BIOLOGICAL INVASIONS IN THE MEDITERRANEAN SEA

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BIOLOGICAL INVASIONS IN THE MEDITERRANEAN SEA

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Editorial: Biological invasions in the Mediterranean Sea

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Editorial on the Research Topic
Biological invasions in the Mediterranean Sea

In an era of climate change and escalating transformations in ecological settings, the Mediterranean Sea is recognized as a hotspot of global biotic and abiotic changes (Moullec et al.). With a semi-enclosed-and-locked configuration affected by physical, oceanic, and atmospheric processes (Bas, 2009), the Basin is characterised by well-defined mosaics of contrasting ecosystems that make it an appealing natural laboratory for the study of natural and human-induced changes (Aurelle et al., 2022).

In the last decades, human activities have caused an imposing upsurge of Non-Indigenous Species (NIS) establishments in the Mediterranean Sea, at rates that have been unprecedented, globally (Zenetos et al., 2022). A major driver of NIS introductions are the successive enlargements of the Suez Canal, from 1960s to date, which have established a permanent sea-level waterway connecting thermophilic species of Indo-Pacific origin from the Red Sea to the Mediterranean Sea (Galil et al., 2017). Introduced species can cause a range of impacts, from ecosystem shifts and biodiversity reductions, to provision of ecosystem services and fishery benefits (Kleitou et al., 2021a; Tsirintanis et al., 2022; Kleitou et al., 2022).

Despite a growing scientific literature published in recent years (Figure 1), the absence of adequate empirical data has been emphasized as a major bottleneck for understanding the different facets and dynamics of Mediterranean bio-invasions (Galil et al., 2018; Kourantidou et al., 2021; Kleitou et al., 2021b). A bibliometric search in Scopus, using the terms “Alien Species” OR “Invasive Species” OR “non-native species” OR “non-indigenous species” OR “non indigenous species” OR “non native species” OR “Invasive Alien Species” AND “Mediterranean Sea”, was made on 07 August 2022 and identified a total of 1329 references with over 80% of the references published after 2010 (Figure 1). Indeed, an analysis, of keywords with at least eight co-occurrences, using the VOSviewer software 1.6.18 (Van Eck and Waltman, 2010), extracted 106 keywords that were mostly related to countries, introduction routes, and taxonomic groups (Figure 1),

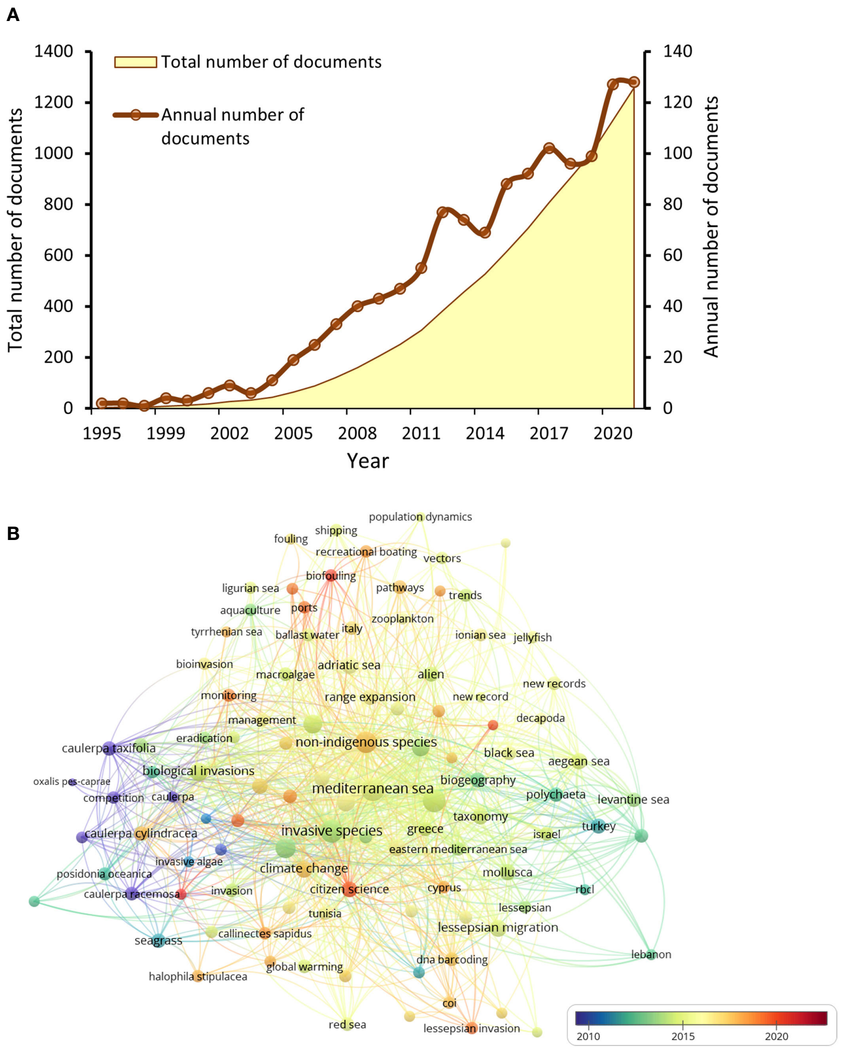

FIGURE 1
(A) Number of documents published in Scopus, extracted using the terms "Alien Species" OR "Invasive Species" OR "non-native species" OR "non-indigenous species" OR "non indigenous species" OR "non native species" OR "Invasive Alien Species" AND "Mediterranean Sea", on 07 August 2022. (B) Connection and average publication year for each keyword (with at least 8 occurrences) used in the extracted documents.

indicating that most studies might focused on detection and distribution monitoring with limited information to elucidate the complex dynamics of biological invasions.

In this context, this Research Topic (RT) intended to provide novel information about NIS biology and ecology in the Mediterranean Sea and promote successful management practices. Within the RT, a total of 12 novel and impactful articles covering diverse topics were published.

Three of the articles focused on lionfish (*Pterois miles*), a recently introduced NIS in the Mediterranean Sea with the potential to cause ample negative effects in the marine ecosystems (Kletou et al., 2016). Gavriel et al. used an acoustic telemetry system to monitor the diel activity and depth preference of lionfish within its native range (Red Sea). During the study, the lionfish demonstrated flexible and wide niche-breadth characteristics that potentially facilitated its invasion success. In a global collaboration of 25 researchers, Ulman et al. reviewed and synthesized experiences from two decades of lionfish management in the Western Atlantic to give policy recommendations for their management in the Mediterranean Sea. A strong highlight of the study was the importance of participatory management to effectively tackle the invasion, including the frequent removal by SCUBA divers, the development of recreational and commercial lionfish fisheries, and the development of a market. In another article, Ulman et al. compiled data from published literature and grey literature to provide a comprehensive review of predators for lionfish and the poisonous and invasive silver-cheeked toadfish (*Lagocephalus sceleratus*). The review identified many natural predators highlighting the importance of renewed effort towards prioritizing the rebuilding of their populations in the Mediterranean Sea. However, the low predation frequency suggested that control may require proactive, targeted human removals of the invasive species [e.g. Kleitou et al. (2021c)].

