (RT) is particularly important for seagrasses because of the ecosystem services they provide and their vulnerability to ongoing declines (Smith et al., 2016). Knowing how long seagrasses take to recover would allow more accurate prediction of how long they might be vulnerable to additional disturbance, or the length of time required for them, and their ecosystem services (Nowicki et al., 2017; Scott et al., 2021), to return following loss. Limited understanding of RT makes it challenging to determine realistic timeframes for management or intervention (O'Brien et al., 2018), particularly if RT is beyond funding cycles. Greater understanding of RT is likely critical in understanding seagrass resilience and protecting these ecosystems into the future.

Equally important is understanding the mechanism for recovery. Seagrasses are known to recover through three primary mechanisms. Firstly, they expand into unoccupied space by vegetative expansion from surrounding, intact meadows (Marbà and Duarte, 1998). This is common to most seagrasses. Second, they may recover from the germination of seeds in a seedbank. This mechanism seems particularly important in colonizing seagrasses (Kilminster et al., 2015), as these species typically produce large numbers of seeds that accumulate in the sediment. However, little is known about the dormancy of these seeds, their longevity and what breaks dormancy, and consequently it is unclear whether seeds could provide a mechanism of recovery throughout the year or only at certain times. Finally, seagrasses can recover from the immigration of fragments created through breakage of plants in other locations due to physical disturbance (McMahon et al., 2014). Understanding the relative importance of these mechanisms for recovery can guide our understanding of how and how quickly recovery might occur, and the nature of its dependence on adjacent meadows.

Our study investigated if grazing intensity affects the RT of a colonizing species of seagrass. We expected that the RT of seagrass would be slower with increasing grazing intensities;

if there is greater removal of seagrass material, there is more area that seagrass needs to replenish to return to the original condition and will subsequently take longer. We also explored which mechanisms explained the patterns in recovery.

Materials and methods

Study location and species

A manipulative field experiment was carried out in the lower Swan-Canning Estuary on the Swan Coastal Plain in southwestern Australia (**Figure 1**). The estuary is permanently open to the ocean and has diurnal oceanic tides with a range of 0.6–0.9 m. Much of the estuary is less than 3 m deep (Thomson et al., 2001). The Swan Coastal Plain also contains transient to permanent wetland areas which provide important habitat for the black swan (*Cygnus atratus* Latham); an iconic herbivorous waterfowl in Australia. Swans are considered "ecosystem engineers" through their impact on the environment which is linked to their behavior, movements, and foraging (Bakker et al., 2016). Swans graze on seagrass meadows in the estuary (**Figure 2**) and are most abundant during autumn and summer (Storey et al., 1993; Choney et al., 2014). Swans, by virtue of their large size (up to 9 kg), have considerable impacts on their food source, such as seagrass (Wood et al., 2012; Bakker et al., 2016). Swan populations have been shown to consume 25% of daily seagrass production in an Australian estuary (Choney et al., 2014), and up to 20% of annual seagrass biomass in a New Zealand estuary (Dos Santos et al., 2012).

Halophila ovalis (R. Br) Hook. F., a colonizing species, is the dominant seagrass in the lower Swan-Canning Estuary (**Figures 1, 2**). In 2011, *H. ovalis* meadows covered 403 hectares, approximately 16% of the lower estuary (Forbes and Kilminster, 2014). Other species are found in the estuary in mixed meadows with *H. ovalis*, which include *Ruppia megacarpa*, *Zostera muelleri*, *Posidonia australis*, and *Halophila decipiens* (Forbes and Kilminster, 2014). Growth of *H. ovalis* in the estuary is highest during summer and peak biomass is generally reached in December, when temperature, salinity, and light conditions are highest (Hillman et al., 1995), typically followed by peak flowering and fruiting (Kilminster and Forbes, 2014). The experiment was conducted from December 2018 to April 2019, a period of maximum seagrass growth likely to capture the fastest possible RT and maximum swan abundance.

Grazing simulation experiment

A block experimental design was used to assess the effects of simulated swan grazing on *H. ovalis* seagrass recovery. Four experiment locations (Blocks, 7 × 5 m) were chosen within monospecific meadows of *H. ovalis* in the estuary at Lucky Bay (32°01′16.2″ S, 115°48′47.5″ E), Pelican Point (31°59′16.5″ S, 115°49′28.8″ E), Point Resolution (32°00′06.3″ S, 115°47′26.2″ E), and Rocky Bay (32°01′30.6″ S, 115°46′21.2″ E) (**Figure 2**). Each block acted as a single replicate location within the estuary. At each location, six levels of simulated grazing were imposed, each in 1 × 1 m experimental plot within the block. As we were not testing for differences among locations, we did not replicate the grazing treatments within each location. Swans were excluded from the experimental blocks to prevent further grazing using exclusion structures. The experiment was established in seagrass meadows with water depths no greater than 1 m and that had no sign of recent grazing activity. Within each block, the placement of grazing treatment plots was randomly configured. The six treatments were (1) Control: no simulated grazing used as a reference to determine the RT of the grazed plots; (2) 25%: removal of 25% of total seagrass biomass from the 1 m^2 as simulated grazing; (3) 50%: removal of 50%; (4) 75%: removal of 75%; (5) 100%: removal of 100%; and (6) Procedural Control (PControl): a 1 m^2 plot marked in the surrounding meadow at least 1 m from the boundary of the block without grazing exclusion and used to test for experimental artifacts in comparison to Control plots inside the structures. Individual swans can devegetate areas with diameters between 0.3 and

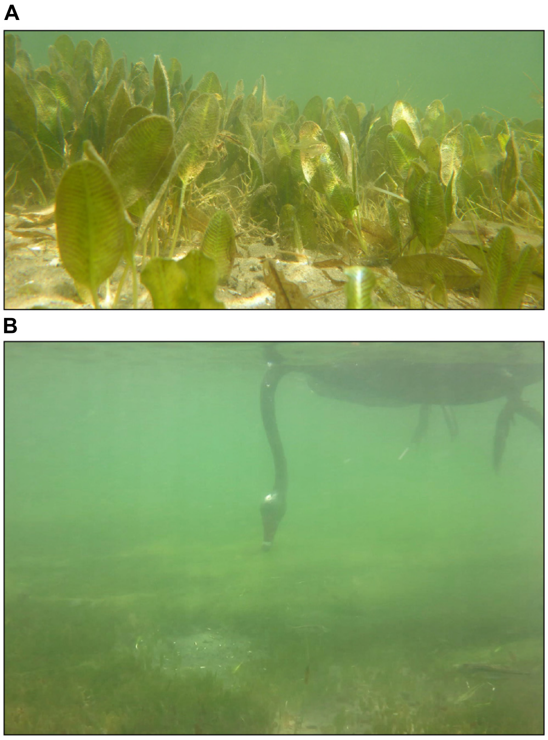

FIGURE 1
Seagrass (*Halophila ovalis)* in the Swan-Canning Estuary **(A)** and black swan (*Cygnus atratus*) grazing **(B)**.

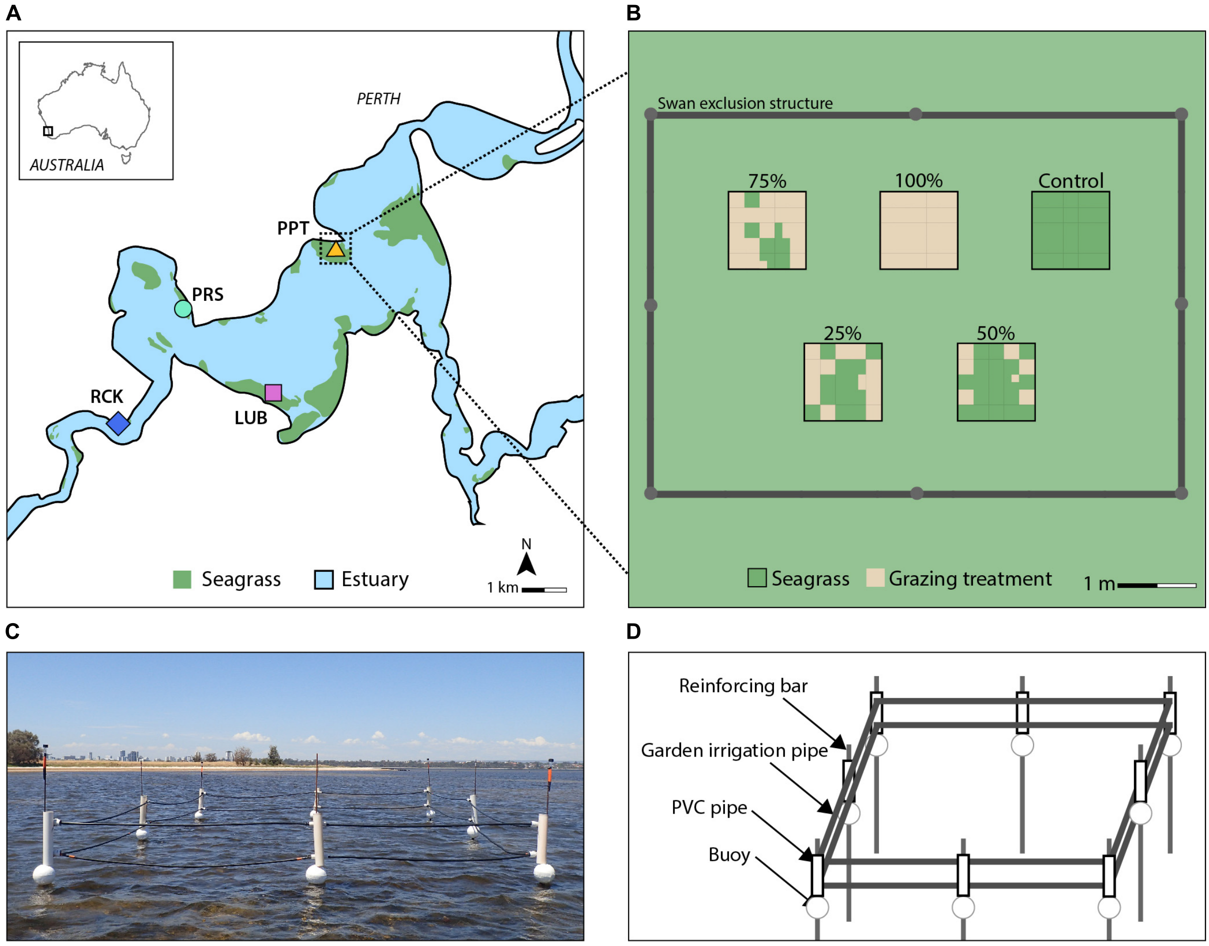

FIGURE 2
Four replicate blocks (colored symbols) were established across seagrass meadows (green area) in the Swan-Canning Estuary in Western Australia **(A)** (adapted from Forbes and Kilminster, 2014), and conceptual diagram of the replicate block design **(B)**. The grazing treatments show the placement of grazed cells (brown color) which were randomly allocated within a treatment, replicated across the four blocks within seagrass meadows (green color). Swan exclusion structures were constructed to prevent interference at each replicate block **(C)**. Each structure comprised eight steel reinforcing bars (3 m height, 12–16 mm diameter) supported by star pickets (0.5–1 m height) placed at least 1 m into the sediment and in water up to 1 m deep **(D)**. A buoy was threaded along each bar, supporting a two-stranded fence made of garden irrigation pipe threaded through modified PVC pipe brackets separated by a spacer.

1.5 m, with an average size of 0.28 m^2 (Dos Santos et al., 2012), represented by the 25% treatment in this experiment and treatments with greater removal represented increased grazing intensity. The seagrass cover in the PControl plots was not different to that in the Control plots over the duration of the experiment (**Supplementary Figure 1** and **Supplementary Table 2**), indicating that the exclusion structures did not impact the cover of seagrass.

The temporary exclusion structures were placed around each replicate "block" (experimental and Control plots, but not the PControl) to prevent natural grazing activity (**Figure 2**). The exclusion design was adapted from a similar experiment conducted in Chesapeake Bay United States to exclude mute swans (*Cygnus olor*, Gmelin) (Tatu et al., 2007) and which had no negative impacts on swans or other fauna (J. Anderson, personal communication). The structure used in the present study was designed to minimize interference with light availability and water flow. Birds were observed occasionally perching and/or roosting on the structures. The structure always remained above the surface of the water to prevent swan access throughout the tidal regime, while ensuring swan welfare. This study was reviewed and approved by the ethics committee at Edith Cowan University (project 21327).

Grazing was simulated on January 17, 2019 by dividing each 1 m^2 treatment plot into 25 equally sized cells (0.04 m^2) and removing seagrass biomass within a random selection of cells from each plot according to the treatment (**Figure 2**). Seagrass was removed using similar methods employed by Eklöf et al. (2009) and Choney (2012): rhizomes were cut with a core (Ø 9.6 cm) which encompassed most of one cell, and the plant material removed by hand. The seagrass was sieved so that most of the sediment was placed back into the cell.

Data collection and analysis

Swan presence

The number of swans within line of sight was assessed visually was recorded at each block and at each sampling time. There was no evidence of natural grazing scars within or near the experiment, except at one block Rocky Bay (RCK) at the end of the experiment period. Due to this interference, data is unavailable for biomass characteristics and recovery mechanisms in the 25% grazing treatment at RCK.

Percent cover

Halophila ovalis cover (%) was measured within each treatment, Control and PControl plot to determine the RT following simulated grazing treatments. Cover was recorded before grazing was simulated (time = 0), immediately following grazing, and then 14, 25, 49, 63, and 77 days following grazing. To measure cover, all 25 cells within each plot were photographed. A single image was taken of each cell and analyzed using SeaGIS TransectMeasure software. A regular grid of 25 measuring points was overlaid on each image and the presence/absence of seagrass at each point was recorded at each point. The points with seagrass present were pooled across all 25 images taken for one plot, and was divided by the total number of points across the plot (625 points measured over 1 m^2 plot) to provide an overall cover (%). Due to low tides which prevented photography, data are unavailable for all treatments on Day 0 at Lucky Bay (LUB) and for 25% at RCK. On rare occasions, treatments were unable to be sampled due to equipment failure: the PControl on Day 49, and 75% on Day 49 and 77 at Pelican Point (PPT); and the 25% on Day 49 at Point Resolution (PRS). Five to seven weeks following simulated grazing, a substantial proportion of *H. ovalis* leaves were shed from the plants as part of regular senescence resulting from reducing temperatures (Hillman et al., 1995). The PControl also followed this trend, indicating the reduction in cover was unlikely a result of the experiment treatments (**Supplementary Figure 1**). The natural grazing that occurred at RCK at the end of the experiment occurred at 77 days and therefore did not affect the collection of this data.

Recovery time

The RT of seagrass in each treatment plot was defined as the number of days required for the cover (%) in a treatment plot to reach the minimum cover observed in the Control plot over the experimental period. Thus, the cover criterion for recovery was set independently at each block, as there was variation in seagrass cover in the Control plots among blocks. RT for each treatment was calculated by plotting the change in cover over time, fitting a curve to this relationship and then using this curve to estimate the earliest time at which the cover of a treatment intercepted the minimum cover of the Control plot at the same block (**Supplementary Figure 2**). In some cases, the cover of seagrass in the treatments did not reach the minimum cover observed in the corresponding Control during the experiment, so the curve was extrapolated beyond the time of the experiment to determine the RT. For many plants, growth in the early life stages often follows an exponential trajectory, as has been demonstrated for the cumulative rhizome length of *H. ovalis* which exhibits linear growth in initial stages then exponential (Marbà and Duarte, 1998). Consequently, an exponential curve was applied to the data to describe the change in cover over time:

$$y \quad ae^{bx}$$

where y = cover (%) at a given time, a = cover (%) at Day 0 following simulated grazing, e = Euler's Number (∼2.7182), b = growth constant or continuous rate of increase in cover, and x = time (days).

Biomass characteristics

The biomass characteristics (leaf density, aboveground biomass and belowground biomass) were assessed 84 days following simulated grazing by destructively sampling within each plot. Three replicate samples were collected using cylindrical cores (9.6 cm diameter × 15 cm depth) in the same three randomly selected cells from each plot. The collected material was placed in calico bags and transported to the laboratory and stored at −5°C until processed. In the laboratory, the plant material was rewashed in estuary water, and the leaves scraped to remove excess sediment and epiphytic material. The leaves and petioles were separated from the rhizomes using a blade, and the leaf density, aboveground (leaf + petiole) and belowground (rhizome + root) biomass (g DW, following drying at 60°C) was recorded per 0.007 m^2. Data are unavailable for RCK in the 25% treatment due to natural grazing occurring before sample collection.

Recovery mechanisms

The experimental sites were monitored on day 14 and day 25, to determine whether the mechanism of recruitment of new vegetative material into grazed areas was through: (a) rhizome extension from surrounding seagrass patches, characterized by unbroken rhizomes extending into "grazed" areas from the adjacent meadow; (b) establishment of vegetative fragments, where there was no rhizome connection between the recruited material and the surrounding meadow; or (c) germination of seeds. The presence of each recovery mechanism was recorded for all "grazed" cells within each treatment. Due to the nature of the simulated grazing treatments, with different treatments having a different number of grazed cells, the data were pooled across the two sampling periods and expressed as a proportion of the number of cells measured in each plot at each location.

The samples collected for the biomass were further processed to determine the characteristics related to potential mechanisms of recovery. These were: (a) node density, (b) the ratio between above and belowground biomass which can indicate the allocation of resources, (c) branching frequency (number of formed branches, as a proportion of node density), and (d) branching potential frequency (number of apical buds, as a proportion of node density) which can all indicate the patterns of clonal growth; (e) flowering and fruiting frequency (as a proportion of nodes), which can indicate potential investment in sexual reproduction.

Statistical analysis

To test for differences in RT, the data were analyzed using a permutation two-way analysis of variance (using Primer v6+ and PRIMER-E) with two factors: (1) Treatment: 25, 50, 75, and 100% grazing nested in Block; and (2) Block: LUB, PPT, PRS, and RCK. The PERMANOVA analysis was run on the resemblance matrix using Euclidean distance (*p*-value = 0.05) with between 822 and 840 unique permutations for each treatment combination. The dispersion of the data was tested on raw data using PERMDISP (*p*-value = 0.05). The null hypothesis for the analysis was there was no difference in RT across the four grazing intensity treatments. Permutation pair-wise tests were performed, following significant mains-test, to determine which levels within each factor were significantly different. A PERMANOVA was conducted on the meadow characteristics and recovery mechanisms to test for significant changes in each variable for Treatment, Block, and interactions (Treatment × Block) as fixed factors. Main and pair-wise tests were run as described for RT.

To test if the aboveground cover (%) of seagrass could predict the characteristics of the meadow (leaf density, aboveground biomass and belowground biomass), we pooled all replicate blocks and treatments and calculated Spearman rank correlation coefficient (ρ) with a *p*-value of 0.05. This was done for each of the three meadow characteristics in each treatment with the seagrass cover at the corresponding time point (**Supplementary Figure 3**).

Results

Swan presence

Swans were observed at all blocks except PRS, at least once during the experiment (**Supplementary Table 3**). Swans were observed the most at LUB on almost 90% of sampling occasions and at PPT more than 60% of the time, with up to 60 and 35 individuals observed at one time, respectively. At RCK, two individuals were observed grazing immediately beside the exclusion structure, but only once (63 days following simulated grazing). Natural grazing was observed in the 25% treatment at RCK on the following sampling occasion.

Cover

Seagrass cover in the control plots was variable over the experiment but was consistently higher than in the treatments, and the cover decreased toward the end of the experiment due to senescence (**Supplementary Figure 1**). The grazing simulation effectively reduced seagrass cover in each treatment and was proportional to the treatment imposed. Cover increased in all treatment plots over the experiment, but the extent of these increases varied among treatments. By the end of the experiment (77 days post-grazing), 13 of the 16 treatment plots were more than 70% of the respective control plots and RTs could be calculated within this timeframe. However, some treatments that did not reach the criteria and the RT was extrapolated (**Figure 3**).

Recovery time

The RT following simulated grazing ranged from 25 to 135 d and, generally, increased with the intensity of grazing (**Figure 4**). PERMANOVA indicated a significant effect of treatment on RT ($F = 12.489$, $p < 0.05$, **Supplementary Table 1**). *Post-hoc* analysis showed no significant difference in the mean RT between the 25 and 50% grazing treatments (**Supplementary Table 4**, $p > 0.05$, **Figure 4**), which averaged 28 ± 1 and 35 ± 3 d, respectively. The 75% grazing treatment had a significantly (**Supplementary Table 4**, $p < 0.05$, **Figure 4**) longer RT (75 ± 13 d), and the longest RT was the 100% treatment (92 ± 13 d) which was significantly different to all other treatments (**Supplementary Table 4**, $p < 0.05$, **Figure 4**). The variability in RT also increased with grazing intensity, with a small coefficient of variation (CV) in the 25% treatment of 6% increasing to 21% in the 50% treatment, to 41% in the 75% treatment and 32% in the 100% treatment. This increase in variability was due, primarily, to the much longer RT for the 75 and 100% grazing treatments at the PRS block, which were nearly two times longer than in the other blocks.

Biomass characteristics

PERMANOVA identified significant differences among treatments in the leaf density ($F = 10.819$, $p < 0.05$), aboveground biomass ($F = 6.473$, $p < 0.05$) and belowground biomass ($F = 15.996$, $p < 0.05$), 84 days after simulated grazing (**Supplementary Table 1**). *Post-hoc* analysis indicated there were no statistically significant differences between the Control and PControl

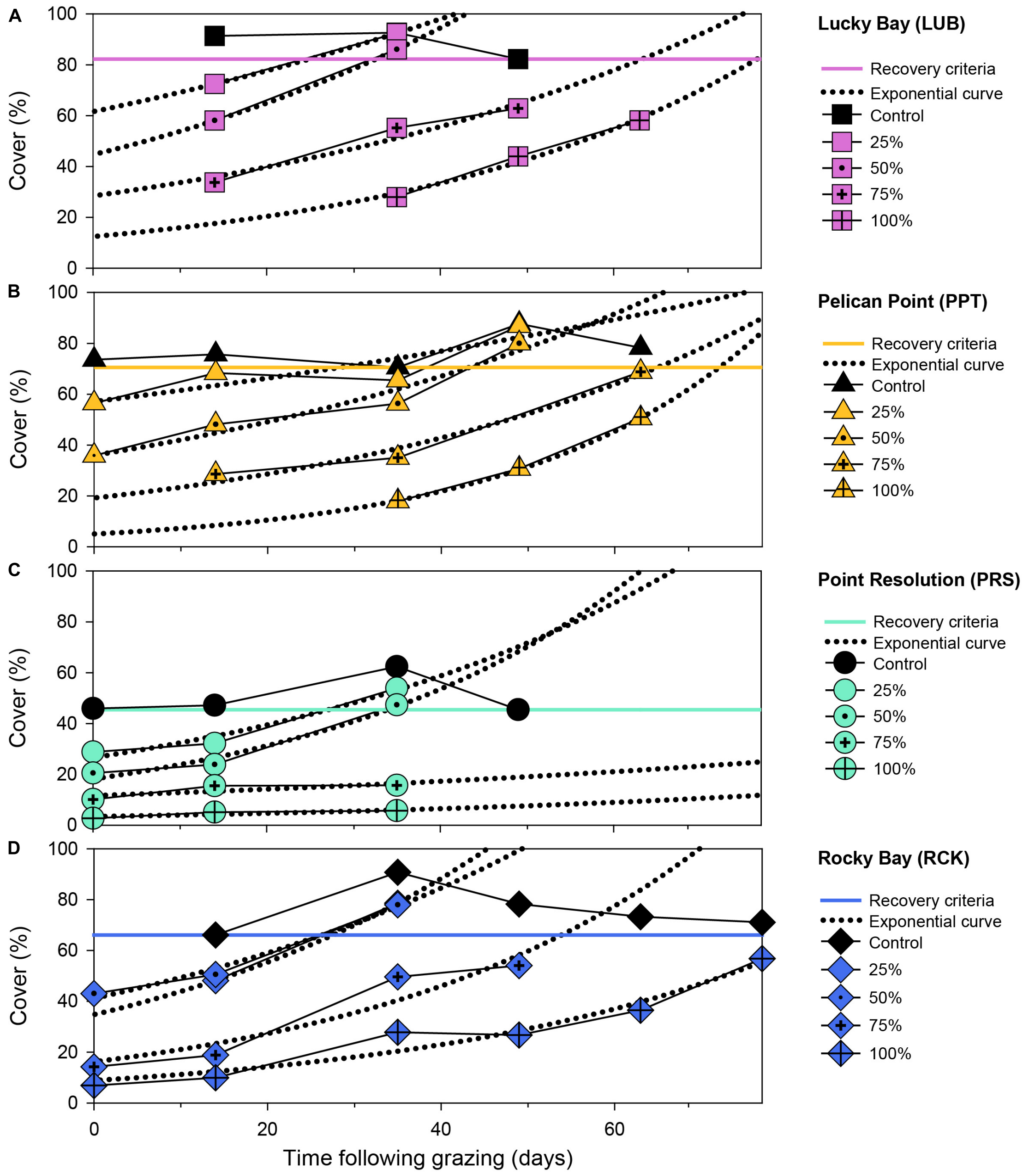

FIGURE 3
Seagrass aboveground cover (%) over time following simulated grazing (simulated on Day 0) at four replicate block locations: Lucky Bay **(A)**, Pelican Point **(B)**, Point Resolution **(C)**, and Rocky Bay **(D)**. Data is the cover of each treatment plot at each replicate block location (solid lines with colored symbols) with an exponential curve applied (dotted black lines). The minimum recovery time (RT) for seagrass cover was determined at the point at which the fitted curve intercepted the minimum cover observed in the control plot (solid colored line). Missing points is where data is unavailable.

plots for these characteristics (Supplementary Table 5, $p > 0.05$), but there were among the Control plots and the higher grazing treatments (Supplementary Table 5, $p < 0.05$, Figure 5). Although there were significant differences among blocks, as the interaction term was not significant, the response of the grazing treatments was consistent among blocks.

By 84 days following simulated grazing, the leaf density and belowground biomass was the same as the Control only in the 25% grazing treatment (Table 1 and Figure 5). For aboveground biomass, there was no difference between the Control and the 25 and 50% grazing treatments (Table 1, Figure 5, and Supplementary Table 5), but all other treatments were lower than the controls, despite the observed or predicted recovery

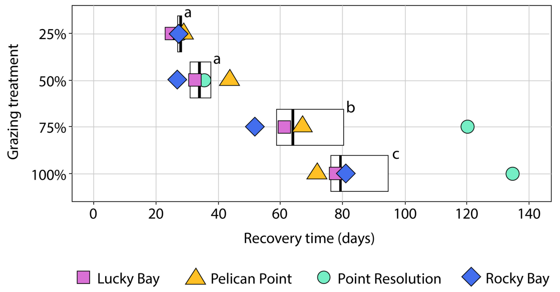

FIGURE 4
Boxplot of the Recovery Time (RT) in days of *Halophila ovalis* based on cover (%) following different intensities of simulated grazing (removal of 25, 50, 75, and 100%). Lower and upper box boundaries are the 25th and 75th percentiles, respectively, the dark vertical line is the median, the lower and upper error bars are the 10th and 90th percentiles, respectively. The symbols indicate the RT at the replicate blocks in the Swan-Canning Estuary: Lucky Bay (LUB), Pelican Point (PPT), Point Resolution (PRS), and Rocky Bay (RCK). Letters indicate statistical significance among RT for treatments based on permutational pair-wise *post-hoc* analysis (**Supplementary Table 4**, $p \leq 0.05$). Where bars share the same letter, there is no significant difference.

of seagrass cover for all treatments (except for the high grazing treatments at PRS) by that time (**Table 1**). The exceptions to this were the 75 and 100% grazing treatments at the PRS block, where recovery based on cover had also not occurred by 84 days.

For pooled data across all replicate blocks and treatments, the Spearman's rho correlation analysis indicated a significant and strong positive correlation between the leaf density and cover ($r = 0.71$, $p < 0.001$), and between aboveground biomass and cover ($r = 0.71$, $p < 0.001$), and significant, moderately positive correlation between the belowground biomass and cover ($r = 0.41$, $p < 0.001$; **Supplementary Figure 3**).

Recovery mechanisms

The 25, 50, and 75% grazing treatments at all replicate blocks had recruitment of *H. ovalis* into all the cleared cells *via* rhizome extension in the first 4 weeks following simulated grazing. There was rhizome extension observed in 70–100% of the cells in the 100% treatments (**Table 2**). Recruitment from the settlement of vegetative fragments was observed in 40–46% of cells in the higher intensity grazing treatments (75 and 100%), and less in the lower grazing treatments (12–19% of cells). No recruitment from seedlings was observed throughout the experiment. Visual inspection in the field and in the cover photographs indicated that grazed cells near the edge of the plot regenerated before grazed cells in the center of the plot.

There were significant differences in node density among treatments (PERMANOVA; $F = 31.441$, $p < 0.05$), branching frequency ($F = 11.941$, $p < 0.05$), and above to belowground biomass ratio ($F = 9.693$, $p < 0.05$) between the treatment and control plots, and sometimes there were also differences among blocks (**Supplementary Table 1**). While *post-hoc* analysis indicated there were significantly less nodes in all treatments compared to the Control plots (**Supplementary Table 6**, $p < 0.05$, **Figure 6**), there was significantly more branching in the grazing treatments (**Supplementary Table 6**, $p < 0.05$, **Figure 7**). The aboveground to belowground biomass ratio was significantly higher in grazed plots than in Controls (**Supplementary Table 6**, $p < 0.05$, **Figure 6**), and this effect generally increased with the intensity of grazing. There were no significant differences observed between the Control plots and treatments for branching potential frequency (**Supplementary Table 6**, $p > 0.05$, **Figure 7**). There was a significant interaction between Block and Treatment observed for the frequency of male flowers (**Supplementary Table 1**, $F = 2.962$, $p < 0.05$), with *post-hoc* analysis showing a greater proportion of male flowers observed in treatments compared to the control but at LUB only (**Supplementary Table 7**, $p < 0.05$, **Figure 8**). There were also significant differences identified at PRS, but in this case it was due to the absence of flowers in the 25% treatments. There were no differences for female flower frequency for any factor (**Supplementary Table 1**, $p > 0.05$, **Figure 8**).

Discussion

The findings supported our hypothesis that greater extent of grazing pressure resulted in slower RT of seagrass cover, with higher intensity grazing requiring between 2.5 and 5 times longer (11–20 weeks) to achieve recovery than lower intensity grazing (4–5 weeks). These findings are broadly consistent with O'Brien et al.'s (2018) generalization that recovery is influenced by the spatial extent of disturbance, with recovery delayed or compromised when a larger area is impacted. These recovery times (RTs) were even greater for other seagrass attributes, such as below-ground biomass. Not only did the RT increase with greater grazing pressure, but so too did the variability in the time required for recovery. Somewhat unexpectedly, there was no recovery from seed germination, instead it was overwhelmingly due to vegetative growth from the surrounding meadow with some contribution from settlement and growth of seagrass fragments. We discuss these findings in context of seagrass resilience and consider their implications for estuarine seagrasses under changing climate regimes.

Grazing is a physical and mechanical disturbance that removes biomass (Valentine and Heck, 1999), and in this study, the initial impact to seagrass cover was proportional to the treatment intensities simulated. As recovery of the disturbed patch requires either vegetative expansion from the surrounding meadow, immigration of fragments parts of adult plants, or the germination of seeds stored in seed banks or immigrated from other areas, when more biomass is removed it takes longer to

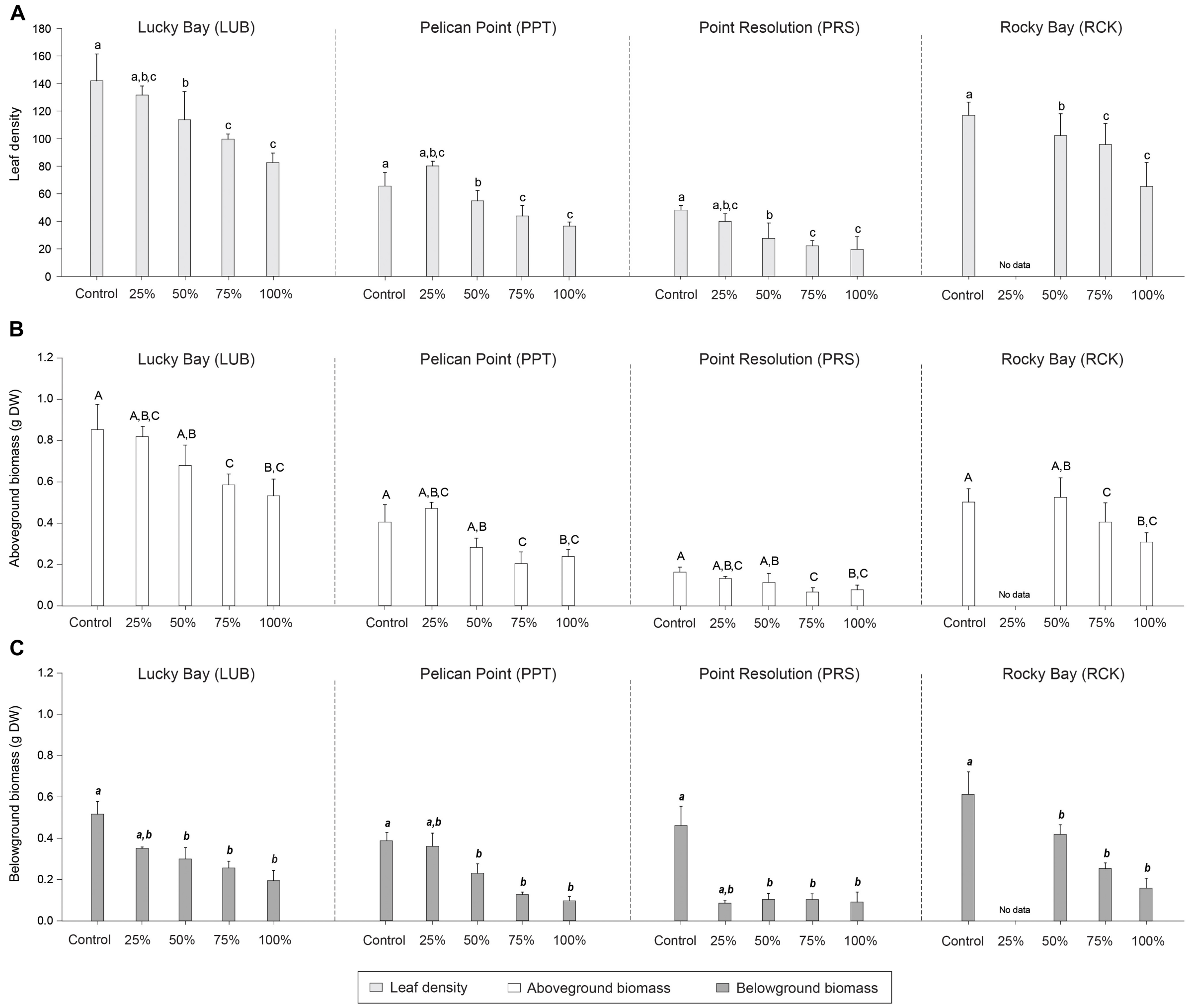

FIGURE 5
Mean leaf density (**A**; light gray bars), aboveground biomass (**B**; white bars), and belowground biomass (**C**; dark gray bars) in the grazing treatment plots (25, 50, 75, and 100% removal) and Control plots at the four replicate block locations across the Swan-Canning Estuary: Lucky Bay (LUB), Pelican Point (PPT), Point Resolution (PRS), and Rocky Bay (RCK). Statistical significance (pairwise test) is indicated in lowercase letters for leaf density, uppercase letters for aboveground biomass, and bold italic letters for belowground biomass based on pairwise *post-hoc* comparison summarized in **Supplementary Table 5** ($p \leq 0.05$). Where bars share the same letter, there is no significant difference. Pairwise testing indicated significant differences among blocks for leaf density (LUB = RCK > PPT > PRS), aboveground biomass (LUB > RCK > PPT > PRS) and belowground biomass (LUB = RCK > PPT > PRS) (**Supplementary Table 5**; $p \leq 0.05$). Data are the means (n = 3) ± standard error.

TABLE 1 **Comparison between the minimum recovery time (RT) (days) based on aboveground cover (%) to the meadow characteristics sampled 84 days post-grazing treatments.**

Grazing treatment	Recovery time (days)				Leaf density or belowground biomass				Aboveground biomass			
	LUB	PPT	PRS	RCK	LUB	PPT	PRS	RCK	LUB	PPT	PRS	RCK
25%	25	29	28	28	✓	✓	✓	✓	✓	✓	✓	✓
50%	33	44	35	27	x	x	x	x	✓	✓	✓	✓
75%	61	67	120	52	x	x	x	x	x	x	x	x
100%	78	72	135	81	x	x	x	x	x	x	x	x

Cell color indicates if the treatment was predicted to have recovered at the time of sample collection (blue = recovered, red = not recovered) and the symbols indicate if the meadow characteristic was recovered based on the difference to the Control plot (tick = characteristic not significantly different to Control, cross = significantly different to Control).

TABLE 2 Proportion (%) of recovery due to (1) rhizome extension and (2) vegetative fragments observed up to 4 weeks following grazing simulation (25, 50, 75, and 100% removal), as a proportion (%) of the number of grazed cells measured in each treatment, per replicate block in the Swan-Canning Estuary: Lucky Bay (LUB), Rocky Bay (RCK), Pelican Point (PPT), and Point Resolution (PRS).

Block	**Treatment**			
	25%	**50%**	**75%**	**100%**
(1) Rhizome extension				
LUB	100	100	100	100
RCK	100	100	100	90
PPT	100	100	100	90
PRS	100	100	100	70
Mean	100 ± 0	100 ± 0	100 ± 0	87.5 ± 6.3
(2) Vegetative fragments				
LUB	0	25	66.7	70
RCK	50	50	66.7	40
PPT	0	0	16.7	30
PRS	0	0	33.3	20
Mean	12.5 ± 12.5	18.75 ± 12.0	45.8 ± 12.5	40 ± 10.8

recover. If recovery mechanisms were driven by immigration, this could reduce the RT, as the immigrants would more likely fill in the space in a random manner, whereas if recovery was driven by growth from the remaining meadow, the timescale of recovery would be longer with a greater extent of disturbance, as it is strongly dependent on the size and spatial orientation of empty patches and in-filling from the edge of disturbance (Rasheed, 2004; Rasheed et al., 2014). The plants in this study relied entirely on vegetative mechanisms to recover, mostly through the expansion of rhizomes from the surrounding ungrazed meadow, which explains the dependence of RT on disturbance intensity. A small proportion of recruitment was from fragments, a novel finding for this species (McMahon et al., 2014), and no recovery from seed. While recovery through sexual reproduction could have been expected for this colonizing species of seagrass (Kilminster et al., 2015), recovery from seed was not observed in this study. While *H. ovalis* can develop large seed bank reserves and this has been documented locally (Kilminster et al., 2015; Webster et al., 2021), germination is most likely to occur in spring (Statton et al., 2017), a period not captured in this study. The time of year when recruitment from seed germination is likely to occur coincides with periods of slower plant growth (Ronald et al., 2014; Cubas, 2020), whereas this study was conducted in summer during the peak growth period for this seagrass (Hillman et al., 1995; Kilminster and Forbes, 2014). The findings indicate that even in a colonizing species of seagrass, regrowth from surrounding meadows may be integral to the recovery, especially during periods when recruitment from seed does not occur and, consequently, recovery could be delayed or prevented in less dense or patchy meadows or following large-scale or repeat disturbance (Preen, 1995; Gangal et al., 2021). If, indeed, the dependence of recovery on vegetative regrowth is a result of the timing of the experiment, and because growth rates are highest at this time of year, then the RTs we report here are likely to be faster than at other times of year when growth rates are slower. However, this assumes that rhizome extension is the primary source of recovery at all times, which is unlikely given that *H. ovalis* produces viable seedbanks (Kuo and Kirkman, 1992). It is possible that at some times of year, the seedbank may provide an alternative source of recovery, though it is not possible from our results to predict how enhanced seed-based recovery might also influence the timeframe of recovery. It is also not completely clear whether recovery from seeds could occur throughout the year, or only at some times. *H. ovalis* seeds are known to have a dormancy period and so release from that dormancy would be required for seed-based recovery (Kuo and Kirkman, 1992). For some seeds, physical disturbance (such as that caused by grazing) is known the break the dormancy of seeds (Peterken and Conacher, 1997), but our findings suggest that this is not the case here. This is consistent with finding of Statton et al. (2017), that *H. ovalis* seeds require changes in temperature to break the dormancy. Thus, we conclude that recovery occurs through vegetative expansion, without excluding the possibility for other mechanisms at other times of year.

Rapid clonal growth and in-filling of patches is facilitated by branching (Marbà and Duarte, 1998; Kilminster et al., 2015). This was evident with 1.8–8.2 times more branching in the recovered grazed patches compared to ungrazed seagrass. This increased formation of branches to facilitate recovery has been recorded for this species following dugong grazing (Preen, 1995; Nakaoka and Aioi, 1999) and other mechanical disturbances like boat anchor scarring (Widmer, 2006). These studies also found similar recovery timeframes of weeks to months. In contrast, RT for other, persistent or opportunistic seagrass species is much longer for disturbance of similar spatial scales. For *Z. muelleri* meadows following simulated swan grazing (Dos Santos et al., 2013), *Thalassia*, *Syringodium*, and *Posidonia* meadows following urchin grazing (Eklöf et al., 2008), and in *Posidonia* meadows following removal from boat moorings (Glasby and West, 2018), the RT is in the order of years to decades. This suggests that recovery timescales are dependent on the species and life history strategy (Kilminster et al., 2015), and rapid recovery cannot be assumed for all seagrass meadows. The species or life history should be an important consideration when determining the timeframe of how monitoring programs set recovery targets. This is particularly important as the condition or persistence of meadows can be influenced by external factors over space and time (O'Brien et al., 2018).

Two possible factors could explain the greater variability in the RTs following higher grazing intensity: (1) disruption of self-sustaining feedbacks following disturbance at larger scales; or

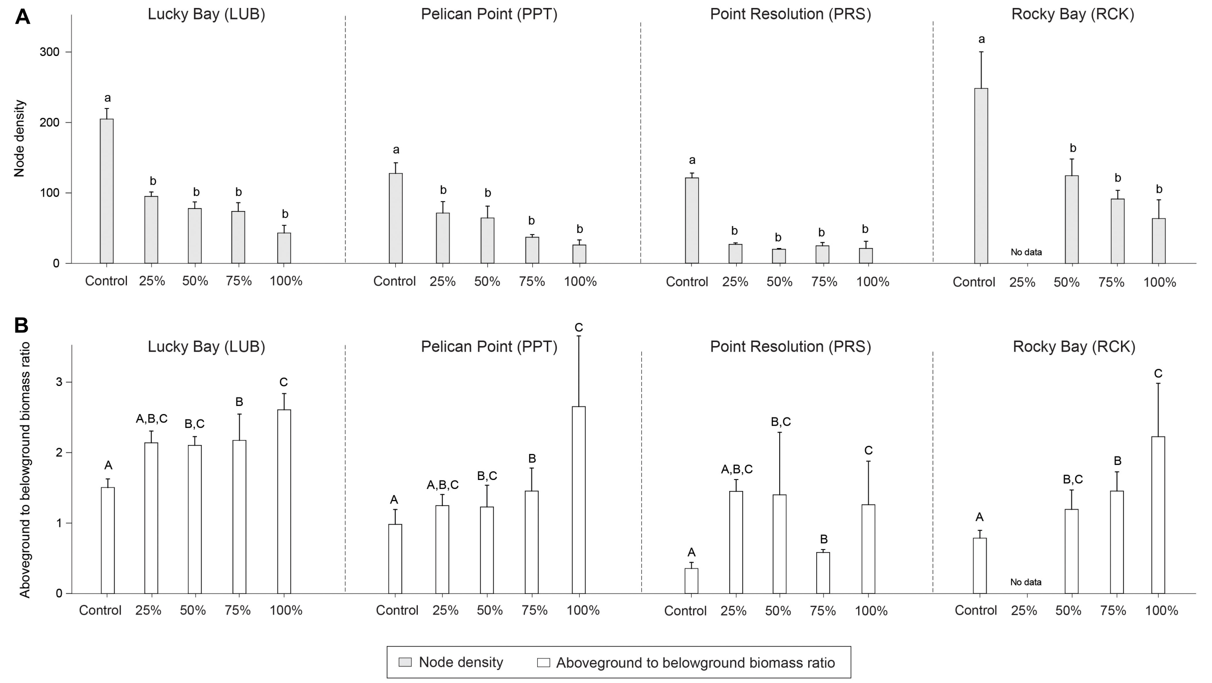

FIGURE 6

Mean node density (**A**; light gray bars) and ratio of aboveground to belowground biomass (g DW) ratio (**B**; white bars) in the grazing treatment plots (25, 50, 75, and 100% removal) and Control plots at the four replicate block locations across the Swan-Canning Estuary: Lucky Bay (LUB), Pelican Point (PPT), Point Resolution (PRS), and Rocky Bay (RCK). Statistical significance (pairwise test) is indicated in lowercase letters for node density and uppercase letters for the aboveground to belowground biomass (g DW) ratio based on pairwise *post-hoc* comparison summarized in **Supplementary Table 6** ($p \leq 0.05$). Where bars share the same letter, there is no significant difference. Pairwise test indicated significant differences among blocks for node density (RCK > LUB > PPT > PRS) and aboveground to belowground biomass (g DW) ratio (LUB > RCK = PPT > PRS) (**Supplementary Table 6**; $p \leq 0.05$). Data are the means ($n = 3$) ± standard error.