Three other articles focused on the *Caulerpa cylindracea*, a species characterised as one of the most invasive NIS in the Mediterranean with a capacity to rapidly colonize and alter native assemblages (Klein and Verlaque, 2008). Caronni et al. investigated gametogenesis and spawning events by *C. cylindracea* in the Tyrrhenian Sea using a combination of a field and laboratory experiments. The authors provided novel information about the timing, intensity, and frequency of the species' sexual reproduction showing that it can play an important role in the dynamics of the species spread. Water motion was the most important abiotic factor for gametogenesis and spawning while temperature had also a secondary additive effect. Santamaria et al. studied the diets of four of the most abundant omnivorous Sparidae species in the Mediterranean Sea to understand the foraging selectivity, magnitude and frequency on *Caulerpa cylindracea*. Low particle-size selection scores indicated that all species avoided feeding on the invasive alga. However, several individual specimens were found to have consumed high amounts of *C. cylindracea* suggesting that they could play a role in controlling its expansion. Miccoli et al. combined diet analysis, phenology, as well as biological and chemo-ecological biomarkers to elucidate potential contribution of *C. cylindracea* digestion in Abnormally Tough Specimen (ATS) anomalies of male white seabream *Diplodus sargus* in the Tyrrhenian Sea. The authors highlighted direct trophic interactions between the two species but further studies are needed to establish whether the algae or its metabolites are directly responsible of the anomaly.

Goldberg et al. used controlled mesocosm experiments and found that the invasive rabbitfish (*Siganus rivulatus*) significantly reduced its food consumption when exposed to chemical alarm cues released from a recently killed conspecific fish. The results signified that a healthy ecosystem with high density of piscivores and hence predation, or an artificial introduction of predation cues, could mitigate the impact of the herbivorous rabbitfish. Edelist et al. combined multidisciplinary approaches including citizen-science, oceanographic models, and molecular tools to track the connectivity and dynamics of the Indo-Pacific jellyfish *Rhopilema nomadica* off Israel. They produced vital information about bloom formations and found decentralized swarm origins with similar genetic structure, interannual differences, and high offshore transport of the swarms. Tamburini et al. used a standardized monitoring protocol to assess spatial and temporal changes in the structure of fouling communities in the Ligurian Sea. Through its application, authors detected many new NIS arrivals and showed its potential for upscaling in the Mediterranean Sea to provide a standard monitoring platform for long-term data collection.

In a novel work, Guastella et al. demonstrated how micropaleontologic analysis of sedimentary cores coupled with radiometric dating can be used to reliably date the first arrival and to reconstruct temporal trends of taxa having mineralized remains such as the alien foraminiferal species *Amphistegina lobifera* and its cryptogenic congener *A. lessonii*. In one of the most comprehensive studies on ascidians in the Mediterranean Sea, Virgili et al. combined bathymetric and habitat mapping, extensive transect and photo-quadrat sampling, and a morphological and molecular study to elucidate the ascidians presence in the marine reserve of the Miseno Lake. The study revealed a massive but so far overlooked presence of NIS and highlighted the importance of monitoring programs to achieve solid NIS management and protect vulnerable marine reserves. Finally, Sala et al. investigated the metabolomics of a colonial non-indigenous tunicate (*Botrylloides niger*) in the Fusaro Lake, and identified nutraceuticals and bioactive natural products that could be exploited to turn the species from a threat into a resource.

Author contributions

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

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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Reconstructing Bioinvasion Dynamics Through Micropaleontologic Analysis Highlights the Role of Temperature Change as a Driver of Alien Foraminifera Invasion

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Invasive alien species threaten biodiversity and ecosystem structure and functioning, but incomplete assessments of their origins and temporal trends impair our ability to understand the relative importance of different factors driving invasion success. Continuous time-series are needed to assess invasion dynamics, but such data are usually difficult to obtain, especially in the case of small-sized taxa that may remain undetected for several decades. In this study, we show how micropaleontologic analysis of sedimentary cores coupled with radiometric dating can be used to date the first arrival and to reconstruct temporal trends of foraminiferal species, focusing on the alien *Amphistegina lobifera* and its cryptogenic congener *A. lessonii* in the Maltese Islands. Our results show that the two species had reached the Central Mediterranean Sea several decades earlier than reported in the literature, with considerable implications for all previous hypotheses of their spreading patterns and rates. By relating the population dynamics of the two foraminifera with trends in sea surface temperature, we document a strong relationship between sea warming and population outbreaks of both species. We conclude that the micropaleontologic approach is a reliable procedure for reconstructing the bioinvasion dynamics of taxa having mineralized remains, and can be added to the toolkit for studying invasions.

Keywords: sea warming, SST, invasive species, foraminifera, radiometric dating, Mediterranean Sea, lessepsian invasion

INTRODUCTION

Global trade and worldwide transport of people and goods have largely altered the natural distribution of species (e.g., Sardain et al., 2019), but large gaps of knowledge remain in assessing spatial and temporal patterns of invasions, especially in the marine environment (Seebens et al., 2017; Ojaveer et al., 2018). Incomplete assessments of temporal trends, origins and drivers of their spread may affect our ability to understand the ecology and history of communities and the relative importance of different factors driving invasion success (Carlton, 2009). Sporadic monitoring and surveillance, weak taxonomic knowledge, or elusive behavior of some species have left several marine invasions undetected for years, decades or even centuries (Carlton, 2009; Griffiths et al., 2010; Zenetos et al., 2019). Several approaches have been attempted to reconstruct the timing of first introduction events and subsequent stages of invasion: re-examination of old museum or herbarium collections (Ahnelt, 2016; Steen et al., 2017), analysis of published descriptions (Zullo, 1992; Galil et al., 2018), interviews to local fishermen (Bariche et al., 2014; Azzurro et al., 2019), molecular tools (Ordóñez et al., 2016; Deldicq et al., 2019) and radiometric dating (Petersen et al., 1992; Albano et al., 2018). In spite of different approaches utilized, the gap still persists because long and continuous time-series are difficult to obtain, due to the often sporadic and intermittent availability of marine biodiversity data; hence several studies are restricted to inter-decadal comparisons (Parravicini et al., 2015) or are based on population model simulations (Clark et al., 2013; Walsh et al., 2016). Moreover, most of the current knowledge of invasions is from larger organisms, whereas very little is known about spatial and temporal patterns of bioinvasions by microscopic taxa such as foraminifera or other unicellular eukaryotes (Langer et al., 2012; Skarlato et al., 2018; Reavie and Cangelosi, 2020). These small-sized invaders can remain unnoticed for several years after their introduction and gain attention from beach visitors or scientists when population sizes reach a certain threshold and become visible to the naked eye (Guastella et al., 2019).