(2) population-specific resilience to grazing. When loss occurs at a larger scale, recovery can become delayed and difficult to predict (O'Brien et al., 2018). In the first case, the RT at one location in the Swan-Canning Estuary was twice as long as other areas, and at this location there is greater exposure to prevailing south-westerly winds and closer proximity to boat traffic. These features could result in hydrodynamic conditions that resuspend sediment, particularly where seagrass is removed, resulting in rhizome disturbance and light limitation that hinders seagrass growth and recovery (Nowicki et al., 2017; O'Brien et al., 2018), a well-known feedback system in seagrass ecosystems (Ralph et al., 2007; Reise and Kohlus, 2008; Adams et al., 2016; Maxwell et al., 2017). Alternatively, the distribution of herbivores across many aquatic and terrestrial ecosystems can be concentrated to areas with preferable forage or habitat conditions (Lefévre and Bellwood, 2011; Owen-Smith, 2014). Dense herbivore populations can intensify grazing activity to certain areas within an ecosystem, affecting the consumption and distribution of plant communities (Lefévre and Bellwood, 2011). Seagrass meadows that host dense herbivore populations could develop population-specific resilience to grazing (White et al., 2011). Terrestrial plant populations with previous exposure to herbivory can develop higher tolerance than populations with low levels of herbivory (Boalt et al., 2010). The seagrass meadows in this study with the fastest RTs have been recorded as swan population "hotspots" (Choney et al., 2014), which suggests these meadows may be more resilient to grazing than others, in the same way that population-specific resilience of seagrass in this system has been observed following exposure to hyposalinity (Webster et al., 2021) and is being actively explored in seagrass ecosystems more generally (Bennett et al., 2021). Irrespective of the cause, the greater variability and reduced predictability of recovery response to higher magnitude of disturbance highlights that RT can be variable at the local scale (Smith et al., 2016). Understanding the existing grazing pressure, local environmental conditions, and recovery mechanisms will help to identify if a seagrass ecosystem is vulnerable (Jenkins et al., 2015), and if it could be appropriate for "future-proofing" restoration efforts (Wood et al., 2019). The results of this study should, however, be taken in context of the small-scale and single occurrence of disturbance, which is unlikely to reflect grazing patterns in nature and more work is needed to upscale these predictions to larger disturbances with confidence.

Increased grazing pressure can occur as a result of increased herbivore populations (Choney et al., 2014; Kollars et al., 2017;

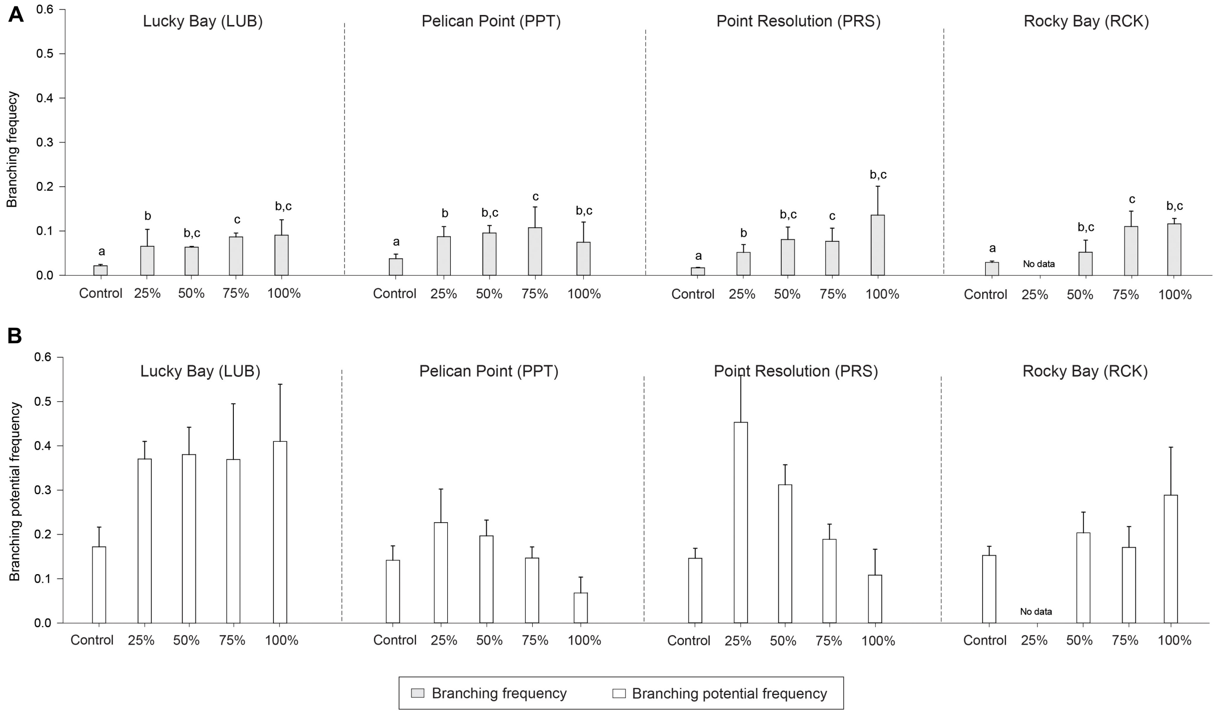

FIGURE 7
Mean branching frequency (**A**; light gray bars) and branching potential frequency (**B**; white bars) in the grazing treatment plots (25, 50, 75, and 100% removal) and Control plots at the four replicate block locations across the Swan-Canning Estuary: Lucky Bay (LUB), Pelican Point (PPT), Point Resolution (PRS), and Rocky Bay (RCK). Statistical significance (pairwise test) is indicated in lowercase letters for branching frequency based on pairwise *post-hoc* comparison summarized in **Supplementary Table 6** ($p \leq 0.05$). Where bars share the same letter, there is no significant difference. If the main PERMANOVA test showed no significant effect, then no pairwise results are included. Pairwise testing indicated significant differences among blocks for branching frequency (LUB > PRS > PPT, LUB > RCK) (**Supplementary Table 6**; $p \leq 0.05$). There was no statistical significance for branching meristem frequency ($p > 0.05$). Data are the means ($n = 3$) ± standard error.

Fourqurean et al., 2019; Buckee et al., 2021), and is already occurring as a result of climate change (Traill et al., 2009). Examples of this include the extension of herbivore distribution ranges due to tropicalisation (Hyndes et al., 2016) or concentration of populations in remaining suitable habitat in drying climates (Chambers, 2008). Meadows lacking an ability to rapidly recover can remain vulnerable if grazing continues or if other disturbances prevail (Dos Santos et al., 2013; O'Brien et al., 2018; Gangal et al., 2021). This can lead to overgrazing and, in extreme cases, the functional extinction of seagrass (Gangal et al., 2021). We have applied our findings to assess the risk of overgrazing of seagrass in this estuarine ecosystem, combining our new understanding of recovery timeframes, together with derived estimates of herbivore density, grazing pressure, and seagrass abundance across the estuary. Dos Santos et al. (2012) estimated a seagrass consumption rate of 394 g DW swan^{-1} day^{-1}. Assuming an estimated swan population of ~300 individuals (M. Bamford, personal communication) and a total biomass of seagrass in the Swan-Canning Estuary of 6×10^9 g DW (~150 g DW m^{-2} multiplied by 4.03 million m^2 of seagrass; Kilminster and Forbes, 2014), then swans would consume 9% of seagrass biomass annually, assuming no recovery growth of the seagrass during that time. Thus, if the population of swans were to more than triple in the estuary, it could take more than 4 years for complete removal of seagrass from the system, in the absence of any recovery (**Supplementary Table 8**). The relatively low abundance of herbivores imposing comparatively low grazing pressure, and the rapid recovery of this seagrass suggests that these meadows are resilient to grazing now and into the future. This is unlikely to be the case for other ecosystems with high and/or dense herbivore populations and slower-growing seagrass species (Buckee et al., 2021; Gangal et al., 2021).

The response of seagrass cover and other meadow attributes that we observed in our study indicate the importance of choosing appropriate seagrass variables as indicators of condition. We defined RT in this study based on the aboveground cover of seagrass, but this did not indicate recovery of other plant attributes or meadow characteristics, despite a significant correlation between seagrass cover and these characteristics. This was evident in the leaf density and belowground biomass only having recovered to the condition of undisturbed seagrass in the least intense grazing treatment, despite our cover-based RT estimates projecting nearly all treatments at all locations to have recovered by the time the

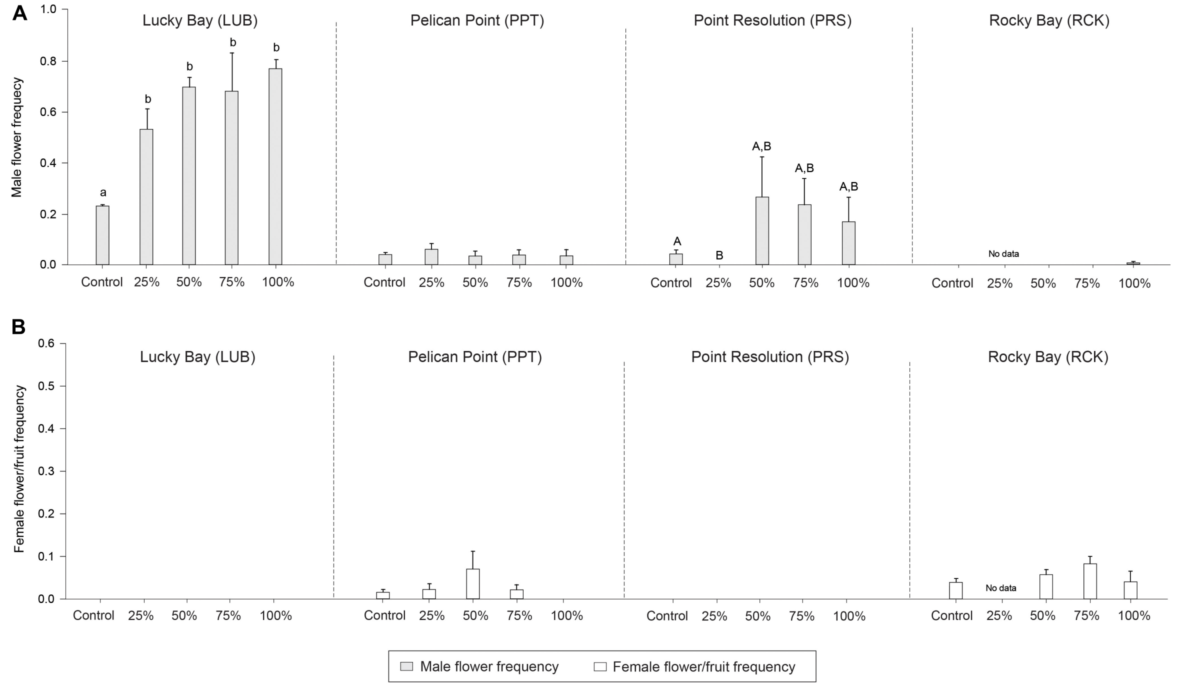

FIGURE 8
Mean male (**A**; light gray bars) and female (**B**; white bars) reproduction frequency in the grazing treatment plots (25, 50, 75, and 100% removal) and Control plots at the four replicate block locations across the Swan-Canning Estuary: Lucky Bay (LUB), Pelican Point (PPT), Point Resolution (PRS), and Rocky Bay (RCK). Statistical significance (pairwise test) is indicated based on pairwise *post-hoc* comparison summarized in **Supplementary Table 7** ($p \leq 0.05$). Pairwise testing also indicated a significant interaction between treatment and block for male reproduction frequency (**Supplementary Table 7**; $p < 0.05$). Where bars share the same letter, there is no significant difference. If the main PERMANOVA test showed no significant effect, then no pairwise results are included. There was no statistical significance for female reproduction frequency ($p > 0.05$). Data are means ($n = 3$) ± standard error.

samples were collected. In this example, aboveground cover underestimated the RT of other seagrass attributes and is unlikely to indicate recovery of ecosystem services provided by seagrasses, such as habitat availability, nutrient cycling, and sediment stabilization (Hemminga and Duarte, 2000; Unsworth et al., 2015). Tiered approaches to monitoring that have clear recovery criteria and that incorporate indicators of recovery at several scales can provide efficient means of detecting and predicting trends in seagrass ecosystems and the services they provide (Neckles et al., 2012). While aboveground cover may underestimate the RT of other seagrass attributes, it can be beneficial as an observational measure of recovery as it is non-destructive.

Conclusion

Aboveground cover of *H. ovalis* recovers within 3 months in the peak growth period from a single grazing event which removed up to 1 m^2 of meadow. Increased extent of swan grazing not only lengthened the time for seagrass to recover, but also increased the variation in RT among different locations in one ecosystem. This suggests that some meadows are more resilient to grazing than others. Overall, seagrass recovery occurred consistently through vegetative growth from intact surrounding meadows, indicating the importance of maintaining healthy seagrass populations to facilitate recovery, though it is possible that at other times of year recovery from seed banks may be important. While recovery of aboveground cover was rapid (weeks to months), it may not accurately predict other seagrass attributes. A combination of recovery indicators can be considered in monitoring programs which aim to track recovery from disturbance. Multiple indicators of seagrass recovery should be incorporated into programs to increase confidence in the conclusion made regarding meadow regeneration. Failure to do so may limit the detection and predictability of seagrass recovery and put these precious ecosystems at risk.

Data availability statement

The datasets generated for this study can be found in the Research Online repository at Edith Cowan University, available at: dx.doi.org/10.25958/fq3d-m857.

Ethics statement

The animal study was reviewed and approved by Ethics Committee, Edith Cowan University (Project 21327).

Author contributions

CO'D: conceptualization, methodology, formal analysis, investigation, data curation, writing – original draft preparation, visualization, project administration, and funding acquisition. PL and KM: conceptualization, methodology, writing – review and editing, and supervision. CW: formal analysis, investigation, data curation, writing – review and editing, and visualization. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.947109/full#supplementary-material

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EDITED BY
Rob Coles,
James Cook University, Australia

REVIEWED BY
Zhijian Jiang,
South China Sea Institute of Oceanology (CAS), China
Richard K. F. Unsworth,
Swansea University, United Kingdom
Seung Hyeon Kim,
Pusan National University, South Korea

*CORRESPONDENCE
Xuan-Vy Nguyen
nguyenxuanvi@gmail.com
Jutta Papenbrock
Jutta.Papenbrock@botanik.uni-hannover.de

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Current advances in seagrass research: A review from Viet Nam

Xuan-Vy Nguyen[1,2*], Thi Thuy Hang Phan[3], Van-Luong Cao[2,4], Nhu-Thuy Nguyen Nhat[1], Trung-Hieu Nguyen[1], Xuan-Thuy Nguyen[1], Va-Khin Lau[1], Cong-Tin Hoang[3], My-Ngan Nguyen-Thi[1], Hung Manh Nguyen[5,6], Viet-Ha Dao[1,2], Mirta Teichberg[7] and Jutta Papenbrock[8*]

[1]Institute of Oceanography, Viet Nam Academy of Science and Technology, Nha Trang, Vietnam, [2]Faculty of Marine Science and Technology, Graduate University of Science and Technology, Ha Noi, Vietnam, [3]University of Sciences, Hue University, Hue, Vietnam, [4]Institute of Marine Environment and Resources, Viet Nam Academy of Science and Technology, Hai Phong, Vietnam, [5]Dead Sea and Arava Science Center, Central Arava Branch, Hatseva, Israel, [6]French Associates Institute for Agriculture and Biotechnology of Dryland, The Jacob Blaustein Institutes for Desert Research, Ben-Gurion University of the Negev, Sede Boqer Campus, Midreshet Ben-Gurion, Israel, [7]Ecosystems Center, Marine Biological Laboratory (MBL), Woodshole, MA, United States, [8]Institute of Botany, Leibniz University Hannover, Hannover, Germany

Seagrass meadows provide valuable ecosystem services but are fragile and threatened ecosystems all over the world. This review highlights the current advances in seagrass research from Viet Nam. One goal is to support decision makers in developing science-based conservation strategies. In recent years, several techniques were applied to estimate the size of seagrass meadows. Independent from the method used, there is an alarming decline in the seagrass area in almost all parts of Viet Nam. Since 1990, a decline of 46.5% or 13,549 ha was found. Only in a few protected and difficult-to-reach areas was an increase observed. Conditions at those sites could be investigated in more detail to make suggestions for conservation and recovery of seagrass meadows. Due to their lifestyle and morphology, seagrasses take up compounds from their environment easily. Phytoremediation processes of *Thalassia hemprichii* and *Enhalus acoroides* are described exemplarily. High accumulation of heavy metals dependent on their concentration in the environment in different organs can be observed. On the one hand, seagrasses play a role in phytoremediation processes in polluted areas; on the other hand, they might suffer at high concentrations, and pollution will contribute to their overall decline. Compared with the neighboring countries, the total C_{org} stock from seagrass beds in Viet Nam was much lower than in the Philippines and Indonesia but higher than that of Malaysia and Myanmar. Due to an exceptionally long latitudinal coastline of 3,260 km covering cool to warm water environments, the seagrass species composition in Viet Nam shows a high diversity and a high plasticity within species boundaries. This leads to challenges in taxonomic issues, especially with the *Halophila* genus, which can be better deduced from genetic diversity/population structures of members of Hydrocharitaceae. Finally, the current seagrass conservation and management efforts in Viet Nam are presented and discussed. Only decisions based on the interdisciplinary cooperation of scientists from all disciplines mentioned will finally lead to conserve this valuable ecosystem for mankind and biodiversity.

KEYWORDS

degradation, distribution, diversity, ecosystem services, seagrass, taxonomy

Introduction

Seagrasses are marine angiosperms that have recolonized the marine habitat approximately 100 million years ago during at least three events (Les et al., 1997). Seagrasses are found in thousands of kilometers of the sedimentary shorelines ranging from tropical to temperate regions. They are found in different aquatic conditions including hypersaline, marine or brackish water at estuarine, nearshore, and subtidal and intertidal sand (Short et al., 2007). They are foundation species and provide essential ecosystem services, e.g., oxygen production, habitat providers, nutrient recycling, among many others (Orth et al., 2006; Fourqurean et al., 2012; Lamb et al., 2017), and represent one of the most significant natural carbon sinks on Earth (Fourqurean et al., 2012; Macreadie and Hardy, 2018). Nonetheless, the seagrass population is suffering a global decline, driven mainly by the growing number of pressures linked directly to human activities (e.g., ocean warming, coastal modification, water quality degradation) (Orth et al., 2006; Waycott et al., 2009). Globally, seagrasses are disappearing at a worrying rate of 110 km^2 per year (Waycott et al., 2009). Based on review from 215 different studies, Waycott et al. (2009) revealed that 29% of the total world seagrass population was lost from 1980 to 2006. Moreover, seagrass ecosystems in Japan, Europe, Australia, and USA have been lost as a result of diseases, deteriorated water quality, and coastal development (Sullivan et al., 2018).

In the Southeast Asia (SEA) region, the review study of Fortes et al. (2018) estimated that the total seagrass cover was about 36,763 km^2. However, McKenzie et al. (2020) indicated that the global seagrass distribution is much lower than that mentioned in previous publications. Sudo et al. (2021) showed that the seagrass distribution in the SEA was about 3,670 km^2. Seagrass bed decline was found in almost all countries from SEA. Recently, Sudo et al. (2021) combined data from Global Distribution of Seagrasses (GDS) issued by UNEP-WCMC (before 2001), and new data (Sudo and Nakaoka, 2020) from 68 sites in nine countries/regions of SEA showed that more than 60% of seagrass meadows declined at an average rate of 10.9% year^{-1}, while 20% of beds increased at an average rate of 8.1% year^{-1}. Therefore, an overall average decline of 4.7% year^{-1} in SEA has been estimated. In particularly, in reports on the status of seagrass beds from Indonesia, Unsworth et al. (2018) indicated that seagrasses across the Indonesian archipelago are in a critical state of decline. In Malaysia, loss of seagrass habitats was recorded at different specific sites (Hossain et al., 2015; Bujang et al., 2016). However, a recent study on a smaller scale at Nakhon Si Thammarat Province, Thailand, showed an increasing area of seagrass beds (Rattanachot et al., 2018). Therefore, there is an urgent need to map existing intertidal seagrasses in Thailand and elsewhere to better understand reasons for both decrease and increase in seagrass meadows (Koedsin et al., 2016). Among the SEA region, Fortes et al. (2018) reported that there are 21 seagrass species, but some of these are still considered taxonomically uncertain. Seagrass species richness from SEA is the highest in the world (Short et al., 2011). Since the last decade, the increase in use of genetic markers has successfully solved some issues in taxonomy and genetic diversity, population structure, and gene flows among seas/oceanic systems (Nguyen et al., 2014; Arriesgado et al., 2015; Wainwright et al., 2018).

Based on the status review, we focus on current research and highlight gaps in knowledge of seagrass ecosystems within Viet Nam. First, the current seagrass distribution from Viet Nam and changes in selected sites are described, including the important role in phytoremediation processes by seagrass. The role of seagrass meadows for blue carbon storage are also discussed with an emphasis on the situation in Viet Nam. Next, the taxonomic issues of *Halophila* and genetic diversity of members of Hydrocharitaceae collected from the Vietnamese waters are presented. Finally, the importance of the interdisciplinary cooperation of scientists from all disciplines is discussed for future works.

Seagrass distributions and changes

Viet Nam is located in the central part of SEA where it is known as the place of the evolutionary origin of seagrasses (Chen et al., 2012). Among 22 ecoregions of seagrass distribution from SEA, Viet Nam consists of three ecoregions including $N_0$20112 (Gulf of Tonkin), $N_0$20115 (Gulf of Thailand), and

N$_0$20116 (southern Viet Nam) (Spalding et al., 2007). The coastline of Viet Nam was divided into four regions including northeast (1), north central (2), south central (3), and southern Viet Nam (4) (see Figure 1 for more details). Cao et al. (2014) reported that in the total area of 2,240 ha, 12 seagrass meadows were found in region 1. For region 2, two seagrass meadows were localized at Tam Giang-Cau Hai lagoon and a nearby area with 2,037 and 618 ha, respectively (Cao et al., 2014; Cao et al., 2020a). For region 3, numerous geographically suitable areas (e.g., lagoons, bays, islands, islets, atolls, and reefs) can be identified along the coast providing a diversity of habitats for the occurrence of seagrasses. The seagrass distribution of region 3 is the most well-studied among the four regions with a total area of 3,109 ha (Cao, 2011; Cao et al., 2019; Nguyen et al., 2021a; Nguyen et al., 2021b). Recently, Nguyen et al. (2021a) showed that the seagrass distribution at Phu Quoc Island (i.e., the biggest island of Viet Nam) was about 7,579 ha and is considered the biggest seagrass bed in Viet Nam (region 4). In addition, another recent study has documented the existence of a 30-ha seagrass bed at Hai Tac archipelago (Do et al., 2020). In Table 1, more details on the distribution of seagrass meadows in Viet Nam are illustrated.

A significant decline in seagrass coverage from Vietnamese waters has been detected across the whole area. The total seagrass area in Viet Nam was estimated to cover about 29,162 ha in 1990 (Trinh and Takeuchi, 2019). By using satellite Landsat TM/OLI image analysis, Vo et al. (2020) indicated that 186.2 ha (equivalent to 35.8%) of the original seagrass beds were lost in the last three decades at Van Phong Bay due to a number of different reasons (Figure 2). The authors identified that typhoons may be the main driver for the loss of seagrass beds at open-sea sites, while human-induced stressors, such as aquaculture activities, excavation, and terrigenous obliteration, may be the main reasons in protected sites. By

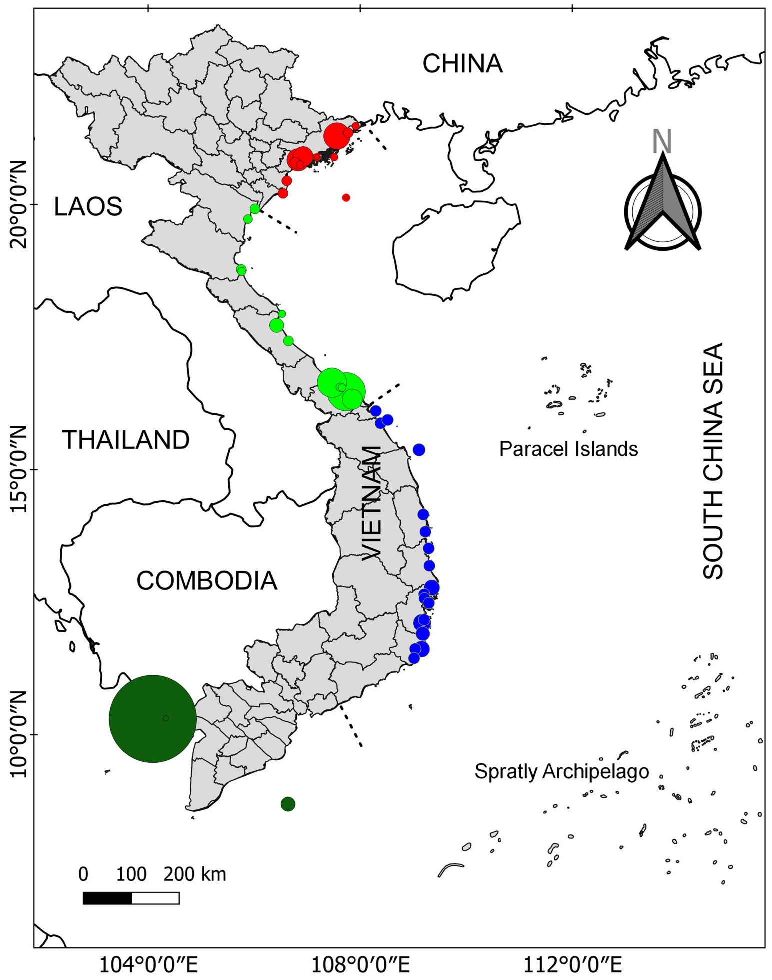

FIGURE 1
Seagrass distribution in Viet Nam. The four different color cycles indicate seagrass distribution in four different regions. Data were combined from various studies. See Table 1 for more details.

TABLE 1 Seagrass distribution (ha) and species diversity at each region.

Regions	Number of provinces/ cities	Distribution (ha)	Species diversity	Sources
1	5	2,240	Hb, Ho, Rm	(Cao et al., 2014)
2	6	2,655	Hb, Hu, Rm, Zj	(Cao et al., 2014; Cao et al. (2020a)
3	8	3,109	Hb, Hd, Ho, Hma Hmi, Hsp, Ea, Th, Tc, Cr, Cs, Hu, Hp, Si, Rm	(Nguyen et al., 2021b), (Cao et al., 2019; Cao et al., 2020b; Nguyen et al., 2021a)
4	9	7,609	Hb, Hd, Ho, Hma "Hmi", Hsp, Ea, Th, Cr, Cs, Hu, Hp, Si	(Do et al., 2020), (Nguyen et al., 2021b), (Nguyen et al., 2021a)
Total	**28**	**15,613**		

Hb, *Halophila beccarii*; Hd, H. *decipiens*; Ho, *H. ovalis*; Hma, *H. major; Hmi, putative H. minor*; Hsp, *Halophila major SL type*; Ea, *Enhalus acoroides*; Th, *Thalassia hemprichii*; Tc, *Thalassodendron ciliatum*; Cr, *Cymodocea rotundata*; Cs, *C. serrulata*; *Hu, Halodule uninervis*; Hp, H. pinifolia; Si, *Syringodium isoetifolium*; Rm, *Ruppia maritima*. Zj, *Zostera japonica*. See Figure 1 for the location of the regions.

using Landsat TM/ETM +/OLI imageries and the ground reference data, Chen et al. (2016) indicated that from 1996 to 2015, the total area of seagrass beds in Cam Ranh Bay had declined by approximately 25% (66 ha), mainly due to coastal development and infrastructure construction. Based on Sentinel-2, Landsat-8, and VNREDSat-1 analyses for the Khanh Hoa coastal area (a part of region 3), submerged aquatic vegetation including seagrass was reduced by 74.2%, while gains in new areas compensated for less than half of these losses (Khanh Ni et al., 2020).

Establishing accurate seagrass distribution maps and long-term monitoring, therefore, are needed to form the basis for the conservation and development of the current and future seagrass ecosystems in Viet Nam (Nguyen et al., 2021a; Nguyen et al., 2022a). Furthermore, recent studies of seagrass distribution from Viet Nam may partly fulfill knowledge gaps on basic information on seagrass habitats in southeast Asia, as mentioned by Fortes et al. (2018). New methods such as spectroscopic methods with a higher resolution of remote sensing images will be helpful to determine species composition within seagrass beds. Although Nguyen et al. (2021) mapped almost all seagrass beds of a significant size from South Viet Nam, the patchy small-scale meadows from offshore islands, lagoons, and inlets were not included. In addition, *Halophila decipiens* beds in deep waters such as close to the Cu Lao Cham Islands, offshore Ninh Thuan (>15 m) may not be detected in remote sensing imageries. Therefore, a combination of several approaches including ultra-high-resolution, multibeam echo-sounder, unmanned aerial vehicles (UAVs) and fieldwork are needed to map seagrass beds in deep waters.

Biodiversity in seagrass beds

Studies of biodiversity of fishes within seagrass beds revealed differences in different habitats and locations. In region 1, results of Nguyen (2013) indicated that the density of fish larvae and juveniles within seagrass beds (327 individuals m^{-2}) was 180 times higher than the bare sediment at Cat Ba Island (1.79 individuals m^{-2}). Taxon diversity of fishes within seagrass beds in this site was only six taxa. For the crustaceans, there is a number of taxa of juveniles including groups of Penaiedae, Alpheidae, Palaemonidae, Aryidae, Squillidae, Sergestidae, and Pandalidae that were commonly found. The density and biomass of zoobenthos showed high variation between seagrass beds and the bare sediment in both dried and rainy seasons. In the rainy season, the density and biomass of zoobenthos in seagrass beds were 434 (individuals m^{-2}) and 169.9 (g m^{-2}), respectively, whereas those values were 128 and 27.6 in the bare sediment. In the dry season, the density and biomass of zoobenthos in seagrass beds were much higher with 1,226 (individuals m^{-2}) and 289.3 (g m^{-2}) (Nguyen et al., 2002).

In region 2, seagrass beds in Tam Giang-Cau Hai lagoon are the largest size. Therefore, there are several studies on animals' diversity including fishes, crustacean, mollusk, zoo, and phytoplankton. Eighty-seven taxon of fish larvae and juveniles were identified from this lagoon. Among them, some high economic species such as *Epinephelus sexfasciatus* Valenciennes, *Lujianus russelii* Bleeker, *Lethrinus* spp., and *Siganus* sp. are the most dominant. Nguyen (2013) showed that there is a positive correlation between above-ground biomass of seagrass and diversity of fish larvae and juveniles in almost all seagrass beds occurring in Tam Giang-Cau Hai lagoon. In addition, Nguyen and Nguyen (2012) listed 177 fish species at Tam Giang-Cau Hai lagoon, the highest species composition compared to other locations. Based on underwater videos, Espadero et al. (2020) identified 59 fish species representing 23 families that were recorded in the 26 video deployments in the seagrass beds from the Philippines. For Crustaceans, 20 taxa of crustacean larvae and juveniles were recorded in this lagoon. Among families, both Penaeidae and Portunidae were the dominant groups. For the diversity of zoobenthos, 203 species including 92 species of mollusk, 51

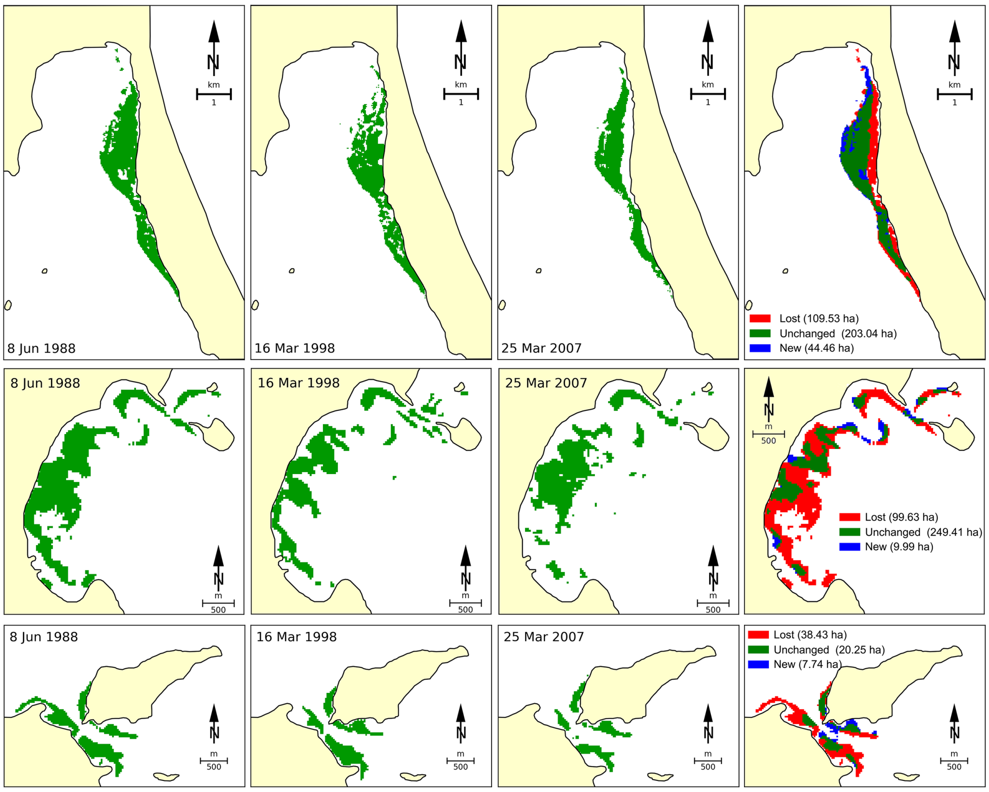

FIGURE 2
Changes of seagrass beds at three sites of Van Phong Bay (region 3) from 1998 to 2018 deduced by using datasets of satellite image Landsat 5 TM and Landsat 8 OLI. The figure was adapted from Vo et al. (2020).

species of crustacean, 49 species of polychaeta, and other species of Echinodermata were found (Nguyen, 2013).

Biodiversity of marine organisms in seagrass beds along the coast of region 3 also showed variation. In general, the diversity of juvenile shrimps and fish species in seagrass beds is higher than in the bare sediment. Nguyen et al. (2000) showed that density of larvae and juveniles of Penaeidae in seagrass beds (78 individuals m^{-3}) is eight times higher than the bare sediment (17.12 individuals m^{-3}) at Thuy Trieu lagoon. For larvae and juvenile of fish in seagrass beds from the in Cua Dai, the results of Nguyen et al. (2008) indicated that the density of these groups in seagrass beds was 2.8 times higher than that in the bare sediment. Fish species composition within seagrass beds at Thuy Trieu lagoon included 87 species belonging to 12 orders and 47 families. Among them, order Perciformes showed the highest family with 30 families (Nguyen and Nguyen, 2012). In addition, there are 68 species of zoobenthos, with 39 species of polychaeta, 18 species of mollusk, 7 species of crustacean, and 4 species of Echinodermata in seagrass beds in Xuan Tu (Nguyen, 2013). Several studies (Surugiu et al., 2021; Barnes, 2022) revealed that seagrass beds support assemblages of macrobenthic invertebrates with different composition and with considerably greater abundance and species density than adjacent areas without this cover.

Seagrass beds in Phu Quoc Island are considered as typical beds in region 4. There are 33 species of larvae and juvenile fish found within seagrass beds. Higher species composition and diversity of larvae and juvenile fish were found in dense seagrass beds. Twenty species of larvae and juveniles of crustacean were also recorded, and the density of this group was up to 350 individual m^{-2} (Nguyen, 2013). Of the 86 fish species that were found in seagrass beds, the family Apogonidae showed the highest diversity with 18 species. A comparison of diversity of marine organisms including larvae and juveniles and fish composition in three typical seagrass beds (lagoon, estuary, and offshore islands) is presented in Table 2. Diversity of zoobenthos in seagrass beds in the southern area (regions 3 and 4, 292 species) was greater than that in the northern area (region 1 and 2, 134 species). Among them, Gastropoda and Bivalvia revealed higher species diversity in the south, whereas species diversity of Annelida and Arthropoda in the North showed higher number of species (Table 3).

Larvae and juveniles strongly differ in morphology from adults, and their identification to the species level remains problematic. In addition, the current guide of Indo-Pacific fish larvae allows identification only to the family level. DNA barcoding method can identify fish larvae samples to genus and species level (Collet et al., 2018). Identification of fish larvae were best conducted with the aid of molecular method in a study on larval fishes collected from Hawaiian waters (Xing et al., 2022). Therefore, validation of diversity of larvae and juveniles within seagrass beds in Viet Nam may be enhanced when we apply these methods.

Phytoremediation processes of seagrass

Anthropogenic activities have increased heavy metal pollution in previously uncontaminated ecosystems, threatening terrestrial and aquatic plant communities (Boquete et al., 2021; Zhang et al., 2021). Most heavy metals are not an essential element for plants, and excessive amounts can cause growth inhibition and even death (Burkholder et al., 2007). In Viet Nam, the number of studies on heavy metal accumulation and phytoremediation processes of seagrasses are very limited. A study on accumulation of different heavy metals in the three different organs of the tropical seagrass species *Enhalus acoroides* collected in different lagoons and bays showed that a significant positive correlation of the bio-concentration factor (BCF) for Cu was observed between sediment and rhizome, while significant positive correlations of BCF for Cu, Pb, and Zn were observed between sediment and roots (Nguyen et al., 2017a). Remarkably, the Cu concentration of *E. acoroides* rhizomes collected near shipyards was approximately 140 µg mg^{-1} DW, higher than at other locations at Khanh Hoa province (<20 µg mg^{-1} DW) (Nguyen et al., 2017b). Dung et al. (2014) indicated that Cu showed an extremely severe enrichment in the marine sediments collected near a shipyard. Phytochelatins (PCs) are considered as an important component of the metal detoxifying mechanisms (Ahmad et al., 2019). PCs occur in plants, algae, and some yeast species that grow at high heavy metal concentrations. PCs are translocated within the plant, transported to the vacuole as PC–metal complexes, and stored as high molecular weight PC–metal complexes (Cobbett and Goldsbrough, 2002; Clemens, 2006). PCs, usually with the structure of (1'-Glu-Cys)$_n$–Gly (n = 2–11), are glutathione-derived metal-binding peptide. PC$_2$ contains two units of gamma-Glu-Cys, while PC$_3$ contains three units of gamma-Glu-Cys. The higher the levels of heavy metals that were accumulated in the tissue, the more units of gamma-Glu-Cys were formed (Cobbett and Goldsbrough, 2002). Results showed that higher PC$_2$, appearance of PC$_3$, and a strong correlation between PC$_2$ and Pb concentration were found in the root organ collected from a Pb-contaminated area from southern Viet Nam (Nguyen et al., 2017a) (Table 4). Metallothioneins (MTs) are defined as low-M$_r$ Cys-rich proteins that bind heavy metals, and nine MT-like sequences from Cu- or Cd-treated *Posidonia oceanica* were isolated and classified into two subgroups (Giordani et al., 2000). It may reveal the roles of MTs in terms of phytoremediation processes of seagrass, which is not well known. Therefore, studies on relationship between heavy metals exposure and expression levels of MTs from some tropical seagrass species are still gaps. Heavy metal accumulation was carried out from *E. acoroides* only, and the heavy metal accumulation of remaining species are still unknown.

Blue carbon storage

Seagrass beds are considered efficient natural carbon sinks among the important coastal blue carbon ecosystems, since they can capture carbon dioxide from the air through photosynthesis and store organic carbon (C_{org}) in their biomass and within the sediment, thereby mitigating climate change (Fourqurean et al., 2012; Greiner et al., 2013; Macreadie et al., 2019; Williamson et al., 2022). As a result, there is growing interest in managing this "blue carbon" ecosystem worldwide. The C_{org} content and stocks in seagrass meadows have been thoroughly studied in many countries, and some of them have been developed at a global level to assess "blue carbon," e.g., the Blue Carbon Initiative. However, basic information on this "blue carbon" habitat is still very limited in Southeast Asia, including Viet Nam (Stankovic et al., 2021).

TABLE 2 Number of families and species of zoobenthos in seagrass beds.

	Tam Giang–Cau Hai[a]	Lap An[a]	Cua Dai[b]	Thuy Trieu[a]	Phu Quy[c]	Phu Quoc[c]
Larvae and juveniles of fishes						
Families	47	15	12	n.d.	19	20
Species	87	15	15	n.d.	24	30
Larvae and juvenile of crustacean						
Families	6	8	2	n.d.	3	8
Species	20	12	8	n.d.	12	20
Fishes						
Families	na	50	32	46	14	34
Species	na	151	55	87	25	86

n.d., not determined. (Source: Nguyen, 2013). [a]lagoon, [b]estuary, [c]offshore island.

TABLE 3 Number of family and species of zoobenthos in seagrass beds.

		Northern part (Regions 1 and 2)	Southern part (Regions 3 and 4)
Phylum Annelida			
	Families	18	13
	Species	40	21
Phylum Mollusca			
	Class Gastropoda		
	Families	15	24
	Species	31	105
	Class Bivalvia		
	Families	15	24
	Species	38	114
	Class Cephalopoda		
	Families	n.d	1
	Species	n.d	1
Phylum Echinodermata			
	Class Asteroidea		
	Families	n.d	4
	Species	n.d	8
	Class Echinoidea		
	Families	n.d	5
	Species	n.d	10
	Class Holothuroidea		
	Families	1	3
	Species	1	12
	Class Ophiuroidea		
	Families	1	1
	Species	2	1
Phylum Arthropoda			
	Families	13	10
	Species	22	20
	Total	**134 species/63 families**	**292 species/85 families**

n.d., not determined (Source: Nguyen, 2013).

In recent decades, various methods have been used to measure C fluxes and C storage in seagrass beds, such as measuring techniques of fluxes of oxygen and carbon dioxide, primary production, and remote sensing (Macreadie et al., 2019). However, Viet Nam's research on carbon storage in seagrasses and other marine plants (e.g., mangroves, seaweeds, and salt marshes) has rarely been published. For example, Cao et al. (2013) calculated the amount of C_{org} in seagrass beds in Tam Giang-Cau Hai lagoon (central Viet Nam, region 2) through dissolved oxygen (DO) content in a light–dark *ex situ* experiment in the seagrass *Halodule pinifolia*. Specifically, the seagrass ecosystem in the lagoon was estimated to produce 25.71 tons of C day^{-1} during the rainy season and 28.93 tons day^{-1} in the dry season. However, it should be noted that the seagrass beds in the lagoon include up to seven seagrass species, and *H. pinifolia* is not one of the dominant species of the lagoon, so the estimate seems biased.

The carbon storage capacity of seagrasses was also evaluated through their biomass in Thi Nai lagoon (region 3), with the total amount of organic carbon and carbon dioxide fixed by seagrass beds estimated at 136.7 and 501 tons ha^{-1}, respectively (Cao and Nguyen, 2017). In addition, Stankovic et al. (2021) recently revealed the potential for seagrass beds' carbon sinks in Southeast Asia as a nature-based solution for climate change mitigation. The study found that the total C_{org} (in both sediment and living biomass) in seagrass beds from Viet Nam was 133.16 ± 36.97 Mg ha^{-1}. When compared with countries in Southeast Asia, this value was higher than in the Philippines, Malaysia, and Indonesia and almost the same as in Thailand and Myanmar (Stankovic et al., 2021). The local and regional C_{org} variation among seagrass beds is controlled by many factors such as seagrass community complexity, fine sediment fraction, seawater depth (Monnier et al., 2022; Stevenson et al., 2022), sediment run-off, and primary production of the seagrass (Serrano et al., 2021).

TABLE 4 Comparison of concentration of selected heavy metals in the rhizosphere sediment, distinct organs, and phytochelatins (PCs) in *Enhalus acoroides* from different locations along the coast of Khanh Hoa province and other locations.

		Cd	Cu	Pb	Zn	PC_2	PC_3
MG-Vie* **	Sediment	0.05	40.77	5.08	40.17	n.d	n.d
	Leaf	0.05	10.23	0.08	37.13	0.29	n.d
	Rhizome	0.08	143.90	0.04	28.60	0.24	n.d
	Root	0.07	9.58	1.22	38.12	0.59	n.d
TT-Vie* **	Sediment	0.14	6.37	19.90	27.97	n.d	n.d
	Leaf	1.37	2.17	0.08	26.03	0.22	n.d
	Rhizome	0.65	3.86	0.04	14.28	1.13	n.d
	Root	0.59	0.65	19.34	11.64	10.32	0.66
TL-Vie* **	Sediment	0.03	1.02	0.81	5.62	n.d	n.d
	Leaf	0.37	2.29	0.08	15.03	0.48	n.d
	Rhizome	0.14	1.47	1.22	13.19	0.80	n.d
	Root	0.16	0.77	4.26	8.87	0.61	n.d
Palau*	Sediment	0.02	8.00	1.00	11.00	n.d	n.d
	Leaf	0.05	3.00	0.50	17.6	n.d	n.d
	Root	0.02	1.10	0.50	8.10	n.d	n.d
India**	Sediment	0.52–5.72	2.76–21.64	4.4–10.36	10.36–127.2	n.d	n.d

MG, My Giang; TT, Thuy Trieu lagoon; TL, Tuan Le; Vie, Viet Nam. Unit = µg g^{-1} DW. n.d., not determined. Source: * Jeong et al. (2021), ** Gopi et al. (2020), *** (Nguyen et al. 2017a).

The average total blue carbon stock in seagrass ecosystems in Viet Nam was estimated to be 2.06–2.95 Tg, and these ecosystems can accumulate 25.18–29.28 Gg C_{org} $year^{-1}$. Compared with the neighboring countries, the total C_{org} stock from seagrass beds in Viet Nam was much lower than in the Philippines (259.17–425.21 Tg) and Indonesia (62.08–107.50 Tg); however, it was higher than that of Malaysia (0.005–0.25 Tg) and Myanmar (0.02–0.04 Tg) (Stankovic et al., 2021). The difference in average total blue carbon stocks in seagrass ecosystems in different countries depends not only on the total C_{org} in the seagrass beds but also on the area of seagrass beds. Thus, although Viet Nam has higher C_{org} in sediments and biomass of seagrass beds than the Philippines and Indonesia, the blue carbon stock from Vietnamese seagrass ecosystems is lower than these two countries because seagrass area of Viet Nam is much less than those two countries (44 and 139 times, respectively) (Stankovic et al., 2021). Thus, studies on blue carbon storage in Vietnamese seagrass beds are very few; further studies such as C_{org} stock estimates, the carbon accumulation rate, and the risk of loss of C stock in seagrass beds are essential to contribute to comprehensive globe blue carbon estimates for the global climate change mitigation strategy.

Ecology and physiology of Vietnamese seagrasses related to different environmental conditions

Viet Nam has a long coastline of approximately 3,260 km (exclusive of the shoreline of islands) stretching a latitudinal gradient from Mong Cai (21° 31′ 28.96″ N 107° 57′ 58.28″ E) in the north to Ha Tien (10° 22′ 59.99″ N 104° 28′ 59.99″ E) in the south (Hanh and Furukawa, 2007). Temperature is generally the most important range-limiting factor to seagrass distribution (Duarte et al., 2018). Therefore, the sea water temperature shows variation among regions. In regions 1 and 2, the sea water temperature ranges from 15.7°C to 29°C (average 24.1°C) in the northeast monsoon (November to February) and from 22.6°C to 30.7°C (average 28.5°C) in the southwest monsoon (June to August). However, regions 3 and 4 showed higher sea water temperature in both monsoons; they are 21.6°C–30.7°C (average 27.5°C) and 22.8°C–31.0°C (average 28.9°C) in northeast monsoon and southwest monsoon, respectively (Yu et al., 2019). The difference in sea water between regions 1, 2 and 3, 4 may reflect the species distribution along the coast of Viet Nam. *Zostera japonica* Asch. & Graebn is a common species in both regions 1 and 2 and is found from 13.8°N northward. In contrast, *E. acoroides* (Linnaeus f.) Royle is found from 16°N southward (regions 3 and 4). There are nine species occurring in regions 1 and 2, while more six species are found in regions 3 and 4 (Nguyen, 2013; Nguyen et al., 2021). In addition, the coastal area of Viet Nam consists of numerous bays, estuaries, and beaches with dynamic variabilities in environmental conditions [e.g., depth, sediment characteristics, light, salinity, levels of anthropogenic pressures, seasonal changes, etc. (Tang et al., 2004; Hanh and Furukawa, 2007; Veettil et al., 2020, authors' observations)]. Therefore, it is expected to have great intra-/inter-species-specific variations in ecological, morphological, and physiological traits among similar/different seagrass species in the country as previously demonstrated in several seagrass species from different regions (Coyer et al., 2004; Jahnke et al., 2019a; Nguyen et al., 2021). To date, however, the number of studies from Viet Nam on this topic remains very limited (Huong et al., 2003; Pham et al., 2006). For example, Huong et al. (2003) investigated seasonal and depth dynamics of two intertidal seagrass species (*Halophila ovalis* and *Z. japonica*) in Ha Long Bay (northern Viet Nam) and demonstrated inter-species specificities between the two species in terms of their tolerances to low-light conditions and desiccation. Interestingly, even occurring in the same area, the two seagrass species sexually reproduced in two distinct timeframes (e.g., November and April for *H. ovalis* and *Z. japonica*, respectively) (Huong et al., 2003). On the other hand, Pham et al. (2006) conducted a year-round monitoring at two nearby bays with different characteristics (especially in depth) in the center of Viet Nam (i.e., Van Phong bay and Cam Ranh bay) focusing on two other seagrass species including *E. acoroides* and *T. hemprichii*. This study showed not only inter-species-specific differences between the two seagrass species from both bays but also intra-specific variations between populations of the same species at both study sites in terms of shoot density, above ground biomass, and leaf production rate (Pham et al., 2006). In a more sheltered environment of a coastal brackish lagoon in central Viet Nam, Phan et al. (2018) revealed that salinity and sediment composition (silk vs. sand) were the two main factors governing the distribution and abundance of seagrasses and other submerged aquatic plants in the lagoon. Overall, studies on the influence of ecological factors on seagrasses in Viet Nam are very few and only small in scope. These pioneer studies have hinted a great dynamic in seagrass ecology of Viet Nam and furthermore emphasize a significant gap in knowledge on this topic to which future studies are strongly encouraged.

Taxonomic issues of *Halophila*

In Viet Nam, the members of *Halophila* were found in different aquatic conditions. *H. beccarii* is found in brackish waters (shallow lagoons), while *H. decipiens* is found in the depth of 5–15 m in offshore islands (Nguyen et al., 2013a; Hoang et al., 2021). The genus *Halophila* is known as one of the most complex taxonomic challenges due to leaf morphological traits that overlap among species (Kuo et al., 2006). *H. johnsonii* Eiseman was first identified as distinct species in paddle-bladed seagrasses of the Hydrocharitaceae (den Hartog, 1970). So far, *Halophila* cf. *johnsonii* was reported from some lagoons in Viet Nam based on the leaf shape (elliptic) (Nguyen et al., 2002). However, the recent detailed analysis of leaf morphology indicated that there were distinctive leaf morphotypes (narrow-leaf type) of *H. ovalis* (Nguyen et al., 2013a). Based on leaf morphology of samples collected from the island of Nha Trang Bay, the trait of distance between intramarginal veins and lamina margin ratio, Nguyen et al. (2013b) introduced the new records of seagrass species—*H. major* for Vietnamese's flora. *H. minor* was recorded for the first time from Viet Nam by Pham-Hoang (1993). Nguyen et al. (2015) found that there were misidentifications of some samples collected from Vietnamese waters labeled as *H. minor*. It should be treated as *H. ovalis* due to its morphological characteristics and genetic analysis. The misidentification between *H. ovalis* and *H. minor* was also reported from the samples collected at Thailand (Kim et al., 2017). For approximately the last 20 years, no new sequences of *H. minor* have been assigned to GenBank since the study of Waycott et al. (2002). Therefore, the occurrence of *H. minor* is still questionable. Recently, Nguyen et al. (2021a) reported that the main characteristic between *H. ovalis* and *H. major* is that distance between the intra-marginal veins and the lamina margin of *H. ovalis* is much wider than *H. major*, and the intra-marginal veins of *H. ovalis* is easily recognized by naked eyes, and other parameters including leaf width, leaf length, number of cross-veins, number of branching cross-veins, space between cross-veins, and the angle between cross veins and mid-veins did not show significant differences between two species. For different habitat types, *H. ovalis* grows in lagoons that experience large salinity differences between the dry and the rainy seasons, low water velocity, and weak wave action, whereas *H. major* is found in the offshore islands (Nguyen et al., 2021b). "*Halophila major* SL type" collected from Nha Trang Bay showed morphological characters consistent with *H. major*; however, Nguyen et al. (2021a) suggested that this population may be the cross-hybridization between *H. ovalis* and *H. major*. Based on phylogenetic analysis of ITS1-5.8S-ITS2, Nguyen et al. (2021a) depicted that *H. major*, *H. ovalis*, and putative hybridization (*H. major* SL type) grouped into three districted clades (Figure 3). The cross-hybridization between *H. ovalis* and *H. major* was reported from materials collected at Sri Lankan waters (Liu et al., 2020) and may be "*Halophila ovalis* Red Sea type" at the Red Sea (Nguyen et al., 2018). Hybridization has been documented in other seagrass species, such as *Posidonia* (Sinclair et al., 2019) and *Halodule* (Ito and Tanaka, 2011). Hence, the genetic relationship between *H. ovalis*, *H. major*, *H. ovalis* Red Sea type, *H. major* SL type, and the hybridization from Sri Lankan waters should be clarified.

Genetic diversity and population structures of selected species of the Hydrocharitaceae family

The seagrass species members of the Hydrocharitaceae in Viet Nam represent a great opportunity to study genetic structure and genetic diversity. Below, we discuss some recent studies that investigated genetic components of seagrass meadows in Viet Nam. For instance, Nguyen and Papenbrock (2019) demonstrated a reduced genetic diversity and a genetic

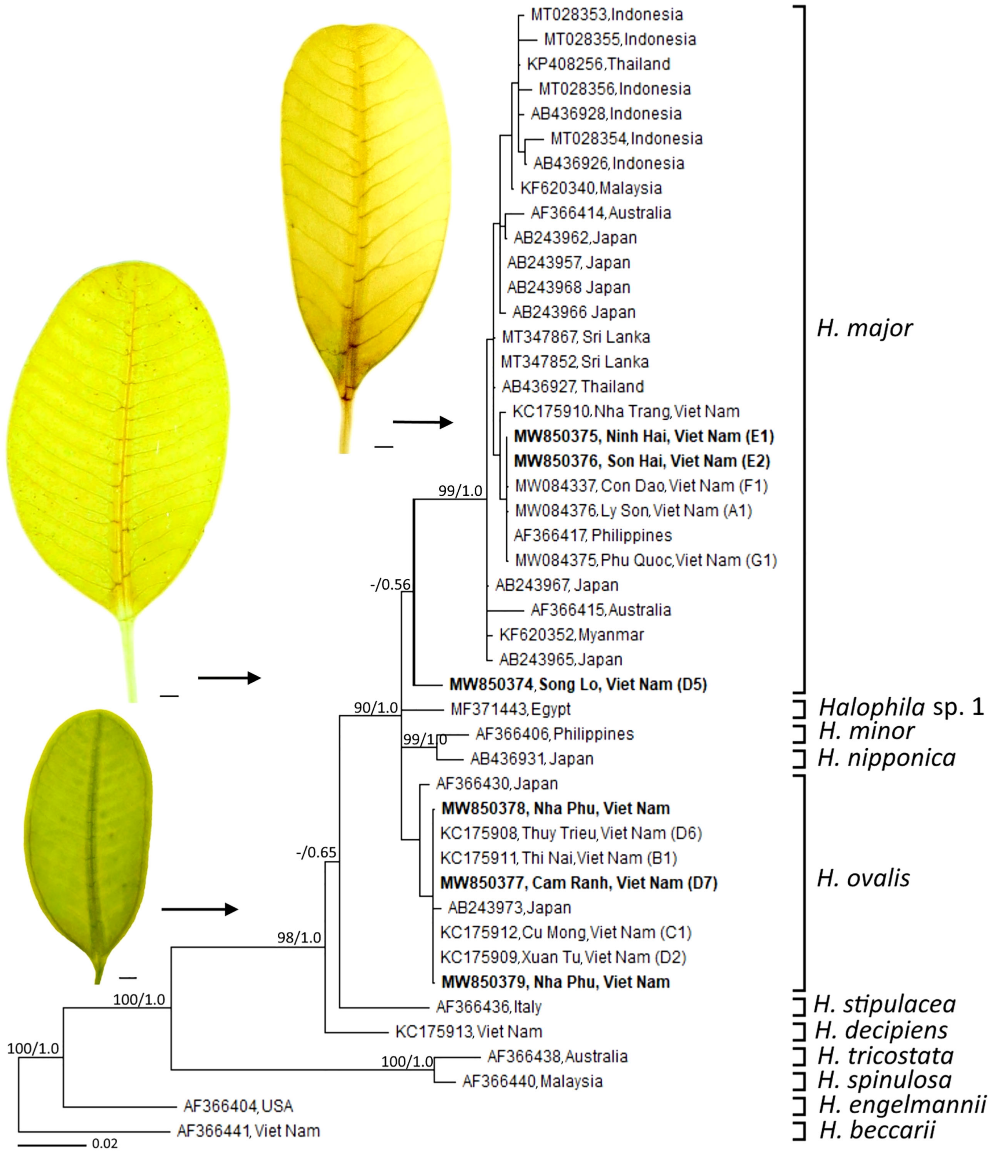

FIGURE 3
Phylogeny of members of the genus *Halophila* 612 bp of the ITS1-5.8S-ITS2 sequence. Leaf morphology of *Halophila* spp. from samples collected in Viet Nam were placed beside the phylogenetic tree. Scale in each leaf figure = 1 mm. The figure was modified from Nguyen et al. (2021a).

differentiation between the lagoon sites versus the open sea sites for *E. acoroides* beds occurring along the coast of the Khanh Hoa Province. Based on the results from studying eight populations of *E. acoroides* using 11 polymorphic microsatellite loci along the South-Central Coast of Viet Nam, Dierick et al. (2021) reported clonal richness and structure, genetic diversity, and levels of dispersal within and between eight populations of *E. acoroides* in four lagoons along the South Central Coast of Viet Nam. The authors showed that lagoons were strongly differentiated and may act as barriers to gene flow and that large resistant genets contribute to the resilience of *E. acoroides* meadows under high levels of disturbance.

Thalassia hemprichii (Ehrenberg) Ascherson is another member of Hydrocharitaceae, which is quite common and widely distributed in the Tropical Indo-Pacific, including the east coast of Africa and the Red Sea (Ferrer-Gallego and Boisset, 2015). This species was used for several studies on genetic diversity, population structure, and gene flow among the populations in the Pacific and Indian Oceans (Hernawan et al., 2017; Wainwright et al., 2018; Jahnke et al., 2019b). However,

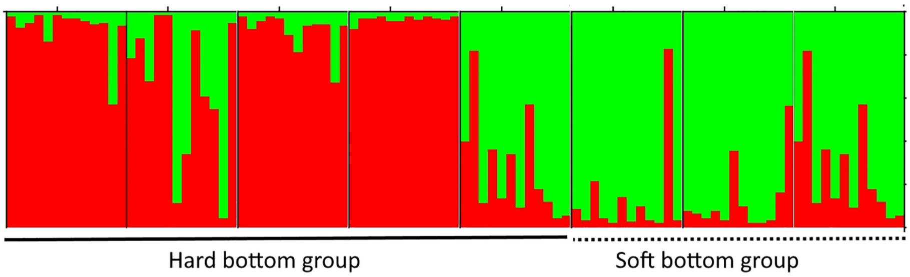

FIGURE 4
The cluster analysis of the *Thalassia hemprichii* population from southern Viet Nam based on nine microsatellite loci. Eight populations were divided into two groups consisting of the open sea and lagoon clusters. Adapted from Nguyen et al. (2022).

reports on the genetic diversity and population structure of *T. hemprichii* in Viet Nam is very limited with only one study done so far (Nguyen et al., 2022). By using 10 loci of microsatellite markers, Nguyen et al. (2022) showed that eight populations were separated into two groups in agreement with the two different habitat types (hard and soft bottoms) (Figure 4), and the western boundary currents in the South China Sea influence the gene flow among *T. hemprichii* populations in southern Viet Nam. The authors also reveal that four populations with a high relative genetic contribution value should have the priority to be conserved (Nguyen et al., 2022).

For the members of the genus *Halophila*, *H. ovalis* is widely distributed in the Indo-Pacific region (Liu and Hsu, 2021). By using five loci of microsatellite markers, Nguyen et al. (2014) indicated that the genetic distances between southern Viet Nam and Gulf of Thailand were lower than other regions (Malaysia, Hong Kong, Andaman Sea). For the haplotype diversity of *H. ovalis*, there are seven haplotypes in the SEA (Nguyen et al., 2018). Along the coast of Viet Nam, the internal transcribed spacer (ITS) analysis indicated that there is only one haplotype, although sample collections were carried in five different locations (Nguyen et al., 2018). It is lower than in other neighbor countries in SEA such as Malaysia and Indonesia. *Halophila major*, the sister species of *H. ovalis*, was reported in offshore islands in Viet Nam. By using rDNA marker (ITS1-5.8S-ITS2), Nguyen et al. (2021b) depicted the haplotype network of *H. major* in the world (Figure 5). There are two haplotypes in southern Viet Nam, one of them is common to those from the Philippines. This number is much lower than in the Wallacea region where eight haplotypes were found (Nguyen et al., 2021b). Another member of the genus *Halophila*, *H. beccarii*, on the other hand, has been listed as a vulnerable species on the IUCN Red List of threatened seagrass species (Short et al., 2011), and it has been locally extirpated in the Philippines. Phan et al. (2017) revealed the low level of genetic and clonal diversity in *H. beccarii* in a Viet Nam lagoon habitat, and sexual reproduction is an important mode besides asexual regrowth in maintaining *H. beccarii* meadows.

Seagrass conservation and management

The Fisheries Law of Viet Nam issued in 2017 included provisions for the protection and growth of aquatic resources, including marine conservation, in the sense of sustainable fisheries development and international integration (Government of Viet Nam, 2017). So far, the approval of the strategy for management of systems of special-use forests, marine reserves, and internal water reserves of Viet Nam through 2020, with a vision toward 2030 was issued in 2014 (Government of Viet Nam, 2014). In Viet Nam, 12 marine protected areas (MPAs) from 10 provinces/cities have been created and operationalized since 2005. It indicated that 35 main seagrass beds are out of protection. Unfortunately, huge seagrass beds including Tam Giang-Cau Hai lagoon in region 2, and seagrass beds along the coast of Khanh Hoa (region 3) are still under threat. As an International Union for Conservation of Nature (IUCN) category-II park, established in 2007, the Phu Quoc MPA (region 4) protects seagrass and coral-reef-based ecosystems (Kien Giang PCC, 2007). However, the results of Tran et al. (2022) showed that the protection of fishes provided by Phu Quoc MPA was ineffective due to lack of difference in species, and functional composition of fish communities was similar between protected and unprotected areas. Therefore, we need further action to optimize MPA design and management to meet conservation goals of seagrass meadows. More potential marine protection areas that include important seagrass beds should be added to the Vietnamese MPA system.

Currently, the management models of seagrass ecosystems in Viet Nam are mostly integrated into the integrated coastal management models to solve the problems of weaknesses that exist in the management, exploitation, and use of natural resources and environmental protection in coastal areas. The model of integrated coastal zone management, by Tran (2011) , is divided according to space separating the western coastal area of Tonkin Gulf; the northern coastal area (Quang Ninh–Ninh Binh), the northeastern coastal sub-region (Quang Ninh–Hai Phong),

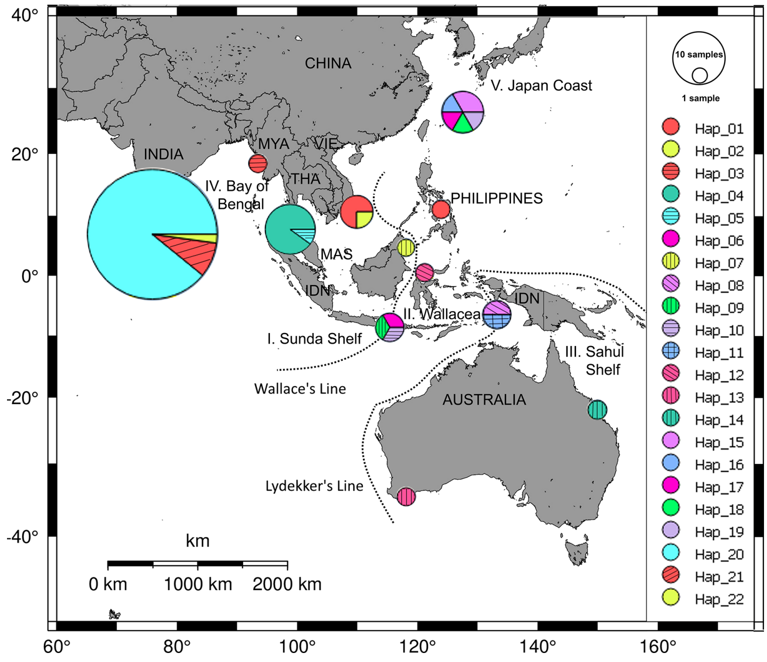

FIGURE 5
Distribution of haplotype frequency of *Halophila major*. Twenty-two haplotypes were found from dataset of 69 ITS sequences of *H. major* collected in five geographic regions: Sunda Shelf (I), Wallacea (II), Sahul Shelf (III), Bay of Bengal (IV), and coast of Japan (V). Source: Nguyen et al. (2021b).

and the coastal area of Hai Phong (the city directly under the central government, the development center of the northern coastal region). This study combined three models of integrated management of the western coastal area of Tonkin Gulf, including managing, rational using of natural resources, and conserving nature and biodiversity; managing, preventing pollution, natural disasters, and environmental incidents; and strengthening institutions, policies, and raising awareness and responsibility for protecting natural resources—environment. In particular, the issues of natural, cultural, and biodiversity conservation include (i) biodiversity conservation, with focus on species diversity and rare and endangered species; (ii) protection and restoration of habitats and ecosystems, with focus on mangroves, coral reefs, seagrass beds, and sandy beaches; (iii) protection of natural landscapes including sea—islands, peninsulas, estuaries, and coastal zones; (iv) planning, construction, and management of nature reserves, including natural heritage sites, biosphere reserves, wetlands of national and international importance, marine protected areas, natural wonders, and natural landscapes (Khanh Ni et al., 2020; Tran, 2011).

Several models of seagrass ecosystem management were integrated into the integrated coastal zone management program in other areas, including the integrated management model of the coastal area in the North Central and Central Coast regions until 2010 and oriented to 2020 according to the Decision No. 158/2007/QĐ-TTg to strengthen management, protection, and utilization capacity of natural resources and environment protection for sustainable development of provinces and cities, and model of community-based and co-management of habitat marine resources and coastal ecosystems, mainly in the form of pilot-model projects implemented by domestic and international organizations (Government of Viet Nam, 2007). Presently, many coastal provinces have implemented the co-management and eco/community tourism models in the marine protected areas (e.g., Quang Ninh, Nam Dinh, and Quang Nam provinces) and co-management of exploiting and protecting aquatic resources (e.g., Quang Ninh, Nam Dinh, Thanh Hoa, Ha Tinh, Thua Thien Hue, Quang Ngai, and Binh Thuan provinces). Therefore, these successful models have been documented and expanded, such as the co-management model in Cu Lao Cham marine protected area, which was implemented from 2011 to the present. This management model has effectively managed resources based on the coordination between local community and management authorities, in which the authorities share management responsibilities with stakeholders and thereby contributing to the protection of coral and seagrass ecosystems (Hoang et al, 2020; Tran, 2011). On the other hand, the local authorities have oriented toward integrated management of seas and islands. However, their roles and responsibilities on the current planning and management practices are still undefined. There is no specific management model for each ecosystem, especially seagrass ecosystems.

Recently, there are very few distinct models of seagrass ecosystem management. One of the projects have been implemented by the Centre for Marinelife Conservation and Community Development (MCD) titled “Strengthening marine ecosystems management and developing local community livelihood, responding to climate change” in four main areas: Giao Thuy-Nam Dinh commune, Nam Phu-Thai Binh commune, Phu Long-Hai Phong commune, and Van Hung-Khanh Hoa commune. MCD builds a combination of community-based livelihood and resource management models such as the model of co-management of protected areas in order to utilize marine resources and sustainably develop those resources according to local regulations and management plans and the community model of sustainable fisheries to support the livelihoods of coastal communities in an environment-friendly practices and without destroying marine ecosystems and marine resources. MCD also diversifies livelihoods, increases income for coastal community, and reduces exploitation pressure on marine resources by developing local ecotourism models (MCD, 2013).

The application of the seagrass ecosystem management models can contribute to the management of exploitation and utilization on coastal resources in the coastal provinces, leading to gradual enhancement of the capacity of climate change adaptation in the future. However, these conservation models are few and mainly implemented in the form of pilot projects by domestic and international organizations.

Future work

Mapping of seagrass beds in the mainland and estimation of the total cover have been done. However, the information on seagrass beds from the offshore islands, for example, some archipelagos in the Gulf of Thailand, is still lacking. Therefore, analyzing and mapping seagrass beds from a few offshore islands in southern Viet Nam are needed. Available remote sensing data are very efficient in the determination of sea grass beds coverage. However, for the species determination within seagrass beds, more in-depth methods need to be applied. The long-term monitoring of seagrass beds will be carried out to extend our understanding of the development and decline of seagrass ecosystems. For the species diversity, the putative hybridization between *H. ovalis* and *H. major* found from Nha Trang Bay should be clarified by using more plastid and nuclear DNA, leaf morphological dimension, and microsatellite DNA loci. Seagrasses are widely used to monitor heavy metal pollution in the nearshore environment and to bio-monitor metal and non-metal contamination in the marine ecosystem. However, only *E. acoroides* was studied in detail from Viet Nam. Therefore, more species should be included. The evaluation of seagrass ecosystem services is also an important step to demonstrate their usefulness to the ecosystem and to humans. Seagrasses can sequester significant amounts of carbon and store it as organic carbon in the sediments for a long time. There is still a need to evaluate the total C_{org} stock from extensive seagrass beds. Sufficient genetic diversity seems to be a key factor to evaluate the health of the seagrass population. In Viet Nam, most of the studies focus onto some members of Hydrocharitaceae. Hence, more species of Cymodoceaceae and Zosteraceae should be carried out. More molecular studies on seagrass response to environmental changes, epigenetics, and holobionts are critical in our planning in the future. Finally, it is important to call for action, especially to speak with stakeholders and policy makers for better management of seagrass beds in Viet Nam.

This review clarified the recent distribution of seagrass from Viet Nam with 156.1 km^2. Change detection of seagrass beds was presented from three specific areas. There are 15 species including putative hybridization of *Halophila*. Only one haplotype of *H. ovalis* and two haplotypes of *H. major* were found along the coast of Viet Nam. Genetic diversity and population structure from four species within Hydrocharitaceae were presented. In addition, other aspects of blue carbon storage and phytoremediation processes of seagrass from Viet Nam were also reported.

Author contributions

X-VN and JP contributed to conceptualization, writing the original draft, review, and editing. TP, N-TN, X-TN, and T-HN contributed to genetic diversity and taxonomy. V-KL and V-LC contributed to mapping and change detection of seagrass. C-TH contributed to seagrass management. TP and V-LC contributed to blue carbon. X-VN contributed to biodiversity within seagrass. HMN and TP contributed to ecology and physiology. M-NN-T and V-HD contributed to project administration. JP, HMN, and MT contributed to review and editing. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

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Grazing preference and isotopic contributions of kelp to *Zostera marina* mesograzers

Angeleen M. Olson[1,2*†], Carolyn Prentice[1†], Zachary L. Monteith[1], Derek VanMaanen[1], Francis Juanes[2] and Margot Hessing-Lewis[1,3]

[1]Nearshore Ecology, Hakai Institute, Heriot Bay, BC, Canada, [2]Fisheries Ecology and Conservation Lab, Department of Biology, University of Victoria, Victoria, BC, Canada, [3]Institute for the Oceans and Fisheries, University of British Columbia, Vancouver, BC, Canada

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Catherine Ann Pfister,
The University of Chicago,
United States

*CORRESPONDENCE
Angeleen M. Olson
angeleen.olson@hakai.org

†These authors have contributed equally to this work

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In seagrass food webs, small invertebrate mesograzers often exert top-down control on algal epiphytes growing on seagrass blades, which in turn releases the seagrass from competition for light and nutrients. Yet, nearshore habitat boundaries are permeable, and allochthonous subsidies can provide alternative food sources to *in-situ* production in seagrass meadows, which may in turn alter mesograzer-epiphyte interactions. We examined the contribution of allochthonous kelp (*Nereocystis luetkeana*), autochthonous epiphytic macroalgal (*Smithora naiadum*), *Ulva lactuca*, and seagrass production to mesograzer diets in a subtidal *Zostera marina* (eelgrass) meadow. In both choice feeding experiments and isotopic analysis, mesograzer diets revealed a preference for allochthonous *N. luetkeana* over *Z. marina*, *S. naiadum*, and *U. lactuca*. Notably, *Idotea resecata* showed an ~20x greater consumption rate for *N. luetkeana* in feeding experiments over other macrophytes. In the meadow, we found a positive relationship between epiphytic *S. naiadum* and gammarid amphipod biomass suggesting weak top-down control on the *S. naiadum* biomass. Epiphyte biomass may be driven by bottom-up factors such as environmental conditions, or the availability and preference of allochthonous kelp, though further work is needed to disentangle these interactions. Additionally, we found that gammarid and caprellid amphipod biomass were positively influenced by adjacency to kelp at seagrass meadow edges. Our findings suggest that *N. luetkeana* kelp subsidies are important to the diets of mesograzers in *Z. marina* meadows. Spatial planning and management of marine areas should consider trophic linkages between kelp and eelgrass habitats as a critical seascape feature if the goal is to conserve nearshore food web structure and function.

KEYWORDS

seascape, epiphytes, stable isotopes, allochthonous subsidies, trophic interactions, eelgrass

1 Introduction

The recognition that ecosystems are connected across boundaries by nutrient and organism flow has broadened our understanding of trophic interactions within and among ecosystems (Loreau et al., 2003; Marczak et al., 2007). Energy that cross ecosystem boundaries - known as allochthonous subsidies - can play key roles in population and community structure of recipient habitats, and in turn, the function and stability of these ecosystems (Polis and Hurd, 1996; Huxel et al., 2002). The importance of allochthonous subsidies is now recognized in nearly all aquatic and terrestrial ecosystems (Lafage et al., 2019), from tropical and temperate rainforest insect communities (Recalde et al., 2016; Recalde et al., 2020; Nakano and Murakami, 2001) to freshwater planktonic communities (Vargas et al., 2011, Adamczuk et al., 2019), subtropical island ecosystems (Spiller et al., 2010) and coastal marine ecosystems (Savage, 2019; Zuercher and Galloway, 2019). While allochthonous subsidies are often overlooked when assessing ecosystem interactions (Buckner et al., 2018; Smale et al., 2018), they are increasingly recognized as having important influences on the composition of food web producers, as well as trophic transfer through food webs.

The availability of allochthonous foods can alter interactions between primary producers and consumers in the recipient ecosystem (Huxel and McCann, 1998; Huxel et al., 2002). For instance, if allochthonous inputs weaken specific autochthonous producer - consumer interactions, then expected trophic cascades could be dampened (Polis and Hurd, 1996; Huxel and McCann, 1998; Rodewald et al., 2011). The influence of an allochthonous subsidy may depend on the characteristics of the trophic subsidy itself (e.g. its duration, timing, spatial extent, palatability) as well as characteristics regulating consumers in the recipient habitat, such as availability of other food sources, consumer size, feeding mode, trophic level and life history stage (Zuercher and Galloway, 2019). Previous work has largely focused on the effect of cross-ecosystem subsidies on nutrient-poor recipient ecosystems such as desert islands (Anderson and Polis, 1999) and sandy beaches (Lastra et al., 2008; Liebowitz et al., 2016), as well as aquatic subsidies into riparian ecosystems (Hocking and Reimchen, 2009; Lafage et al., 2019). Growing evidence suggests cross-ecosystem energy transfer may be important to highly productive recipient ecosystems, such as mangroves (Slim et al., 1996), coral reefs (Carreón-Palau et al., 2013) and seagrass meadows (Hyndes et al., 2012, Cartraud et al., 2021).

In seagrass ecosystems, a central tenet of food web structuring is the top-down role of small invertebrate herbivores (herein 'mesograzers') in consuming algal epiphytes, which releases seagrass from negative impacts of algal shading and/or nutrient competition and maintains a seagrass-dominated ecosystem (Orth and Van Montfrans, 1984; Valentine and Duffy, 2006; Cook et al., 2011). Empirical evidence for this indirect positive effect of mesograzers on seagrass productivity has been demonstrated thoroughly (Orth and Van Montfrans, 1984; Hughes et al., 2004; Moksnes et al., 2008; Whalen et al., 2013) and is increasingly important to our understanding of bottom-up and top-down human disturbances to seagrass meadows (e.g., eutrophication and overfishing, respectively). Although negative relationships between mesograzers and epiphytes are widely generalized, their strength and direction can depend on seasonality (Whalen et al., 2013), the source of nutrient inputs (Hessing-Lewis and Hacker, 2013), mesograzer species composition (Duffy and Harvilicz, 2001; Jaschinski and Sommer, 2008), and predation rates on mesograzers (Moksnes et al., 2008; Hughes et al., 2013).

Seagrass habitats have high *in-situ* productivity and are known to contribute significant amounts of biomass to adjacent ecosystems such as the deep sea and sandy beaches (Heck et al., 2008; Liebowitz et al., 2016; Duarte and Krause-Jensen, 2017). Yet, their role as recipients of allochthonous materials has received relatively less attention. Seagrass meadows often occur in sheltered coastal environments, where their canopies facilitate the deposition of materials from the water column, leading to enhanced accumulation of allochthonous materials (Peterson et al., 2004; Hendriks et al., 2008). Among many potential allochthonous inputs, the role of kelp is emerging as an important food source for organisms in temperate seagrass meadows (Smit et al., 2006; Hyndes et al., 2012; Hyndes et al., 2014; Olson et al., 2019). For example, along the Atlantic coast of Canada, an estimated 82% of annual kelp productivity enters detrital pathways, which can enhance secondary production in recipient food webs (Krumhansl and Scheibling, 2012). Accumulation of kelp biomass in seagrass meadows can be substantial with transportation to meadows occurring from considerable distances away (Wernberg et al., 2006). However, the extent to which kelp may be incorporated into recipient seagrass food webs, and if they can alter mesograzer-producer interactions, remains unclear.

In this study, we examined the contribution of allochthonous bull kelp (*Nereocystis luetkeana*) to the diets of mesograzers in a temperate seagrass *Zostera marina* (common name 'eelgrass'*)* relative to other ubiquitous macrophytes. We quantified the preference for allochthonous vs. autochthonous sources to mesograzers using choice feeding experiments. Next, we assessed the contributions of these same macrophytes to *in-situ* mesograzer diets using natural isotopic tracers. Because of the widespread presence of kelp forests in this seascape, and their high nutrient quality, we expected kelp subsidies to make up a significant proportion of mesograzer diets. Finally, kelp subsidies may mediate mesograzer – macrophyte interactions. As a first step in assessing the trophic influence of kelp on the *Z. marina* food web (see Hessing-Lewis et al., 2018), we examined relationships between common mesograzers and a dominant *Z. marina* epiphyte (*Smithora naiadum*), as well as the effect of meadow location on mesograzer biomass.

2 Methods

British Columbia's (B.C.) coastline is characterized by high complexity, including exposed outer-coast islands, sheltered bays, estuaries, and steep fjords. On the central coast of B.C., the nearshore environment is a heterogenous seascape consisting of kelp forests, seagrass meadows, benthic algae, rocky reefs, and sandy habitats. Our study was conducted in a large subtidal *Z. marina* meadow located in Choked Passage on the northern shore of Calvert Island in the summer of 2015 (Figure 1). This *Z. marina* meadow is predominantly surrounded by shallow rocky reefs, bare sand habitats, annual *N. luetkeana* kelp forests, and to a lesser extent perennial *Macrocystis pyrifera* kelp forests.

2.1 Choice feeding experiments

In August 2015, we conducted a multiple-choice feeding experiment with mesograzers collected from the Choked Passage meadow to determine their dietary preference among allochthonous (*N. luetkeana*) and autochthonous (*Z. marina, S. naiadum*) macrophytes, as well as *Ulva lactuca* (both allochthonous and autochthonous). We focused on mesograzers with larger body size because of their ubiquity in local *Z. marina* meadows and adjacent kelp forests and ease to work with. Mesograzers and macrophytes were collected haphazardly from the meadow, focusing on isopods, *I. resecata*, (mean 23 ± 4 mm length (SD) and 116.3 ± 33.6 mg biomass in our study) and cryptic kelp crabs, *Pugettia richii*, a larger body size grazer (max. size 44 mm, Lamb and Hanby, 2005) averaging 5962.9 ± 4421.9 mg in our study.

The experiment took place in a natural flow-through seawater system. Mesograzers were starved for 35 hours prior to the feeding experiments, weighed, and placed in separate small containers with fine mesh windows open to seawater off the Hakai Institute Observatory dock in Pruth Bay directly adjacent to another *Z. marina* meadow. For the experiment, mesograzers were placed in an experimental container (946 mL volume) which included four macrophytes of equal surface area (2 x 2 cm square): *Z. marina, N.*

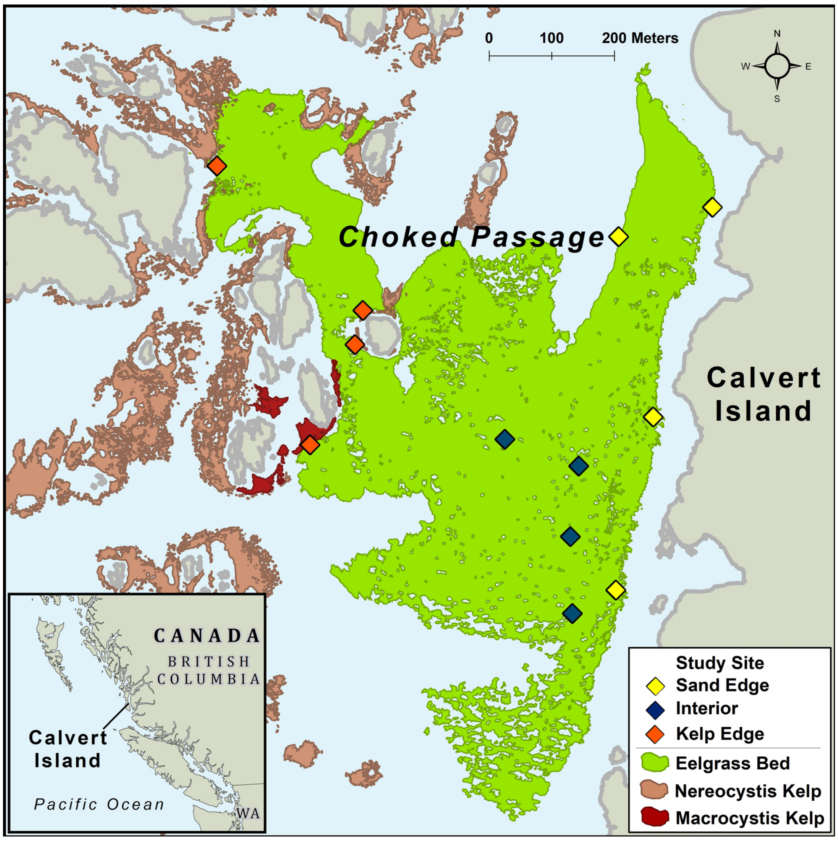

FIGURE 1
Study area off Calvert Island, British Columbia, Canada. Sites were established in a *Zostera marina* meadow in Choked Passage (green) which is located in a nearshore seascape surrounded by canopy forming kelp, primarily *Nereocystis luetkeana* (light red) in proximity to some *Macrocystis pyrifera* (dark red).

luetkeana, *S. naiadum* and *U. lactuca*. The containers were suspended off the dock and subject to natural daylight patterns and temperatures (Supplementary Figure 1). Replicate trials were conducted for each mesograzer type along with simultaneous control trials with no mesograzers present (n = 7). Experimental containers were otherwise bare (e.g., no sand or habitat substitutes). Macrophytes were blotted dry and weighed on a microbalance (mg) before and after the duration of the feeding trials, which were run for 18 hours. Macrophyte consumption rates by each mesograzer were calculated using Equation 1 (Taylor and Brown, 2006; Sampaio et al., 2017), which accounted for consumer size (i.e., biomass in mg) and the change in the weight of the macrophyte relative to a grazer-free control:

$$Consumption\ \ Rate\ =\ \frac{Ti\ *\ (Cf/Ci)-\ Tf}{n\ \ bio\ *\ t} \tag{1}$$

where T_i is the initial producer blotted wet weight (bww), T_f is the final bww, C_i is the initial control bww, C_f is the final control bww, n_{bio} is the grazer biomass (i.e., size) at the end of the experiment (g), and t = duration of the experiment in days. Consumption rate is thus expressed in mg of macrophyte consumed per mesograzer biomass per day (mg PP/mg grazer/day). A one-way analysis of variance (ANOVA) was used to test for differences in the consumption rates for both *I. resecata* and *P. richii* on the four macrophytes. If significant differences were detected ($P < 0.05$), a Tukey's *post-hoc* test was used to examine pairwise comparisons amongst all macrophytes used in the feeding trials.

2.2 Stable isotopes and mixing models

We collected mesograzers from *Z. marina* shoots during a 2-week period in late July- early August 2015 from the kelp edge, sand edge, and interior sites. Mesograzers were frozen until laboratory processing for isotope analysis. Due to their small body sizes, numerous individuals of gammarid amphipods (multiple species in the family Gammaridae) and Lacuna snails (multiple species of the *Lacuna* genus,) were pooled within a single sample (n = 10 individuals/sample, 5 samples total). Other mesograzers were large enough that an individual's biomass filled a sample: Caprellid amphipods (multiple species of the Caprellidae family, n = 13), *I. resecata* (n = 13), and *P. richii* (n = 3). Isotope signatures of most macrophytes were obtained from a concurrent study (see Olson et al., 2019), which included *Z. marina* and *S. naiadum* as *in-situ* meadow production and *N. luetkeana* from surrounding kelp forests. For this study, *U. lactuca* (n = 20) was also collected adrift in the *Z. marina* meadow. Small amounts of *U. lactuca* were found growing in the seagrass meadow and also was commonly found growing in the surrounding reef or sand habitats rather than within the meadow (authors' personal obs.), and thus could be considered as both autochthonous and/or allochthonous production.

Samples were prepared for isotope analysis by defrosting and removing surface debris. Lacuna snail bodies were pulled out of their shells for processing. Whole bodies of the other mesograzers (including stomachs) were rinsed in two baths of deionized water and dried at 60°C. $\delta^{13}C$ and $\delta^{15}N$ were analyzed at the Mazumder Lab at the University of Victoria on a Delta IV Isotope Ratio Mass Spectrometer, as the ratio of heavy to light isotope with values denoted in δ:

$$\delta(‰)=\left(\frac{(Rsample)}{(Rstandard)}-1\right)\ \times 1000 \tag{2}$$

where R represents the ratios 13C/12C and 15N/14N of the sample or laboratory standard. Mass ratios of carbon to nitrogen (C:N) were also determined for macrophytes, and were used to assess their relative palatability, where lower values of C:N represent relatively higher nutritional content. An ANOVA test was used to assess differences in the C:N among macrophytes, with a Tukey's *post-hoc* test to further contrast between each combination.