An example is provided by two algal symbiont-bearing benthic foraminifera: *Amphistegina lobifera* Larsen, 1976, an Indo-Pacific species that entered the Mediterranean via the Suez Canal (Prazeres et al., 2020), and its congener *A. lessonii* d'Orbigny, 1826, a species of uncertain native origin. Discussions on the non-indigenous status of *A. lobifera* and cryptogenic status (*sensu* Carlton, 2009) of *A. lessonii* are provided in Guastella et al. (2019). In particular, fossil specimens of *A. lobifera* have never been found in ancient Mediterranean records, while fossil remains of *A. lessonii* are common in Mediterranean shallow-water strata, late Pliocene to early Pleistocene in age (Di Bella et al., 2005). Both species are currently abundant in the Red Sea, but only *A. lobifera* is successfully spreading in the Mediterranean Sea, following a westward colonization process (Langer et al., 2012; Caruso and Cosentino, 2014; Langer and Mouanga, 2016; Guastella et al., 2019). On the contrary, *A. lessonii* is extremely rare in the Mediterranean Sea, and is mainly characterized by smaller-sized specimens compared to *A. lobifera* (Guastella et al., 2019). This sporadic occurrence and size range has been attributed to its different sensitivity to colder temperature that may limit test calcification (Titelboim et al., 2019).

Both species commonly proliferate in shallow-waters (<20 m depth) of warm tropical and subtropical areas, and their latitudinal distribution is strongly regulated by sea surface temperatures, with winter minimum temperatures around 18°C (Hallock, 1984; Langer and Hottinger, 2000; Hohenegger, 2004; Renema, 2018). In the Mediterranean Sea, their geographic distribution and population abundance is believed to be primarily controlled by wintry sea surface temperature (winter isotherm 15°C, Hollaus and Hottinger, 1997). However, little is known about *Amphistegina* spp. populations established in the Central Mediterranean region, which may have been selected for their higher tolerance to colder temperatures, compared to Red Sea and Eastern Mediterranean populations (Schmidt et al., 2016a; Titelboim et al., 2019). Nonetheless, a further spread of amphisteginids into the Western Mediterranean region could be expected in the near future, also promoted by the ongoing sea surface warming (Langer et al., 2013; Guastella et al., 2019). Profound ecological changes have been documented in the Eastern Mediterranean, where *A. lobifera* populations have become the dominant component of benthic foraminiferal assemblages. Massive deposits of amphisteginids produced an increased accumulation of carbonatic sands, which progressively altered native substrates. This, in turn, reduced biodiversity and caused the displacement of previously established foraminiferal biota (Langer et al., 2012; Mouanga and Langer, 2014).

In this study, we aim to reconstruct the invasion history of *A. lobifera* and *A. lessonii* using a multidisciplinary approach based on the analysis of sediment cores by techniques of micropaleontology, paleoecology, and radiometric dating. We document their population abundance along the timeline, by analyzing two sedimentary cores (CORE18 and CORE19) collected from Malta island (Central Mediterranean Sea). Additionally, we relate the temporal dynamics of the two populations with the trends of sea surface temperature (SST) measured in the area in the last 60 years, in order to assess whether sea warming has driven their invasion pattern. Finally, we elaborate hypotheses on the possible future spreading of both species in the Mediterranean Sea.

MATERIALS AND METHODS

Study Area, Selection of Sampling Site, and Sample Collection

The Maltese Archipelago is composed of three main islands (Malta, Gozo and Comino) and is located within the Sicily channel in the Central Mediterranean Sea, at a latitude of 35°48′28″–36°05′00″ North and a longitude of 14°11′04″–14°34′37″ East (**Figure 1A**). The coasts of the archipelago, with a total perimeter of about 271 km, are mainly characterized by high rocky shores; only a few natural bays are present and most of them exhibit high levels of anthropogenic impact (Magri, 2006).

In order to identify suitable sites for collecting the sediment cores, where the sea-floor could reasonably reflect the pattern of sedimentation that occurred in the past decades and thus the history of colonization of *Amphistegina* spp. in the area, we complied with the following criteria: (I) occurrence of a well-established population of *A. lobifera* and subordinately of *A. lessonii* (**Figure 1B**); (II) absence of fossil amphisteginids in neighboring outcropping rocks, in order to avoid their reworked occurrence in the studied cores, that may confuse the record of current foraminiferal assemblages of marine sediments. To satisfy requirement II, relative abundance of reworked foraminiferal

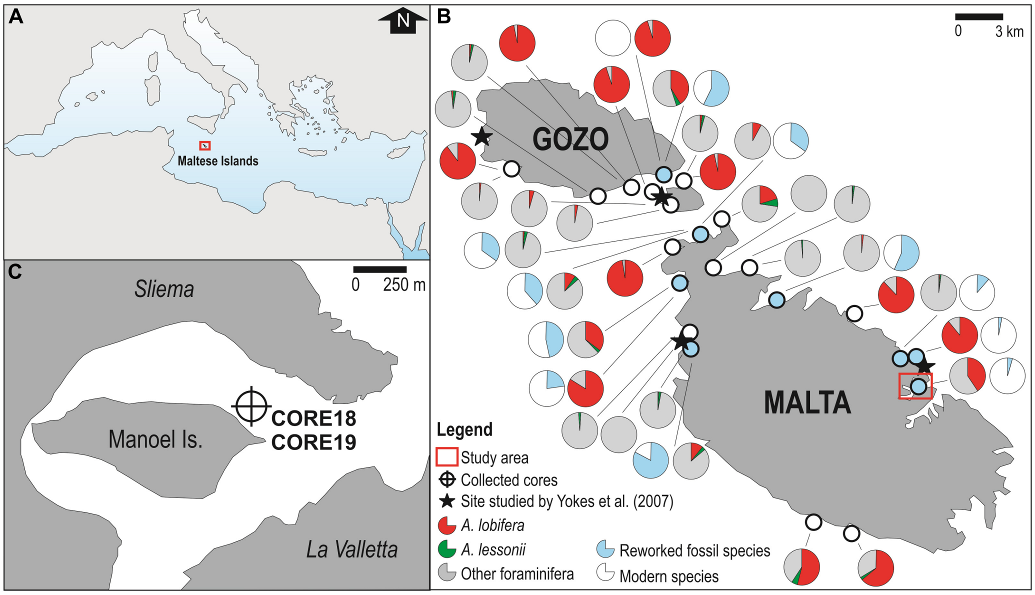

FIGURE 1 | (A) Study area (Central Mediterranean Sea); **(B)** Current distribution and relative abundance of *A. lobifera* (in red) and *A. lessonii* (in green) along the coasts of the Maltese Islands. The pie charts in this panel derived from data available in Guastella et al. (2019) and are supplemented with new data regarding relative abundance of reworked fossil species (in light-blue); **(C)** Sampling site in Marsamxett Harbor. In the selected site, the relative abundance of *A. lobifera* and of reworked fossil species are about 40 and 3%, respectively. Additionally, the collection site is close to one site previously studied (Yokeş et al., 2007) (black star). Maps derived from Google Earth (https://www.google.it/intl/it/earth/; data providers: Google Landsat/Copernicus Data SIO, NOAA, U.S. Navy, NGA, GEBCO IBCAO) and modified with GIMP v. 2.10.20 (https://www.gimp.org/).

species present in sediment samples was calculated through the comparison with fossil assemblages preserved in seven rocky samples collected in Malta from Miocene outcrops: three rocky samples were collected from the *Globigerina* Limestone Formation and prepared as washed residues, and four rocky samples were collected from the Upper Coralline Limestone Formation and prepared as thin rock sections (**Figure 1B** and **Supplementary Table 1** available on-line as **Supplementary Data**); (III) proximity to one of the four sites of the earliest record of *A. lobifera* in Malta (Yokeş et al., 2007), in order to have a temporal constraint for the reconstruction of the invasion dynamics; (IV) prevalence of fine-grained sediments, first requirement necessary for ^{210}Pb dating, a widely applied method to date recent sediments and to assess sediment accumulation rates (Tylmann et al., 2016; Andersen, 2017); (V) location within an enclosed bay, to ensure protection from storm waves and littoral currents that would alter the normal sedimentation process. In fact, the presence of an undisturbed sediment deposition is the second requirement to correctly apply ^{210}Pb-chronology (Tylmann et al., 2016); (VI) absence of dredging or beach nourishment operations in the area, for the same reason as above; (VII) depth >10 m to avoid sediment mixing by waves, as above, and <20 m, the maximum depth where *A. lobifera* has been observed to occur (Hallock, 1984; Hohenegger, 1995; Guastella et al., 2019).

Despite many efforts and surveys to search for multiple suitable sites along the coasts of the Maltese Archipelago, only a single locality satisfied all the stringent conditions listed above: Marsamxett Harbor (35°54′16.7″N; 14°30′27.5″E; **Figure 1C**). Located near station “4” of Yokeş et al. (2007) and known to have in 2018 a relative abundance of *A. lobifera* of ∼ 40% (Guastella et al., 2019), this natural bay displays the required condition of sediment grain size, shelter, depth and absence of human activities that could have altered sedimentation on the sea-floor. Furthermore, *Amphistegina* spp. populations are well established (Guastella et al., 2019) and the rocky outcrops around the selected site are exclusively characterized by rocks belonging to the *Globigerina* Limestone Formation (Aquitanian-early Langhian in age), formed in outer shelf to upper slope environments (Baldassini and Di Stefano, 2017). Thus, no fossil amphisteginids were recorded and the relative abundance of the other reworked fossil foraminifera (mainly deep-water taxa such as *Cibicides pseudoungerianus*, *Heterolepa bellincionii*, *Neoeponides schreibersii*, *Reussella spinulosa,* and *Spiroplectammina carinata* and the planktonic genera *Globigerina*, *Globigerinoides,* and *Globoquadrina*) is only 3% (**Figure 1B** and **Supplementary Table 2** available on-line as **Supplementary Data**).

Within Marsamxett bay, two sediment cores were collected a few meters away from each other using a hand-corer operated by a scuba diver. The first core (CORE18) was sampled on 8th May 2018 at 16 m depth and the second one (CORE19) was collected on 4th September 2019 at 17 m depth. CORE18 was collected as a “pilot” sample in order to evaluate if all the stringent conditions listed above were respected and if the collected data had a high quality; CORE19 was sampled in order to have a replicate that could confirm the reconstruction of invasion dynamic provided by CORE18.

CORE18 is 41 cm long and CORE19 is 50 cm long, both of them mostly made up by fine-grained sediments (fine sands, silts and clays). After collection, both cores were longitudinally sectioned in two halves and then crosscut at each centimeter, obtaining 41 samples from the first one and 50 samples from the second one, respectively. Sediment samples were used for both grain-size and radiometric analyses and prepared for the micropaleontologic analyses.

Grain-Size and Radiometric Analyses

Grain-size analysis was carried out for CORE18 in order to verify its compliance with two prerequisites for radiometric dating: fine-grained sediments and undisturbed record (not mixed vertically). Sediment samples were oven-dried at 40°C for 1 day, weighed (up to a maximum of 17 g) and separated by wet sieving using five overlapped sieves (meshes, respectively of 1 mm, 500, 250, 125, and 63 μm) as indicated in Blott and Pye (2012). The fraction retained on each sieve was oven-dried and weighed again, obtaining weight percentage data in the form of cumulative curves (distinguishing between very coarse, coarse, medium, fine and very fine sands and indistinct mud fraction); the weight of the mud fraction (silts plus clays) was calculated by difference of the total weight.

Both cores were chronologically constrained by measuring activities of ^{210}Pb and ^{137}Cs isotopes along the sedimentary record. ^{210}Pb is a natural radionuclide belonging to the ^{238}U decay series and is characterized by a half-life of 22.3 years. The total ^{210}Pb activity in marine sediments has two components: supported ^{210}Pb activity derived from the decay of *in situ* ^{226}Ra, and unsupported ^{210}Pb activity derived from atmospheric fallout (Kosnik et al., 2015; Andersen, 2017). In marine environments, unsupported ^{210}Pb deposits associated with muddy particles accumulate at the sediment-water interface as excess ^{210}Pb. The dating is based upon the determination of the vertical distribution of unsupported ^{210}Pb, by subtracting supported ^{210}Pb activity from the total activity of ^{210}Pb (Hollins et al., 2011).

In the studied core samples, ^{210}Pb activity was measured at the CNR-ISMAR Institute (Bologna, Italy) via alpha counting of its daughter isotope ^{210}Po, assuming secular equilibrium between the two isotopes as described in Rizzo et al. (2009) and Incarbona et al. (2016). ^{210}Po was extracted from the sediment using hot HNO_3 and H_2O_2, and spiked with ^{209}Po (NIST standard SRM 4326, diluted to 0.43 Bq g^{-1}) used as a yield monitor. After separation of the leachate from the residue, the solution was evaporated to near dryness and the nitric acid was eliminated using concentrated HCl, the residue was then dissolved in 1.5 N HCl and Iron was reduced using ascorbic acid. Finally, Po isotopes were plated onto a silver disk overnight, at room temperature. Repeated measurements of a certain number of sediment samples was carried out to estimate the analytical precision. Sedimentation rates for the last decades were calculated based on the decreasing concentration of excess ^{210}Pb, following the CF:CS model (Constant Flux: Constant Sedimentation) (Robbins, 1978), which assumes that both the atmospheric flux of excess ^{210}Pb to the sediment-water interface and the sediment supply remain constant over time (Abril and Brunskill, 2014).

^{137}Cs is an artificial radionuclide derived from nuclear fission and is commonly used as independent tracer for validation of the ^{210}Pb chronologies (Smith, 2001). Its main sources in the atmosphere were the nuclear weapons testing (peaked in 1963) and in some parts of the northern hemisphere the input from the accident that occurred in Chernobyl in 1986. Dried samples for ^{137}Cs measurements were placed in plastic containers and counted by gamma spectrometry. Gamma emissions of ^{137}Cs were counted at 661.7 keV photo-peak for 24–72 h using Ortec germanium detectors (OrtecHPGeGMX-20195P and GEM-20200) calibrated against a sediment spiked with the Amersham reference standard solution QCY48A by using the same counting geometry. The detectors were coupled to a multi-channel analyzer and shielded by a 10 cm thick layer of lead.