We used a Bayesian isotopic mixing model mixSIAR (Moore and Semmens, 2008, Stock et al., 2018) to assess the relative contribution of the macrophytes to mesograzer diets. When predators consume prey energy, the heavy isotope is favoured over the light isotope due to discrimination from metabolic processes, which causes an enrichment of isotope values with trophic level. Thus diet–tissue discrimination factors (DTDFs) are used when estimating prey contribution to a predator's diet. Because mixing model results are highly sensitive to DTDFs, we assessed two options from the literature: 0.4 ± 0.12‰ for $\delta^{13}C$ and 2.0 ± 0.20‰ for $\delta^{15}N$ (McCutchan et al., 2003) and 0.4 ± 1.14‰ for $\delta^{13}C$ and 3.4 ± 1.0‰ for $\delta^{15}N$ (Post, 2002). After visual assessment of the consumer isotopic values alongside macrophyte values post correction (Phillips et al., 2014), DTDFs from Post et al. (2002) were chosen because of their better fit (i.e., consumer values fell within the range of macrophyte mean and standard deviation values). During this analysis, one caprellid amphipod outlier with depleted $\delta^{13}C$ values outside the macrophyte isotope ranges was removed.

2.3 Establishing mesograzer-epiphyte relationships

To assess biomass and abundance of macrophytes and mesograzers, twelve sites were established in the *Z. marina* meadow (total area ~367,000m^2; Figure 1). 40 m transects were set in the *Z. marina* meadow adjacent to *N. luetkeana* kelp forests (n=4 transects), adjacent to sand habitats (n=4), and in the interior of the meadow (n=4) at depths that ranged from 1.52m – 4.99m. We collected *Z. marina* shoots at 10 m intervals (n = 5 shoots per transect) by scuba. Divers carefully covered shoots with a plastic bag, detached the shoot from the rhizome, and sealed the bag before moving to the next collection point.

Sampling occurred in May, July, and August 2015. Shoot samples were subsequently processed in the laboratory. Bag contents were sieved through a 500um filter, capturing mesograzers > 500um. *Z. marina* shoots were gently scraped of epiphytes and mesograzers. All components were oven dried at 60°C for biomass measurements.

We used a binomial - gamma hurdle generalized linear model (GLM) to assess the relationship between mesograzer biomass and *S. naiadum* biomass in R (R Core Team, 2021). Mesograzers and *S. naiadum* dry biomass (g) were standardized by dry *Z. marina* blade biomass (g) from which they were collected. We first modeled the probability of presence or absence of each mesograzer's biomass related to *S. naiadum* biomass with a binomial distribution. A gamma model was then used on non-zero grazer biomass to estimate the relationship between grazers and *S. naiadum* biomass. If residual plots indicated high leverage points in preliminary model analysis, these data were removed by a Cook's Distance cut off.

3 Results

3.1 Feeding experiment results: Mesograzer preferences

I. resecata and *P. richii* consumed all four macrophytes provided to them (*N. luetkeana*, *U. lactuca*, *Z. marina* and *S. naiadum*) to some extent. Control replicates (no grazers present) showed little loss of biomass and any changes that did occur were accounted for in consumption rate calculations (Equation 1). Preferences for macroalgae over *Z. marina* were demonstrated by both mesograzers, as well as a notably high consumption rate of *N. luetkeana* kelp by *I. resecata* isopods - 0.8 mg kelp/mg grazer/day (Figure 2A).

I. resecata consumed the four macrophytes at different rates (Figure 2A, ANOVA: $F_{(3,\ 24)} = 17.21$, $P < 0.001$). Specifically, they consumed *N. luetkeana* at a greater rate than *U. lactuca*, *S. naiadum* and *Z. marina* ($P < 0.001$ for all pairwise comparisons); consumption rates were ~20x higher for *N. luetkeana* relative to the other three options. Further, there was no difference in their consumption rate of each combination of *S. naiadum*, *U. lactuca* and *Z. marina* (Figure 2A). Similar to *I. resecata*, *P. richii* consumed macrophytes at different rates (Figure 2B, ANOVA: $F_{(3,\ 24)} = 4.271$, $P = 0.015$). The largest difference in consumption rate by *P. richii* was observed between *N. luetkeana* and *Z. marina* ($P = 0.014$). No other pairwise comparisons of consumption rates for *P. richii* were significantly different (Figure 2B).

3.2 Isotopic results and feeding observations from the field

The isotopic composition of mesograzer in the *Z. marina* meadow was variable particularly in $\delta^{13}C$ (Figure 3). Caprellid

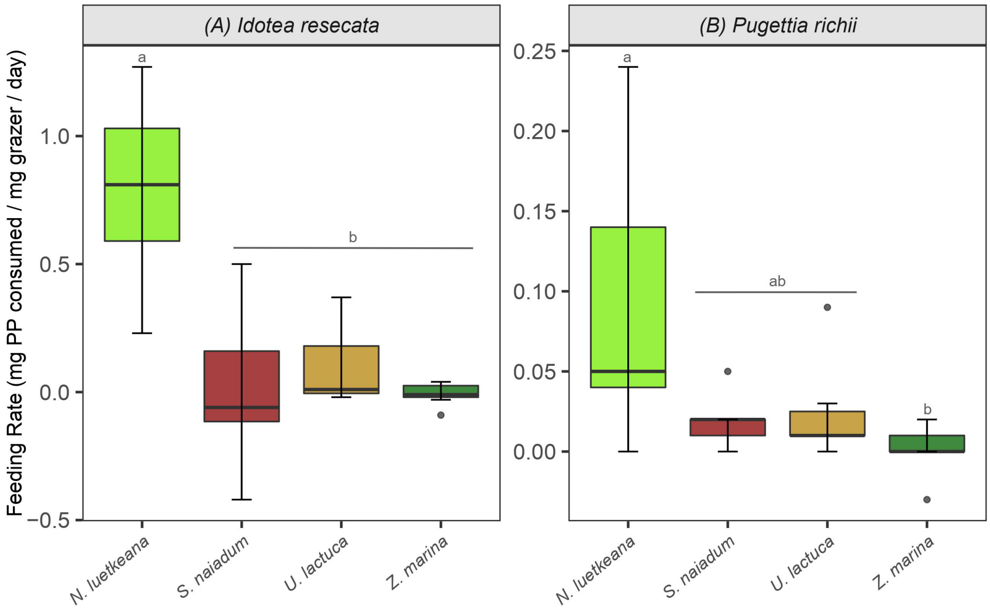

FIGURE 2
Consumption rates (mean and standard error) by **(A)** *I. resecata* isopods and **(B)** *P. richii* crabs on macrophytes from the feeding trials - *N. luetkeana*, *S. naiadum*, *U. lactuca* and *Z. marina*. Note the differing scales for each mesograzer. Letters indicate treatments that are significantly different from one another. The figure represents the full and raw dataset, and negative values derived from the consumption equation were retained for completeness.

amphipods had the most depleted $\delta^{13}C$ signatures, while Lacuna snails were most enriched. $\delta^{15}N$ signatures of the mesograzers were much closer in range, where gammarid amphipods and *P. richii* were more enriched relative to *I. resecata*, caprellid amphipods, and Lacuna snails (Figure 3). $\delta^{13}C$ and $\delta^{15}N$ values of *U. lactuca* fell in between *Z. marina* (most enriched) and *S. naiadum* (most depleted). *N. luetkeana*, *S. naiadum*, and *U. lactuca* macroalgae had lower C:N ratios than *Z. marina* (pairwise comparisons, $P<0.001$). Macrophytes differed in palatibility as measured by C:N ratios (Table 1, ANOVA: $F_{(3,63)} = 158.1$, $P< 0.001$). *S. naiadum* had the lowest C:N ratios of all macrophytes examined ($P<0.05$), specifically ~ 3x lower than the other autochthonous macrophyte, *Z. marina*. We found no difference between *N. luetkeana* and *U. lactuca* C:N ratios ($P = 0.29$).

Contributions of allochthonous vs. autochthonous macrophytes to diets varied by mesograzer (Figure 4). Allochthonous sources were high in *I. resecata* and caprellid amphipods, whereas autochthonous sources contributed more to *P. richii*, gammarid amphipod, and Lacuna snail diets. In *I. resecata* diets, *N. luetkeana* had the highest contribution (54.3%, Figure 4A), followed by *U. lactuca* (17.6%) and *S. naiadum* (16.3%). *Z. marina* had the lowest contribution to *I. resecata* diets (12%). For caprellid amphipod diets (Figure 4B), *S. naiadum* (35.2%) and *N. luetkeana* (33.6%) showed the highest contributions, whereas *U. lactuca* (21.3%) and *Z. marina* (10.4%) had low dietary contributions.

Autochthonous meadow sources dominated *P. richii* diets: *S. naiadum* (28.1%) and *Z. marina* (26.1%) (Figure 4C). Contributions from *U. lactuca* (27.4%) were also high, while contributions from *N. luetkeana* were lowest (18.4%). Similarly, gammarid amphipods diets (Figure 4D) had highest contributions from *S. naiadum* (32.7%) followed by *U. lactuca* (28%), *Z. marina* (22.5%) and the lowest by *N. luetkeana* (16.7%). Lacuna snail diets consisted primarily of *Z. marina* (46%), with lower contributions of *N. luetkeana* (22.6%), *U. lactuca* (19.1%), and *S. naiadum* (12.4%).

The relative uptake of primary production by mesograzers did not consistently mirror their relative palatability as assessed by C:N ratio (Table 1, Figure 4). Caprellid amphipods were the only mesograzer that closely matched their diets to palatability *via* C:N ratio. *S. naiadum* was favoured by most mesograzers (Figures 4B–D). *N. luetkeana* contributed more than expected (based on C:N) to *I. resecata* and Caprellids amphipods. *Z. marina* also had higher than expected contributions, as seen in Lacuna snails and *P. richii* crabs (Figures 4E, C, respectively).

3.3 Field observations of the grazer-producer biomass relationship

The sub-tidal *Z. marina* shoots weighed on average 2.3 ± 0.95 g (SD, dry weight, n = 178), and were characterized as long (146.5 ± 36.0 cm) and wide (0.85 ± 0.15 cm) from n = 115 intact longest

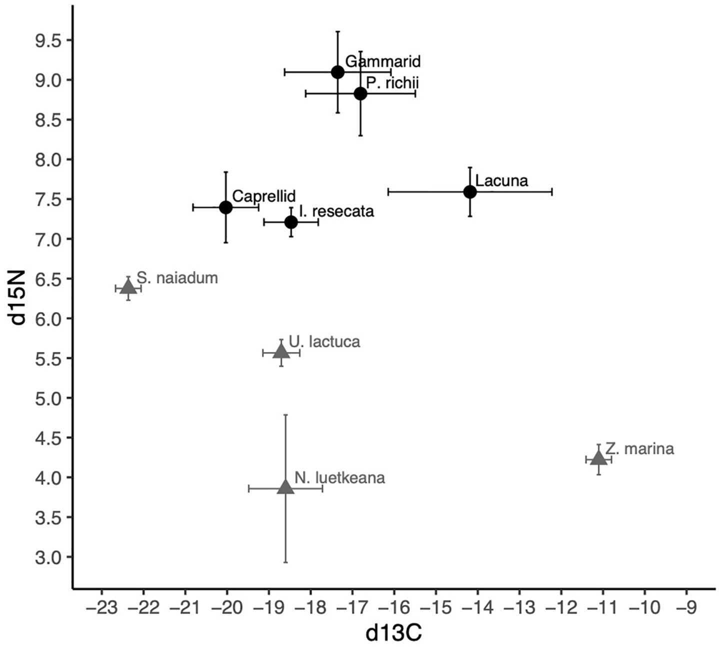

FIGURE 3
$\delta^{13}C$ and $\delta^{15}N$ signatures (mean and standard deviation) of invertebrate mesograzers (black) and macrophytes (grey) in the Choked Pass *Z. marina* meadow.

TABLE 1 Macrophyte carbon to nitrogen ratios (C:N) in the *Z. marina* meadow indicating their relative palatability.

Macrophyte	Mean C:N	SD C:N
Nereocystis luetkeana	10.61	0.44
Zostera marina	18.16	2.34
Smithora naiadum	7.40	0.93
Ulva lactuca	9.03	1.66

Lower C:N values indicate higher palatability.

blades. The dominant epiphyte across the meadow was the red alga *S. naiadum*. Lobed blades of *S. naiadum* growing from encrusted basal cushions were extensive across the meadow: present on 66% of the blades with an average biomass of 0.82 ± 1.1 g shoot^{-1} up to a maximum value of 5.1 g shoot^{-1}. *Punctaria* spp. and *Ulva* spp. epiphytes were present but less abundant - when present, they had minimal biomass on blades (mean of 0.20 ± 0.16 g shoot^{-1} and 0.07 ± 0.05 g shoot^{-1}, respectively).

Results from the gamma hurdle model indicate that gammarid amphipods and *S. naiadum* biomass had a positive relationship (GLM intercept = -6.30 ± 0.25; *b* = 2.21 ± 0.28, P<0.001) in the eelgrass meadow (Figure 5A). The other

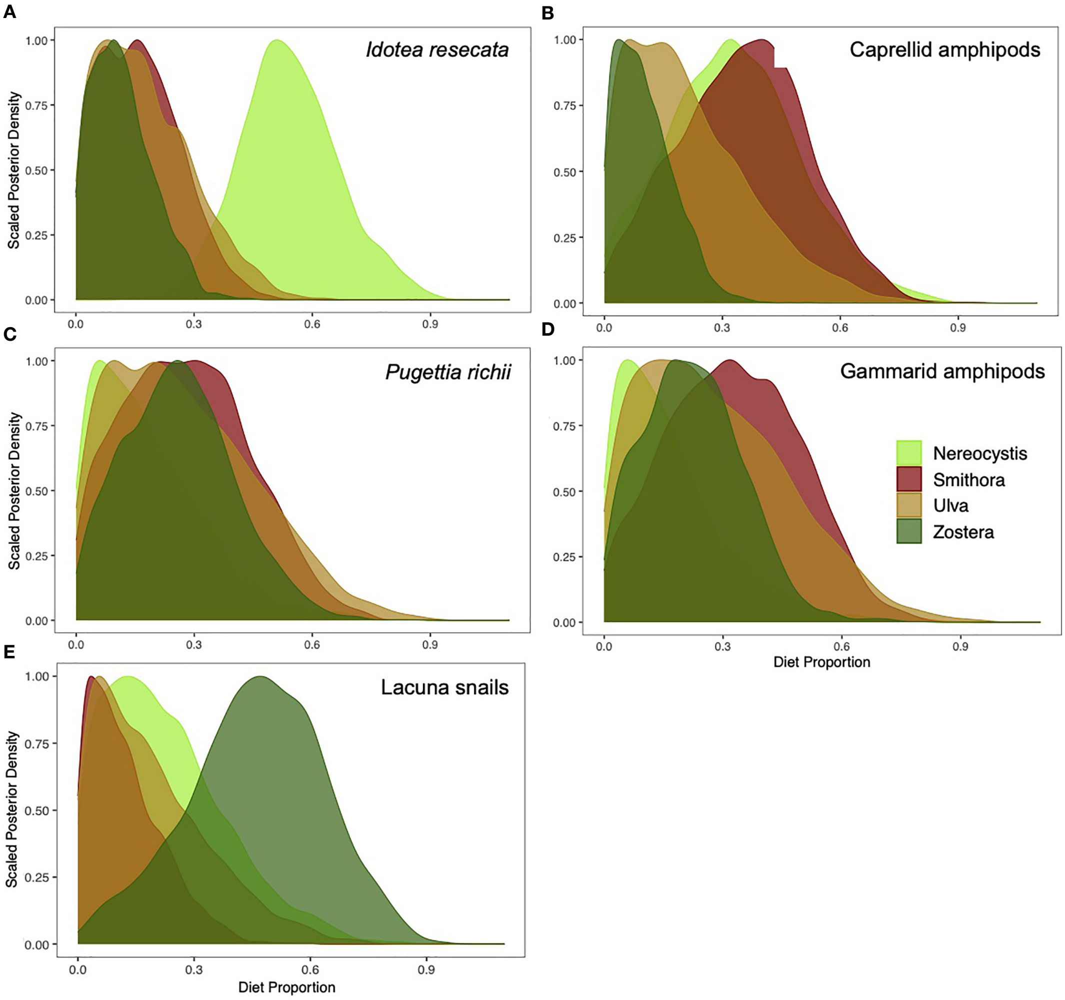

FIGURE 4
Proportion of *N. luetkeana*, *S. naiadum*, *U. lactuca*, and *Z. marina* that contributed to mesograzers diets: **(A)** *I. resecata*, **(B)** caprellid amphipods **(C)** *P. richii* crabs, **(D)** gammarid amphipods, and **(E)** Lacuna snails.

mesograzers examined did not demonstrate a significant relationship with *S. naiadum* (Supplementary Figure 2). The biomass distribution of mesograzers was uneven across the meadow (Figures 5B–D). Gammarid and caprellid amphipods had significantly higher biomass at the kelp edge relative to the sand edges and interior sites (GLM b = 1.63 ± 0.35, $P<0.001$, b = 3.35 ± 1.11, P =0.004, respectively). In contrast, *I. resecata* biomass was significantly lower at kelp edges than the interior and sand sites (P = 0.020). Lacuna snail biomass was consistent throughout the meadow (Supplementary Figure 2D).

4 Discussion

4.1 Overall findings

Using choice feeding experiments and stable isotopes, we found that mesograzers in a *Z. marina* meadow were consuming allochthonous kelp. Kelp was the preferred food in experiments involving *I. resecata* and *P. richii* mesograzers relative to other primary producers (*S. naiadum, Z. marina, U. lactuca*). Yet, stable isotope results revealed a more varied uptake of allochthonous vs. autochthonous food depending on the mesograzer. *N. luetkeana* was an important contribution to the diets of *I. resecata*, Caprellid amphipods, and Lacuna snails, but less so to *P. richii* and gammarid amphipod diets. Epiphytic *S. naiadum* was the most consistent *in-situ* meadow macrophyte source isotopically integrated into mesograzer diets, highlighting the importance of this epiphyte to the *Z. marina* food web. *S. naiadum* also had the highest palatability *via* C:N ratio which may explain its favourability. We observed a positive relationship between gammarid amphipods and *S. naiadum* epiphytes, suggesting that there may be weak top-down control on epiphyte abundance by grazers in this system (see food web structure in Hessing-Lewis et al., 2018). We hypothesize that bottom-up drivers may play a key role in

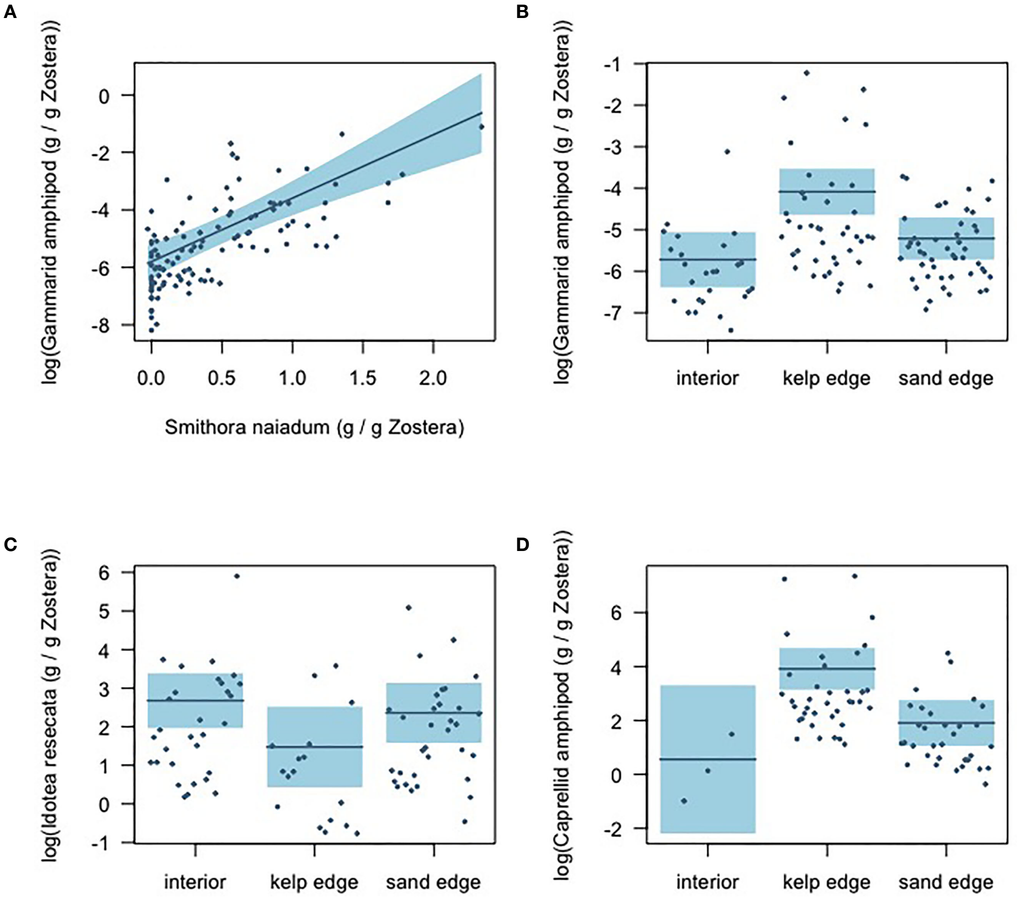

FIGURE 5
Gamma-hurdle model results showing the **(A)** relationship between *S. naiadum* epiphyte biomass and gammarid amphipod biomass (standardized by *Z. marina* biomass); and the mesograzer biomass distributions across the meadow sites: **(B)** gammarid amphipods **(C)** *I. resecata*, and **(D)** caprellid amphipods. Shaded blue indicates 95% confidence intervals.

structuring this *Z. marina* food web, however experiments controlling for potential bottom-up (e.g., currents, allochthonous subsidies, edge effects) and top-down factors (e.g., grazing pressures, species composition) are needed to disentangle those complex interactions in this ecosystem.

4.2 Mesograzer feeding preferences and why kelp may be preferentially consumed

While our results suggest the incorporation of kelp into mesograzer diets for some species, we also observed high variability in diets among mesograzer species, as has been found elsewhere (Duffy and Harvilicz, 2001; Douglass et al., 2011). Mesograzers often have the choice of a variety of food sources (e.g., eelgrass, periphyton, bladed epiphytes, detritus/drift algae) that vary in availability and palatability through time and space. Different food sources may be more or less available to mesograzers depending on their mode of feeding (e.g., filter feeders vs. grazing invertebrates). For *I. resecata*, a common eelgrass-dwelling mesograzer, the feeding trial and isotopic data aligned well and suggested a strong preference for *N. luetkeana* over autochthonous *Z. marina* and *S. naiadum* (Figures 2, 4A). This result makes sense as *I. resecata* are highly mobile and able to consume detritus and larger plant material, and are likely to feed on kelp when available. *I. resecata* is known to consume *Z. marina* and microalgae (Best and Stachowicz, 2012; Lewis and Boyer, 2014). Less is known about *I. resecata* feeding preferences for *N. luetkeana*, however they are a well-acknowledged grazer in *M. pyrifera* kelp forests (Ng and Micheli, 2020). Feeding trial results for *P. richii* suggest a strong preference for *N. luetkeana* (Figure 2). However, the isotope results suggested a different longer-term trend, with larger contributions of *S. naiadum*, *U. lactuca* and *Z. marina* than *N. luetkeana* (Figure 4C).

Mesograzer preference for *N. luetkeana* over autochthonous primary production in *Z. marina* meadows may be explained by its bioavailability and palatability. Although both mesograzer species used for the feeding trials (*I. resecata* and *P. richii*) showed a strong preference for kelp in the lab, *N. luetkeana* subsidies may be temporally and spatially limited in the *Z. marina* meadow. *P. richii* crabs are often in the eelgrass canopy, where access to *Z. marina*, *S. naiadum*, and *U. lactuca* is plentiful. *N. luetkeana* may be less common in their preferred habitat, which may explain why it was less dominant in the isotope results. Additionally, because isotopic analysis captures a longer time-integrated window of feeding activity (e.g., days) (Zanden et al., 2015) relative to the feeding trials which represent a snapshot in time, it is reasonable to expect the isotope results to show a more equal distribution among macrophytes (Figure 4).

Kelp can bioaccumulate in large quantities within seagrass meadows at certain times of the year (Wernberg et al., 2006; Krumhansl and Scheibling, 2012), and mesograzers may be able to respond to these pulses for their nutritional benefit, as demonstrated in the lab experiments. The *Z. marina* meadow examined here is surrounded by both *N. luetkeana* and *M. pyrifera* kelp forests (Figure 1) which culminate to large quantities of sea wrack biomass in the area (Wickham et al., 2020) and is thus likely available *via* detrital and POM pathways to mesograzers. Sediment isotopic analysis in this region has found kelp in sediment carbon pools (Prentice et al., 2019), indicating its accumulation in the not necessary meadow. *N. luetkeana* is an annual species that exhibits higher rates of productivity in the summer months (Maxell and Miller, 1996), when breakage and sloughing can produce the drift material found in seagrass beds. Major exports of kelp in the fall occur past the peak *S. naiadum* growing season, and may fill an important part of mesograzers fall and winter diets.

Examining the C:N ratios of macrophytes, macroalgae (*S. naiadum*, *U. lactuca*, and *N. luetkeana*) had lower C:N ratios, suggesting relatively higher palatability compared to *Z. marina* (Table 1). Given that *S. naiadum* epiphyte loads can be high (up to 5.1g dry weight per eelgrass shoot), our results indicate that *S. naiadum* is an important autochthonous contributor to mesograzer diets. Other nutritional properties are not captured in the C:N ratio that may make *N. luetkeana* a desirable food source, such as low levels of polyphenolic defense compounds (Steinberg, 1985; Pennings et al., 2000) or increased fatty acids or polysaccharides. More generally, *N. luetkeana* appears to be a preferred food choice for a variety of nearshore mesograzers, as seen in not necessary *Tegula funebralis* (Steinberg, 1985) (Steinberg, 1985), *Pugettia producta* (Dobkowski et al., 2017), *Idotea wosnesenskii* (Dethier et al., 2014), and *Strongylocentrotus droebachiensis* larvae (Feehan et al., 2018).

Although we did not look at it explicitly due to its relatively low biomass in the area, the giant kelp *M. pyrifera*, integrate into the food web at similar isotopic values to *N. luetkeana* (e.g, Monteray Bay δ13C ranging from -14.93 ± 0.52 ‰ to -20.54 ± 0.81‰, Drobnitch et al., 2018). Given the dominance of *N. luetkeana* in the study area (Figure 1) it is likely we captured a representative take on mesograzer diets, however examining temporal feed preferences of *M. pyrifera* alongside *N. luetkeana* would be a worthwhile, particularly because *M. pyrifera* the biomass available year-round.

While we did not examine periphyton (e.g., diatoms) as a potential food source it represents another ubiquitous and sometimes abundant food source that should be considered. Epiphytic microalgae may be a more important food source for gastropod mesograzers than to arthropod mesograzers, due to their low mobility (Doropoulos et al., 2009). This also may explain why *Z. marina* was found to be the dominant contribution to Lacuna snail diets (Figure 4E), as they may ingest surface layers of *Z. marina* tissue while scraping the blades for periphyton. Studies elsewhere have shown that these epiphytes can have similar δ^{13}C values to seagrass, examples ranging from -11.3 ± 0.81‰ (Jaschinski et al., 2008) to −15 ± 1.5

‰ (Mittermayr et al., 2014), whereas *Z. marina* can range from -9.64 ± 0.65‰ (Jaschinski et al., 2008) to -13.4 ± 3.3 ‰ (Mittermayr et al., 2014).

In other nearshore regions, allochthonous kelp subsidies has been found in the diets of seagrass meadow mesograzers. In Australia, the kelp *Ecklonia radiata* made a notable contribution to the diets of two seagrass-dwelling gastropods (Doropoulos et al., 2009), and gastropod species were found to assimilate isotopically-labeled $\delta^{15}N$ kelp under both field and laboratory conditions (Hyndes et al., 2012). Further, *in-situ* addition of kelp to *Posidonia sinuosa* seagrass plots increased the densities and biomass of the gastropod *Strigosella lepidus* and shrimp (Cartraud et al., 2021). Finally, the trophic incorporation of kelp subsidies by seagrass dwelling species has been demonstrated at higher levels of the food web such as fish (Wernberg et al., 2006; Olson et al., 2019).

4.3 Trophic implications of kelp subsidies to eelgrass meadows

When allochthonous inputs are high, there is potential for the recipient ecosystem's food web structure to be altered (Zuercher and Galloway, 2019). In seagrass ecosystems, the relationship between mesograzers and epiphytes is important for maintaining meadow health, as mesograzers prevent epiphytes from outcompeting seagrass for light and nutrients. Based on this typical seagrass trophic structure, if mesograzers were primarily consuming epiphytes, we would expect to observe an inverse relationship between mesograzer abundance and epiphyte biomass. A preliminary glance at the trophic structure in Choked Passage revealed a positive relationship between gammarid amphipods and *S. naiadum* (Figure 5A).

In addition to being a food source, *S. naiadum* may be providing habitat for some mesograzer species, supporting the positive relationship observed with gammarid amphipods. Epiphytes are known to add structural complexity to meadows which can increase mesograzer abundance and diversity (Viejo, 1999). We anecdotally observed amphipods exhibiting tube-building behavior within the *S. naiadum* epiphytes, and unpublished gammarid amphipod-*S. naiadum* feeding trials revealed very little consumption of *S. naiadum* and only a minor increase in mass lost with an increase in amphipod number. Thus, there may be a number of biotic and abiotic factors, including the availability of allochthonous food sources, that may be driving this positive mesograzer-epiphyte relationship. Moreover, gammarid and caprellid amphipod biomass was highest at transects next to kelp, suggesting potential edge effects from kelp - increasing food for mesograzers; or adding habitat from increased structural complexity (Olson et al., 2019). These results support bottom-up structuring in this system with allochthonous subsidies playing some role in shaping this ecosystem, however it may be minor compared to the environmental drivers.

Matched seasonal dynamics and bottom-up control of epiphytes and mesograzers may be also at play (Fong et al., 2000; O'Connor et al., 2022). High currents in the study area likely contribute to the persistence of *S. naiadum*, as the constant replenishment of water can supply plentiful nutrients to both seagrass and epiphytes, and maintains a low turbidity water column which reduces competition for light (O'Connor et al., 2022). Our inference of kelp's role in structuring the food web is also limited by the observational nature of our study data. *In situ* experimental addition or exclusion of kelp subsidies would add more insight on the causal effects of kelp on mesograzer-epiphyte relationships (e.g., Cartraud et al., 2021). The expectation of a negative relationship may be more applicable to interactions with smaller epiphytes or diatoms where a reduction in biomass could be reduced expeditiously by a similar abundance of mesograzers. A subsidy effect may further vary based on characteristics of the focal epiphyte (e.g., habitat-forming, nutritional quality, availability) and mesograzers (e.g., size, mobility, feeding behavior). Further research to decipher mechanisms of bottom-up drivers is needed to understand the full effect of kelp to seagrass food webs.

5 Conclusion

Seagrass meadows are highly productive ecosystems that not only export large quantities of biomass, but can also receive energy *via* allochthonous kelp from neighboring habitats. Our results suggest that inputs of allochthonous kelp are important to recipient *Z. marina* food webs through mesograzer consumption. Seagrass and kelp forests face concurrent challenges across their ranges; thus gaining a better understanding of the prevalence and magnitude of linkages among marine ecosystems is timely. Further characterizing the flows of allochthonous energy into and out of seagrass habitats can help us better understand their roles in climate change mitigation and habitat provisioning.

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

AO, CP, MHL, FJ participated in sampling design. AO, CP, DV collected the data, processed samples. AO, CP, ZM analyzed data. AO, CP, and MHL wrote the manuscript, and all authors provided valuable feedback on the manuscript. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.991744/full#supplementary-material

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EDITED BY
Mirta Teichberg,
Marine Biological Laboratory (MBL),
United States

REVIEWED BY
Ana Alexandre,
University of Algarve, Portugal
Muslima Khatun,
National Institute of Biotechnology
(NIB), Bangladesh

*CORRESPONDENCE
R.K.F. Unsworth
r.k.f.unsworth@swansea.ac.uk

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Nutrient additions to seagrass seed planting improve seedling emergence and growth

R.K.F. Unsworth[1,2]*, S.C. Rees[1,2], C.M. Bertelli[1], N.E. Esteban[1], E.J. Furness[1,2] and B. Walter[2]

[1]Seagrass Ecosystem Research Group, Swansea University, Swansea, United Kingdom, [2]Project Seagrass, The Yard, Cardiff, Wales, Bridgend, United Kingdom

To maximize the opportunities of seagrass as a nature-based solution requires restoration to occur on a large scale. New methods and knowledge are required that can solve ecological bottlenecks, improving its reliability and effectiveness. Although there is increasing interest in the use of seeds for seagrass restoration there exists a limited understanding of how best to plant them with the most knowledge on germination and seedling emergence coming from laboratory studies. Here we present the results of a novel field study on the emergence success of seeds of the seagrass *Zostera marina* when subjected to varied planting treatments. Seeds were planted into hessian bags according to a factorial design of three treatments (sediment type, detritus addition, and nutrient addition). By adding nutrients to natural sediment, the present study provides some evidence of seagrass shoot emergence and maximum shoot length doubling. The present study provides evidence that even in heavily nutrient-rich environments, seagrass sediments may require additional nutrients to improve seedling emergence and growth. It also highlights the highly variable nature of planting seagrass seeds in shallow coastal environments. Critically this study provides increasing levels of evidence that small subtleties in the method can have large consequences for seagrass restoration and that for restoration to scale to levels that are relevant for nature-based solutions there remain many unknowns that require consideration.

KEYWORDS

***Zostera*, nature-based solution (NBS), marine, eelgrass, microbiome**

Introduction

Seagrass restoration is increasingly recognized as a means of creating nature-based solutions to a changing climate, whilst also improving biodiversity, increasing coastal nutrient cycling and supporting wellbeing (Unsworth et al., 2022). Global loss of seagrass has resulted in large areas of soft sediment marine habitat creating new opportunities for restoration. With an increasing understanding of the environmental window for seagrass growth, extensive habitat suitability modelling is taking place to propose targeted areas now potentially suitable for seagrass restoration (Van Der Heide et al., 2009). To maximize these opportunities at large scale, new methods and knowledge are required that can solve ecological bottlenecks (Unsworth et al., 2019). This is particularly relevant as only 37% of published seagrass restoration trials were recorded to be successful 3 years after planting (van Katwijk et al., 2015). This figure is likely much lower given the propensity of academic papers to favour positive restoration results. Across varied environments, we need to understand what methods are suitable for upscaling and the relative costs and benefits of using such approaches, particularly in the context of factors that become bottlenecks to successful restoration such as negative feedbacks.

The high seed production by some species of seagrass at densities of over 1000 seeds^{m-2} (Greve et al., 2005; Jarvis and Moore, 2010) creates a huge opportunity for seagrass restoration, as the production is thought to largely be underutilized and lost to deeper waters. The use of seagrass seeds for restoration is becoming a far more accepted method of conducting this work (Van Katwijk et al., 2016) with projects in the Chesapeake Bay showing project success over scales of thousands of hectares (Orth et al., 2020). When seeds are in high abundance they create a means of preserving and improving genetic diversity within projects and reducing impacts upon donor populations (Reynolds et al., 2013).

The challenge with the use of seeds in restoration projects is that germination is poor, seed loss is high and seedling survival often low (Orth et al., 2006; Eriander et al., 2016; Infantes et al., 2016). Germination can often be lower than 5% (Orth et al., 2006) and seedlings can be highly vulnerable to negative feedbacks in their early development (Maxwell et al., 2017). Identifying the factors limiting the seed to seedling transition is a critical step to understanding seagrasses population dynamics and developing seed-based restoration techniques (Wang et al., 2017). Numerous factors have been proposed as potential cues for seagrass germination and seedling emergence (Orth et al., 2000), with sediment anoxia and low salinity two of the most common triggers recorded to improve success rate (Probert and Brenchley, 1999; Orth et al., 2000).

In addition to studies showing anoxia, salinity and temperature as being potential factors influencing seed germination, there is increasing evidence that other factors can come into play, particularly in the context of the emergence of the seedling and seed loss. Seed predation and burial can also be major drivers of seed loss (Infantes et al., 2016), leading some authors to propose the use of burlap/hessian bags to reduce seed loss (Unsworth et al., 2019). Recent studies have also indicated that nutrients and light may also be important triggers of germination and seedling emergence too (Wang et al., 2017; Alexandre et al., 2018). Nutrients are commonly seen as a negative influence on seagrass, however nutrients may also be a limiting factor in some seagrass systems (Powell et al., 1991), particularly within sediments (Udy and Dennison, 1997). But in many environments, restoration is improved by fertilization, lessening nutrient limitations and improving growth of desired species (Macdonnell et al., 2022). Recent studies on the addition of nutrients to experimental seagrass restoration mesocosms indicate that they have a positive effect on seedling emergence rates (Wang et al., 2017; Macdonnell et al., 2022). Sediment type may also impact upon the availability of nutrients, together with the organic content, and potentially the microbial community present (Fahimipour et al., 2017). Recent use of seagrass detritus as a form of nutrient and microbial dosing within seagrass seed planting has shown potential promise in enhancing seedling emergence, however this has received limited investigation (Unsworth et al., 2019).

Although there exists growing knowledge on the triggers of seagrass germination and seedling emergence (Orth et al., 2000; Xu et al., 2021) the majority of this knowledge comes from laboratory/mesocosm controlled experiments with limited field validation. Applying many of the factors determined within the laboratory to real world restoration projects is not always possible, meaning that knowledge development that is happening is not resulting in as much improved restoration success as should be the case. Only a handful of studies exist where seed planting has been conducted over manipulated experimental conditions. Such experiments present in the literature focus on aspects such as seed density, environmental gradients (Orth and Moore, 1983; Infantes et al., 2016) or seasonality (Coolidge Churchill, 1983) rather than manipulation of environmental conditions.

The use of hessian/burlap bags for planting seagrass seeds not only provides a means of controlling feedbacks during seagrass restoration (Unsworth et al., 2019), but also provides a means of bridging the knowledge gaps between laboratory understanding of seagrass growth and that of field-based restoration. This is because the vesicle that the bags create provides a receptacle enabling manipulation of conditions in the field environment.

The present study provided a highly novel field-based investigation into the use of different substrate and nutrient additions commonly utilized in seagrass seed-based restoration to potentially improve seagrass seed germination and survival. The novelty is not the design or the treatments, but the attempt to manipulate these conditions in the field within ‘real-world’ restoration environments. This was done using hessian bags as a vesicle for sediment, seeds and additional treatments (nutrients and seagrass detritus).

Methods

In December 2018 an experimental seagrass restoration trial was planted in Dale in West Wales to understand the relative effects of different planting media used within seagrass (*Zostera* *marina*) seed planting. All sites were within Dale Roads within the Milford Haven Waterway, an area observed to have a large tidal range (7.68m) resulting large flushing and be dominated by fine and silt. Dale has an annual seawater temperature range of 8°C-17°C peaking in August and contains one small-isolated patch (5 m^2) of natural seagrass approximately 50m north of the experimental sites. The nearest large meadow of *Z. marina* meadows exists in Littlewick (Dale and Gelliswick 6.5km from the dale site and commonly referred to as gelliswick) which has been dated back over 100 years (Kay, 1998) but has been observed to be suffering from elevated nutrients and declining density (Jones and Unsworth, 2016; Bertelli et al., 2021).

Viable seagrass seeds (50 in each) (Infantes and Moksnes, 2018) (were placed in hessian bags and exposed to one of 8 different treatments of additional nutrients, sediment types and detrital inoculant (see Table 1). Nutrients were added to the bags using Osmocote^{TM} (5 balls), this contained 15% N, 3.9% P, 10% K, 1.2% Mg, 0.45% Fe, 0.06$ Mn, 0.02% B, 0.05% Cu, 0.02% Mo, and 0.015% Zn. 200ml of sediment was also added to each bag, this was either sterile play sand or locally collected marine sand and bags were either inoculated or not with fresh wet seagrass detritus (50ml) collected from the rotting down stage of seed separation. The use of childs play sand provides a clean alterative to natural local marine sand that doesn't require such excessive licensing to use and is free of any biosecurity hazards. The inclusion of play sand provides a means of adding in a sand without any additional microbiome of nutrients. One replicate (hessian bag) of each of eight different treatments were placed randomly along an 8m transect line with each bag spread 1m apart. Twelve of these transects were established, creating 12 replicates of each treatment. These were spread over 3 plots (of four lines) immediately adjacent to each other so that they cannot be considered independent sites. Each bag contained 50 seeds, meaning 600 seeds were treated to each treatment. Seeds were collected from Porthdinllaen in North Wales during August 2018 and separated within laboratories at Swansea University (during September). During October and November, seeds were stored in recirculating seawater at ambient light and temperatures.

The bags were hand planted onto the seabed and attached to thin lines (with labels) to enable relocation of treatments and to prevent any being washed away. The bags remained partially sunk, but not beneath the sediment. These bags were then observed in April and July 2019 when divers counted shoot number on each bag and measured the longest shoot.

In 2019, collections of seagrass tissue (n=3) for nutrient analysis were conducted in adjacent natural seagrass patches (Jones and Unsworth, 2016). This allowed for the analysis of elemental C, N and P. In addition, analysis of sediment pore water was also conducted at the site to understand background concentrations of nutrients. Seven sediment core samples were taken in close proximity to the experimental plots in Dale using 50ml syringes. The tip of the syringes was cut off and injected into the sediment down to 10cm depth. The content collected within sediment cores were centrifuged. The supernatant was taken of the samples and filtered through a 0.2µm filter. Total oxidised nitrogen (TON), ammonium (NH4(low)+(high), and phosphate (PO4(low)3-(high)) was quantified using a Seal Analytical Continuous Flow system (AA3; SEAL Analytical, Norderstedt, Germany) following the methods of Grasshoff et al. (1983).

Statistical analyses were conducted in PRIMER v7 (Clarke and Gorley, 2006). Data were transformed (square root) where appropriate for count data, to reduce variance of heterogeneity. Univariate analysis consisted of a three-factor (nutrient, detritus and sediment) permutational multivariate analysis of variance (PERMANOVA+; Anderson, 2017) using a Euclidean resemblance matrix to test for differences in relative abundance and shoot length between treatments (Table 1).

Results

In April 2019, seagrass tissue within adjacent patches was recorded to contain 3.5% gDW-1(high) nitrogen and 0.43% g DW-1(high) phosphorus. These values exceed the average values of seagrass throughout the UK (N = 3.58 ± 0.95, P = 0.21 ± 0.07 $^{\% \text{ gDW-1}}$) (Jones and Unsworth, 2016), indicating the nutrient rich environment of the site. Porewater samples taken around the site in Dale contain 280 ± 15µmoll-1 TON, 25 ± 0.2µmoll^{-1} ammonium and 30 ± 0.1µmol l-1 (high) phosphate.

TABLE 1 Treatment designs used in experimental sites in Dale, Pembrokeshire.

Treatment code	Sediment type	Nutrient addition	Organic inoculant	Number of replicates/bags	Number of seeds
1	Sterile	Yes	Yes	12	600
2	Sterile	No	Yes	12	600
3	Natural	Yes	Yes	12	600
4	Natural	No	Yes	12	600
5	Sterile	Yes	No	12	600
6	Sterile	No	No	12	600
7	Natural	Yes	No	12	600
8	Natural	No	No	12	600

In April 2019, 86 of the 96 bags could be observed and reliably quantified. Of these 86, 32 contained seagrass (37%) equating to an average density of 1.11± 2.13 shoots per bag and longest leaf of 1.34 ± 2.01mm see (Scheme 1). By July 2019, 84 of the 96 bags could be observed and reliably quantified with 40 of these showing seagrass shoots (48%). These shoots had a density of 1.43± 2.37 shoots per bag and longest leaf of 6.38 ± 9.66mm.

Shoot density was found to directly correlate with increasing maximum shoot length in both July and April (Figure 1).

In April there was limited differentiation between the density of shoots and the leaf length with respect to the treatments. No significant effects of any of the treatments on seagrass shoot density (P>0.05) or shoot length (P>0.05) were recorded (Figures 2, 3).

By July there were some differences in density and shoot length present between treatments with 44% of natural sediment bags contained seedlings whilst this was higher at 52% for sterile sediment.