Micropaleontologic Analyses

Core samples were oven-dried at 40°C for 1 day, weighed (∼7 g), washed on a 63 μm sieve and then oven-dried again at 40°C for 1 day. Washed residues were separated in discrete aliquots using a precision micro-splitter and finally analyzed at the stereomicroscope for the analysis of foraminiferal content. Since it is quite difficult to morphologically distinguish between the two target species when specimens are small in size (diameter <500 μm; Hohenegger et al., 1999), the counting was limited only to those specimens that clearly displayed distinctive morphological features (following Hottinger et al., 1993) and for which the specific attribution was unambiguous. Specimens were attributed to *A. lobifera* (**Figure 2**, images 1–3) when characterized by tests with a more rounded margin, if observed in profile (p), with lobulated sutures (lobes usually visible on both sides) that were bent backwards forming an unbroken arch in dorsal view (d), while they were mainly sigmoidal in ventral view (v). On the contrary, specimens were attributed to *A. lessonii* (**Figure 2**, images 4–6) when their tests were: (I) more flattened and always characterized by an angular peripheral margin if observed in profile (p); (II) not as rounded as in similar-sized *A. lobifera* specimens; (III) with sutures without any folding nor complications, which sharply bent backwards forming a typical falciform arch in dorsal view (d), while they were slightly depressed in the last chambers but always maintaining the typical sickle shape, in ventral view (v).

The absolute abundances of *A. lobifera* and *A. lessonii* were calculated along the cores as number of individuals recorded per gram of dry sediment (N g^{-1}).

Additionally, the absolute abundance of calcareous nannoplankton was calculated in smear-slides as total number of individuals recorded per mm^2 and then used as proxy for hydrodynamism. Since nannoplankton deposition occurs only in low-energy systems (particle size <30 μm), this independent proxy was used to qualitatively estimate if the energy at the sampling site was low enough to permit deposition of fine-grained particles and, consequently, of radionuclides used for the radiometric dating.

Sea Surface Temperature (SST) Dataset

In order to verify the relation between the abundance of *Amphistegina* spp. and the water temperature, Sea Surface

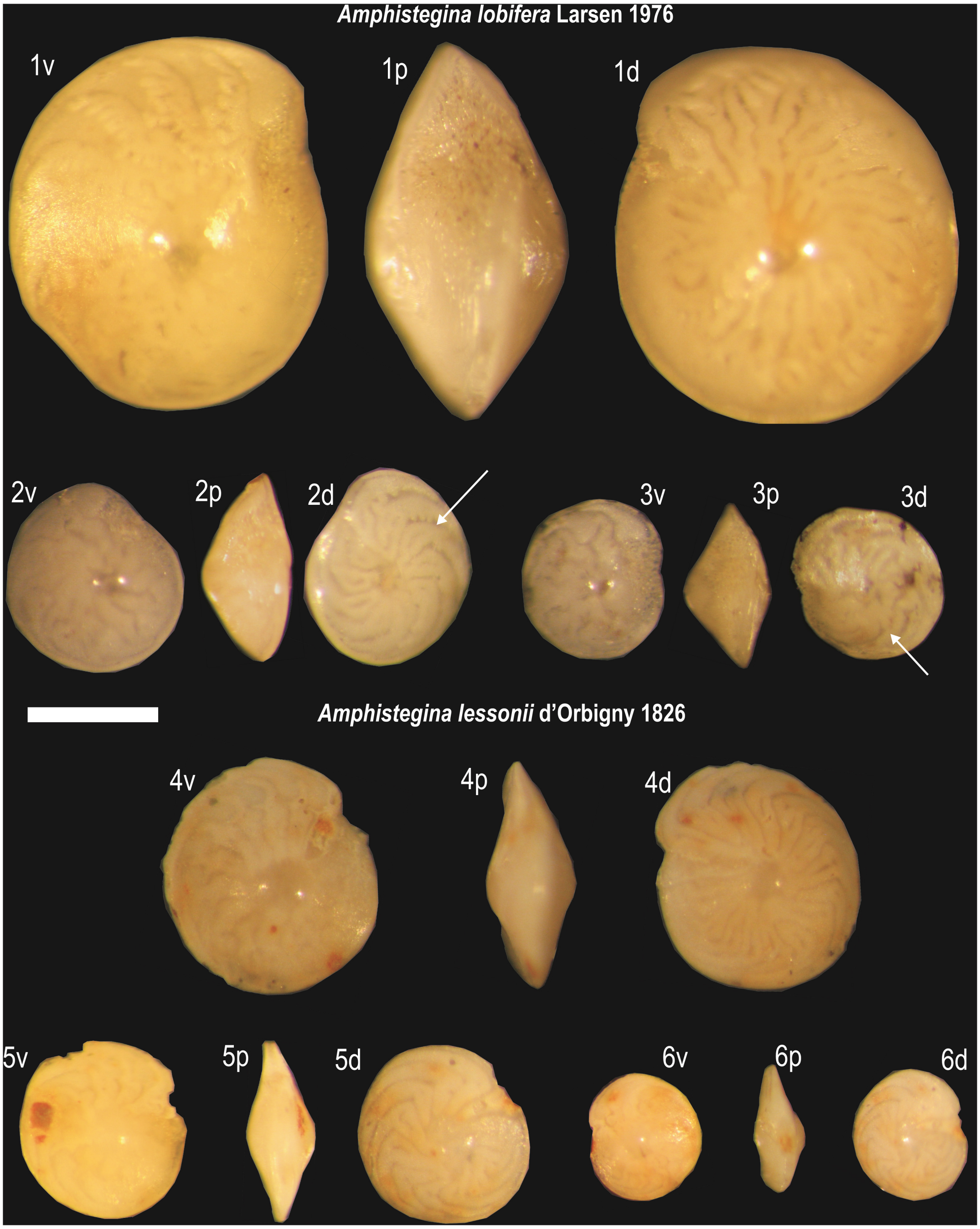

FIGURE 2 | Photomicrographs of adult and juvenile specimens of *Amphistegina lobifera* (images 1–3) and *Amphistegina lessonii* (4–6) collected from cm 2 to 3 bsf of CORE19. v) ventral view; p) profile; d) dorsal view. Note in juvenile specimens of *A. lobifera* (diameter size <300 μm, images 2–3) the presence of typical lobulated sutures (arrows) and the more rounded profile with respect to juvenile specimens of *A. lessonii* (5–6) characterized by more flattened tests with falciform sutures without any folding nor complication. Scale bar 300 μm.

Temperature (SST) of Sicily Channel was considered. SST was extracted from 3D temperature simulated by the reanalyses of Mediterranean Sea (ref. MEDSEA_REANALYSIS_PHY_006_009, horizontal spatial resolution ~ 6 km), available as monthly averages covering the period 1955–2015. The quality of the MEDSEA_REANALYSIS_PHY_006_009 was assessed for the entire period by comparing results with available observations, consolidated climatological products and current knowledge of the ocean circulation. The reanalysis data were simulated by means of the assimilation of *in situ* temperature profile and sea level anomaly in the Mediterranean basin.

In the framework of this study, the sea temperature was analyzed at 16 m depth in the grid point of the model closest to the sampling site of sediment cores [Lat. 35°56′15′′N; Long. 14°30′0′′E]. Starting from available data, curves of annual average SST and annual wintry average SST were obtained. The first one was elaborated by calculating the average SST for each year since 1955–2015, and the second one by calculating the average SST from values simulated during the winter season (January, February, and March). The average annual SST and wintry SST were plotted against the absolute abundances of *A. lobifera* and *A. lessonii*. In addition, the SST annual and wintry anomalies were computed taking as a reference the SST mean over the whole period and over the winter season, respectively. In particular, the annual anomalies were obtained by subtracting the SST averaged over the period 1955–2015 from each annual mean, while the wintry anomalies were obtained subtracting to each wintry mean the SST averaged over all January, February and March in the 1955–2015 period. All relationships were analyzed with Spearman correlation analysis using the software PAST v4.01 (Hammer et al., 2001). All graphs were generated in Microsoft Excel for Microsoft 365 MSO, and modified with GIMP v.2.10.20[1].

RESULTS

Radiometric Dating

Granulometric curves obtained from CORE18 (see **Figure 1** for map) show that the sample meets both prerequisites for radiometric dating through ^{210}Pb: the mud fraction (particle size <63 μm) is continuously present with discrete abundances between 8 and 40%, and high-energy episodes (e.g., storm waves) that could have altered the normal deposition can be reasonably excluded, due to the absence of abrupt variations down core and the continuous occurrence of nannoplankton (**Figure 3**).

The curves of ^{210}Pb decay are very similar in the two cores, indicating a good replicability of the collected data. Both curves show the typical activity-depth profile, with higher activities at the core top that rapidly decrease down core (**Figure 4** and **Supplementary Table 3** available on-line as **Supplementary Data**) halving within the first 25 cm below sea floor (bsf). This interval was used to calculate a constant Sediment Accumulation Rate (SAR) of about 0.22 cm yr^{-1} (**Figure 4A**); the derived age model provides an estimated time interval of about 4.5 years for each centimeter of sediment. Unfortunately, no ^{137}Cs was recorded in either core, thus an independent validation through this method was not possible. While the absence of the Chernobyl peak of ^{137}Cs is common in sediments collected in the southern part of the Mediterranean Sea due to the dispersion pattern of ^{137}Cs fallout that followed the accident, it is surprising to have no signal of nuclear bomb experiments. Nevertheless, the absence of ^{137}Cs in both cores supports the finding that in this area the ^{137}Cs supply is negligible, as also reported by other works (e.g., Hassen et al., 2019).

Amphistegina Content Down-Core

Along the studied cores, the absolute abundance of both *A. lobifera* and *A. lessonii* is characterized by a decreasing trend starting from the core top to the lowest occurrence (**Figure 4** and **Supplementary Table 4** available on-line as **Supplementary Data**). All curves show the same distribution pattern down core indicating that the two records are well replicated and have not suffered episodes of mixing by high energy events. In CORE18, *A. lobifera* is continuously present from cm 0–1 to cm 14–15 bsf, while in CORE19 its lowest occurrence is slightly deeper and corresponds to cm 16–17 bsf. The highest abundances were recorded in the upper portion of sediment cores. In both cores, the absolute abundances of *A. lobifera* decrease abruptly starting from cm 6–7 bsf down to the lowest occurrences, where the minimum values were recorded. Similarly, *A. lessonii* shows the highest abundances in the upper portion of the cores and an abrupt decrease starting from cm 6–7 bsf down to the lowest occurrences, which correspond to cm 14–15 bsf in CORE18 and to cm 17–18 bsf in CORE19.

Sequential Stages of Invasion and Comparison With SST Trends

Based on the age model, where 1 cm of sediment corresponds to a time interval of 4.5 years, the first occurrence of *A. lobifera* in Marsamxett Harbor can be dated to the mid-1940s (**Figure 5**). During the first decades of its colonization, up to the end of the 1980s, *A. lobifera* is present but with very low abundances, probably in response to environmental conditions still not completely favorable for the growth of dense populations. Starting from the 1990s, *A. lobifera* increases more rapidly, reaching the maximum peak of abundance between 2005 and 2010, when it was recorded in the Maltese Islands by Yokeş et al. (2007) for the first time. Thus, the first occurrence of *A. lobifera* was reported in 2006 with a lag time of about 60 years from its true first arrival; additionally, the establishment of dense populations and their spread along the Maltese coasts were reported by Guastella et al. (2019) with a lag time of 30 years (**Figure 5**).

The first occurrence of *A. lessonii* in Marsamxett Harbor can be dated at the end of 1930s. Although with lower abundances than *A. lobifera*, *A. lessonii* displays a similar invasion dynamic: its population is restricted to a few specimens during the first decades, then rapidly increases in abundance starting from the 1990s, up to the first record made in 2018 (Guastella et al., 2019). Thus, the first record of *A. lessonii* in Malta was reported with a lag time of 80 years, while the establishment of permanent populations with a lag time of 30 years (**Figure 5**).

From the comparison of the increasing abundances of *A. lobifera* and *A. lessonii* recorded along the sediment cores with the annual average SST measured during the last 60 years, a strong similarity emerges (**Figure 6** and **Supplementary Table 5** available on-line as **Supplementary Data**). Between the 1980s and the 1990s, a progressive rise of the annual average SST is recorded, with an average overall increase of ∼ 1°C; in the same timeframe, *A. lobifera* and *A. lessonii* abundances start to increase as well. The same pattern results when considering the annual