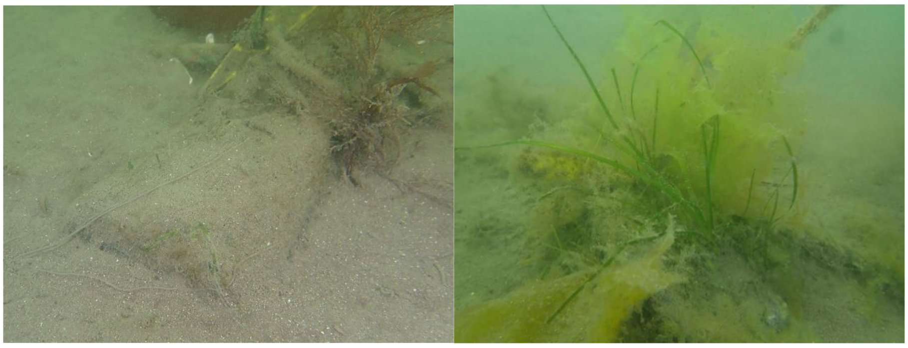
66% of bags containing natural sediment together with additional nutrients contained seagrass shoots. This increased to 72% of bags containing shoots with the addition of detritus. In contrast, natural sediment without any additions had the lowest emergence rates of shoots at 20%. All sterile treatments were consistently between 45 and 54%. In July, mean shoot density ranged from 3.27 to 4.15 in the natural, nutrient and detritus treatment compared to 0.4 to 0.97 in the natural no detritus and no nutrient treatment. These differences between treatments were found to be significant with respect to nutrient additions on both seagrass shoot density ($P<0.05$, $F_{1,77} = 4.33$) and longest leaf length ($P<0.05$, $F_{1,77} = 4.35$), both of these parameters had significant interactions with sediment ($P<0.05$). Detritus addition and sediment type did not significantly affect seagrass shoot density or length (Figures 2, 3).

The highest maximum shoot density and shoot length values across the individual bags (July) were mostly recorded in treatments containing nutrient and detritus additions. The lowest maximum values (excluding zeros) were recorded in sterile sediments without additions.

Discussion

The present study provides a novel field-based insight into the planting of seagrass seeds into the natural environment and the role of different sediment types and supplements in the emergence of seedlings. We found that in a water body characterised by elevated nutrients and potential areas of eutrophication (Jones and Unsworth, 2016; Bertelli et al., 2021), seagrass seed emergence was still enhanced by nutrient additions into the sediment when planting. This indicates that sedimentary pore waters may not have had optimal nutrients available for seagrass growth. By adding nutrients, the present study provides evidence of seagrass shoot emergence increasing ≈2 fold and maximum shoot length also doubling, however this result only happens in the presence of natural sediment. Others have recorded planted seagrass to benefit from the addition of nutrients (Wang et al., 2017; Macdonnell et al., 2022), with nutrients outweighing the influence of light cues (Wang et al., 2017) and the slow release of nutrients also found to be beneficial (Macdonnell et al., 2022), but this is the first field study to demonstrate this with seeds.

In tropical calcareous environments typical of low nutrient conditions, sediment nutrient additions have been recorded to enhance seagrass growth (Udy et al., 1999), this is typically considered to be due to phosphorus limitation (Short, 1987). However, conditions within the present study are those of terrigenous sediments where phosphorus limitation is unlikely. In line with the results of the present study, some authors have demonstrated terrigenous sediments to be replete in nitrogen



SCHEME 1
Seagrass shoots emerging from hessian bags in May (left) and July (right) 2018 following experimental planting of seeds.

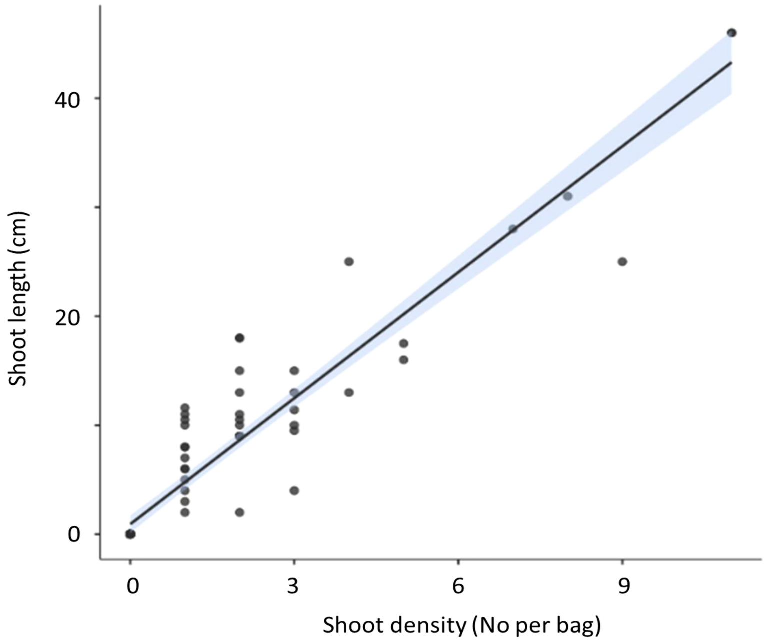


FIGURE 1
Correlation (± 95% CI) between seagrass shoot density and maximum shoot length of the seagrass *Zostera marina (Z. marina)* in hessian bags in Dale, Wales following experimental planting. Seagrass seeds were planted into hessian bags according to a factorial design of three treatments (sediment type, detritus addition, and nutrient addition).

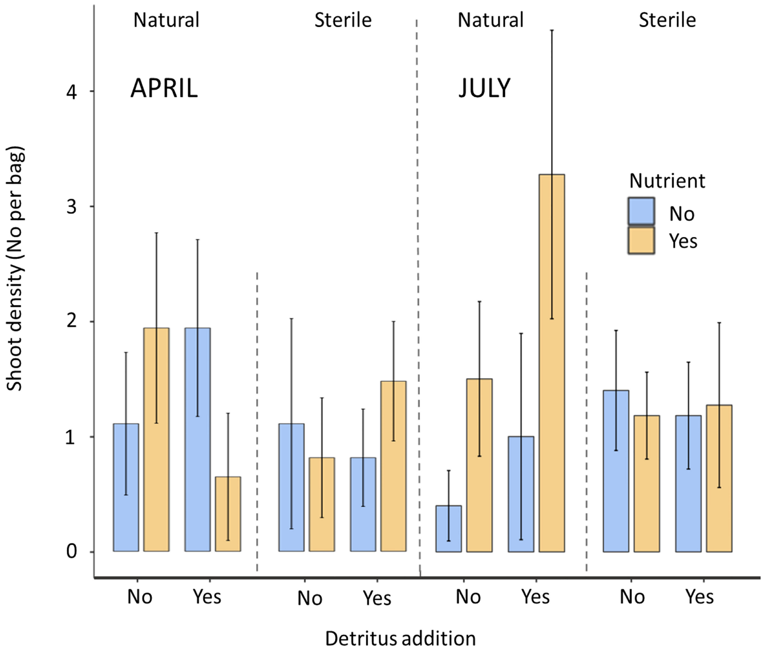


FIGURE 2
Mean (± SD) shoot density of the seagrass *Zostera marina* in hessian bags in Dale, Wales following experimental planting. Seagrass seeds were planted into hessian bags according to a factorial design of three treatments (sediment type, detritus addition, nutrient addition).

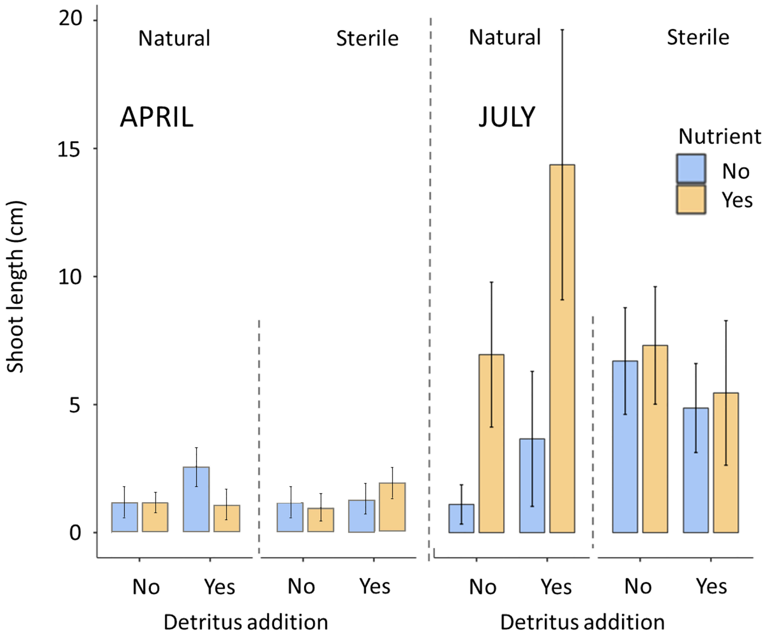


FIGURE 3
Maximum shoot length (mean ± SD) of seagrass (*Zostera marina*) recorded in hessian bags in Dale, Wales following experimental planting. Seagrass seeds were planted into hessian bags according to a factorial design of three treatments (sediment type, detritus addition, nutrient addition).

(Short, 1987), even though the associated water body is enriched with elevated nitrogen.

Enhanced seagrass growth with additional nitrogen provision to the sediments was not ubiquitous across the treatments and had a significant interaction with sediment type. This positive effect was only observed within natural sediments rather than sterile ones. The reason for this lack of response in sterile sediment (play sand) is unclear, however we suggest two potential hypotheses, high permeability of the play sand not maintaining the additional nutrients, or the role of a natural microbiome helping facilitate nutrient uptake in natural sediments. The play sand used here contained no organics, as it provides a means of ensuring no alien or invasive species are spread with seed planting. The origin of this sand is that it's 'child's play sand' with no builders additives. As a result of its origin the particle sizes are far more consistent than natural sediment, increasing permeability due to the lack of fines. We hypothesize that this permeability may lead to the loss and dilution of nutrients from adjacent background porewater.

Although we have as yet limited knowledge of how the sediment and rhizosphere microbiome is influencing seagrass function, there is clear data in the literature showing a distinct microbiome on the roots of *Z. marina* relative to surrounding environments (Fahimipour et al., 2017). The taxa of the microbiome has a high dominance of organisms thought to have functional roles in nutrient cycling. Although we don't have any cause and effect here we do know that the natural sediment came from a nearby location within 50m of extensive seagrass that may have inoculated seedlings early on potentially providing a microbiome that could utilize more effectively the nutrient additions.

This study is marked by high levels of variability between and within treatments, leading to only marginal statistical differences between treatments. This variability is in contrast to the majority of laboratory/mesocosm experiments on seed planting and germination where external influences are controlled in a manner not possible *in situ*. Many lab studies have investigated seagrass seed burial depth as a key determinant of success and a depth of 2cm appears to be broadly consistent across studies (Marion and Orth, 2012; Xu et al., 2021). Seeds planted in bags aren't at a controlled depth even though bags were planted in a controlled manner, this is because upon placement in the bag whilst in the lab, mixing and disturbance happens on route, adding an unquantified level of variability into the design.

Previous methodological development work had included seagrass detritus as a means of inoculating the microbiological environment of the seagrass seeds to assist with seedling development and survival (Unsworth et al., 2019). In the present study, addition of detritus as a potential stimulant of the microbiome did not show any influence on the success of seeds and their emergence.

In conclusion, this present study provides evidence that even in heavily nutrient rich environments, seagrass sediments may require additional nutrients to improve seedling emergence and growth. It also highlights the highly variable nature of planting seagrass in shallow coastal environments. Critically this study provides increasing levels of evidence that small subtleties in method can have large consequences for seagrass restoration and that for restoration to scale to levels that are relevant for nature-based solutions there remain many unknowns. We find that small changes to the sediment surrounding seeds may have a significant impact upon seedling emergence and success.

Data availability statement

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

Author contributions

RU undertook all elements of the project. SR and CB helped design the experiment. All authors undertook the experiment and commented on the manuscript.

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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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Jutta Papenbrock,
Leibniz University Hannover, Germany

REVIEWED BY
Aditya Hikmat Nugraha,
UMRAH, Indonesia
Muta Zakaria,
Putra Malaysia University, Malaysia

*CORRESPONDENCE
Daoru Wang
wangdaoru_hnhky@163.com

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Physiological basis and differentially expressed genes in the salt tolerance mechanism of *Thalassia hemprichii*

Jie Shen[1], Zhongjie Wu[1], Lei Yin[2], Shiquan Chen[1], Zefu Cai[1], Xiaoxiao Geng[2] and Daoru Wang[1*]

[1]Hainan Academy of Ocean and Fisheries Sciences, Haikou, China, [2]Key laboratory of Utilization and Conservation for Tropical Marine Bioresources, Hainan Tropical Ocean University, Ministry of Education, Sanya, China

Seagrass plays a vital role in the stability of marine ecology. The human development of marine resources has greatly affected the survival of seagrass. Seawater salinity is one of the important factors affecting its survival. Seagrass can survive in high saline environments for a long time and has evolved a variety of effective tolerance mechanisms. However, little is known about the molecular mechanisms underlying salinity tolerance by seagrass. *Thalassia hemprichii* is a seagrass species with a global distribution. It is also an ecologically important plant species in coastal waters. Nevertheless, the continuous environmental deterioration has gradually reduced the ecological niche of seagrasses. In this study, experiments were conducted to examine the effects of salinity changes on *T. hemprichii*. The result showed that the optimal salinity for *T. hemprichii* is 25 to 35 PSU. Although it can survive under high and low salinity, high mortality rates are common in such environments. Further analyses revealed that high salinity induces growth and developmental retardation in *T. hemprichii* and further causes yellowing. The parenchyma cells in *T. hemprichii* also collapse, the structure changes, soluble sugar accumulates rapidly, soluble proteins accumulate rapidly, the malondialdehyde (MDA) content reduces, and lipid peroxidation reduces in plant membranes. The molecular mechanisms of salt tolerance differ significantly between marine and terrestrial plants. We found 319 differentially expressed genes (DEGs). These genes regulate transport and metabolism, promoting environmental adaptation. The expression of these genes changed rapidly upon exposure of *T. hemprichii* to salinity stress for three hours. This is the first report on the physiological and biochemical changes and gene expression regulation of *T. hemprichii* under different salinity conditions. The findings of this study well deepen our understanding of *T. hemprichii* adaptations to changes in the shoal living environment.

KEYWORDS

***Thalassia hemprichii*, salinity, differentially expressed genes, soluble sugar, soluble protein, malondialdehyde**

Introduction

Seagrasses are widely distributed along temperate and tropical coastlines of the world. They provide numerous ecosystem services: they can purify water quality, slow water flow, accumulate and stabilitate the sediment. In addition, they are habitats, breeding places, and food sources for marine life (Edgar et al., 1994; Orth et al., 2006). The global seagrass coverage is about 10% of the offshore area or 0.15% of the ocean area, yet the most productive and biodiverse marine ecosystem. To date, 74 seagrass species have been reported and widely distributed worldwide (Hemminga and Duarte, 2000; Huang et al., 2018). Global climate change and human activities severely affect seagrass beds (Waycott et al., 2009; Williams et al., 2016). Of these, salinity affects the health of seagrass beds and their long-term development, thus one of the critical factors in seagrass decline (Waycott et al., 2009). Rapidly expanding low-salinity aquaculture activity along shallow coastlines discharges low-salinity water with a high concentration of nutrients (Funge-Smith and Briggs, 1998; Heil, 2000; Cao et al., 2007). In addition, climate change has increased pulse-type heavy rainfall events (IPCC, 2007), dramatically increasing freshwater loads (Faxneld et al., 2010) and possibly affecting coastal seagrasses (Waycott et al., 2007). Such an extreme event has caused large-scale losses of seagrass habitats in Eastern Africa (Bandeira and Gell, 2003), Queensland of Australia (Campbell and McKenzie, 2004), and Venezuela (Chollett et al., 2007). On the other side, global warming has accelerated lagoon evaporations, and the silting at the mouth reduces seawater exchange, leading to high salinity in the lagoons. Seawater salinity affects the osmotic pressure of seagrass plant cells, disrupting their physiological and biochemical characteristics, thus, affecting seagrass survival (Munns, 2002). For example, high salinity stress decreases nonstructural sugars content, glutamate synthase activity and growth potential of *Posidonia oceanica* (Duarte et al., 2010). High salinity promotes the predominance of salt-tolerant algae, negatively affecting seagrass ecosystems (Huang et al., 2006).

To cope with salt stress, plants have evolved various strategies, such as changes in signaling pathways, accumulation of reactive oxygen species (ROS), activations in antioxidant systems, scavenging and repairing of redox balance, and induction or degradation of osmotic regulation, etc. Seagrasses originated on land, migrated to fresh or brackish water, and are the only higher plant living wholly submerged in the marine environment (Wissler et al., 2011). Salinity tolerance is a major evolutionary factor in physiological adaptation separating seagrasses from their freshwater relatives. The salt tolerance of *Zostera marina* is higher than that of terrestrial salt-tolerant plants. Meanwhile, its evolutionary position is higher than other lower salt-tolerant plants in the ocean. Salinity is a key ecological factor affecting the survival, growth, and distribution of seagrass. Due to their long-term survival in a high salinity environment, seagrasses have evolved various high-efficiency salt tolerance mechanisms to adapt to salinity fluctuations by modulating distinct physiological responses from intracellular ion concentration (e.g., organic osmotic regulators, etc.) to cell wall elasticity (Larkum et al., 2006; Touchette, 2007; Deng and Lü, 2018). Current research on the mechanism underlying salt tolerance in higher plants mainly focuses on terrestrial halophytes. However, the physiological and molecular mechanisms underlying the salt tolerance of seagrasses are poorly studied (Deng and Lü, 2018).

T. hemprichii is widely distributed in shallow coastal areas in the tropics and subtropics of the eastern Atlantic and Indo-Pacific, between 28°Sand 32°N (Den, 1970; Phillips and Me˜nez, 1988; Spalding et al., 2003). In the South of China Sea, *T. hemprichii* plays a vital role in balancing the ecological environment in the coastal waters. The presence of *T. hemprichii* affects marine ecology, biogeography, and genetic diversity (Lee et al., 2016; Nordlund et al., 2017; Jahnke et al., 2019). With the further deterioration of the marine ecological environment, extreme salinity fluctuations in coastal areas have led to restrained growth or even death of *T. hemprichii* (Sinclair et al., 2016; Wainwright et al., 2018; Shen et al., 2021). However, at present, the tolerance and mechanisms underlying the response of *T. hemprichii* to salinity stress have not been adequately studied. This study compared and analyzed the growth and development, measured physiological and biochemical indexes, and analyzed transcriptome information of *T. hemprichii* under different salinity conditions. The result of this study provides a research basis for the investigation of seagrass adaptability to salinity changes in a large environment.

Materials and methods

Plant materials and collection

T. hemprichii was collected from the Lingshui Xincun port seagrass special protected area, Hainan, China. Xincun Bay, a lagoon of about 21.97 km^2, is located in the southeast of Lingshui Li Autonomous County, Hainan Province, and south to Li'an port. It is about 4 km long from north to south, with a port door from Xincunjiao (18°24′42′′N,109°57′58′′E) to Shitoucun Shazui (18°24′34′′N, 109°57′42′′E) (Figure 1B), an area exhibiting typically oceanic salinity around 28 to 32 PSU. Intact and robust plants were selected, rinsed with tap water to remove sediment and attachments on the plants, and taken to the laboratory.

The collected intact *T. hemprichii* plants were transplanted into a glass aquarium. The bottom of the glass aquarium was covered with aquarium soil and coral sand (1:1) and filled with 60 L seawater (salinity = 30 PSU). The culture temperature was 26 ± 1°C, and the light-dark cycle was 12L/12D. Salinity was

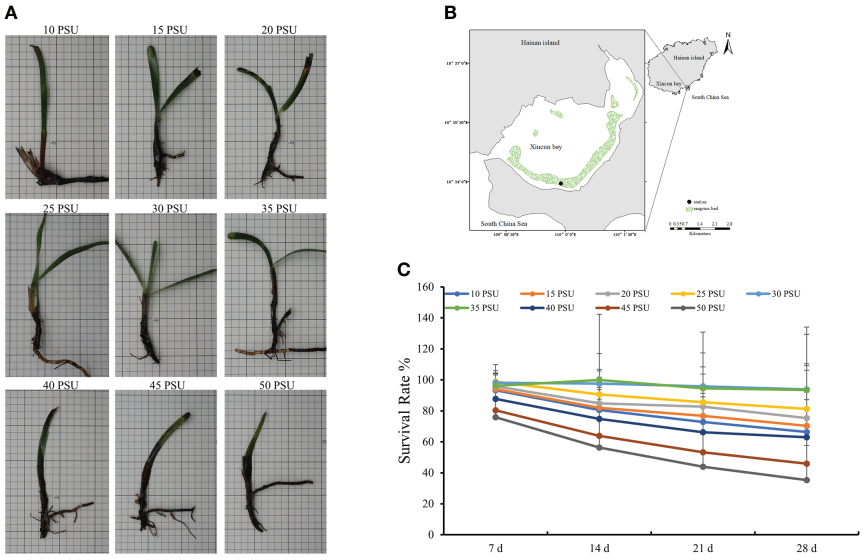

FIGURE 1
The growth state and survival rate of *T. hemprichii* under different salinity conditions. **(A)** Growth state; **(B)** Location of sampling site, Xincun Bay, South China Sea; **(C)** Survival Rate. The statistical significance was assessed using a one-way analysis of variance (ANOVA), $P < 0.01$.

increased or decreased using Instant Ocean salts and Deionization Water, and monitored twice daily using a Lab Salinometer (SYA2-2, National Ocean Technology Center, China). The medium was carefully renewed daily (Jiang et al., 2013).

Plant treatments with different salinity conditions

Twenty-five plants were planted in each treatment, the culture temperature was 26 ± 1°C, and the light-dark cycle was 12L/12D. The *T. hemprichii* plants were harvested after 28 days of laboratory culture under 10, 15, 20, 25, 30, 35, 40, 45, and 50 PSU.

The salt tolerance threshold was determined every 7days. The salt tolerance threshold is the salt concentration of 50% survival rate (plants grow within this range; more than 50% of the plants can grow normally in this range. When salinity exceeds this threshold, the growth of more than 50% of the plants is restricted, reducing the yield). In this study, statistical analyses were performed based on the percentage of surviving leaves (dropped leaves and black leaves were considered dead) against the total number of leaves using fifteen plants per treatment. Proportional data were arcsine square root-transformed before analysis. All statistical analyses (student-t, $p < 0.05$) were performed using SPSS (v.21.0).

Ultrastructure observation of plant

Three treatments with 15, 30, and 45 PSU were selected to observe the structure by optical microscope, scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The underground stems and leaves of 0, 7, 14, 21, and 28 days were selected for sectioning to observe their microstructure and ultrastucture. Dehydrating, embedding, sectioning, and staining were performed by Wuhan Sevier Biotechnology Co., Ltd., Wuhan, China.

The specimens were observed under NIKON ECLIPSE E100 positive optical microscope (Nikon, Tokyo, Japan), HITACHI scanning electron microscope SU8100 (Hitachi, Tokyo, Japan), and HITACHI transmission electron microscope HT7800/HT7700 (Hitachi, Tokyo, Japan).

Determination of plant resistance indicators

Three treatments with 15, 30, and 45 PSU salinity were selected to determine plant resistance characteristics. Samples were taken every 7 days after different salinity treatments. After sampling the leaves and wiping off the water, 0.1 g was weighed, quick-frozen in liquid nitrogen, and frozen at -80 °C to determine various physiological and biochemical indicators of *T. hemprichii*. The test period was 21 days. The experiments were replicated three times.

Physiological indicators, such as soluble protein, soluble sugar, malondialdehyde (MDA), catalase (CAT), peroxidase (POD), and superoxidase (SOD), were measured using the total protein assay kit (with standard: BCA method) (A045-3-2), plant soluble sugar content test kit (A145-1-1), malondialdehyde (MDA) assay kit (TBA method) (A003-1-2), catalase (CAT) assay kit (Visible light) (A007-1-1), peroxidase assay kit (A084-3-1), and total superoxide dismutase (T-SOD) assay kit (Hydroxylamine method) (A001-1-2) (Nanjing Jiancheng Biotechnology Engineering Institute, Nanjing, China), respectively.

RNA-sequencing and analysis

Samples treated with 15, 30, and 45 PSU were selected for comparative transcriptome sequencing. After treatments, the samples were taken at 0, 3, 6, 12, 24, and 48 hours, respectively. Sequencing was conducted by Beijing Genomics institution (BGI-Shenzhen, China).

The mRNA library construction and transcriptome sequencing were performed by BGI, and the single end of the amplified flow cell was sequenced by the BGSEQ-500 Platform (BGI-Shenzhen, China). The raw reads were filtered using Trimmonatic software to get clean reads, then assembled by SOAPdenovo software. The transcripts were de-redundant to obtain unigenes. Next, the unigene sequences were compared with the protein databases, including the National Center for Biotechnology Information (NCBI) non-redundant (NR), Swiss-Prot, Kyoto Encyclopedia of Genes and Genomes (KEGG), and Clusters of Orthologous Groups of proteins (COG) by blastX (e-value < 0.00001). Sequence orientation of unigenes was determined using the protein sequences with the best comparison results. The sequence orientation of unigenes was determined in the priority of NR, Swiss-Prot, KEGG, and COG when results from different databases conflicted. Unigenes which could not be compared with any of the above four libraries were predicted using the ETScan software.

The expression levels of unigenes were calculated using the FPKM (Fragments Per Kilobase of transcript per Million) method, and differentially expressed genes (DEGs) between samples were determined by log2 (Fold change). Genes with log2 (Fold change) ≥ 1 and P-value < 0.05 were classified as up-regulated genes, while genes with log2 (Fold change) < 1 and P-value < 0.05 were classified as down-regulated genes.

Quantitative real-time PCR

Quantitative real-time PCR assays for multiple genes were performed using the SYBR® Premix Ex Taq™ II (Takara, Shiga, Japan). Two or three primer pairs were designed for all the amplification segments to ensure the qPCR quality. However, only one pair was used in the final test. The primer sequences are given in Table S1. Melting-curve analyses were performed for all the primers. The 18S rRNA was used as an internal reference gene to normalize the Ct values for each gene. Quantitative real-time PCR was performed by a Mastercycler® ep realplex (Eppendorf, Hamburg, Germany). All qPCR assays were replicated three times to eliminate mechanical errors.

Data analysis

Gene expression was analyzed using a one-way analysis of variance (ANOVA) with Tukey adjustments. The qPCR reactions and data were analyzed according to the methods of Livak and Schmittgen (2001) and Bustin et al. (2009). The data were analyzed with ANOVA to determine the treatment effects relative to the control.

Results

Growth and development of *T. hemprichii* under different salinity stresses

T. hemprichii had stunted growth, with yellow leaves under low salinity stress, and the plants died rapidly under high salinity stress (Figure 1A). The mortality rates of *T. hemprichii* under the three treatments with the salinity of 40, 45, and 50 PSU were over 40% after 28 days of treatments. With low salinity of 10, 15, and 20 PSU, plant death occurred after 21 days of treatments, and the mortality rates were above 30%. The mortality rates of *T. hemprichii* under salinity of 25 to 35 PSU did not exceed 10% (Figure 1C), and all plants grew normally with green leaves (Supplementary Figure S1).

Ultrastructure of *T. hemprichii* under different salinity stresses

The parenchyma cells between the vascular bundle and the epidermis of the *T. hemprichii* stem are filled with parenchyma, forming regularly scattered airways. The diameter of the airways is slightly larger than that of the parenchyma cells. In seawater with 30 PSU salinity, the size of the airways was uniformly distributed in a regular arrangement. A single airway hole was regular and circular, with a clear boundary from the central vascular bundle. Under the treatment with a high salinity of 45 PSU, the airway structure shrank and was damaged, the airway holes were deformed and atrophied, and the cell walls were thickened (Figure 2 and Supplementary Figure S2). Under the treatment with a low salinity of 15 PSU, the airway diameter was smaller, the stomata expanded significantly, and the walls were thinner than those under the treatment of 30 PSU.

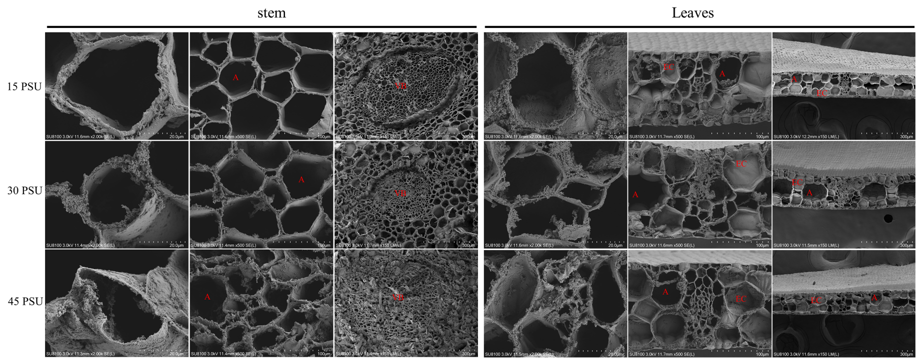

FIGURE 2
Comparative analysis of the internal structure of stems and leaves at 15 PSU, 30 PSU, and 45 PSU salinity stress by scanning electron microscope (SEM). (A) Aerenchyma; (VB) Vascular bundle; (EC) Epidemic cell.

The microstructure of *T. hemprichii* leaves revealed only one layer of small and dense epidermal cells, which gradually thickened with the increase in salinity. When exposed to high salinity, the proliferative tissue of *T. hemprichii* was prominent, significantly increasing airway diameter. The parenchyma cells increased in size, and the airway shrunk. While the parenchyma cells and the airway were smaller under low salt stress, less proliferative tissues were observed. The epidermal cell wall of *T. hemprichii* leaves was denser and thicker than the cell walls of other parts. Under the treatment with a low salinity of 15 PSU, the degree of epidermal cell wall thickening was less, and the results were similar in the other two treatments (Figures 2, 3B).

The primary functional cells of the stem are epidermal cells, and most of their parenchyma cells contain vacuoles. With a salinity of 30 PSU, the epidermal cells had many organelles, most of them were mitochondria, vacuoles in epidermal cells were small and few. After a low salinity treatment of 15 PSU, the vacuoles in the epidermal cells were significantly increased in quantity and enlarged in volume, and the organelles were concentrated close to the plasma membrane. A 45 PSU treatment caused significant degeneration of the intracellular organelles in epidermal cells, what remains were mainly large vacuoles, and the cell wall thickened significantly. This phenomenon revealed that the effect of hypertonicity was more significant than hypotonic (Figure 3A).

T. hemprichii cell wall thickened with an increase in salinity. The plasma membrane of epidermal cells was folded, bulged of different sizes and shapes, which is the transmission cell-like structure. The chloroplasts of *T. hemprichii* leaves are mainly concentrated in epidermal cells and mesophyll cytoplasm closing to the epidermal cells. The chloroplasts in epidermal cells of *T. hemprichii* treated with 30 PSU salinity had significantly more numbers than those treated with 15 and 45 PSU salinity. In addition, their shape was plump, kidney-shaped, fusiform, or arcuate; the grana lamellae were neatly arranged; there were a few osmiophilic granules; the structure was compact, and the shape was normal. After low and high salinity treatments, chloroplasts were deformed and distorted; the shape became longer than in 30 PSU, or one end were swollen, irregular, or nearly circular; the grana were irregularly arranged; the lamella spacing increased; the structure were loose; the density was reduced; the number of osmiophilic granules increased (Supplementary Figure S3).

Physiological and biochemical changes under different salinity stresses

Soluble protein content

The soluble protein content of *T. hemprichii* first decreased and then increased under salinity stresses. After the three salinity treatments (15, 30, and 45 PSU), the soluble protein content of *T. hemprichii* was the lowest at 12 hours of stress, reduced by 30.26%, 24.01%, and 21.33% respectively compared with at 0 hours. The soluble protein content under a 45 PSU high salinity treatment was significantly higher than that of a 15 PSU low salinity treatment. With the prolonged stress time, the soluble protein content under 15 and 45 PSU salinity treatments were significantly higher than at 0 hours ($P<0.01$). The soluble protein content under a 45 PSU high salinity treatment was significantly higher than that with a 15 PSU low salinity treatment (Figure 4A). High salinity stress rapidly increased the osmotic regulation ability of *T. hemprichii* by increasing soluble protein after stress to reduce salt damage.

Soluble sugar content

Organic osmotic protective substances, such as proline, soluble sugar, and soluble protein, can maintain the cellular osmotic

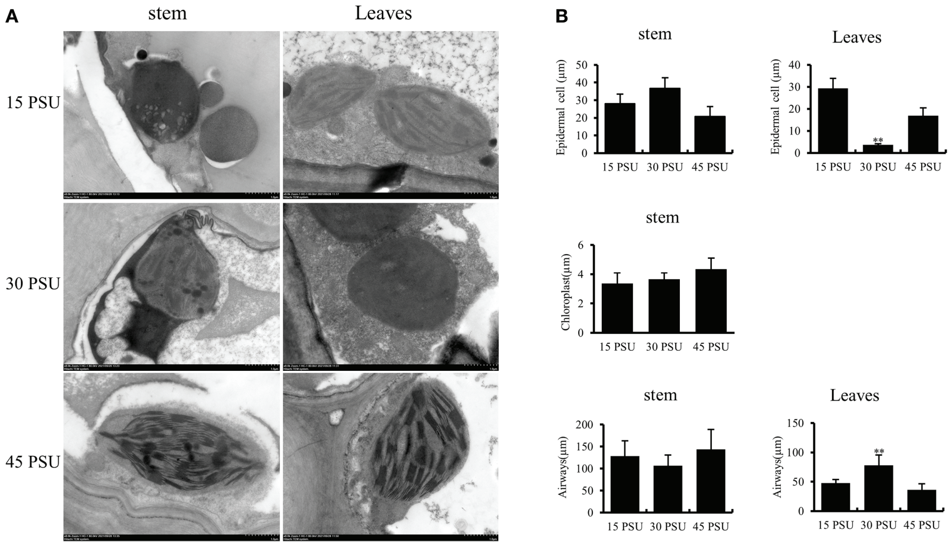

FIGURE 3
Images of the structure of *T. hemprichi* observed under Transmission electron microscopy (TEM). **(A)** Transmission electron microscopy (TEM) result; **(B)** (EC) Epidemic cell, Chloroplast, and Airways analysis. The statistical significance was assessed using Student's *t* multiple comparisons test. $^{**}P < 0.01$.

potential under salinity stress and prevent dehydration. In addition, they play an important role on stabilizing and protecting the structure and function of biological macromolecules. As an intermediate product of energy metabolism in cells, soluble sugar maintains the stability of intracellular pH and ion balance and is a critical osmotic regulator of plant salinity stress. The soluble sugar content of *T. hemprichii* was increased under the three salinity treatments of 15, 30, and 45 PSU. The soluble sugar content increased significantly under a 15 PSU salinity treatment for 6 hours than 0 hours (P<0.05). The maximum soluble sugar content was at 12 hours, which was 64.17% higher than at 0 hours. The soluble sugar content under treatments with the salinity of 30 and 45 PSU for 3 hours was significantly higher than that at 0 hours (P<0.01), and the maximum soluble sugar content was at 6 hours and 48 hours, 69.84% and 115.04% higher than at 0 hours respectively. The soluble sugar content under 45 PSU salinity

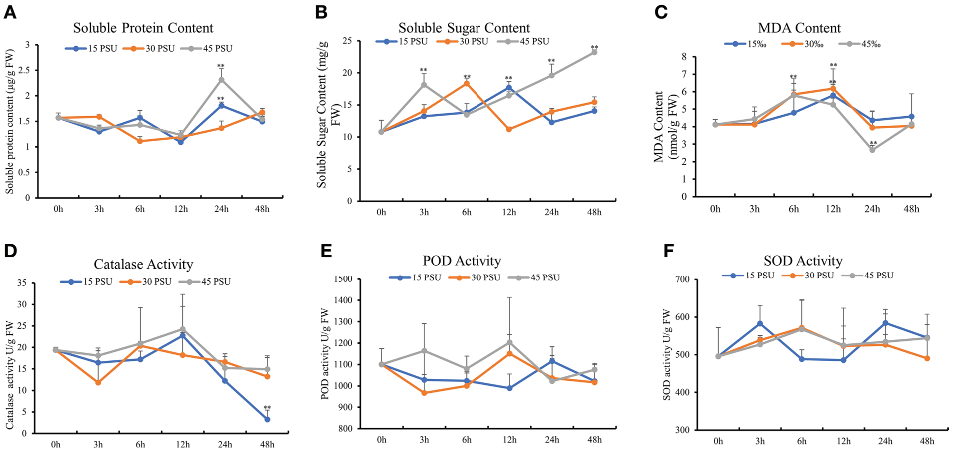

FIGURE 4
Physiological and biochemical index changes under different salinity conditions. **(A)** Soluble protein content; **(B)** soluble sugar content; **(C)** malondialdehyde (MDA) content; **(D)** catalase activity; **(E)** peroxidase (POD) activity; **(F)** superoxide dismutase (SOD) activity. The statistical significance was assessed using Student's *t* multiple comparisons test. $^{**}P < 0.01$.

treatment for 3 hours was significantly higher than that under treatments with the salinity of 15 and 30 PSU, revealing a significant difference under 24 hours and 48 hours stress compared to the salinity of 15 and 30 PSU treatments (Figure 4B). The soluble sugar content of *T. hemprichii* was high under prolonged stress time. High salinity treatment significantly increased soluble sugar content, indicating that *T. hemprichii* had strong salt tolerance. Therefore, high salinity stress promotes the accumulation of soluble sugars.

MDA content

The MDA content reflects the degree of damage to plants under salinity stress. The MDA content of *T. hemprichii* was significantly higher under 30 and 45 PSU treatments for 6 and 12 hours than at 0 hours. Compared with the control group, the MDA content of *T. hemprichii* treated with 30 and 45 PSU salinity for 6 hours increased by 42.01% and 40.61%, respectively. while the MDA content of *T. hemprichii* treated with 30 and 45 PSU salinity for 12 hours increased by 49.97% and 27.72%respectively. The MDA content under treatments with higher salinity of 30 and 45 PSU for 6 hours increased by 21.95% and 20.74%, compared with that under treatment with a salinity of 15 PSU. Therefore, salinity stress damaged the biofilm, causing membrane lipid peroxidation and increasing MDA content significantly. With the prolonged stress time, the MDA content in *T. hemprichii* significantly reduced under treatment with a salinity of 45 PSU for 24 hours (Figure 4C). This could be caused by the plant resistance mechanisms of the high salinity stress. The reduction of MDA content affected membrane lipid peroxidation, protected the cell membrane system and alleviated the effects of salinity stress on plants. We also detected the activities of CAT, POD, and SOD. However, no significant difference under different salinity stresses was observed (Figures 4D-F).

Gene expression variations under different salinity stresses

Sequencing statistics

A total of 47 samples were measured using the DNBSEQ platform, producing an average of 21.49 M data each (Supplementary Table 1). The clean reads were aligned to the reference gene sequence using Bowtie2. A total of 190,908 transcripts were detected, and the ratio of mapping to the reference sequence (unpublished) was 73.01% ~ 91.91%. The average mapped ratio was 80.24% (Supplementary Table 2). The overall transcriptome sequencing analysis, including randomness, coverage, and sequencing saturation statistics, revealed that the sequencing results were reliable. The lengths of the obtained transcripts were more than 300 bp, 108,002 of which were between 300 and 1000 bp, accounting for 56.57% of the total transcripts (Supplementary Table S3). The correlation analysis revealed a high correlation between samples, the result consistented with the gene expression level (Supplementary Figure S4). Also, the expression distribution of each sample presented a normal distribution (Supplementary Figure S5). The assembled sequence data for these raw reads were deposited at the NCBI Sequence Read Archive (SRA, http://www.ncbi.nlm.nih.gov/Traces/sra) under the accession number PRJNA848129.

Temporal characteristics under different salinity stresses

The clustering analysis of 108,002 unigenes was performed across 45 samples to define the temporal characteristics of the complete transcriptome dataset by Mfuzz, and all the unigenes were divided into 12 clusters. Different salinity treatments greatly affected the gene expression pattern of *T. hemprichii*. Under the low salinity treatment of 15 PSU, the gene expression pattern varied gently (Figure 5A, clusters 5, 7, 8, 9, 10) or greatly at one of the time points (Figure 5A, clusters 1, 2, 3, 4, 6, 11, 12). Compared with the treatment of 30 PSU, no clusters with large variations in gene expression were observed (Figure 5B, clusters 9, 11). We observed more variation patterns and more intense variation in gene differential expression patterns under a high salinity treatment of 45 PSU. The numbers of intense variation clusters under a 45 PSU treatment were significantly more than under a 30 PSU treatment (Figure 5C, cluster 8,9,11,12). Therefore, hypertonicity significantly alters gene expression patterns, while hypotonicity is weaker than hypertonicity.

Differential expression analysis

Compared with the control group, both the 45 PSU and 15 PSU treatments had up-regulated genes significantly more than the down-regulated genes (Supplementary Figure S6A). The DEGs under 15 PSU and 45 PSU treatments of *T. hemprichii* were significantly more than under 30 PSU treatments for 3 and 6 hours. Then, the number of DEGs gradually decreased to the same level as the treatment with a salinity of 30 PSU (Supplementary Figure S6B). From the proportion of DEGs, up-regulated genes were more than down-regulated genes. Regardless of high salinity or low salinity stress, the up-regulated genes were treble down-regulated genes after 3 hours of treatments. After 6 hours of treatments, the ratio of up- and down-regulated genes under low salinity treatment increased, while the ratios of up- and down-regulated genes were similar under both 30 PSU and 45 PSU treatments. Subsequently, the ratio of up- and down-regulated genes under 15 PSU treatment was equivalent to that under 30 PSU treatment. However, the ratio of up- and down-regulated genes under the high salinity treatment fluctuated periodically at 24 hours, which may be

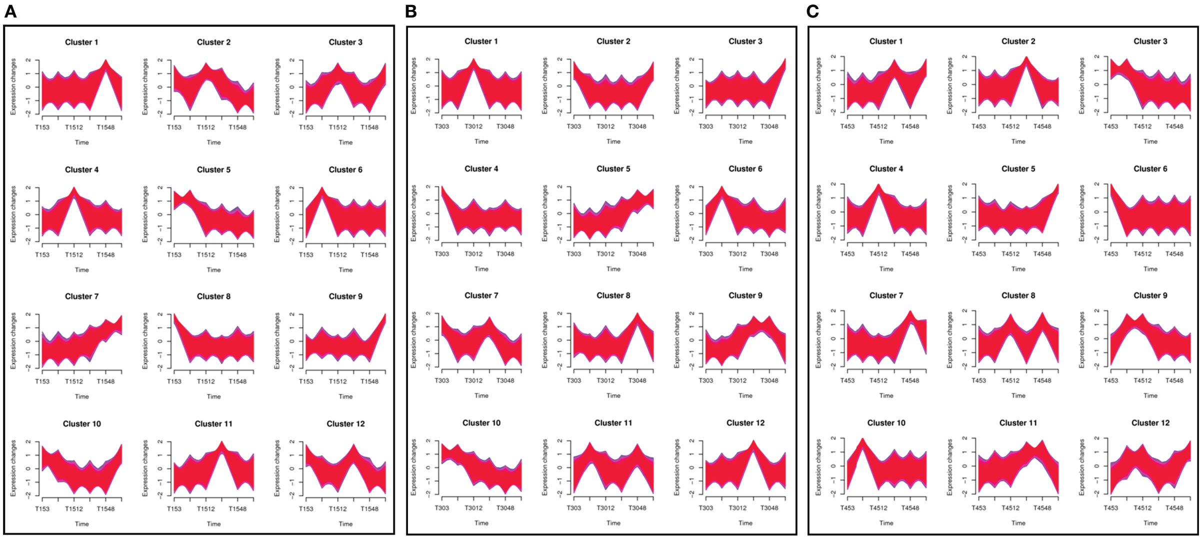

FIGURE 5
Clustering analysis of differentially expressed genes (DEGs) and time-series analysis of DEGs under different salinity conditions. A total of 108,002 unigenes were classified into 12 clusters using the Mfuzz package in R. A. The horizontal axis shows the different groups, while the vertical axis shows the time series of the gene expression levels. **(A)** Under a 15 PSU treatment; **(B)** Under a 30 PSU treatment; **(C)** Under a 45 PSU treatment.

related to the growth rhythm of *T. hemprichii* under hypertonic conditions (Supplementary Figure S6C).

Subsequently, DGEs were analyzed. Only eight co-expressed genes were present at each time point under treatment with a salinity of 30 PSU (Figure 6A; Supplementary Table 4). The co-expressed genes were probable *methyltransferase*, *putative glucose-6-phosphate 1-epimerase*, *putative bystin*, *cytochrome c1-2*, *protein stay-green-like chloroplastic*, *protein LHCP translocation defect*, *MLP-like protein 423* and *mitochondrial inner membrane protein mitofilin*. At each time point under 15 PSU treatment, the specifically expressed genes were the majority. A total of 690 genes were co-expressed after 3 and 6 hours of treatments, and only 15 unigenes were expressed at each time point (Figure 6B; Supplementary Table S4). A lot of genes were specifically expressed at each time point under a high salinity treatment of 45 PSU, and rhythmic changes appeared with prolonged time. A total of 27 co-expressed genes were observed at six-time points (Figure 6C; Supplementary Table S5), significantly more than under the low and normal salinity treatments. Commonly expressed genes under the three salinity treatments were totally different, but most of them were associated with salinity stress.