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[1] https://www.gimp.org/

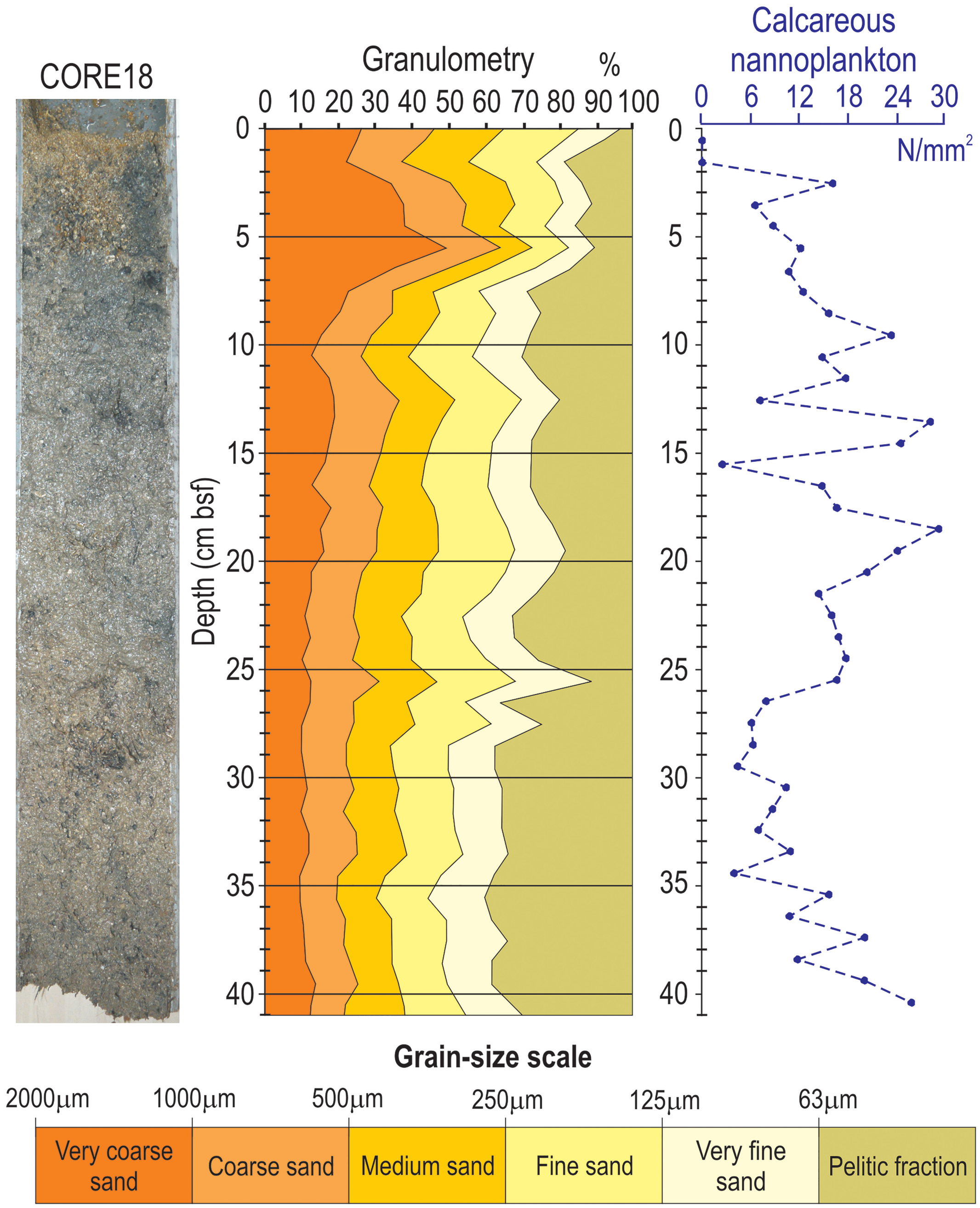

FIGURE 3 | From left to right: photo of CORE18, granulometric curves according to Blott and Pye (2012), and curve of absolute abundance of calcareous nannoplankton down core. The content of finest particles continuously recorded without abrupt variations along the core is mandatory in order to proceed with radiometric dating.

average wintry SST, for both *A. lobifera* and *A. lessonii*. Since the 1980s, the average SST increased rapidly during the winter, exceeding 15°C at the beginning of the 1990s and leading to an average overall rise of ~ 0.7°C (**Figure 6**). The aforementioned relation is further supported by the time series of annual SST anomalies evaluated against the 1955–2015 average, at 16 m depth in the grid point of the model closest to the sampling site of sediment cores in Malta (**Figure 7** and **Supplementary Table 5**). In fact, **Figure 7** shows that both *A. lobifera* and *A. lessonii* increase their abundance only when the annual SST anomaly curve exhibits positive values, corresponding to the warmer phase (with temperature higher than 1955–2015 average) starting from 1985 and still in progress (Marullo et al., 2011), and finally, the pattern is similar when considering also the annual wintry SST anomaly. All correlations between absolute abundance of both foraminiferal species and annual average SST, wintry SST,