We analyzed co-expressed genes under different salinity stresses at the same time point by the same method. The co-expressed genes were up to 182 after 6 hours of treatments and then gradually decreased at 12 hours (40), 24 hours (35), and 48 hours (18) (Figures 6D–H). As salinity treatments of 15 PSU and 30 PSU, the genes in *T. hemprichii* varied rapidly, and gradually formed a stable tolerance mechanism over time, while the gene varied drastically all the time under high salinity stress. Subsequently, we performed a Venn diagram analysis of these co-expressed genes. No co-expressed genes at different time points were observed. Only six genes were co-expressed at three-time points: isoform_44713 (*cytochrome b561* and *DOMON domain-containing protein*), isoform_99546 (*mitochondrial inner membrane protein*), isoform_40878 (no annotation), isoform_107180 (*protein gigantea-like*), isoform_11135 (*probable methyltransferase*), and isoform_138425 (*putative bystin*) (Figure 7). Combined with quantitative reverse-transcription PCR (RT-qPCR) technology, we detected the expression patterns of the six genes at different time points under treatment with salinity of 30 PSU. The results were consistent with the transcriptome sequencing (Figure 8).

KEGG and GO cluster analyses were performed on the 319 genes. The genes involving in stress response were enriched in KEGG and GO classifications (Supplementary Figure S7). From the KEGG pathway, the DEGs at all stress time points were enriched in six pathways: “transport and catabolism”, “carbohydrate metabolism”, “global and overview maps”, “biosynthesis of other secondary”, “metabolites”, and “environmental adaptation”. From GO enrichment analysis, the DEGs at all stress time points were enriched in ten pathways: “cellular anatomical entity”, “intracellular binding”, “transcription regulator activity”, “cellular process”, “metabolic process”, “catalytic activity”, “localization”, “protein-containing complex” and “biological regulation”. Although these common DEGs varied widely among these time points, the enrichment

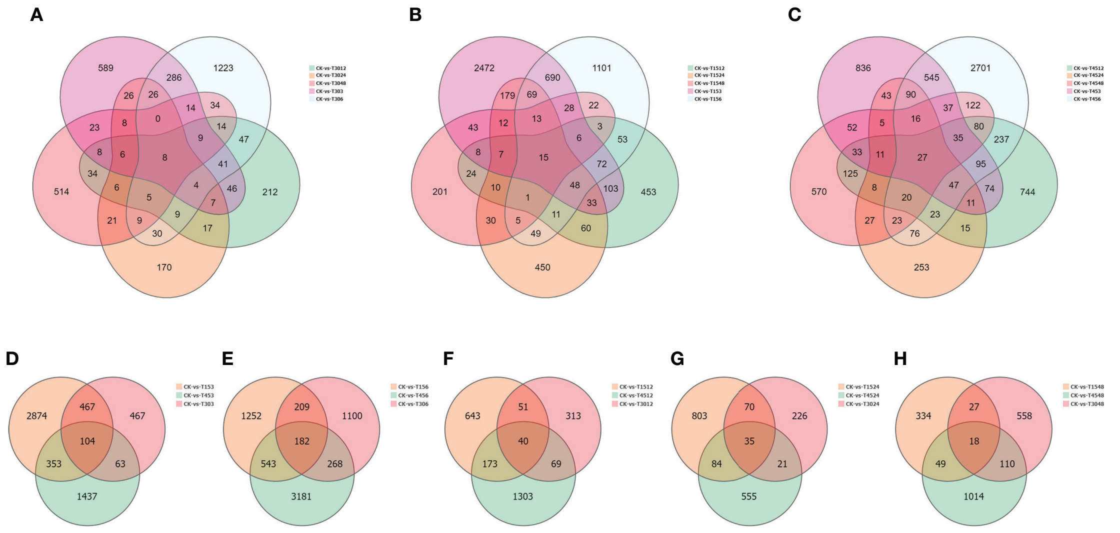

FIGURE 6
Differentially expressed genes (DEGs) under different treatments conditions. **(A)** Comparative analysis of DEGs under the same salinity condition (15 PSU) and different time conditions (3, 6, 12, 24, and 48 hours); **(B)** Comparative analysis of DEGs under the same salinity condition(30 PSU) and different time conditions (3, 6, 12, 24, 48 hours); **(C)** Comparative analysis of DEGs under the same salinity condition(45 PSU) and different time conditions (3, 6, 12, 24, 48 hours); **(D)** Comparative analysis of DEGs under different salinities after 3 hour treatment; **(E)** Comparative analysis of DEGs under different salinities after 6 hour treatment; **(F)** Comparative analysis of DEGs under different salinities after 12 hour treatment; **(G)** Comparative analysis of DEGs under different salinities after 24 hour treatment; **(H)** Comparative analysis of DEGs under different salinities after 48 hour treatment.

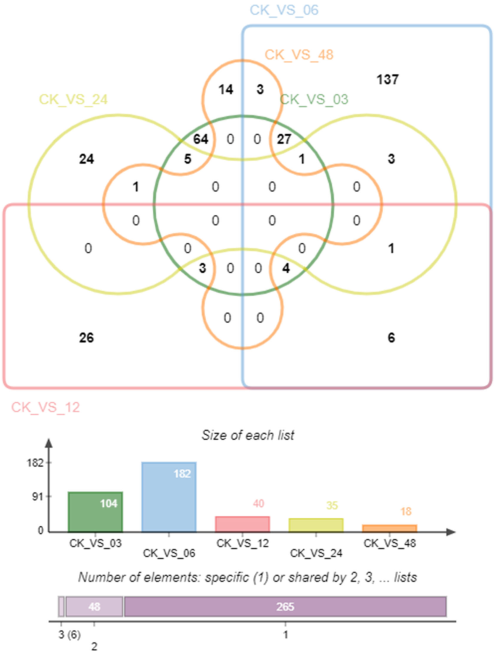

FIGURE 7
Distribution of differentially expressed genes (DEGs) at different time points.

classification was very concentrated, suggesting that these pathways may be key factors in salt tolerance.

Discussion

Salinity stress is a key factor that affects the healthy growth of plants by inhibiting photosynthesis (Munns, 1993). Plant tolerance to salinity promotes its growth in adverse environments. For example, the marine angiosperm *Zostera marina* had a lower survival rate under varying salinity (Johnson et al., 2021). However, marine plants are more tolerant of salinity than terrestrial plants. The optimum salinity range for Thalassia is 24 to 35 PSU (Phillips, 1960; McMillan and Moseley, 1967; Zieman, 1975). However, it tolerates a broad salinity range for very brief exposures, ranging from 3.5 to 60 PSU (McMillan and Moseley, 1967). Nevertheless, such exposures commonly result in leaf loss.

In situ salinity of the seawater in the seagrass bed of Xincun Bay was approximately 30 PSU (Yang and Yang, 2009), acting as control levels in the laboratory experiments. In this study, the salinity of 25 to 35 PSU promoted the growth potential of *T. hemprichii* under treatments after 28 days, the survival rate was between 81.22% and 93.77%. On the other hand, the survival rate of *T. hemprichii* was 35.35% when the salinity was 50 PSU, and the survival rate was significantly affected under low salinity stress, showing that *T. hemprichii* could grow in a wide range of salinity. Similar

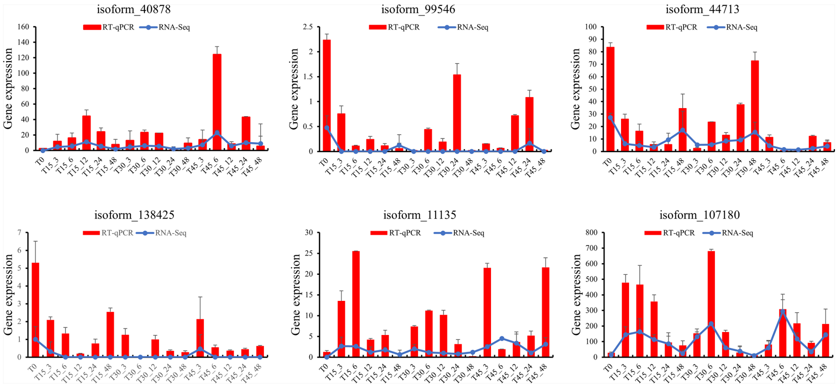

FIGURE 8
Comparative analysis of the quantitative polymerase chain reaction (qPCR) and transcriptome data for six genes.

conclusions were foundin other aquatic plants. For example, the optimal salinity for the growthof *Halophia baccarii* is 25 to 30 PSU, however, the length and width of the leaves are significantly shorter and narrower under 0.52 to 4 PSU and45 PSU salinity stress (Fakhrulddin et al., 2013). Under 30 PSU and sufficient light conditions, *Halophila ovalis* grows better than in other salinity conditions (Bujang et al., 2008; Ralph, 1998).

Microstructural results showed that, like other marine plants, *T. hemprichii* had many dissociative parenchyma cells, or the whole cells collapsed into cavities and air cells, forming many airways. The airways and parenchyma cells in *T. hemprichii* showed significant structural variation with the salinity changes (Watkin et al., 1998). The effect of high salinity on the structure of airways were significantly higher than that of low salinity, indicating that salinity is one of the key factors affecting the structure of airways. High salinity thickened hyperplasia tissues around the stem of *T. hemprichii*, similar structures were found on the leaves surface. Several organelles were observed in the epidermal cells of stems, such as mitochondria. Changes in mitochondrial morphology reflect the tolerance to salinity stress in plants, the structures of mitochondrial is destroyed under high salinity stress in sensitive cells (Domondon et al., 2020; Munns et al., 2020). The volume of the mitochondrial expanded and the structure were significantly damaged under 45 PSU stress, while the mitochondrial structure were relatively stable under 30 PSU stress. Therefore, the stems and leaves of *T. hemprichii* absorbed and accumulated a higher concentration of inorganic ions from the vacuoles of the parenchyma cells, reducing the water potential and adjusting the osmotic pressure. A lot of airways and parenchyma cells in the stem disintegrated with the prolonged time under 45 PSU high salinity stress, and the functional cells of the epidermis were damaged.

Plant stems under salinity stress reduce water loss by thickening the cuticle, epidermis, and mechanical tissue (Stelzer and Luchli, 1978). The microstructure revealed that, due to high salinity, epidermal cell wall of *T. hemprichii* leaves thickened. Chloroplast is one of the most salinity-sensitive organelles in plant cells (Chen et al., 2021), and the chloroplast ultrastructure is closely related to salt tolerance (Wu et al., 2021; Zhu et al., 2021). Many halophyte leaves have osmiophilic granules, which can reduce the osmotic potential, increase the cytoplasmic concentration, and ensure the absorption of water and inorganic salts (Mamane and Gottlieb, 1992; He et al., 2022). The increase of osmiophilic granules is one of the biomarkers for plant salt tolerance. Many osmiophilic granules were found in *T. hemprichii* under different salinity stresses, indicated that osmiophilic granules were ubiquitous in *T. hemprichii* cells. The chloroplast structure was relatively intact, with fewer osmiophilic granules under 30 PSU salinity stress, indicating that optimal salinity conditions are required for the normal growth of *T. hemprichii*.Organic osmotic protective substances, such as proline, soluble sugar, and soluble protein, maintain osmotic potential of plant cell to prevent dehydration under salinity stress. Thus, they stabilize and protect the structure and function of biological macromolecules. For example, high salinity stress damaged corn seedlings, reduced their regulation ability, the soluble sugar and protein content first increased and then decreased with the increase of salinity (Huang et al., 2022). Tomato seedlings subjected to salinity stress revealed similar results (Chaudhary et al., 2020; Ali et al., 2021). *T. hemprichii* is a marine plant and different from terrestrial plants. The content of soluble sugar and soluble protein increased with a long-term salinity stress, especially under a high salinity stress.The MDA content reducing significantly may avoid oxidative damage, which is an important physiological indicator of plant resistance to salinity stress. The MDA content of *T. hemprichii* was high after the prolonged treatment under salinity stress, increasing by 49.97% after 12 hours treatment. However, the MDA content decreased significantly after 24 hours of high salinity treatment, which may be due to the generation of the resistance mechanism of *T. hemprichii* under high salinity stress. The low MDA content reduced the lipid peroxidation of plant membranes, protected the cell membrane system of plants, and alleviated salinity stress effects on plants in the degree. Different to terrestrial plants, the POD, SOD, and CAT contents remained almost unchanged in *T. hemprichii* under different salinity stresses. High salinity stress promoted the rapid accumulation of soluble sugar and soluble protein. Furthermore, the decrease of MDA content in *T. hemprichii* reduced the lipid peroxidation of plant membranes, formed the high adaptability of *T. hemprichii* to a wide range of salinity changes.

The molecular mechanisms underlying the response of plants to salinity stress are complex. Many studies have focused on the changes in gene expression in terrestrial plants after salinity stress (Chanwala et al., 2020; Yuan et al., 2021). However, few studies have been conducted on marine plants. In this study, the typical marine plant *T. hemprichii* was treated with different salinity levels, and its early gene response patterns were analyzed. After salinity treatments, *T. hemprichii* had fewer DEGs in overall number. The pathways in response to high salinity environments of terrestrial plants could be divided into abscisic acid-responsive salinity stress pathways and abscisic acid-independent salinity stress pathways (Punia et al., 2021). In this study, the differential genes of *T. hemprichii* were not enriched in the abscisic acid signaling pathway, but concentrated in the pathways associated with transportation, metabolism, and environmental adaptation after treatments. The commonality analysis of DEGs at each time point was also in line with the results. Among the 319 commonly expressed DGEs, no single gene was consistently differentially expressed at different concentrations and time points. The changes of gene expression in *T. hemprichii* were sharp in the early stage after suffering from salinity stress. From the time-series analysis, the variations of gene expression were drastically after 3 and 6 hours of treatments, while changes were more intense with high salinity stress.

Herein, we studied the changes in physiological characteristics and microstructure of the shoal marine plant *T. hemprichii* under different salinity stresses. The comparative transcriptome analysis under different salinity stresses was also analyzed. This is the first systematic study on the response of *T. hemprichii* to different salinity stresses. This study revealed that *T. hemprichii* had a high fitness to salinity, with more than 35% survival rate under 50 PSU treatment for 28 days. However, under the high and low salinity conditions, the growth and development of *T. hemprichii* showed of retardation and yellowing in different degrees. Meanwhile, the parenchyma cells in *T. hemprichii* collapsed, the contents of soluble sugar, soluble protein and MDA changed, indicating that *T. hemprichii* was sensitive to salinity variation. The growth and development of *T. hemprichii* were significantly affected, although without plant death. From the transcriptome results, its molecular characteristics were different from the salt tolerance mechanism of terrestrial plants. This study provided a basis for a deep study on the mechanism underlying the response of *T. hemprichii* to shoal living environment changes and data support for the study of the molecular mechanism of *T. hemprichii* in response to salinity stress.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: NCBI, PRJNA848129.

Author contributions

JS, DW designed the experiments. JS, ZW, and LY analyzed the data. SC and ZC collected the samples. XG collected the Electron microscope picture. JS, DW wrote the paper. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.975251/full#supplementary-material

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EDITED BY
Mirta Teichberg,
Marine Biological Laboratory (MBL),
United States

REVIEWED BY
Elena L. Peredo,
Marine Biological Laboratory (MBL),
United States
Jessica Pazzaglia,
Anton Dohrn Zoological Station Naples,
Italy

*CORRESPONDENCE
Jun Chen
chenjun271@163.com
Xuexi Tang
tangxx@ouc.edu.cn

[†]These authors have contributed equally to this work

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Insights into the regulation of energy metabolism during the seed-to-seedling transition in marine angiosperm *Zostera marina* L.: Integrated metabolomic and transcriptomic analysis

Meiling Zhu[1†], Yu Zang[2†], Xuelei Zhang[2], Shuai Shang[3], Song Xue[1], Jun Chen[1*] and Xuexi Tang[1*]

[1]College of Marine Life Sciences, Ocean University of China, Qingdao, Shandong, China, [2]Key Laboratory of Marine Eco-Environmental Science and Technology, First Institute of Oceanography, Ministry of Natural Resources, Qingdao, Shandong, China, [3]College of Biological and Environmental Engineering, Binzhou University, Binzhou, Shandong, China

Seed development is a crucial phase in the life cycle of seed-propagated plants. As the only group of angiosperms that evolved from terrestrial plants to complete their life cycle submerged in marine environments, the mechanisms underlying seed development in seagrasses are still largely unknown. In the present study, we attempted to combine transcriptomic, metabolomic, and physiological data to comprehensively analyze the molecular mechanism that regulates energy metabolism in *Zostera marina* seeds at the four major developmental stages. Our results demonstrated that seed metabolism was reprogrammed with significant alteration of starch and sucrose metabolism, glycolysis, the tricarboxylic acid cycle (TCA cycle), and the pentose phosphate pathway during the transition from seed formation to seedling establishment. The interconversion of starch and sugar provided energy storage substances in mature seeds and further acted as energy sources to support seed germination and seedling growth. The glycolysis pathway was active during *Z. marina* germination and seedling establishment, which provided pyruvate for TCA cycle by decomposing soluble sugar. Notably, the biological processes of glycolysis were severely inhibited during *Z. marina* seed maturation may have a positive effect on seed germination, maintaining a low level of metabolic activity during seed maturation to preserve seed viability. Increased acetyl-CoA and ATP contents were accompanied with the higher TCA cycle activity during seed germination and seedling establishment, indicating that the accumulations of precursor and intermediates metabolite that can strengthen the TCA cycle and facilitate energy supply for *Z. marina* seed germination and seedling growth. The large amount of oxidatively generated sugar phosphate promotes fructose 1,6-bisphosphate synthesis to feed back to glycolysis during seed germination, indicating that the pentose phosphate pathway not only provides energy for germination, but also complements the glycolytic pathway. Collectively, our findings suggest these energy metabolism pathways cooperate with each other in the process of seed transformation from maturity to seedling establishment, transforming seed from storage tissue to highly active metabolic tissue to meet the energy requirement seed development. These findings provide insights into the roles of the energy metabolism pathway in the complete developmental process of *Z. marina* seeds from different perspectives, which could facilitate habitat restoration of *Z. marina* meadows *via* seeds.

KEYWORDS

seagrass, seed development, starch and sucrose metabolism, glycolysis, TCA cycle energy metabolism during seed development

1 Introduction

Seagrasses are the only flowering plants (angiosperms) adapted to grow and reproduce entirely submerged in seawater (Tol et al., 2017). Similar to land angiosperms, seagrasses can produce seeds by reproducing sexually, which is a critical stage in the life cycle of seed-propagated plants (Tarquinio et al., 2021). *Zostera marina* is an important foundation species for coastal ecosystems in the northern hemisphere that provides essential ecosystem functions, including stabilizing sediments, providing nursery grounds for juvenile fish, and sequestering carbon (Fourqurean et al., 2012; Bengtsson et al., 2017). Despite their ecological importance, *Z. marina* meadows are degrading at an breakneck rate under anthropogenic impacts and natural threats (Orth et al., 2006; Waycott et al., 2009; Short et al., 2011). Thus, effective protection and active restoration engineering are becoming increasingly important. Seed restoration is the main method to repair damaged seagrass meadows because it has a low cost, maintains population genetic diversity, and causes less harm to the original seagrass meadow (Yue et al., 2019). However, such restoration is limited by the low germination rate of seedlings and poor seedling establishment after seed germination (Orth et al., 2010). Therefore, determining the changes in key physiological processes during seed development for seagrasses is important for promoting the successful regeneration of seagrass meadows.

Seed maturation, germination, and seedling establishment are vital stages during a plant's life cycle (Ma et al., 2017). These stages are precisely regulated by various biological processes involving signaling transduction, phytohormone regulation, and energy metabolism (Hao et al., 2022). Previous studies have shown that energy metabolism plays a dominant role in regulating seed development; it provides the material and energy basis for seed development by regulating the synthesis, hydrolysis, and conversion of storage compounds, such as starch, storage proteins, and lipids (Liu et al., 2015; Ali and Elozeiri, 2017; Ma et al., 2017). Starch is the major form of carbohydrates and energy carriers in plant storage organs (Ma et al., 2020). The amylolytic breakdown of stored starch in seeds during germination is an important biochemical event that provides reducing sugars as energy sources to support the early stages of seed germination in angiosperm plants (Chen et al., 2019). Seed germination and seedling establishment are accompanied by intense and complex physiological metabolic activity (Zienkiewicz et al., 2014). Most storage substances begin to be mobilized, and three respiratory pathways namely glycolysis, the pentose phosphate pathway, and the tricarboxylic acid cycle (TCA cycle) are activated to provide materials and energy for subsequent seedling growth and development (Song et al., 2021). For example, glycolysis provides basic intermediates and energy upon seed germination, and the TCA cycle is crucial for energy generation during seed germination by supplying reducing equivalents for the operation of the respiratory chain (Tan et al., 2013). Moreover, the pentose phosphate pathway can provide not only pentose phosphate for nucleotide metabolism, but also NADH for different biosynthesis reactions to promote seed germination and seedling growth (He et al., 2011). However, some studies have indicated that changes in the enzymes involved in the TCA cycle are not detected during the germination of cereal seeds, and the energy demand seems to be fulfilled mainly by glycolysis (Yang et al., 2007). In addition, other studies have demonstrated that glycolysis and the TCA cycle provide most of the energy for seed germination, while the pentose phosphate pathway plays a small contributory role in some angiosperm seed development (Han et al., 2014; He et al., 2019).

Although the number of studies about the complex series of physiological changes in terrestrial plants during seed development is gradually increasing (Yan and Chen, 2017; Lee et al., 2018), physiological responses and molecular regulatory mechanisms in the process of seed development for marine angiosperms still need to be further explored (Sugiura et al., 2009). In particular, the physiological changes in the complete seed development process have not been studied rigorously. To gain a further understanding of the role and regulation of energy metabolic characteristics during seed maturation and germination in *Z. marina*, we analyzed the morphological and physiological characteristics, as well as the changes in transcription and metabolism at four different stages, from seed maturation to seedling establishment. This study provides novel insights into the roles of the energy metabolism pathway during the crucial developmental phase in *Z. marina* seeds and facilitates its future application in promoting seed germination to improve seagrass conservation effectiveness.

2 Materials and methods

2.1 Plant materials and growth conditions

Zostera marina seeds were randomly collected from May to September 2021 from a shallow seagrass meadow in Changdao County, Yantai City, China. To avoid potential bias in seed vigor due to aging, only the seeds produced from the current year were used in this study. Immature seeds were distinguished from those of the different developmental stages by selecting soft and green seeds that were still attached to spathes at the time of collection. Mature reproductive shoots were picked and placed in a 600-µm mesh bag, which was suspended in seawater at sampling sites for 1 month until mature seeds were released from the rotten shoots. Mature seeds were sieved (2-mm) from the decaying shoots and brought back to the laboratory in natural seawater. The mature seeds were rinsed repeatedly with seawater and then planted in a large box containing sieved sediment at 2–3 cm deep. The box was placed in aerated flow-through tanks filled with natural seawater at a salinity of 30 ± 0.2 psu and a temperature of 15°C. The seawater was changed weekly to observe the germination of *Z. marina* seeds.

Yellow seeds with soft in texture and neatly arranged in the spathe were chosen as immature stage (stage I). Black or brown seeds with a very firm and hard texture were defined as mature stage (stage II). The cotyledons broke through the seed coat was considered as germinated stage (stage III). Seeds that grew their first young leaves in aquariums were marked as the seedling establishment stage (stage IV). Healthy seeds were collected at different stages, including stage I, II, III, and IV, and were frozen in liquid nitrogen for further analysis.

2.2 Morphological and histological observations

Light microscope was prepared for observing the morphological alterations of *Z. marina* seeds at differernt stage. For histological procedures, three biological replications (cotyledon of three seeds) were performed for periodic acid–schiff (PAS) stainning at each developmental stage. Samples were fixed by FAA and embedded by paraffin to make paraffin sections with 6–10µm slices using a LEICA2150 rotary microtome (Leica, Germany). After dewaxing, the slices were stained in PAS solution for the observation of starch changes using an Olympus BX-61 microscope (Olympus, Japan).

2.3 Physiochemical analysis

The 0.1g frozen seeds/seedlings from each repetition were weighed and ground into fine powder in liquid nitrogen for subsequent indicator determination. The starch content was quantified using the anthrone sulfuric acid method (Wang et al., 2016b). The starch was decomposed into glucose by acid hydrolysis and then reacted with anthrone, that has the absorption peak at a wavelength of 620 nm. Sucrose and fructose contents were tested by resorcinol and colorimetric methods (Du et al., 2020). The product of the reaction of fructose and resorcinol has a maximum absorption value at 480 nm and further calculated the content of sucrose, which could be hydrolyzed to fructose under acidic conditions. The glucose content was measured according to the glucose oxidase/peroxidase method (Kabasakalian et al., 1974). After 0.1g frozen seed samples was homogenated with 1 mL of distilled water and extracted at 100°C for 10min, the supernatant was used to determining the content of glucose after centrifugation at 8000 g at 25°C for 10 min by glucose detection kit (Solarbio, Beijing, China). For measuring the amino acids (AAs) content, the ninhydrin assay method was used (Lee et al., 2003). α-amino group with ninhydrin results in bluish violet compounds with a characteristic absorption peak at 570 nm. Pyruvic acid, total proteins, and ATP content by pyruvate content assay kit, BCA protein kit and ATP content assay kit obtained from Solarbio Company (Beijing, China). The free fatty acid (FFA) content was using a FFA assay kit (Comin Biotechnology Co. Ltd, Suzhou, China).

Frozen samples weighing 0.1 g were ground with liquid nitrogen to make tissue homogenates for enzyme activity analysis. The activities of α-amylase and ADP-glucose pyrophosphorylase (AGPase) were conducted using amylase activity assay kit (Comin) and AGP activity assay kit (Comin). The seeds for each stage were measured across three biological replicates, where each biological replicate included three technical replicates.

2.4 Transcriptome analysis and data processing

Trizol reagent was used to extract total RNA from each stage. Four biological repetitions were performed for each stage, and each repetition contained 200 mg of *Z. marina* seeds. The 2100 Bioanalyzer (Agilent) was used to assess RNA quality, and RNA quantification was confirmed by ND-2000 (NanoDrop Technologies). The cDNA library construction was processed using a TruSeq^{TM} RNA sample preparation kit (Illumina, SanDiego, Canada) and was sequenced with an Illumina NovaSeq 6000 sequencer (2×150 bp read length).

Clean paired-end reads were aligned against the *Z. marina* reference genome (*Z. marina*_668_v3.1) using HISAT2 (http://ccb.jhu.edu/software/hisat2/index.shtml, version 2.1.0) to get mapped reads, and gene abundances were quantified *via* RSEM (http://deweylab.biostat.wisc.edu/rsem/, version 1.3.3) to calculate FPKM values. We annotated the DEGs with the genome annotation file "*Z. marina*_668_v3.1.annotation_info.txt" from the Joint Genome Institute (JGI) database. The DEGs between any two adjacent developmental stages were screen out using DESeq2 (http://bioconductor.org/packages/stats/bioc/DEScq2, version 1.10.1) with $|\log_2 FC| > 1$ and FDR <0.05 based on FPKM rasults. The biological function of these DEGs was further determined by the KEGG enrichment analysis (http://www.genome.jp/kegg/, version 2017.08), and a P-value ≤ 0.05 was considered significant enrichment.

2.5 Metabolite extraction and LC-MS/MS analysis

To further measure the change in energy metabolites, we performed liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. *Z. marina* seeds from the four stages were harvested with four independent biological replicates. A total of 50 mg of each repetition was used for the extraction of metabolites with 500 μL precooled methanol at -20°C. Then the mixture was homogenized by vortexing for 3min and centrifuged for 10 min (12,000 g, 4°C) to collect the supernatant. The loading solution was obtained by passing 200 μL of the supernatant over the protein pellet plate for further analysis by Q-Trap 6500+ mass spectrometer linked to UPLC system. The ACQUITY UPLC BEH Amide column (1.7 μm, 100 mm×2.1 mm i.d.) was used (column temperature: 40°C, injection volume: 2 μL). Ultra-pure water (phase A) + 90% acetonitrile (phase B) were used to constitute the mobile phase, which was run at 0.40 mL/min.

Based on the standard-built database MWDB, the mass spectrometry detection data were qualitatively analyzed, and a quantitative analysis was performed in MRM mode. Then, significantly differential metabolites between groups were filtered under the condition of VIP value ≥ 1 and *P*-value ≤ 0.05.

2.6 Statistical analysis

Heatmap and clustergram analysis ("heatmap" R package, version 1.0) for four replicates were performed on the log 10 (FPKM) values of genes. Statistical analyses of physiological indicators and metabolite content were analyzed using SPSS 25.0. Measurement data were presented as means ± SD for three replicates. All data were first tested for homogeneity of variance using Levene's tests and for normality using K-S test. The differences between adjacent stages were compared by a one-way analysis of variance (ANOVA), followed by the SNK test to evaluate statistical significance. Significance levels for all tests were set at a *P*-value < 0.05.

3 Results

3.1 Morphologic and cellular structure changes of seeds

In this experiment, we observed the morphological changes in the complete *Z. marina* seeds development process to better understand the characteristics of physiological metabolism changes during seed maturity, germination, and seedling establishment (Figure 1A). The seeds were neatly arranged in spathes, yellow-green and soft in texture at the immature seed stage. Mature seeds were released from spathes, had a hard texture, and were brown or black. Seed germination began with cracking in the seed coat and incomplete embryo exposure. The germ embryo of germinated seeds further developed and differentiated into one young leaf during the seedling establishment process.

The changes in starch granules in the cotyledons during the four stages were observed under a microscope after the adoption of PAS. The number of starch grains increased significantly from stage I to II, while the number of starch grains consistently decreased from stage II to IV (Figure 1B). This indicated that the starch content changed dramatically from seed maturity to seedling establishment.

3.2 Physicochemical index changes in the energy metabolism of seeds

To better understand changes in energy metabolism during the different seed developmental stages, we further measured the content of related metabolites. The sucrose, starch, and total proteins contents remarkably increased by 40, 31 and 46%, respectively from stage I to II ($P < 0.05$). However, after seed

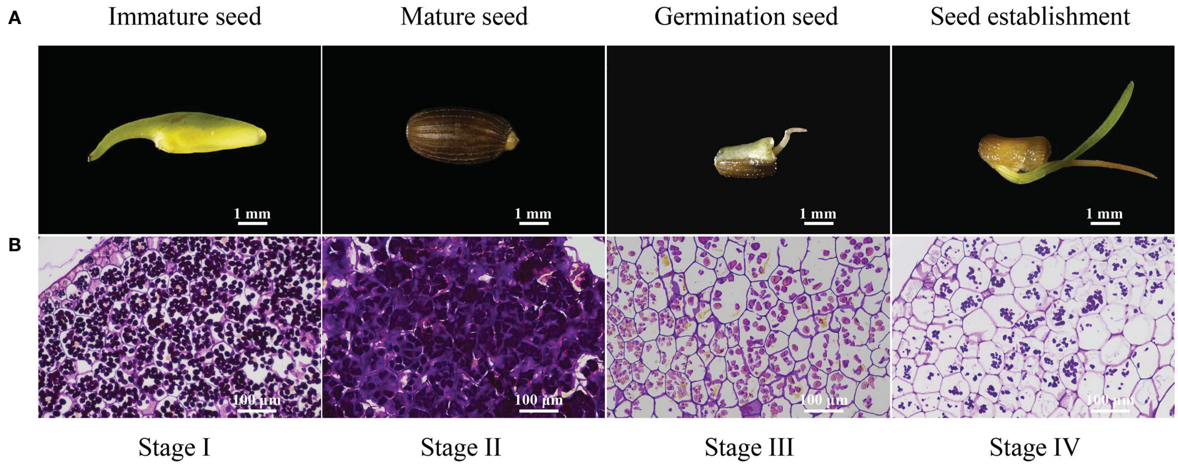

FIGURE 1
Morphology and histology of developing *Zostera marina* seeds. **(A)** *Zostera marina* seed at different developmental stages. **(B)** During *Zostera marina* seed development, starch was stained with PAS. The rulers indicate 1 mm in **(A)** and 200 μm in **(B)**.

maturation, the starch, sucrose, and total protein contents were reduced by 59, 36, and 23%, respectively (Figures 2A, B, E). Both glucose and fructose contents decreased significantly at stages I–II ($P < 0.05$), followed by a marked increase at stages II–III (Figures 2C, D). In addition, the amino acids (AAs) and free fatty acid (FFA) contents decreased from stage I to II, while the contents increased from stage II to IV (Figures 2F, G). The ATP content markedly decreased from stage I to II but increased significantly from stage II to IV, the maximum occurred at stage I (Figure 2H). This suggested that changes in energy metabolism occurred in different seed developmental stages.

Significant changes in enzyme activity were found in *Z. marina* seeds at different developmental stages ($P < 0.05$). AGPase activity was markedly increased from stage I to II and then sustainably decreased, with the minimum occurring at stage IV (Supplementary Figure 1A). In addition, the activity of α-amylase was not significantly changed from stage I to II and then slightly rose in stage III, followed by a marked increase at stage IV (Supplementary Figure 1B).

3.3 Global analysis of dynamic changes during *Z. marina* seed development by transcriptomic and metabolic profiling

To identify key genes involved in regulating *Z. marina* seed maturity, germination, and transition to seedling establishment, we first carried out RNA-seq analysis in samples from four stages. A total of 121.16 Gb of clean sequencing data from 16 samples were produced, and the average clean data from each sample were more than 6.69 Gb. The rate of alignment between sequencing reads and the reference genome fell in the range of 82.51–93.88%, the GC content (44.1–46.77%) and high Q30 value (94.58–95.57%) enabled the subsequent differential expression analysis (Supplementary Table 1). Principal component analysis (PCA) showed significant differences among the four different stages and good intra-group repeatability (Figure 3A). Differentially expressed genes (DEGs) were screened based on two aspects: log2 (fold change) and the statistical significance level. Pairwise differential expression profiling analysis was used to count the number of up- or down-regulated DEGs between any two neighboring stages. There were 14390 DEGs identified based on pairwise comparison analysis, inculding 10636 (4475 up- and 6161 downregulated) in stage I to stage II, 8070 (4737 up- and 3333 downregulated) in stage II to stage III and 5379 (3559 up- and 1820 downregulated) DEGs were screened out between stage III and stage IV, indicating a significant difference in different stages (Figure 3B, Supplementary Table 2). And a Venn diagram showedthe stage-specific expression genes between two neighboring stages, indicated the each stage specificity (Supplementary Figure 2).

To better explore the dynamics of energy metabolism from *Z. marina* seed mature to seedling establishment at the metabolic level, we performed targeted metabolomic profiling analysis for research in energy metabolomics using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) platform. 57 metabolites related to energy metabolism in *Z. marina* seeds of four stage were detected. PCA revealed that different stages formed a significant separate cluster, indicating that metabolic changes occurred in different seed developmental stages (Figure 3C). Various numbers of significant metabolites among neighboring stages were identified. Similar to differential gene expression analysis, the number of significantly differential metabolites was relatively large between stage I and stage II (35 differential metabolites), followed by 22 the differential

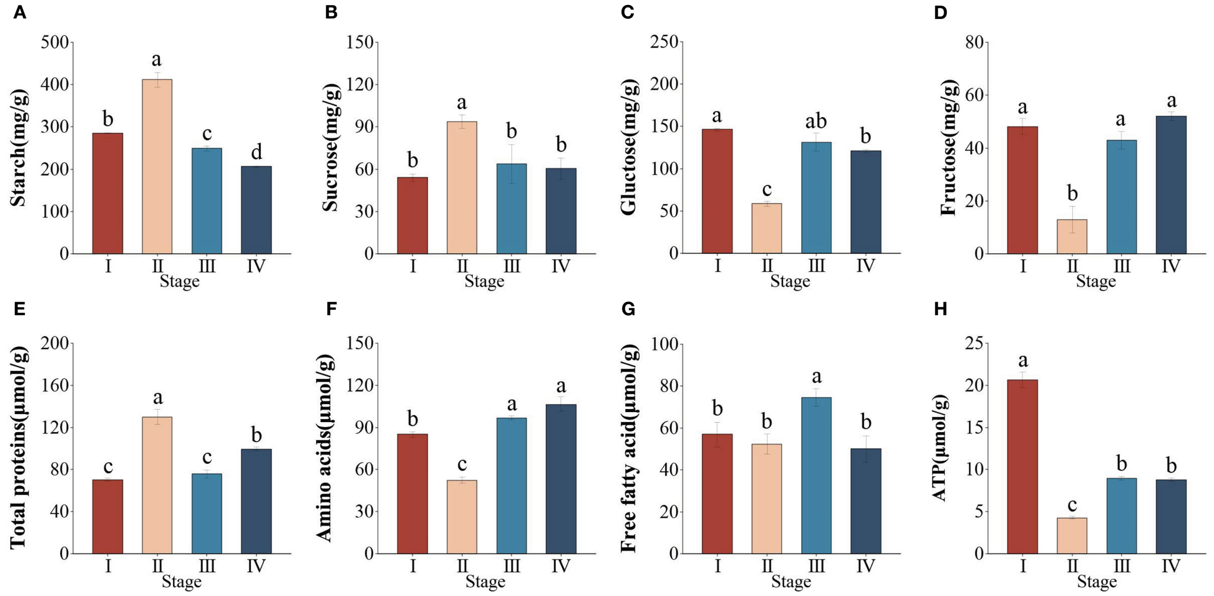

FIGURE 2
Changes in the content of energy metabolism metablites during different developmental stages in *Zostera marina* seeds. **(A)** Starch, **(B)** sucrose, **(C)** glucose, **(D)** fructose, **(E)** total proteins, **(F)** amino acids, **(G)** free fatty acids, **(H)** ATP. Values are the means ± standard deviation (n = 3). Different letters represent significant differences ($P < 0.05$).

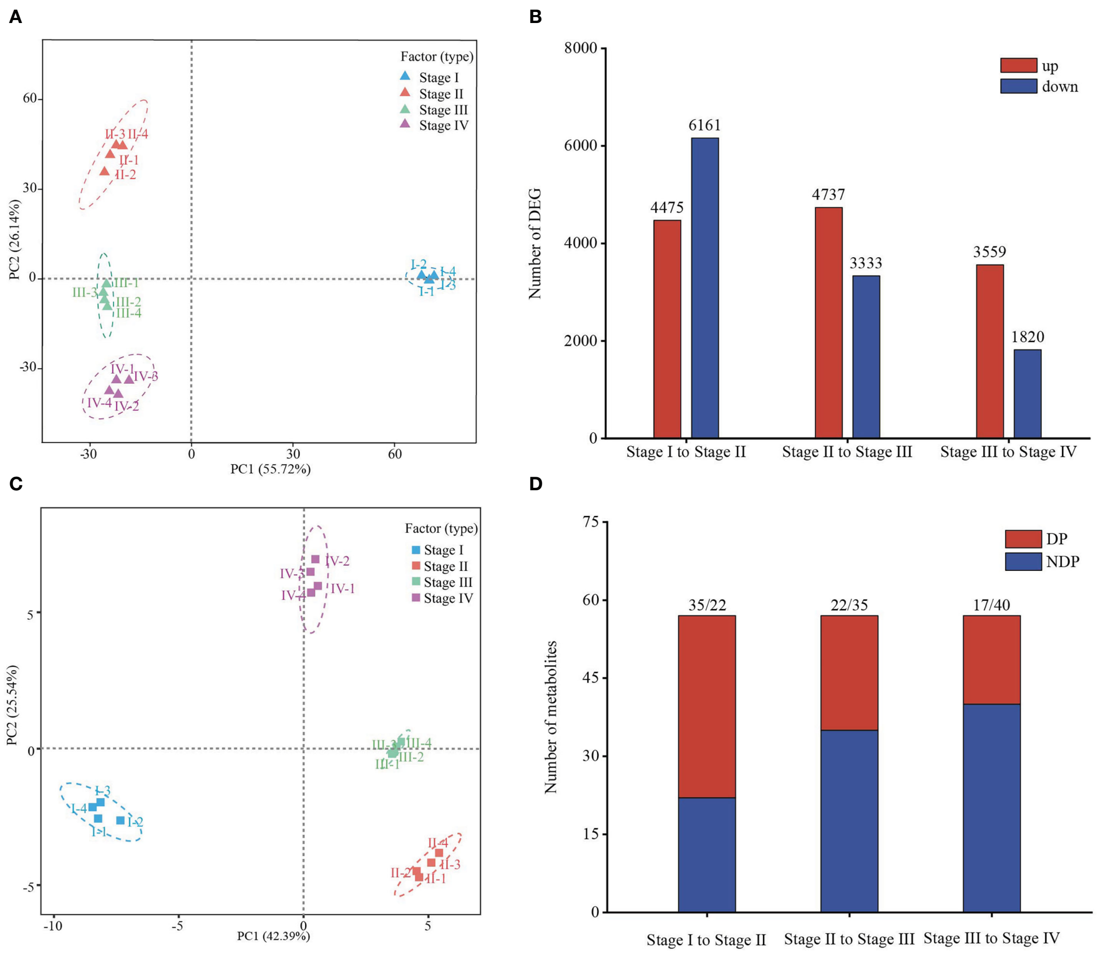

FIGURE 3

Transcriptomic and metabolic analyses reveal a dynamic transition during *Zostera marina* seed development. **(A, C)** PCA of transcriptome and metabolome during *Zostera marina* seed development. **(B)** Numbers of up- and down- regulated genes at different stages. **(D)** Number of differential metabolites during the transition between development stages. DP, differential metabolites. NDP, non-differential metabolites.

metabolites were identified in stage II to stage III, and 17 the differential metabolites in stage III to stage IV, providing important information combined with transcriptome data for the further exploration of energy metabolism dynamics (Figure 3D, Supplementary Table 3).

3.4 KEGG enrichment analysis of DEGs during *Z. marina* seed development

To understand the most involved metabolic pathways during *Z. marina* seed development, we tried to map the processes by functionally annotating the DEGs against the KEGG database. KEGG enrichment analysis found that from stage I to II (Figure 4A), glycolysis/gluconeogenesis was significantly enriched, following by photosynthesis-antenna proteins, inositol phosphate metabolism and citrate cycle (TCA cycle) ($p<0.01$). In addition, the number of DEGs was also very high in starch and sucrose metabolism ($p<0.05$). There were ten extremely significant enrichment pathways from stage II to III ($p<0.01$): such as glycolysis/gluconeogenesis, pentose phosphate pathway and fructose and mannose metabolism. Followed by the starh and sucrose metabolism also was a significant enrichment pathway ($p<0.05$) (Figure 4B). As shown in Figure 4C, there are 19 significantly enriched metabolic pathways from stage III to IV ($p<0.05$), such as the largest KEGG term was “ribosome” with 114 genes. Additionally, 48 genes were associated with the “glycolysis/gluconeogenesis pathway,” 40 with “starch and sucrose metabolism,” and 53 with “MAPK signaling pathway-plant.” KEGG analysis showed that pathways related to energy metabolism dominated, such as starch and sucrose metabolism, glycolysis, the TCA cycle, and the pentose phosphate pathway.

3.5 Dynamic regulation of key genes and metabolites in starch and sucrose metabolism during seed development

To identify important genes regulating energy metabolism during *Z. marina* seed developmental processes, we searched the major components in the starch and sucrose metabolism pathway based on KEGG enrichment analysis (Figure 5A). A total of nine

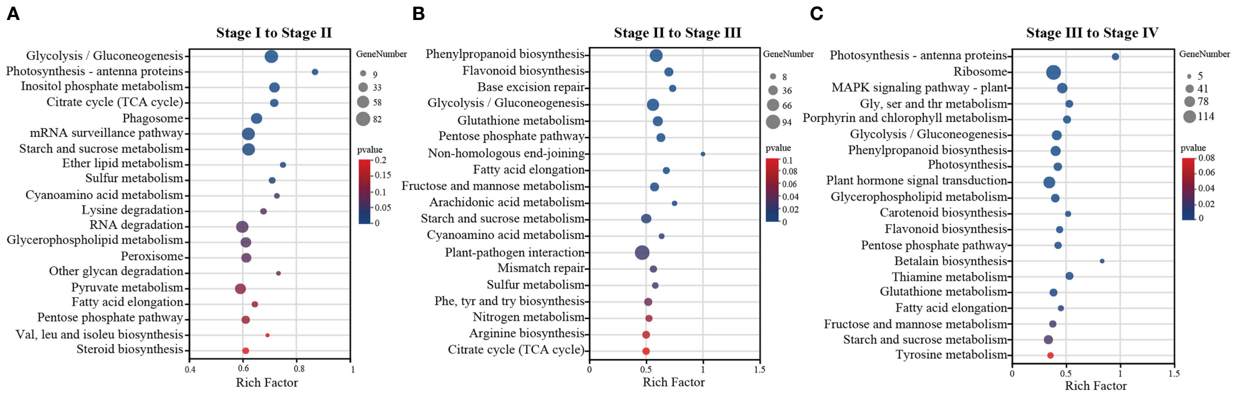

FIGURE 4
KEGG enrichment analysis of DEGs identified by pairwise comparison were performed by R package (version 1.0) clusterProfiler. **(A)** stage I to stage II, **(B)** stage II to stage III, **(C)** stage III to stage IV. The y-axis is the name of KEGG pathway, x-axis represents the ratio of the number of genes annotated in this pathway to background differentially expressed genes in this pathway. Size of circles indicates the number of enriched genes in each pathway, and the color represents the level of significance.

genes were annotated as *ZmSuSy* encoding sucrose synthase (SUS); six were upregulated at stage II, and five were upregulated at stage III, suggesting that these genes might play differential roles in sucrose synthesis and degradation. The *ZmUGP*, *ZmSS*, and *ZmAGP* genes encoding UDP-glucose pyrophosphorylase (UGPase), starch synthase (SS), and AGPase, respectively, had a high expression level in stages I and II, while the expression levels of *ZmSS* and *ZmAGP* were significantly downregulated after stage II. In addition, the *ZmAMY*, *ZmbglX*, and *ZmINV* genes encoding α-amylase (AMY), β-glucosidase (bglX), and invertase (INV), respectively, were overall upregulated after stage II (Figure 5B, Supplementary Table 4). However, at the metabolome level, the glucose 1-phosphate (G1P), glucose 6-phosphate (G6P) and fructose 1,6-bisphosphate (FDP) content decreased, while the UDP-glucose (UDPG) and starch content increased from stage I to II. However, the G1P, G6P and FDP content increased, but UDPG content decreased from stage II to IV (Figure 5C). It was possible that more G1P was used to synthesize UDPG and thus starch.

3.6 Dynamic regulation of key genes and metabolites in glycolysis and the pentose phosphate pathway during seed development

KEGG analysis showed that glycolysis and pentose phosphate processes were significantly enriched in the seed maturation to seedling establishment and the relevant components in the pathway were drawn (Figure 6A). To better verify the molecular regulation of energy metabolism during seed development, we examined the DEGs involved in multiple processes, including genes functioning in glycolysis and pentose phosphate (Figures 6B, D, Supplementary Table 4). In the glycolytic pathway, 67 DEGs were enriched. The expression of most transcripts (e.g., *ZmENO*, *ZmPFK*, *ZmGAPDH*, and *ZmPK*) was downregulated from stage I to II, while the expression of these genes was significantly upregulated from stage II to IV (Figure 6B). Glycolysis establishes links to the pentose phosphate pathway *via* G6P. The important components in the pentose phosphate pathway include *ZmtktA*, *ZmG6PD*, *ZmPGD*, and *ZmtalA*. The expression of these genes was continually upregulated from stage I to IV (Figure 6D).

The changes in the metabolome were roughly consistent with transcriptional changes. In glycolysis, the content of pyruvic acid decreased from stage I to II, while there was no significant change in the content of PEP and 2-Phospho-D-glyceric acid (2-PGA). However, the content of these metabolites significantly increased from stage II to IV (Figure 6C). In the pentose phosphate pathway, our metabolic profiling analysis showed that the content of erythrose 4-phosphate and ribulose 5-phosphate were decreased from stage I to II, followed by an increase from stage II to IV. In addition, the content of sedoheptulose 7-phosphate was continually increased from stage I to IV (Figure 6C).

3.7 Dynamic regulation of key genes and metabolites in the TCA cycle pathway during seed development

According to the results of KEGG enrichment analysis, the TCA cycle pathway diagram was drawn (Figure 7A). A total of 37 DEGs were significantly enriched in this pathway in *Z. marina* seed development (Figure 7B, Supplementary Table 4). *ZmMDH*, *ZmPDHA*, *ZmFUMC*, and *ZmACO* were significantly downregulated from stage I to II, while the expression of these genes were continuously upregulated from stage II to IV (Figure 7B). The metabolome data were roughly consistent with the expression data, the content of succinic acid and fumaric acid decreased from stage I to II, while the content of citric acid and isocitric acid increased. However, from stage II to IV, in addition to the decrease in the citric acid content, the content of succinic acid, fumaric acid, and isocitric acid increased (Figure 7C).

In addition, most AAs are important source of substrates for the TCA cycle. Our results showed that the content of lysine (Lys),

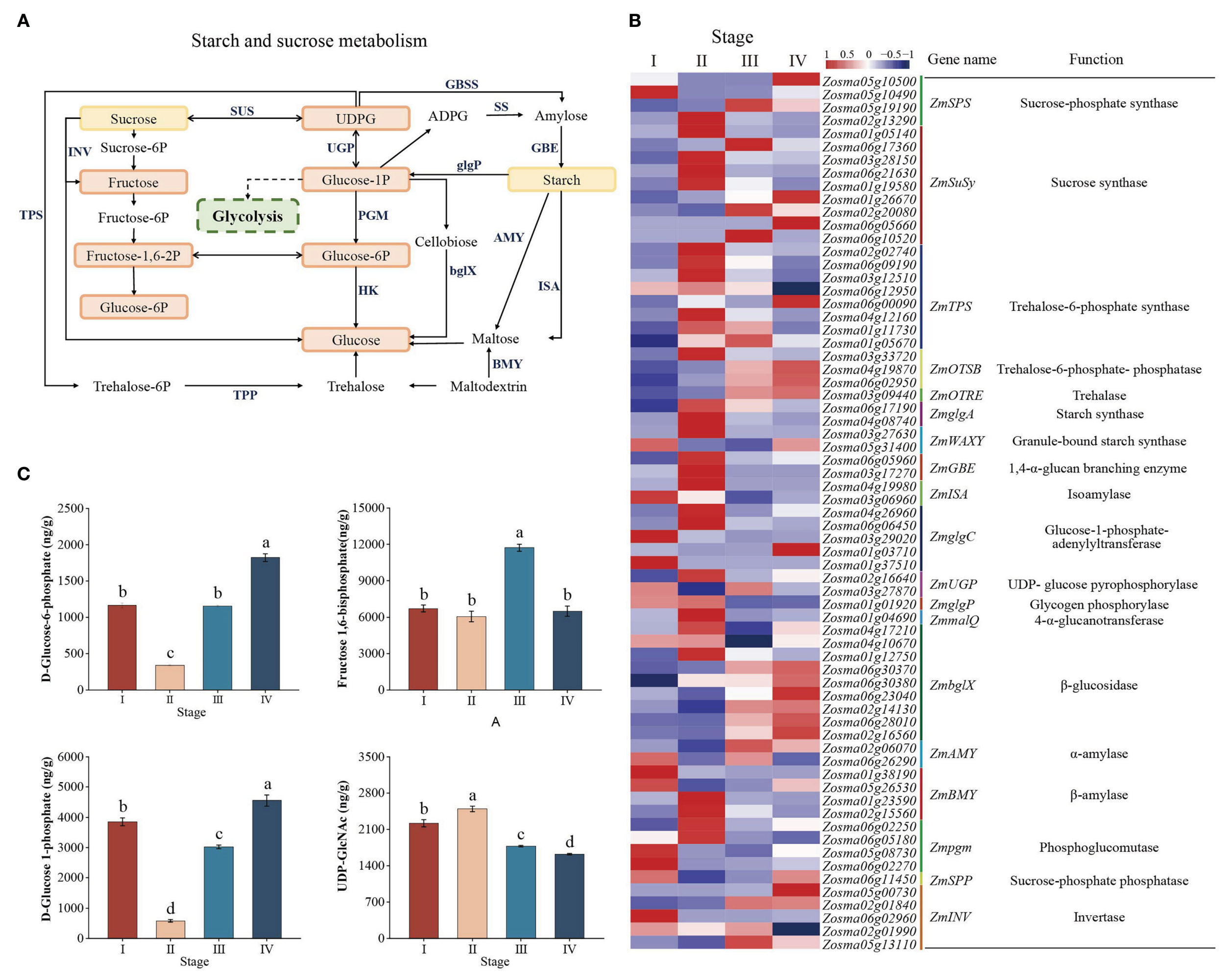

FIGURE 5
Starch and sucrose metabolism pathway. **(A)** Model and key components of starch and sucrose metabolism in *Zostera marina* seeds. The highlighted boxes indicate the metabolites quantified from the LC–MS/MS results. **(B)** Heatmap analysis of DEGs (log 10 (FPKM) values) involved in starch and sucrose metabolism. **(C)** Analysis of changes in the metabolites represented in starch and sucrose metabolism during *Zostera marina* seed development.

threonine (Thr), asparagine (Asn), and tyrosine (Tyr) remained higher from stage III to IV (Figure 7C). This means that different AAs are catabolized and enter the TCA cycle, thereby increasing flux toward the TCA cycle.

4 Discussion

4.1 Interconversion of starch and sugar plays an important role during *Z. marina* seed development

Starch and sucrose regulatory pathway is crucial in maintaining the dynamic balance of sugar substances and energy homeostasis in plant growth and development by regulating the interconversion of starch and sugar (Zhao et al., 2022). Some studies have suggested that sucrose serves as a substrate for the formation of storage reserves and as a main nutrient and energy source for plant development (Yu et al., 2016; Ma et al., 2020). In the metabolic pathways of *Nelumbo nucifera*, sucrose is mainly produced in leaves by photosynthesis, transported to seeds, and then accumulated as energy reserves for seed maturation (Wang et al., 2016a). In the early seed germination stages, sucrose is predominantly hydrolyzed to generate glucose and fructose to provide energy and precursors for seed germination (Silva-Sanchez et al., 2013; Wang et al., 2016a; Mala et al., 2021). In accordance with a previous study, sucrose was considered to be one of the major carbohydrate reserves in mature *Z. marina* seeds (Touchette and Burkholder, 2000). In this study, the content of sucrose significantly increased from stage I to II, indicating that sucrose produced by leaf photosynthesis may be transported to the seed during *Z. marina* seed maturation. In addition, the increase in sucrose content might further regulated starch synthesis. In contrast, the content of sucrose decreased as the content of glucose and fructose increased from stage II to IV, indicating that sucrose can be hydrolyzed into hexose and further provide energy for seed germination and growth.

Sucrose synthase (SUS) has reversible activity, where it catalyzes sucrose synthesis and degradation, but hydrolytic activity is suggested to play a leading role in guaranteeing a steady concentration of UDP-glucose (UDPG) and providing energy for

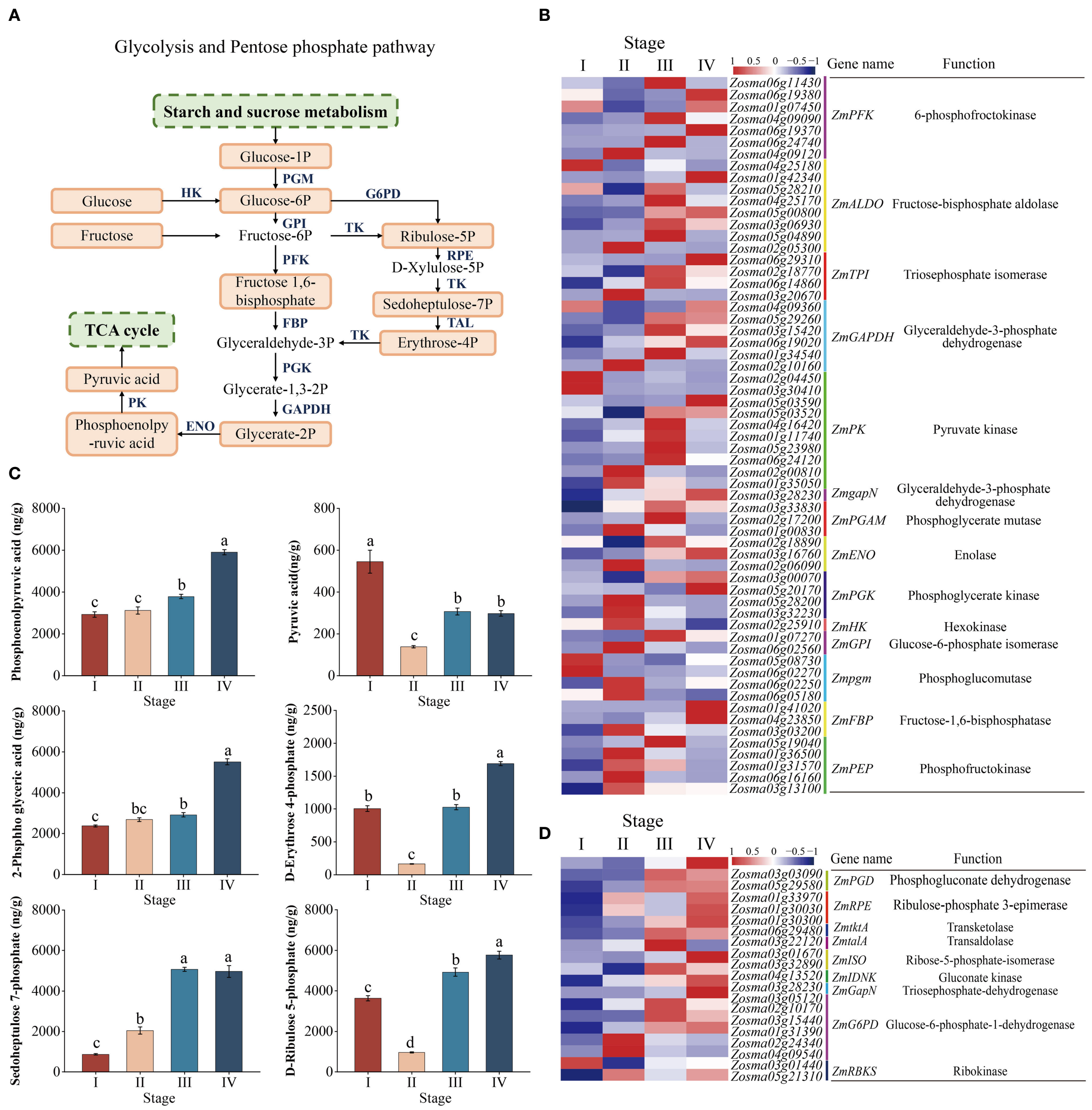

FIGURE 6

Glycolysis pathway and pentose phosphate pathway. **(A)** Model and key components of the glycolysis pathway and the pentose phosphate pathway in *Zostera marina* seeds. The highlighted boxes indicate the metabolites quantified from the LC−MS/MS results. **(B)** Heatmap analysis of DEGs (log 10 (FPKM) values) involved in the glycolysis pathway. **(C)** Analysis of changes in the metabolites represented in the glycolysis and pentose phosphate pathways during *Zostera marina* seed development. **(D)** Heatmap analysis of DEGs involved in the pentose and phosphate pathways.

plant growth (Zhang et al., 2016). However, our results showed that the expression of *ZmSuSys* affected sucrose homeostasis. Nine genes were annotated as *ZmSuSy* encoding sucrose synthase (SUS); six of these genes were upregulated at stage II, and five *ZmSuSys* were upregulated at stage III, suggesting that *ZmSuSy* might play differential roles in sucrose synthesis and degradation during different development stages. Invertase (INV) irreversibly cleaves sucrose into glucose and fructose and is very active in rapidly elongated meristems, as well as in rapidly developed young organs or tissues (Zhu et al., 2018). Here, the expression of *ZmINV* tended to increase at stages III and IV, which indicated that *ZmINV* acted as a regulator to provide glucose and fructose for seed germination and seedling establishment.

Starch acts as the major carbohydrate storage form in mature seeds for degradating to generating energy and metabolites during germination and seedling growth (Tang et al., 2017). In the present study, the starch content significantly increased together with the decreased content of glucose from stage I to II. These results revealed a higher starch synthesis metabolism in seed maturation, thus ensuring an energy reserve for germinating seedlings. These findings are similar to studies on the seed development process in *Posidonia oceanica*, abundant seed storage (starch) appears to be a

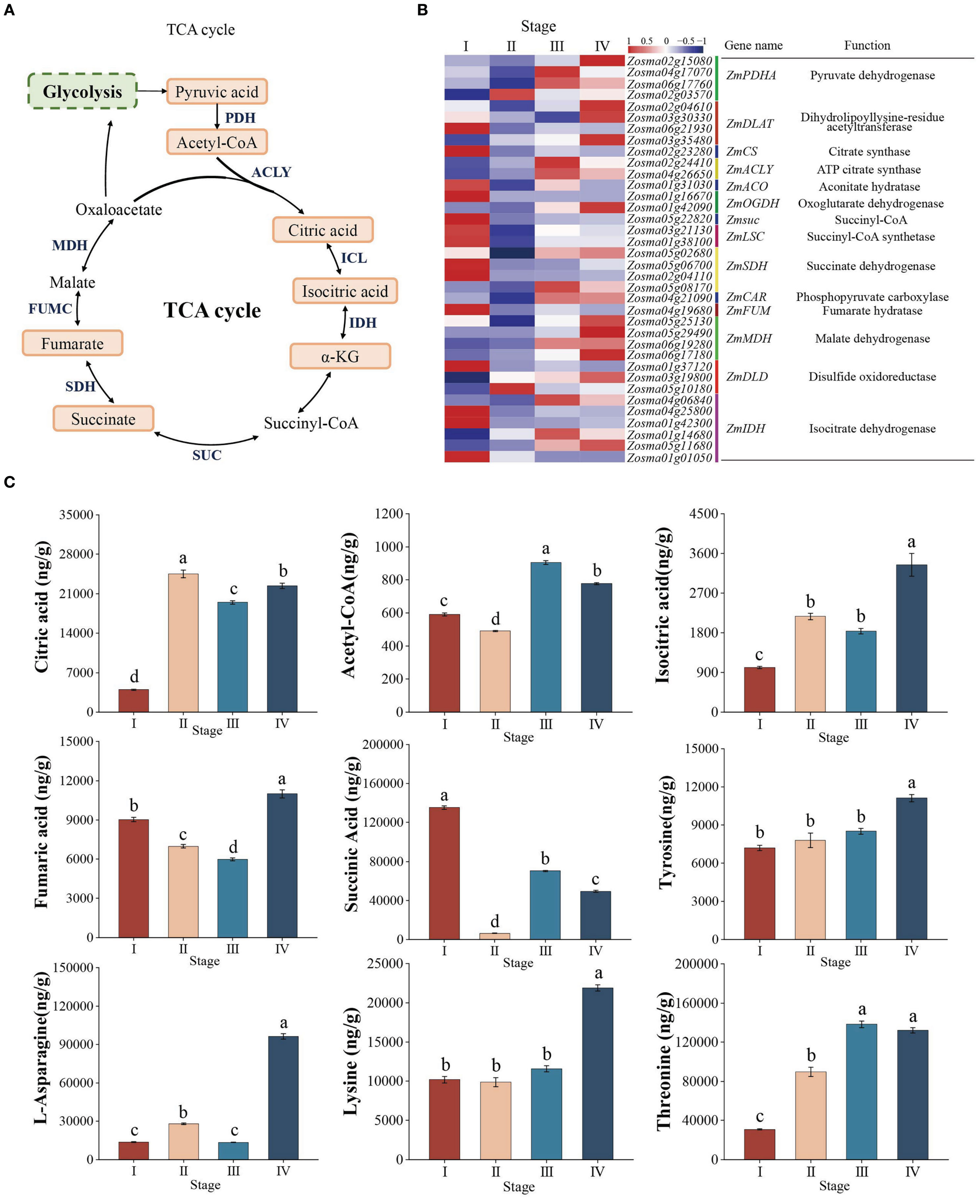

FIGURE 7
TCA cycle. **(A)** Model and key components of the TCA cycle in *Zostera marina* seeds. The highlighted boxes indicate the metabolites quantified from the LC–MS/MS results. **(B)** Heatmap analysis of DEGs (log 10 (FPKM) values) involved in the TCA cycle. **(C)** Analysis of changes in the represented metabolites in the TCA cycle during *Zostera marina* seed development.

characteristic, and should be favorable for seedling establishment in a marine environment (Belzunce et al., 2005). Previous studies on the development of *Glycine max* seeds showed that the decrease in hexose phosphates (G6P, G1P, and FDP) and the nucleotide sugar UDPG, which were precursors to carbohydrate production, was accompanied by the accumulation of starch in the mature seed (Kambhampati et al., 2021). Our study showed that G6P, G1P and FDP decreased first from stage I to II and then increased from stage II to IV, while the content of UDPG and starch showed the opposite trend. This suggests that starch–sugar interconversion occurs throughout *Z. marina* seed development.

Starch synthesis occurs through the activity of three major enzymes: AGPase, SS, and UGPase. This systematic interaction of multienzymes gradually leads to synthesized starch (Chopra et al., 2005; Smith and Zeeman, 2020). Studies have demonstrated that the activity of AGPase and UGPase is correlated with the starch content and that starch synthase activity increases with the accumulation of starch during the early development of mung bean seeds (Chopra et al., 2005). We identified six genes encoding the main enzymes of starch biosynthesis. *ZmAGP*s and *ZmSS*s were upregulated at stage II and the activity of AGPase increased, which enhanced the carbon flux into starch. β-glucosidase (bglX) and amylase are the key enzymes involved in the process of starch decomposition, affecting seed germination and seedling growth (Zhao et al., 2022). α-amylase is a major carbohydrase that catalyzes the hydrolysis of α-(1, 4) glycosidic linkages in starch, glycogen, and related polysaccharides (Huang et al., 2019). The enzymes bglX belong to the glycosylhydrolase family, which catalyze the hydrolysis of glycosidic bonds and hydrolyze compounds such as cellobiose into glucose to regulate plant growth and development (Huang et al., 2019). In this study, most *ZmbglX*s along with *ZmAMY*s were upregulated, and α-amyase activity was significantly increased from stage II to IV, thereby accelerating starch metabolism and generating glucose. The glucose content increased to the highest level at stage IV, indicating that the processes of starch consumption and glucose accumulation were active at this stage. The findings was similar to previous findings in other seagrass seeds. The starch stored in mature seagrass seeds (*Thalassia hemprichii* and *Posidonia* species: *P. australis*, *P. sinuosa* and *P.coriacea*) were presumably hydrolyzed to sugar and usedto nourish early seedling growth (Kuo and Mccomb, 1989; Kuo and Kirkman, 1990; Kuo et al., 1990; Kuo et al., 1991).

4.2 Glycolysis provides an important pivotal substance for the seed germination process

Glycolysis is a main catabolic pathway of glucose metabolism and can provide pyruvate to the TCA cycle and mitochondrial respiration for further ATP production (Zhao and Assmann, 2011; Chang et al., 2017). Glucose 6-phosphate (G6P) and phosphoenolpyruvate (PEP) can be readily converted to pyruvate (Lim et al., 2019). A previous study indicated that the overall pyruvate level is the key indicator in the control of glycolysis, entering the TCA cycle and releasing ATP to stimulate plant growth (Lim et al., 2019). In our study, glucose consumption increased with an increase in G6P, PEP, and pyruvate at stages III and IV, suggesting that the glycolysis pathway was active during germination and seedling establishment, while it was severely inhibited during maturation.

Phosphofructokinase (PFK) and pyruvate kinase (PK) are important control points in the glycolytic pathway, as they catalyze two irreversible steps (He et al., 2019). PFK regulates the level of fructose-1,6-bisphosphate, a major pathway that generates ATP from glucose (Li et al., 2013). PK catalyzes the conversion of phosphoenolpyruvate into pyruvate during glycolysis (Zeng et al., 2019). A previous study demonstrated that *OsPK1* was involved in plant morphological development, and the disruption of *OsPK5* function resulted in slow germination and seedling growth, blocked glycolytic metabolism, caused glucose accumulation, and decreased energy levels (Yang et al., 2022). In this experiment, from stage I to II, *ZmPFK* and *ZmPK* expression was downregulated, followed by a rapid increase in expression until stage IV when G6P, pyruvate, and PEP accumulated, implying the critical roles of these genes in glycolytic metabolite accumulation during *Z. marina* seed germination and seedling growth.

In addition, overexpression of a cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (*OsGAPC3*) leads to a high germination rate in rice seeds (Tan et al., 2013). Enolase (ENO) catalyzes the synthesis of 2-Phospho-D-glyceric acid (2-PGA) and PEP in the glycolytic pathway, thereby providing organic acids for seed germination (Østergaard et al., 2004; Liu et al., 2018). In our study, *ZmGAPDH*s and *ZmENO*s were actively expressed at stages III and IV, which may have accelerated glycolytic metabolism to provide 2-PGA and PEP for *Z. marina* seed germination and seedling growth. Notably, genes and metabolites associated with the biological processes of glycolysis were downregulated during *Z. marina* seed maturation and then upregulated during germination. It can be speculated that these changes may have a positive effect on seed germination and are thus repressed, maintaining a low level of metabolic activity during seed maturation to preserve seed viability and enable optimal operation.

4.3 TCA cycle becomes the main source of energy during *Z. marina* seed development

The TCA cycle is the final metabolic pathway for the decomposition of pyruvate and fatty acids, which enter as acetyl-CoA and are further oxidized to produce ATP (Liu et al., 2019; Dong et al., 2021; Gostomska-Pampuch et al., 2021). In this study, an increase in acetyl-CoA was accompanied by an increase in free fatty acids from stage II to IV, indicating that free fatty acids released from triacylglycerol are degraded to acetyl-CoA with the release of energy during *Z. marina* seed germination. Interestingly, although the content of pyruvate increased at stages III and IV, the pyruvate content decreased significantly compared with stage I, indicating that a large amount of pyruvate produced by glycolysis was broken down into acetyl-CoA and entered the TCA cycle. Meanwhile, acetyl-CoA entering the TCA cycle was oxidized to organic acids and produced large amounts of ATP, which provided energy for plant growth and development (Zhang and Fernie, 2018; Liu et al., 2019). In our study, the contents of organic acids (succinic acid, fumaric acid, citric acid, and isocitric acid) and ATP significantly increased at stages III and IV, indicating that the TCA cycle provides many intermediates (organic acids) and energy sources for *Z. marina* seed germination and seedling growth.

The expression of *MDH*, *ACO*, *FUMC*, or *PDH* encoding malate dehydrogenase, aconitase, fumarate hydratase, and pyruvate dehydrogenase, respectively, improved respiratory activity by regulating organic acid synthesis during seed germination and promoted germination (Liu et al., 2019). Our results showed that *ZmACO*s, *ZmFUMC*s, *ZmMDH*s, and *ZmPDC*s maintained a high expression level along with massive organic acid accumulation at stages III and IV, implying the critical roles of these genes in regulating the efficiency of the TCA cycle to increase energy production during seed germination.

Apart from decomposition of the glucose and fatty acids, another pathway feeding the TCA cycle is the protein catabolism (Mei et al., 2021); proteins derive from seagrasses seeds were hydrolyzed into amino acids by proteases during seed germination (Kuo et al., 1990). Acetyl CoA was not only an oxidative breakdown product of sugars and fatty acids, but also a metabolite of certain amino acids (Galili et al., 2014; Mei et al., 2021). Our study showed that the content AAs generated from protein breakdown w significantly increased from stage II to IV. Several studies have found that the added aspartic acid may enter the TCA cycle, thereby used to replenish intermediates for the TCA cycle (Vigani et al., 2016; Gao et al., 2019; Zhou et al., 2020). For example, the aspartic acid (Asp) family such as Lys, Thr, and Asn provide carbon skeletons to synthetic acetyl-CoA, which may enhance the cycle flux for the TCA cycle (Vigani et al., 2016; Su et al., 2017). In addition, catabolism of Tyr also had important effects on the synthesis of the TCA cycle metabolite fumarate succinate (He et al., 2019). In our study, the content of these amino acids was significantly increased from stage II to IV, indicating that the catabolism of amino acids provided the supply of materials to ensure efficient operation of the TCA cycle during *Z. marina* seed germination and seedling establishment.

4.4 Pentose phosphate pathway is activated during *Z. marina* seed germination and seedling establishment

The pentose phosphate pathway is a supplementary source of cellular energy that can directly oxidize sugars and complement glycolysis to provide the cell with NADPH and materials to promote seed germination (Wang et al., 2021). During the development of *Arabidopsis*, G6P is metabolized to erythrose 4-phosphate, ribulose 5-phosphate, and sedoheptulose 7-phosphate with the release of NADPH and ATP through the pentose phosphate pathway to promote plant development (Andriotis and Smith, 2019). Subsequently, sugar phosphate is converted to fructose 1,6-bisphosphate and then feed back into glycolysis (Wang et al., 2015; He et al., 2018). From stage II to IV, an increase in the content of erythrose 4-phosphate, ribulose 5-phosphate, and sedoheptulose 7-phosphate was accompanied by an increase in the intermediate metabolites of glycolysis, indicating that the pentose phosphate pathway was also an energy supply pathway to a certain extent during *Z. marina* seed germination.

Glucose 6 phosphate dehydrogenase (G6PD) and phosphogluconic acid dehydrogenase (PGD) are rate-limiting enzymes of glucose oxidative decomposition in the pentose phosphate pathway; and their activity reflects the activation state of this pathway (Yang et al., 2019). Other studies have found that an increase in G6PD, PGD, transketolase (TK), and transaldolase (TAL) activity can promote the transition of *Euscaphis konishii* seeds from dormancy to germination (Zhang et al., 2015). In our study, transcriptome analysis showed that from stage II to IV, the expression levels of *ZmG6PD*, *ZmPGD*, *ZmtktA*, and *ZmtalA* were upregulated when erythrose 4-phosphate, ribulose 5-phosphate, and sedoheptulose 7-phosphate accumulated, implying that the pentose phosphate pathway mought be activated during *Z. marina* seed germination.

5 Conclusion

In this study on the entire developmental process of *Z. marina* seed, we used transcriptomic and metabolomic approaches to analyze the dynamic physiological processes and molecular mechanisms, unveiling many novel findings. Starch and sucrose metabolism plays an indispensable role in maintaining the dynamic balance of sugar substances and energy homeostasis for the entire developmental process of *Z. marina* seed by regulating the interconversion of starch and sugar. The glycolysis pathway was inhibited at maturation but was activated and produced pyruvate to enter the TCA cycle at germination and seedling establishment. The TCA cycle was proved to be the more effective pathway to supply energy for *Z. marina* germination and seedling growth through an upregulation in a large number of genes and metabolites. The pentose phosphate pathway, as a metabolic bypass of glycolysis, receives its substrate from glycolysis and feeds its products back into glycolysis, thereby playing a supplementary pathway role during seed germination and seedling establishment. Collectively, the results provides new insights into changes in metabolic pathways throughout the development of *Z. marina* seeds, and provides solid evidence for large-scale restoration of seagrass beds using seed methods.

Data availability statement

The original contributions presented in this study can be found in the article/Supplementary Material. The RNA-Seq raw sequence data presented in the study are deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) repository, accession number SRP425505.

Author contributions

MZ: investigation, methodology, data curation, formal analysis, software, writing – original draft. YZ: investigation, methodology, formal analysis, conceptualization, funding acquisition, resources, validation, supervision, writing – review & editing. JC: software, visualization. SS: software, visualization. SX: software, visualization. XZ: investigation, methodology. XT: data curation, conceptualization, funding acquisition, project administration, validation, supervision, writing – review & editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1130292/full#supplementary-material

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EDITED BY
Glenn Hyndes,
Edith Cowan University, Australia

REVIEWED BY
Panayiotis G. Dimitrakopoulos,
University of the Aegean, Greece
Wensheng Bu,
Jiangxi Agricultural University, China

*CORRESPONDENCE
Mirta Teichberg
✉ mteichberg@mbl.edu

†PRESENT ADDRESS
Agustín Moreira-Saporiti,
The Ecosystems Center, Marine Biological
Laboratory, Woods Hole, MA, United States
Mirta Teichberg,
The Ecosystems Center, Marine Biological
Laboratory, Woods Hole, MA, United States

‡These authors share last authorship

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A trait-based framework for seagrass ecology: Trends and prospects

Agustín Moreira-Saporiti[1,2†], Mirta Teichberg[2*†], Eric Garnier[3], J. Hans C. Cornelissen[4], Teresa Alcoverro[5], Mats Björk[6], Christoffer Boström[7], Emanuela Dattolo[8], Johan S. Eklöf[6], Harald Hasler-Sheetal[9], Nuria Marbà[10], Lázaro Marín-Guirao[8,11], Lukas Meysick[7,12,13], Irene Olivé[8,14], Thorsten B. H. Reusch[15], Miriam Ruocco[8], João Silva[16], Ana I. Sousa[17], Gabriele Procaccini[8‡] and Rui Santos[16‡]

[1]Faculty for Biology and Chemistry, University of Bremen, Bremen, Germany, [2]Algae and Seagrass Ecology Group, Department of Ecology, Leibniz Centre for Tropical Marine Research, Bremen, Germany, [3]CEFE, Univ Montpellier, CNRS, EPHE, IRD, Montpellier, France, [4]Systems Ecology, A-LIFE, Vrije Universiteit Amsterdam, Amsterdam, Netherlands, [5]Centre d'Estudis Avançats de Blanes, Blanes, Spain, [6]Department of Ecology, Environment and Plant Sciences (DEEP), Stockholm University, Stockholm, Sweden, [7]Åbo Akademi University, Environmental and Marine Biology, Åbo, Finland, [8]Department of Integrative Marine Ecology, Stazione Zoologica Anton Dohrn, Naples, Italy, [9]University of Southern Denmark, Odense, Denmark, [10]Global Change Research Group, Institut Mediterrani d'Estudis Avançats (IMEDEA, CSIC-UIB), Esporles Illes Balears, Spain, [11]Oceanographic Center of Murcia, Spanish Institute of Oceanography (IEO-CSIC), Murcia, Spain, [12]Helmholtz Institute for Functional Marine Biodiversity (HIFMB) at the University of Oldenburg, Oldenburg, Germany, [13]Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research, Bremerhaven, Germany, [14]School of Geographical and Earth Sciences, University of Glasgow, Glasgow, United Kingdom, [15]Marine Evolutionary Ecology, Division of Marine Ecology, GEOMAR Helmholtz Center for Ocean Research Kiel, Kiel, Germany, [16]Centro de Ciências do Mar, Universidade do Algarve, Campus de Gambelas, Faro, Portugal, [17]CESAM – Centre for Environmental and Marine Studies, Department of Biology, University of Aveiro, Campus Universitário de Santiago, Aveiro, Portugal

In the last three decades, quantitative approaches that rely on organism traits instead of taxonomy have advanced different fields of ecological research through establishing the mechanistic links between environmental drivers, functional traits, and ecosystem functions. A research subfield where trait-based approaches have been frequently used but poorly synthesized is the ecology of seagrasses; marine angiosperms that colonized the ocean 100M YA and today make up productive yet threatened coastal ecosystems globally. Here, we compiled a comprehensive trait-based response-effect framework (TBF) which builds on previous concepts and ideas, including the use of traits for the study of community assembly processes, from dispersal and response to abiotic and biotic factors, to ecosystem function and service provision. We then apply this framework to the global seagrass literature, using a systematic review to identify the strengths, gaps, and opportunities of the field. Seagrass trait research has mostly focused on the effect of environmental drivers on traits, i.e., "environmental filtering" (72%), whereas links between traits and functions are less common (26.9%). Despite the richness of trait-based data available, concepts related to TBFs are rare in the seagrass literature (15% of studies), including the relative importance of neutral and niche assembly processes, or the influence of trait dominance or complementarity in ecosystem function provision. These knowledge gaps indicate ample potential for further research, highlighting the need to understand the links between the unique traits of seagrasses and the ecosystem services they provide.

KEYWORDS

functional ecology, trait-based approach, seagrass traits database, ecosystem service vulnerability, response-effect framework

1 Introduction

Trait-based response-effect frameworks (hereafter TBFs) have been extensively used in terrestrial plant ecology (Suding et al., 2008; Díaz et al., 2013). TBFs are based on the study of traits, which capture the form and function of organisms, and are defined as "any morphological, physiological or phenological heritable feature measurable at the individual level, from the cell to the whole organism, without reference to the environment or any other level of organization" (Violle et al., 2007 as modified by Garnier et al., 2016). Traits are categorized into response and effect traits. Hence, the structure of a plant community is the result of the environmental filters and biotic interactions that exclude phenotypes that do not possess appropriate response trait values (Weiher and Keddy, 1995; Díaz et al., 1998; Belyea and Lancaster, 1999). Effect traits, on the other hand, influence how the organism affects ecosystem functions and they are therefore controlled by the distribution of trait values shaping the community (Garnier et al., 2016).

There are many examples of the use of TBFs in terrestrial plant ecology. Functional trait diversity explains more variation of community biomass than species richness (Roscher et al., 2012); community-weighted means of leaf dry matter content can be used to explain variations in digestibility, which is a critical component of herbage nutritive value, a major service delivered by grasslands (Gardarin et al., 2014); litter decomposition is not only controlled by the abiotic environment, but mostly by species-level plant traits (Cornwell et al., 2008; Tardif et al., 2014). The general relevance of the TBF to the study of terrestrial plant ecology has triggered its development in marine ecology (e.g. Solan et al., 2004; Follows et al., 2007; Andersen and Pedersen, 2009; Edwards et al., 2013; Elleouet et al., 2014). TBFs enable generalized predictions of community composition and function of any type of ecosystem across organizational and spatial scales, independent of taxonomy (Shipley et al., 2016), which allows for the testing of a variety of ecological hypotheses. To illustrate the concepts that have been developed in trait-based research, a conceptual TBF has been compiled (Figure 1), based on the seminal works by Lavorel and Garnier (2002) and Suding et al. (2008), which also considers phylogeny (Díaz et al., 2013) and intraspecific variability (Violle et al., 2012) using modern analytical methods (Mouillot et al., 2013).

First, community assembly processes encompass the mechanisms underlying the composition and structure of communities in response to environmental variation (McGill et al., 2006; Weiher et al., 2011; Grime and Pierce, 2012), from dispersal to the influence of abiotic and biotic factors. For plants, dispersal into a local community (Figure 1: Filter 1) is partly controlled by stochastic processes occurring at a geographical scale and random local events, which drive colonization and local extinctions respectively and are poorly related to the traits of organisms (Weiher et al., 2011). The abiotic filter (Figure 1: Filter 2) determines which species can establish due to the influence of local environmental conditions, the availability of resources, and the disturbance regime (Wilson, 2011). This defines the fundamental niche of the species. The biotic filter (Figure 1: Filter 3) corresponds to the positive and negative interactions between living organisms within communities and determines the set of coexisting neighboring species (Tilman, 1985). It is the realized niche of the species along the range of possibilities from competitive exclusion (Gause, 1937) to facilitation (Maxwell et al., 2017). These niche assembly processes define how local communities assemble from the regional species pool through the filtering of abiotic and biotic factors (Keddy, 1992) that, together with stochastic processes, explain the characteristics of local communities (Vellend, 2010).

To understand which metrics might be useful for detecting which assembly process predominates in shaping a community, it is helpful to envisage species trait values as coordinates (e.g. along axes of variation in multivariate analysis such as principal component analysis) locating species in the functional space (see Mouillot et al., 2013). Studying whether the functional coordinates of a species are sorted out from the local pool is random or the consequence of their response to the environmental drivers provides the grounds to test the niche and neutral assembly theories (Garnier et al., 2016) (Figure 1: Hypothesis #1).

The functional trait structure of the local community can be convergent (showing high similarity among functional traits in co-existing species) or divergent (showing dissimilarity among functional traits in co-existing species) (Grime, 2006; Cornwell and Ackerly, 2009; Bernard-Verdier et al., 2012; Gross et al., 2013) depending on the relative importance of the abiotic and biotic filtering on the community (Figure 1: Hypothesis #2). Abiotic factors tend to dominate the trait distributions when they set major physico-chemical constraints on the ecosystem, which then leads to a convergent distribution, whereas biotic factors dominate when there are few or weak abiotic constraints and there is room for increasing competition (Weiher et al., 1998; Grime, 2006), which

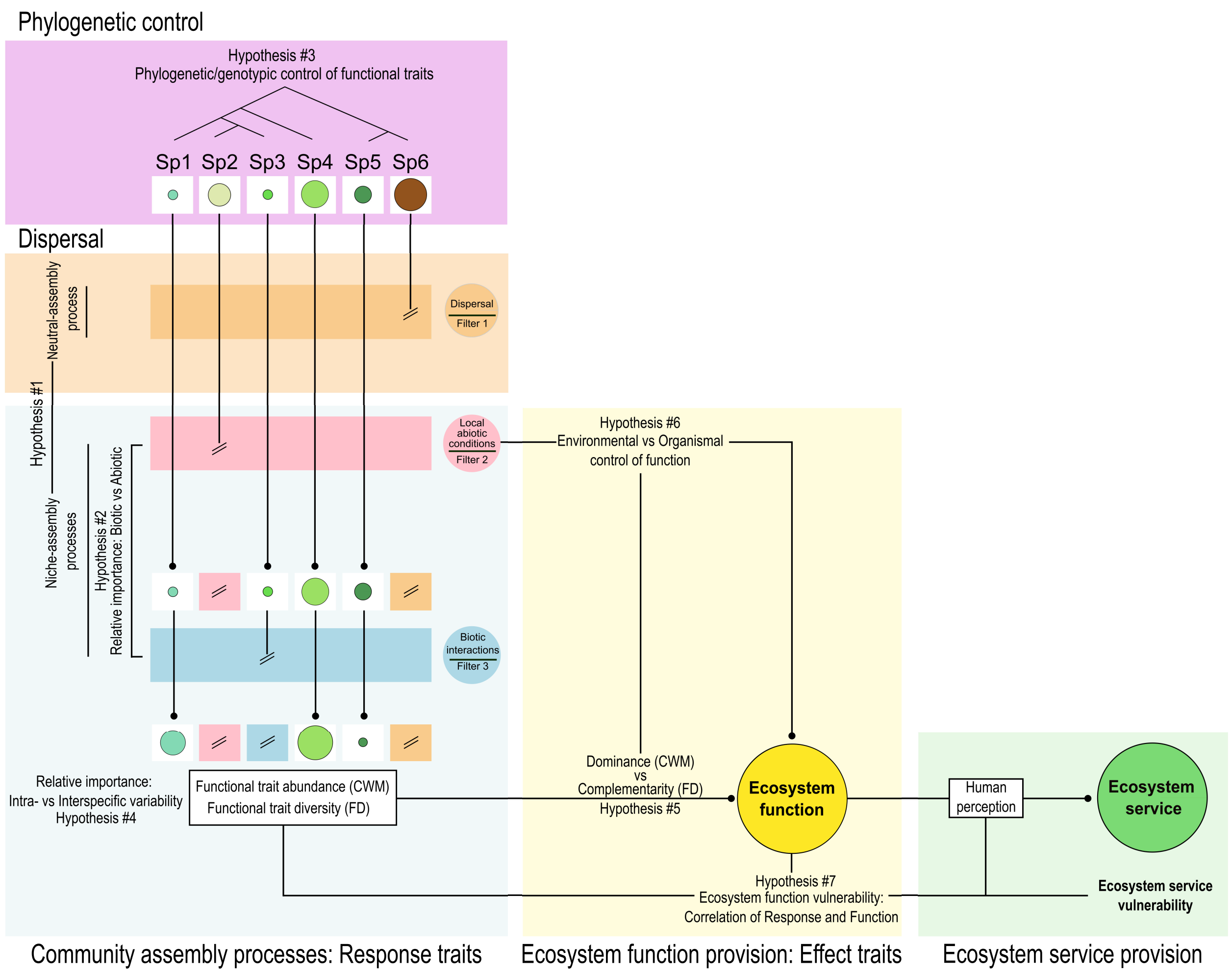

FIGURE 1
Conceptual TBF (trait-based response-effect framework) synthetized combining the concepts introduced by Lavorel and Garnier (2002); Suding et al. (2008); Violle et al. (2012); Díaz et al. (2013) and Mouillot et al. (2013). A given array of species at the regional level (Sp1-Sp6) are influenced by several filters until the final local community assemblage. The circles indicate the species abundance at the regional level (top) and at the local level after the filters (bottom). Traits can be used to study if community assemblage is a neutral or niche assembly process, meaning that it is mainly stochastic or affected by environmental drivers (Filters 1 and 2, Hypothesis #1). Once settled, the relative importance of the abiotic filter (i.e. environmental drivers) and the biotic filter (Filter 3) (i.e. competition for resources and biotic interactions) can be studied through trait convergence and divergence (Hypothesis #2). Functional trait abundance (CWM) and diversity (FD) can be calculated in a community to study the influence of traits in ecosystem function delivery (Hypothesis #5). Functional traits can, however, be phylogenetically controlled (Hypothesis #3) and their inter- and intraspecific variability may change among species and communities (Hypothesis #4). Ecosystem function delivery not only depends on traits, but also the environmental constraints may play a central role in it (Hypothesis #6). The correlation of the ecosystem function delivery and response will determine the function vulnerability (Hypothesis #7). Finally, ecosystem functions and their perception by humankind determine the ecosystem services provided and, therefore, their vulnerability.

tends to lead to competitive exclusion and thereby a divergent distribution (but see discussion in Mayfield and Levine, 2010).

Functional traits can be phylogenetically conserved or they can diverge strongly at the tips of the phylogeny, thereby reflecting relatively recent evolutionary trait change (Figure 1: Hypothesis #3). Therefore, the measurement of phylogenetic diversity (PD) can be an indicator of functional trait diversity (FD) (Forest et al., 2007), even though there is a considerable debate on this topic (Garnier et al., 2016). Indeed, the correlation between PD and FD is not universal, and high PD can generate many assemblages that have a lower FD than randomly chosen sets of species (Mazel et al., 2018).

Intraspecific trait variability can constitute a relatively large part of overall community-level trait variability (Violle et al., 2012). Therefore, it is fundamental to determine the relative importance of inter- vs intraspecific variability (Figure 1: Hypothesis #4). Violle et al. (2012) showed the importance of including intraspecific variability to get a better understanding of the environmental filters acting on the vegetated community rather than using mean trait values per species present in the community. This was a revision of the concepts of alpha and beta niches (Pickett and Bazzaz, 1978), which allow understanding the effects of environmental filters on intraspecific and interspecific trait variability (Ackerly and Cornwell, 2007).

Effect traits allow to scale up from the functioning of an individual to that of ecosystems (Grime, 1998; Chapin et al., 2000; Lavorel and Garnier, 2002; Díaz et al., 2007). Two different and non-exclusive hypotheses have been formulated to relate the functional structure of communities to ecosystem properties: dominance (mass-ratio effect) and niche complementarity. The dominance hypothesis stipulates that the functional traits of the dominant species will be the predominant influence on the ecosystem function (Grime, 1998; Smith and Knapp, 2003), this being proportional to its abundance in the community (Garnier et al., 2004; Díaz et al., 2007; Violle et al., 2007). The metric used to test this hypothesis is the community weighted mean (CWM). By contrast, the niche complementarity hypothesis stipulates that the presence of functionally different species, which use environmental resources in a complementary manner, will positively influence ecosystem functioning (Loreau and Hector, 2001; Tilman, 2001; Eviner and Chapin, 2003; Díaz et al., 2006; Petchey and Gaston, 2006). It is therefore hypothesized that positive relations exist between ecosystem functions and functional diversity (FD). These two hypotheses are not mutually exclusive, and it is possible that both are important in influencing ecosystem functions (Díaz et al., 2007, Figure 1: Hypothesis #5). More evidence has been found, however, for a relation between dominance and function (Garnier et al., 2016). A drawback in the study of function provision is that some functions may not be correlated with traits under constraining environmental factors, not allowing for the determination of causality between trait and function. Environmental factors should be, therefore, controlled for in a "common garden" or statistically with structured equation models (Grace et al., 2007; Shipley, 2010) (Figure 1: Hypothesis #6) to disentangle the links between environment, trait and function.

Ecosystem services are defined as the capacity of natural processes and components to provide goods and services that satisfy human needs, directly or indirectly (de Groot et al., 2002). The definition of an ecosystem service is contingent upon human perception and needs, and therefore each ecosystem service has underlying functions that are biologically measurable. The importance of the concept of ecosystem service is the possibility to integrate ecosystem functions in management and policy. Díaz et al. (2013) introduced the concept of ecosystem service vulnerability, based on the idea that the security of ecosystem functions depends on how the effects and tolerances of organisms (which both depend on combinations of functional traits) correlate across species. Therefore, the correlation of the response and effect traits of organisms can determine the vulnerability of an ecosystem function (Figure 1: Hypothesis #7). The final step in the TBF proposed above is the translation of effect traits from ecosystem functions to ecosystem services. Effect traits driving ecosystem service provision are, therefore, a tool to understand the link between organism, function and service, and the vulnerability of the service provision under a changing environment.

Despite the wide application of TBFs in terrestrial plant ecology, its application has been very scarce in seagrasses. Seagrasses are a polyphyletic group of basal monocotyledonous angiosperms belonging to four families in the Alismatales: Posidoniaceae, Zosteraceae, Cymodoceaceae and Hydrocharitaceae. Limited to coastal areas, they occupy a global surface of about 160 387 km^2 (<0.2% of the ocean's surface, McKenzie et al., 2020). The colonization of marine habitats from terrestrial wetland habitats occurred exclusively from this monocotyledonous order and took place in four independent and parallel evolutionary events (Les et al., 1997; Janssen and Bremer, 2004; Waycott et al., 2006). From an evolutionary timescale perspective, this colonization was contingent upon a number of critical adaptations, which partially reverted many of the original key adaptations of flowering plants to terrestrial life. These adaptations are reflected in specific genomic losses and gains (Golicz et al., 2015; Lee et al., 2016; Olsen et al., 2016; Lee et al., 2018), with adaptive changes in sets of genes associated with central biological pathways (Wissler et al., 2011). Despite their successful adaptation to the marine realm and wide distribution in most coastal areas around the world, seagrasses exhibit very low species richness (60-70 species) compared to other groups in the Alismatales, which is possibly partially compensated by pronounced local adaptation (or intraspecific variability) within species (e.g. Jueterbock et al., 2016; Dattolo et al., 2017; Jahnke et al., 2019).

All seagrass species share a similar morphology with basal meristems that form strap-like leaves grouped in shoots connected by rooted rhizomes in the sediment. Their low morphological diversity is possibly the result of a convergent evolution to the submerged lifestyle in a hydrodynamically active and saline environment (Arber, 1920; Les et al., 1997). Unfortunately, the coastal habitat colonized by seagrasses is under high and increasing anthropogenic pressure. Consequently, seagrasses are under decline worldwide due to multiple local (Burkholder et al., 2007; Unsworth et al., 2018; Moreira-Saporiti et al., 2021a) and global pressures (Orth et al., 2006; Waycott et al., 2009; Turschwell et al., 2021). Reversal of this negative trend, however, is possible (Lefcheck et al., 2018; de los Santos et al., 2019; Sousa et al., 2019; Dunic et al., 2021; Turschwell et al., 2021) when appropriate management and conservation actions are implemented.

Much seagrass research to date has measured responses in various plant traits to environmental variation, to i) better understand seagrass biology and ecology (Sousa et al., 2017), ii) prevent their decline (Fernandes et al., 2019), iii) restore degraded ecosystems (Paulo et al., 2019; Lange et al., 2022), or iv) predict their fate under future global change scenarios (Hyndes et al., 2016). Synthesis of the existing data on seagrass response to the environment has been used to identify potential indicators for assessing the health of seagrass ecosystems (Roca et al., 2016). Additionally, there is a good understanding that the sole presence of seagrass is enough for the provisioning of functions like invertebrate habitat (Virnstein et al., 1983) or the modification of the inorganic carbon system (Unsworth et al., 2012). The provisioning of these functions, however, must be underpinned by the traits of the component species or genotypes, but the link between seagrass traits and functions has been resolved in only a handful of examples (e.g. Fonseca and Callahan, 1992; Hendriks et al., 2008; Gustafsson and Boström, 2011; Hendriks et al., 2014). At present, we lack a comprehensive picture and predictive framework of how key seagrass traits underpin the resistance and resilience of seagrass species to current and future pressures, and their relation to ecosystem functions and services.

In order to push seagrass research forward, we compiled the existing knowledge on seagrass trait research and pointed its knowledge gaps and research possibilities. We carried out a systematic review of the seagrass literature with the goal of quantifying the use of TBFs in the assessment of seagrass responses, ecosystem functions and services and to identify the gaps of knowledge in this field, including (1) how frequently trait-based research has been adopted in seagrass ecological research and how many of these studies could be classified as TBFs (as defined by the seminal work from Lavorel and Garnier, 2002), (2) which of the methodologies, hypotheses and theories introduced by TBFs have been already studied in seagrass communities in relation to their traits and (3) identify under- and over-studied traits, drivers and functions in seagrass research, with examples from the literature. The conceptualization of the results of the literature review under a TBF will allow the exploration of the research gaps and indicate future research pathways in seagrass ecology, specifically focusing on the ecosystem function and service provision and vulnerability.

2 Methods

We followed the ROSES protocol (Haddaway et al., 2018) for a literature review (metadata of the review can be found in the Supplementary Material 1). We identified 21,100 publications of potential relevance within the Google Scholar database using the query "Seagrass trait" and "*Seagrass species* trait" ("*Seagrass species*" being the currently accepted names of all seagrass species). To guarantee that the focus of the publication was on the study of trait-based research, the word trait had to be present in the title, abstract and/or keywords of the publication, elsewise the publication was not included in the review process. We acknowledge that this search query would leave out literature studying seagrass traits, but not using the terminology "trait". However, this was the only way to ensure the focus of the review in the study of seagrass traits and trait-based research. The number of publications was limited to those in English. The temporal range of the sample was restricted to the limitations of the database itself, i.e. publications included the range from 1988 through March 2022. Using the above screening criteria, the initial number of publications was reduced to 380. From these 380 publications, 137 were discarded as they referred to the study of seagrass-associated fauna, benthic macroalgae within seagrass meadows and seagrass epiphytes; 12 duplicates and 19 misclassified publications were also discarded. 19 more publications were discarded as they were gray literature. The final database was sized down to 193 relevant publications. The complete database with the categorization of the publications can be found in the Supplementary Material 2.

For goal (1), we counted the number of studies including the word "trait" and the number of studies in which an existing TBF (as defined by Lavorel and Garnier, 2002) was used to test a hypothesis or research question. For goal (2), we categorized the studies in *a priori* categories derived from the TBF presented above (Figure 1). Lastly, for goal (3), we created an *a priori* classification of seagrass traits and *a posteriori* classification with the environmental drivers and ecosystems functions found in the literature. We made the final figures using the software R with the package ggplot (Wickham, 2016; R Core Team, 2022) and InkScape (v 0.92).

3 Results and discussion

3.1 Seagrass TBF studies and studies including the word trait

Trait-based response-effect frameworks, TBFs, are currently underexplored in seagrass research. The number of studies including the word "trait" increased steadily since the first study from the year 1988, reaching a maximum in the last five years (n=73, Figure 2A). Only 29 studies were found to use existing TBFs, accounting for only 15% of the total. The "trait-based approach" was first developed in 2002 (Lavorel and Garnier, 2002) for terrestrial plants, and it does not appear in the seagrass literature until 2012. This indicates that the body of knowledge available from terrestrial plant ecology has been under-utilized by seagrass researchers.

Studies focusing on seagrass traits have been mainly developed in the Mediterranean bioregion (27%, Figure 2B), while the Tropical Atlantic (9%) and Temperate Southern Oceans (11%), showed the lowest number of studies. TBF studies have been homogeneously performed in all bioregions, with the Temperate North Pacific showing the highest number (24%). Differences across bioregions could be attributed to the differential use of the term "trait" across research groups and the seagrass species that are the focus of their study. As a consequence, while the widespread species *Zostera marina* accounted for 30% of trait studies, *Posidonia oceanica*, endemic to the Mediterranean Sea, accounted for 19% (Figure 2C). This result indicates a certain bias in the use of the trait nomenclature in certain species like *P. oceanica*, while it simultaneously highlights the problem of research bias and inference from unique species to the others, specifically when trait responses (Viana et al., 2020) can be species-specific.

3.2 Dispersal and settlement in seagrass communities: Challenging the neutral assembly theory

We found seven studies that linked seagrass dispersal and settlement to traits (3.6% of the total, Figure 3), suggesting that seagrass dispersal can be predicted by the traits. Target traits can change according to the life stage of the seagrass plant (seed or vegetative fragment) (Orth et al., 2007; McMahon et al., 2014; Bryan-Brown et al., 2017). In the case of seeds, traits such as flotation capacity and digestibility determine the distance they can disperse and the effectiveness of using animal vectors, respectively (Wu et al., 2016). Seeds also show a high degree of intraspecific variability in size, which determines their settling velocity and dispersal potential (Delefosse et al., 2016). When it comes to settlement, traits like germination rate can be site specific and negatively affected by increasing temperature in *Z. marina* (Cabaço and Santos, 2010). In addition, the current velocity in the settlement area and the stiffness and flexibility of surrounding shoots limit the settlement capacity of seagrass seeds (Bouma et al., 2009). In the case of vegetative fragments, the plant

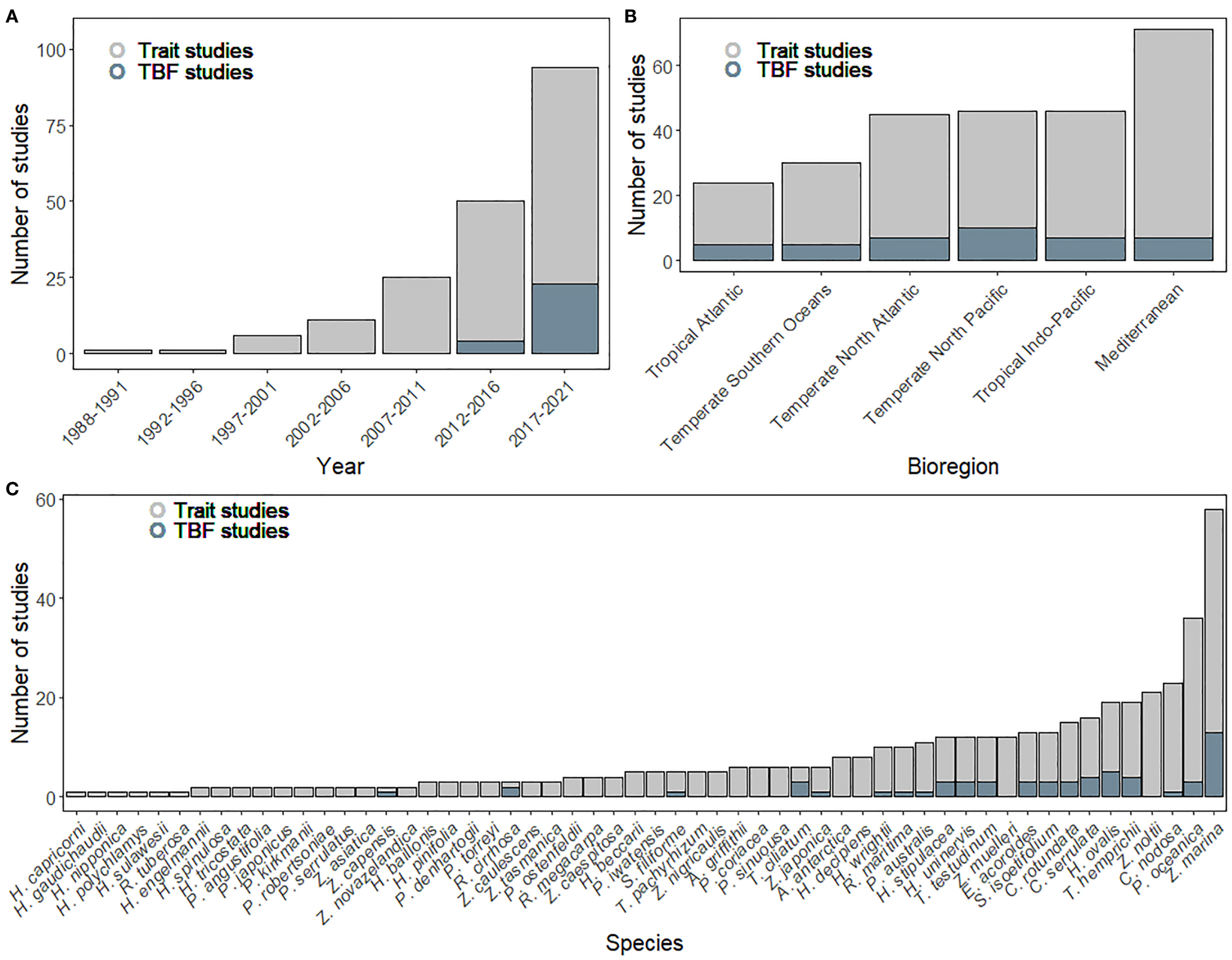

FIGURE 2

Number of studies included in the systematic review classified by **(A)** Year, **(B)** Bioregion and **(C)** Seagrass species. The light gray color indicates the number of studies including the word "trait" and the dark gray color the number of studies classified as using a trait-based frameworks (TBF). Studies from the year 2022 (n=5 until March 31st) are not included in this figure.

morphology can partially control their dislodgement resistance, whereas the age and rooting rate determine their capacity for settlement (Lai et al., 2018). Vegetative fragments have the potential for long distance dispersal thanks to long lasting shoot buoyancy and survival, allowing the colonization of new areas (Berković et al., 2014). Additionally, fruits of certain seagrass species allow for long distance dispersal as well, as it is the case of *Posidonia australis* (10s to 100s of kilometers, Ruiz-Montoya et al., 2015).

The identification of traits controlling dispersal and settlement of seeds and vegetative fragments challenges the perception of dispersal as a stochastic and unpredictable process. In addition, abiotic (temperature, current velocity, wave disturbance and exposure) and biotic (animal vectors, shoot stiffness) factors as well as a combination of these exemplified by the seascape mosaic formed by the plants on an unvegetated substrate affect their dispersal and settlement success. The neutral assembly process hypothesis (Figure 1: Hypothesis #1), while not formally tested, seems to be false in the case of seagrasses (Table 1). However, only a small number of studies investigated this question. Further research is needed to test this hypothesis at different scales, with null models as a fundamental tool to assess the relative importance of purely stochastic and niche assembly processes (Mori et al., 2015). This hypothesis has been tested in other marine organisms, including fish communities (Ford and Roberts, 2018), which assemble neutrally at the regional scale but not at the local scale, and woody plants, whose dispersal and settlement are primarily trait-driven (Duarte et al., 2010).

3.3 Community assembly processes: The abiotic and biotic filters in seagrass communities

Much research has been performed on the responses of seagrass traits to environmental drivers, making a total of 72% of the studies found in the literature review. There was, however, a large imbalance between the study of seagrass traits under the abiotic (89%) vs the biotic (11%) filter (Figure 3). This indicates that the knowledge of seagrasses is focused on the study of its fundamental

FIGURE 3
Results of the systematic literature review adapted to the conceptual TBF proposed by this manuscript. The studies were classified in "Study types" (top), "Study subcategories" (middle, *a posteriori* classification with the environmental drivers and ecosystem functions found in the review process) and "Trait categories" (bottom, trait classification decided before the review process). The size of the circle indicates the number of trait studies. The darker circles within indicate the number of studies classified as using TBFs. The horizontal lines indicate the number of connections among the "Study types". The darker lines indicate the number of connections among study types using a TBF.

niche, i.e. the major physico-chemical constraints in the system. For example, the vertical zonation of tropical seagrasses was explained by physiological traits controlling their ability to tolerate high irradiances and nutrient inputs (Björk et al., 1999). Other examples focus on the assessment of the fundamental niche of individual species. *Halophila decipiens* occupies a wide range of irradiances and temperatures, due to its phenotypic plasticity (Gorman et al., 2016). *Z. marina* has a low niche specialization in the Baltic Sea, allowing this species to exist under variable environmental conditions in comparison to other macrophytes (Herkül et al., 2018). The three most frequent abiotic drivers studied were temperature (28.3%), nutrients (24.4%) and light (17.3%), whereas the least studied include the effect of freshwater input (1.5%) or metal pollution (2.4%).

Traits have been therefore used as indicators of environmental change, and their response is both driver and species-specific. This can be illustrated using the example of temperature. An increase in temperature within the optimal range fosters leaf growth (*Thalassia hemprichii*: Viana et al., 2020; *Enhalus acoroides*: Artika et al., 2020) and leaf size (*Z. marina*: Young Kim and Seob Choi, 2004; Ondiviela Eizaguirre et al., 2018; DuBois et al., 2019; *Halodule wrightii*: Sordo et al., 2011; *Zostera noltei*: Ondiviela Eizaguirre et al.,

TABLE 1 Studies included in the literature review that linked dispersal and settlement with seagrass traits.

Hypothesis #1 Neutral assembly process: Preliminary rejected					
Process	Dispersal unit	Relevant traits	Abiotic filter	Biotic filter	Sources
Dispersal	Seed	Sinking rate, seed size	Current velocity	Animal vectors	Berković et al. (2014); Delefosse et al. (2016); Wu et al. (2016); Lai et al. (2018)
	Vegetative fragment	Shoot buoyancy, shoot viability, fragment breakage, shoot growth rate, spathe release rate, dislodgement resistance	Current velocity, burial conditions	–	
Settlement	Seed	Seed weight, germination rate	Temperature, current velocity, scouring	Shoot size and stiffness of surrounding seagrass	Bouma et al. (2009); Cabaço and Santos (2010); Lai et al. (2018)
	Vegetative fragment	Fragment age, rooting rate	Current velocity	–	

2018; *T. hemprichii*: Viana et al., 2020; *Cymodocea serrulata*: Viana et al., 2020; *E. acoroides*: Artika et al., 2020; Artika et al., 2021; *Zostera capensis*: Beltrand et al., 2022). However, when the optimal temperature for a species is surpassed, heat stress reduces these two traits (*Posidonia oceanica*: Traboni et al., 2018; *Halophila ovalis*: Ontoria et al., 2020, *Halophila stipulacea*: Viana et al., 2020). Co-inhabiting species can have different thermal optima (Collier et al., 2011), and their trait responses can give fundamental information on how future warming will affect seagrass communities. Traits can therefore inform about the responses of seagrass to environmental change, these responses being species-specific or general among seagrass species. The diversification of research to different species and bioregions is therefore fundamental to predict how seagrasses will deal with future global change scenarios, as even co-inhabiting species may respond in different ways (Agawin et al., 2001).

The prevalence of the use of morphological traits (55.9%) among all other trait categories is worth mentioning (biochemical 31.5%; physiological 29.1%; growth 28.3%; mechanical 15.7%; reproductive 10.2%). This is likely explained by their relatively easy and inexpensive measurements compared to physiological measurements of e.g., photosynthetic efficiency (Hernán et al., 2016; Llagostera et al., 2016) or enzymatic activity (Alexandre et al., 2004; Alexandre et al., 2010), which require specialized equipment, technical staff and laboratories. There are, therefore, extrinsic economical and technical reasons that constrain scientific questions in seagrass research. This trait type imbalance may impede a deeper understanding of responses of seagrasses to abiotic drivers, as physiological and biochemical indicators are recommended over morphological ones for early stress detection in seagrasses (Roca et al., 2016).

The study of morphological (52%), biochemical (50%) and growth traits (34.8%) under the biotic filter was more balanced compared to the abiotic filter. Most of the traits were studied in response to eutrophication (36.9%) and/or herbivory (23.9%). Eutrophication is considered one of the main threats to seagrasses, as it can lead to a phase shift in primary producers from seagrass to macroalgal dominance (Duarte, 1995; Orth et al., 2006; Burkholder et al., 2007; Waycott et al., 2009). Eutrophication affects different compartments of the ecosystem (seagrass, micro- and macroalgae, epiphytic organisms), changing their relative abundances and causing changes in the light penetration or the redox potential in the sediment (Burkholder et al., 2007). Seagrass species respond to eutrophication through their traits, again highlighting their value as environmental change indicators. Under eutrophication, *Cymodocea nodosa* increases the nutrient content in its leaves, while reducing its fiber content and biomechanical properties (Jiménez-Ramos et al., 2018a). Similarly, *P. oceanica* increases the nutritional quality of its leaves under fertilization, while reducing plant growth (Ravaglioli et al., 2018). In the case of herbivory, seagrass plants respond to this driver using a comprehensive array of traits, including growth compensation, changes in their nitrogen content or mobilization of carbohydrates (Sanmartí et al., 2014) and their morphology and growth form can predict grazing impacts on a global scale (Poore et al., 2012).

One of the main questions posed in the conceptual TBF is the relative importance of the abiotic *vs* the biotic filters (Figure 1: Hypothesis 2) to better understand the fundamental and realized niches of seagrass species. The available trait-based studies have focused separately on the fundamental and realized niches of seagrass species. We found that only 17.1% of the studies included both biotic and abiotic factors simultaneously, which indicates the existence of a knowledge gap in this topic. Trait-based approaches suggest the study of the convergence and divergence of trait distributions to unravel the relative effects of the abiotic and biotic filters (see review by Grime, 2006). Herbaceous plant communities tend to diverge at the species level but converge at the trait level (Fukami et al., 2005), indicating that environmental forces select for functional groups but not for species identities, which are historically contingent. This finding exemplifies the two schools of thought in ecological community assembly. On one hand, it has been suggested that different species coexist, occupying different niches (Diamond, 1975). On the other hand, members of the same plant community tend to exhibit similarity in plant traits, therefore showing overlapping niches (Clements, 1916).

The drivers that shape communities, namely environmental drivers, competition, and disturbance regime, can act at different spatial scales (Díaz et al., 1998; Pierce et al., 2007). To disentangle these effects, it is necessary to calculate the functional diversity (FD) and, specifically, functional trait dissimilarity among species within and among communities (Petchey and Gaston, 2002; De Bello et al., 2009). If the functional dissimilarity is lower than a set of random species (null model, see Götzenberger et al., 2016), this indicates trait convergence due to environmental filtering, whereas the opposite indicates trait divergence, and therefore a dominance of competition and/or a disturbance regime allowing for differential life-history strategies (Mason et al., 2007; Mouillot et al., 2007; Petchey et al., 2007). Despite the knowledge that seagrass traits change under environmental drivers (Roca et al., 2016) and that these traits affect interspecific competition (Moreira-Saporiti et al., 2021b), there are only a handful of examples in which trait convergence and divergence (Figure 1: Hypothesis #2) have been tested, and uniquely in *Z. marina* at the intraspecific level. *Z. marina* communities have a higher trait diversity with higher genetic relatedness among genotypes, indicating that trait divergence may be selected among competing genotypes (Stachowicz et al., 2013). Similarly, niche differentiation through trait divergence is suggested as an explanation for the positive correlation between its genotypic and trait diversity (Abbott et al., 2017). However, transplant experiments have shown that the species morphology changes under local environmental conditions, resembling the morphology of local populations (Ruesink, 2018). The results of these studies indicate that divergence occurs at the local level among competing plants, while convergence seems to occur at a larger scale.

The large body of literature on response traits under abiotic and biotic factors shows that there is a wealth of data that can be reassessed to answer questions in the context of the TBF presented here. In addition, the study of response traits uses a variety of nomenclature that could not be included in this review (e.g. indicators, responses…), further increasing the volume of data available. Despite the presentation of neutral and niche assembly theories separately, both are not incompatible. While some species can be stochastically eliminated from the local community (not reaching a suitable habitat or a random event leading to local extinction, see Sp6 in Figure 1), the subset of species that successfully colonized a community undergo a process of niche assembly. The disentanglement of fundamental and realized niches under a TBF is currently unexplored in seagrasses, providing an opportunity to answer fundamental research questions under global change that includes both abiotic and biotic drivers.

3.4 Phylogenetic and genotypic control of seagrass traits

The study of the phylogenetic and genotypic control of traits is quite prevalent in the seagrass literature (23.3% of studies, Figure 3). There is ample evidence that genotypic richness covaries with phenotypic variation in functionally relevant traits, such as leaf morphology and shoot productivity within *P. australis* (Evans et al., 2016). In contrast, genetic diversity is a poor proxy for trait differentiation in *Z. marina* (Abbott et al., 2018). In *P. oceanica* a reproductive trait like flower abundance was negatively correlated to genotypic diversity and positively correlated to heterozygosity (Jahnke et al., 2015a), while there was a correlation of genetic indices and their response to environmental conditions (Jahnke et al., 2015b). Ecosystem functions like the accumulation of biomass and susceptibility to herbivory are also genotypically controlled in *Z. marina* (Tomas et al., 2011), with genotypes differing in key traits related to these processes. Similarly, nutrient uptake rates differed among genotypes of *Z. marina* (Hughes et al., 2009).

These studies confirm that there is genetic control of seagrass traits (Figure 1: Hypothesis #3) and, consequently, of ecosystem functions and services (Díaz et al., 2013). However, this control is species-specific. Knowledge gaps in this area lay in the lack of information from most of the seagrass species, as the genus *Zostera* and *Posidonia* accumulate 82.2% of the studies.

3.5 Intraspecific trait variability in seagrasses is key to their survival

In seagrass ecosystems, characterized by low plant species richness, intraspecific variation is likely to play a more important role than in terrestrial ecosystems. In comparison to terrestrial plant lineages, the taxonomic diversity of seagrass is low with all species belonging to four Alismatales families. Indeed, many temperate meadows are monospecific, and most tropical meadows consist of only a handful of co-occurring species (Short et al., 2007).

Species and populations can differ for the level of plasticity (i.e., amplitude of the genotypes' reaction norm), which is a fundamental trait affecting genotype persistence in changing environments (Pazzaglia et al., 2021). The plasticity of populations and genotypes is given by different levels of genetic variability, encompassing clonal somatic mutations and epigenetic changes. Several studies have indicated that the intraspecific trait variability of seagrass species is key for their survival. For example, the plant size of *Z. marina* (the predominant species in the northern hemisphere) spans more than two orders of magnitude across its distribution range (Ruesink, 2018), and different genotypes show large differences in nutrient uptake capacity and key photosynthetic parameters when grown in a "common garden" (Hughes et al., 2009). Even putatively less plastic species such as *P. oceanica* display a large variation in the acclimation to environmental factors (e.g. heat, Marín-Guirao et al., 2018). This feature, potentially supported by high intra-specific and intra-clonal (epi-)genetic diversity, enables seagrasses to cope with major environmental changes (Maxwell et al., 2014) and has most likely contributed to their successful colonization of shallow coastal zones along five continents, despite their low taxonomic diversity. Intraspecific variability in traits does not only occur at the species level, but also at the shoot, rhizome and clone levels. Epigenetic differences are even present within the same rhizome, which foster clonal persistence both within the same shoot (Ruocco et al., 2021) and within the same leaf (Ruocco et al., 2019a; Ruocco et al., 2019b). Recent evidence even points out that within single clones, somatic mutations lead to differentiation of ramets (= clone mates), with the potential to result in phenotypic differences within clones (Yu et al., 2020).

This body of literature highlights the importance of intraspecific trait variability in the response of seagrasses to disturbances, their resilience and capacity for ecosystem functions provision. However, we found only one example of the simultaneous study of intra- and interspecific variability of structural and nutritional traits, which drive palatability and herbivory in seagrasses (Jiménez-Ramos et al., 2018b). Future research assessing the relative importance of inter- vs intraspecific variability in both response and effect traits (Figure 1: Hypothesis #4) will be necessary to understand the relative role of intra- and interspecific diversity in seagrass ecosystem functions.

3.6 Effect traits and seagrass ecosystem functions: Understanding complementarity, dominance, and environmental control of ecosystem functions

The links between effect traits and ecosystem functions were tested in 26.9% of the studies (Figure 3). Herbivory (38.4%) and primary production (23%) were the most studied functions. Morphological (53.8%) and biochemical (46.1%) traits were the most used in the assessment of functions (Figure 3). Examples include wave attenuation, which is explained by a combination of morphological and mechanical traits including blade stiffness, shoot density and leaf length (Bouma et al., 2005; Paul et al., 2012) or herbivory of *Z. noltei*, which is mediated by both structural and nutritional leaf traits (Martínez-Crego et al., 2016). Examples of more nuanced, indirect interactions between traits and functions include the reduction of the canopy height in *P. oceanica* by grazing, thereby increasing the predation risk on associated sea urchins (Pagès et al., 2012).

There are, therefore, clear mechanistic links between seagrass effect traits and ecosystem functions. However, at the community level, there is the question of whether effect traits control ecosystem functions through dominance (CWM) or complementarity (FD) (Figure 1: Hypothesis #5). In addition, the link between traits and ecosystem functions can be environmentally constrained (Figure 1: Hypothesis #6). A great number of studies on ecosystem functions included environmental metrics (71.1%).

The hypothesis of the control of ecosystem functions by functional complementarity (FD) versus dominance (CWM) have been barely tested in seagrass ecosystems, with only a handful of examples found in the literature review (Table 2). Regarding the dominance hypothesis, CWM has been found as a reliable predictor of primary production in marine and brackish plant communities, including *Z. marina* (Gustafsson and Norkko, 2019). Particularly, plant height had positive effects on primary production, while the effects of other traits were environmentally constrained (Table 2). In the case of carbon storage, geophysical attributes seem to constrain any effect of seagrass traits (Belshe et al., 2018). Complementarity alone was tested in one study (Abbott et al., 2017, Table 2), showing that the Rao quadratic entropy index of trait diversity can predict invertebrate abundance. We found only two studies assessing simultaneously the effect of dominance and complementarity on ecosystem functions. In the case of habitat provision for fishes, trait complementarity had no effect, while the dominance of structurally more complex plants positively affected fish abundance (Jones et al., 2021). In the case of primary production, dominance of taller plants with bigger leaves positively affected production (Angove et al., 2020), while complementarity was discarded as a significant driver.

There were, however, several studies on *Z. marina* assessing both the dominance and complementarity effects of genotypic diversity on ecosystem functions. Primary production is influenced by genotypic diversity of *Z. marina* at the plot level (Abbott et al., 2017). There is also evidence of intraspecific niche complementarity in the partitioned nutrient uptake of genotypes of *Z. marina* (Hughes et al., 2009). Dominance and complementarity hypotheses have been tested simultaneously in one study in *Z. marina* (Hughes and Stachowicz, 2011). Biomass production was higher in polycultures (i.e., higher complementarity) at high disturbance levels, whereas under no disturbance, monocultures (i.e., dominance) outperformed polycultures. Additionally, polycultures outperformed monocultures in shoot and biomass production under a macroalgal bloom. It is worth mentioning that, despite not being included in the literature review due to not being focused on the study of traits, there have been studies in communities including *Z. marina* linking taxonomic richness to resistance to shading (Gustafsson and Boström, 2013) and complementarity to increased biomass production (Salo et al., 2009).

The scarcity of trait complementarity *vs* dominance data on seagrasses highlights the complexity of assessing their relative importance, particularly under a changing environment. To test the dominance and complementarity hypotheses it is fundamental to find effect traits with proven mechanistic relationships with ecosystem functions. These relationships may be environmentally controlled and therefore it is necessary to include relevant environmental metrics in the study of ecosystem functions (van der Plas et al., 2020). This has been barely tested in seagrass communities and only in the case of three ecosystem functions (primary production, habitat provision for invertebrates and fishes and carbon storage, see Table 2). There is therefore a big knowledge gap in our understanding of how the functional traits of seagrass communities are linked to ecosystem functions, and how this provision will be altered under global change.

3.7 Vulnerability of seagrass ecosystem function and service provision under global change

The worldwide rate of seagrass loss and the numerous threats to seagrass ecosystems (Orth et al., 2006; Waycott et al., 2009) call for the assessment of the vulnerability of the ecosystem functions provided by seagrasses. Therefore, it is necessary to study the correlation between response and effect in seagrass ecosystems (Figure 1: Hypothesis #7).

As stated in previous sections, the study of the response of traits to environmental drivers is common, particularly in the case of temperature or light (Tanaka and Nakaoka, 2006; Mota et al., 2018). Traits are sensitive indicators of plant stress under environmental change (Roca et al., 2016). When it comes to ecosystem functions, their vulnerability is generally discussed in terms of seagrass loss, i.e., the loss of the seagrass meadows would mean the end of certain ecosystem function provision (Trevathan-Tackett et al., 2018). However, one important missing link is the identification of

TABLE 2 Studies included in the literature review which test ecosystem function provision by seagrass communities through trait dominance, complementarity, and environmental constraints (Figure 1: Hypothesis #5 and 6).

Ecosystem function	Seagrass species	Effect trait(s)	Hypothesis tested			Conclusion	Reference
			Dominance	Complementarity	Environmental constraint		
Carbon storage	*T. ciliatum*, *C. serrulata*, *C. rotundata*, *T. hemprichii*, *S. isoetifolium*, *H. uninervis*, *H. ovalis*, *H. stipulacea*	Above- and belowground biomass, nitrogen content, shoot density	Yes	No	Geophysical attributes	**Environmentally constrained**. No trait dominance effects	Belshe et al. (2018)
Primary production	*Z. marina*, *R. cirrhosa*, other brackish plant species	Maximum vegetative height, specific leaf area, leaf and root nitrogen, leaf and root δ^{15}N and δ^{13}C, maximum root length	Yes	No	Exposure gradient	**Dominance effect and environmental constraints.** Vegetative height had a positive effect on primary production. Effects of root N and leaf δ^{15}N were constrained by the exposure gradient.	Gustafsson and Norkko (2019)
	Z. marina, algae species, brackish plant species	Life habit (longevity, environmental position), morphology (growth form, size), tolerance (salinity and wave exposure tolerance) traits	Yes	No	No	**Dominance effect** of the three trait categories included.	Jänes et al. (2017)
	Z. marina, *R. cirrhosa*, other brackish plant species	Median height, leaf area, median maximum root length, specific root length, leaf nitrogen content, leaf δ^{15}N and δ^{13}C	Yes	Yes	No	**Dominance effect** of plant height and leaf area. Effect of functional richness due to presence of extreme trait values, not because of complementarity effect.	Angove et al. (2020)
Habitat provision for fishes	*T. ciliatum*, *C. serrulata*, *C. rotundata*, *T. hemprichii*, *S. isoetifolium*, *H. uninervis*, *H. ovalis*, *H. stipulacea*, *E. acoroides*	Meadow structure (shoot density, leaves per shoot, canopy height, leaf length, leaf width), seagrass cover	Yes	Yes	Depth	**Dominance effect** of meadow structural complexity. **Environmental effect** of depth. No complementarity effects.	Jones et al. (2021)
Habitat provision for invertebrate grazers	*Z. marina* (intraspecific study using different *Z. marina* genotypes)	17 traits, summarized: biomass accumulation, growth rate, morphology, nutrient uptake rate, leaf phenolic content, photosynthetic parameters	No	Yes	No	**Complementarity effect** (Rao quadratic entropy) of trait diversity on invertebrate grazer abundance.	Abbott et al. (2017)

response traits that drive function effects. The rationale is that, despite the presence of seagrasses, a change in their trait values or the replacement by another species or taxa with different traits may alter ecosystem function provision. This hypothesis has not been explicitly tested in seagrass ecosystems (Figure 1: Hypothesis #7).

The concept of ecosystem service has gained increasing importance in the last two decades, as a tool to couple science with environmental policymaking and management (Costanza et al., 1997; Costanza et al., 2017). The identification of effect traits responsible for the provision of ecosystem services is of fundamental importance to develop a taxon-independent metric that could be incorporated into policymaking and guide coastal management strategies. Ruiz-Frau et al. (2017) classified ecosystem functions performed by seagrass in ecosystem services based on the TEEB - The Economics of Ecosystems and Biodiversity categorization created in TEEB (2009). For example, fisheries are classified as food provision, while carbon burial and storage are classified as gas and climate regulation. Knowing which functions underlie each ecosystem service, and how to relate simple trait metrics to ecosystem function and service vulnerability, is fundamental to achieve a holistic view of seagrass response, function provision and service provision under a changing environment.

4 Conclusion

TBFs (trait-based response-effect frameworks) are a powerful tool to address ecological questions in all fields of study, both terrestrial and marine. The synthesis of a comprehensive TBF based on previous knowledge allowed for a holistic view of traits, from their response to environmental drivers to ecosystem service provision.

The proposal to apply a TBF to seagrass ecology acknowledges the importance of considering the scientific advances of other research fields in order to push marine research forward. The application of a TBF to seagrasses appears as a powerful avenue to unveil new insights on the functioning of these important ecosystems, particularly in face of their special evolutionary history and narrow phylogenetic origin. We revealed that there is a wealth of data on seagrass response and effect traits, and on seagrass ecosystem functions, which allow a great potential to re-analyze existing data under a TBF perspective so that new research questions and hypotheses may be tested. In addition, there is a variety of nomenclature to refer to traits in seagrass research, further increasing the volume of data that could be reassessed under a TBF perspective but was not included in this review.

Most of the hypotheses of the TBF have not yet been formally tested. There is much evidence that stochastic processes (Hypothesis #1) have a lower relative importance than niche-based processes, both in the dispersal and vegetative stages of community assembly (Hypothesis #2). Additionally, traits are under a certain level of genotypic control (Hypothesis #3), but this could be highly trait dependent. Intraspecific diversity seems to be one of the mechanisms by which seagrasses respond to environmental drivers, and its understanding will prove fundamental to predict the response of seagrass to global change (Hypothesis #4). Ecosystem function provision by seagrass communities is generally controlled by trait dominance, but genotypic complementarity has also been shown to affect ecosystem functions, showing the need to understand the link between genotypic and functional trait diversity (Hypothesis #5). Additionally, only a handful of functions have been studied and the importance of dominance or complementarity can be environmentally constrained, as it is the case for primary production and carbon storage (Hypothesis #6).

Despite the positive signs of seagrass recovery in Europe and the United States, we cannot ignore the fact that the world is experiencing fast and unprecedented changes. The use of a TBF that assesses the vulnerability of ecosystem function and service provision (Hypothesis #7) can help to understand which ecosystem services may be compromised by the changes in species traits or species abundances. Therefore, the translation of biological and ecological seagrass research into a framework explicitly considering ecosystem services will prove fundamental for the development of comprehensive policies and for the informed management of seagrass ecosystems. However, mechanistic links between traits, functions and services will have to be resolved, further indicating the need for the mechanistic understanding of the traits that underpin ecosystem functions and services.

In an era in which global open data storage and sharing is becoming a central part of research, there is real need for a seagrass trait database, which has been developed at the Centro de Ciências do Mar (CCMAR, Portugal) in collaboration with the Portuguese national bioinformatics research infrastructure (http://biodata.pt/Elixir.pt). The Seagrass TraitDB (https://bio.tools/seagrasstraitdb) adopts standardized file formats, metadata, vocabularies, and identifiers so that it is compatible with global plant trait databases such as TRY (Kattge et al., 2020). It validates, stores, and disseminates MIAPPE-compliant data (https://www.miappe.org) and uses plant trait ontology to describe phenotypic traits of seagrasses. This tool will prove fundamental for the development of holistic and global research on seagrasses and a great opportunity for the application of the proposed TBF. We urge seagrass scientists to contribute to this data base.

We believe that the adoption of the concepts presented in this manuscript in seagrass research will aid the assessment of ecosystem services provision, improving the awareness of humankind on the importance of seagrass meadows worldwide.

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

This review and conceptual TBF was initiated as part of the Euromarine workshop “TRAITGRASS” led by GP and RS. All the authors contributed to the initial discussion that led to the production of this manuscript. AM-S did the systematic review for the manuscript. AM-S wrote the initial draft with significant contributions from all the authors. All the authors critically revised the different versions of the manuscript. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1088643/full#supplementary-material

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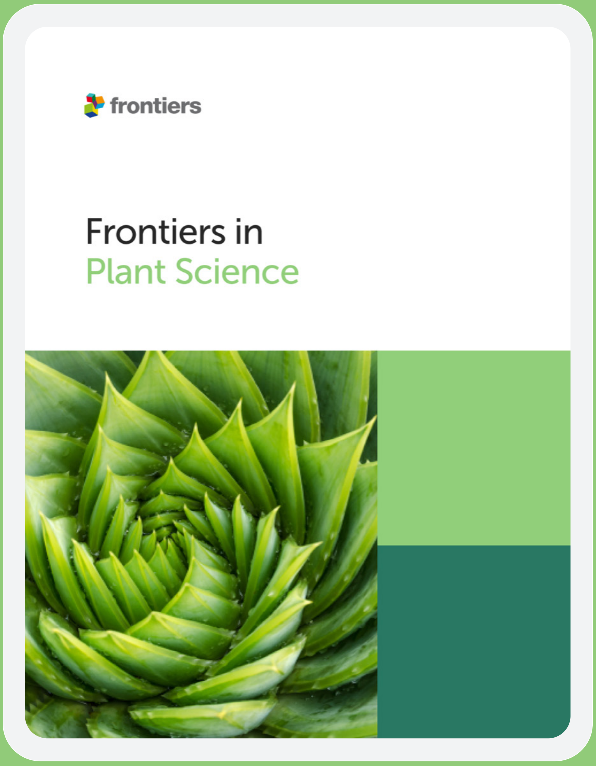

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