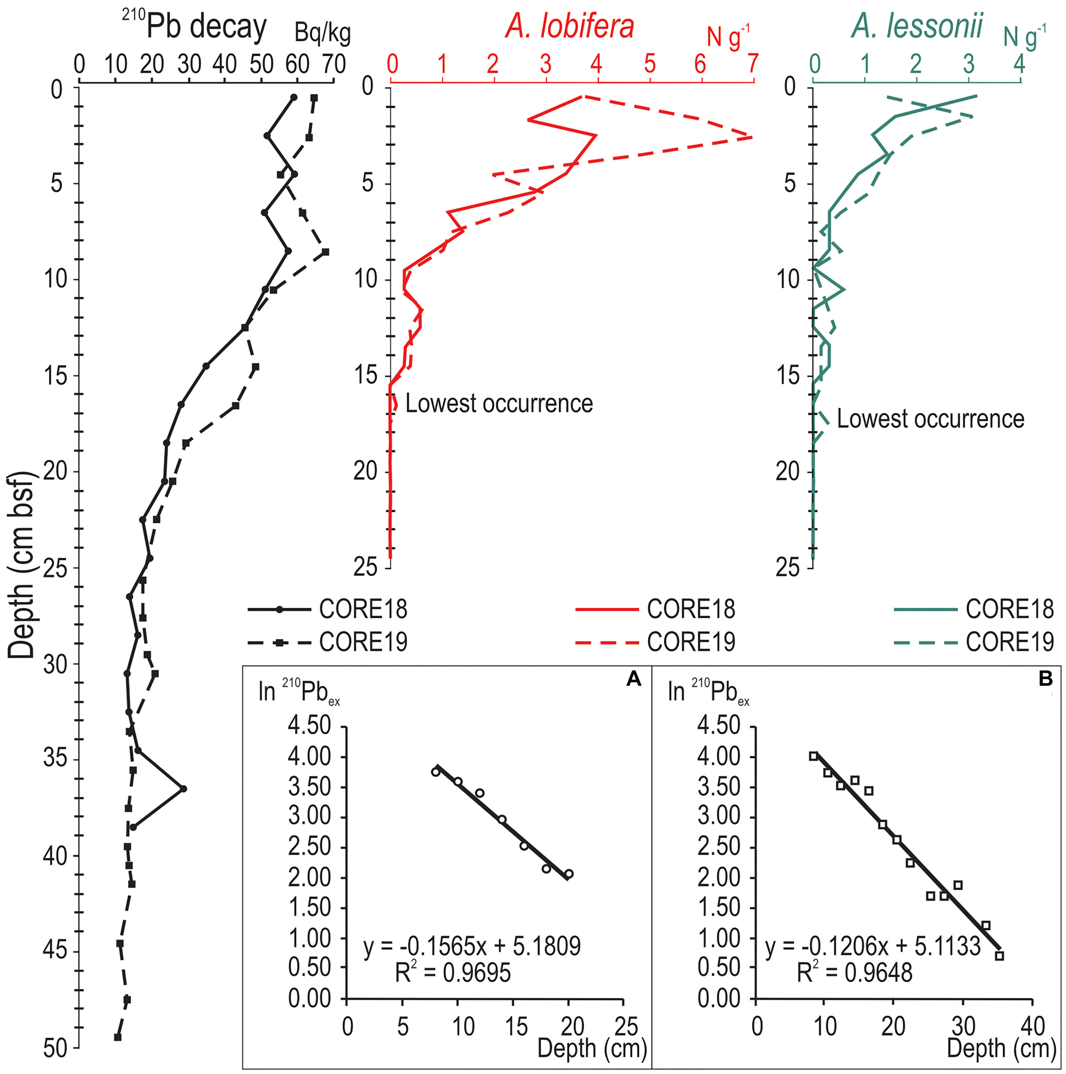

FIGURE 4 | Curves reporting from left to right: ^{210}Pb decay, showing the typical activity profile decreasing with depth, and absolute abundances of *A. lobifera* (in red) and *A. lessonii* (in green) recorded in both cores with a characteristic decreasing trend moving down core. The boxes on the bottom report a constant SAR, respectively of 0.20 cm yr^{-1} for CORE18 **(A)** and 0.22 cm yr^{-1} for CORE19 **(B)**; this last value was utilized in the applied age model, which leads to an estimated time interval of about 4.5 years for each cm of sediment (see the text for further explanations).

SST anomaly and wintry SST anomaly resulted positive and significant in both core samples, with an average correlation coefficient of 0.74 (**Figures 6**, **7** and **Supplementary Table 6** available on-line as **Supplementary Data**).

DISCUSSION

The Micropaleontologic Approach as a Reliable Procedure in Studies of Invasion Dynamics

This study is one of the few works (e.g., Ribeiro et al., 2012; Lishawa et al., 2013; Albano et al., 2018; Deldicq et al., 2019; Holman et al., 2019) applying analyses and methods commonly used in Micropaleontology and Paleoecology for the investigation of a relatively recent phenomenon, such as marine bioinvasions. Here we unified the recent fossil record of two alien foraminiferal species preserved in sediment cores with field observations, radiometric dating and environmental variables, such as SST trends. We also documented a possible causality link between temperature increase and population outbreaks.

Advantages of this approach include: (I) possibility of analyzing long historical records (up to about one century) on a continuous base; (II) possibility of analyzing early stages of invasions (e.g., see Walsh et al., 2016), which are very often unknown because several alien species tend to remain overlooked for a long time after their first arrival (Carlton, 2009); (III) possibility of directly exploring the response of the target species to environmental parameters, provided that long time-series are

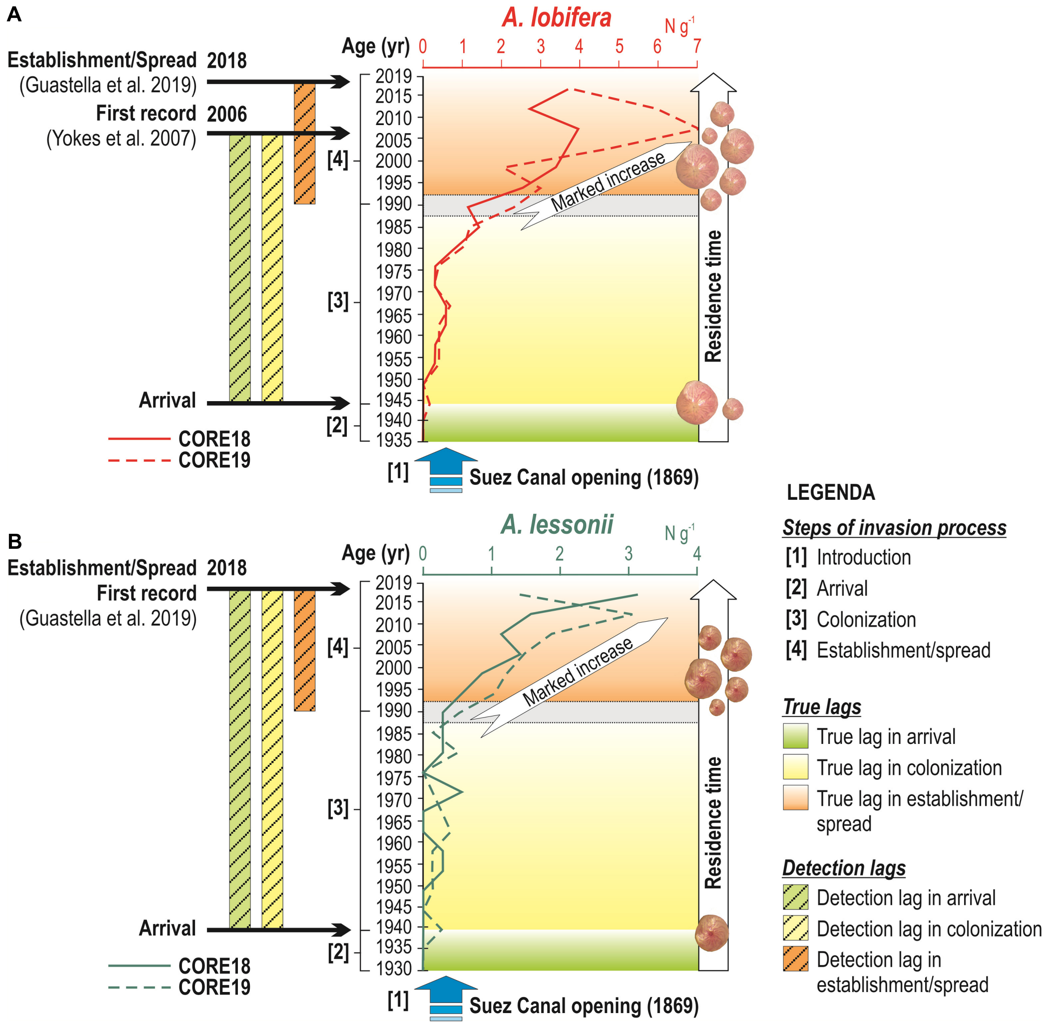

FIGURE 5 | Curves of absolute abundance of *A. lobifera* **(A)** and *A. lessonii* **(B)** with the different steps of invasion process (on the left): (1) introduction of the species after the opening of Suez Canal in 1869; (2) first arrival of *A. lobifera* occurred in the mid-1940s and of *A. lessonii* at the end of 1930s, respectively; (3) subsequent gradual colonization and (4) the establishment and population increase since the 1990s for both species. Compared to the real first arrival and the beginning of colonization process of *A. lobifera*, the first record documented in 2006 (Yokeş et al., 2007) occurred with a lag time of about 60 years. Guastella et al. (2019) reported the first record of *A. lessonii* in Malta in 2018, with a lag time of about 80 years and the establishment/spread of *A. lobifera* and *A. lessonii* with a lag time of about 30 years.

available. More in general, the analysis of sediment cores has proven useful in detecting anthropogenic change in a variety of coastal and marine communities, by reconstructing ecological scenarios before human intervention (e.g., Yamamuro and Kanai, 2005; de Boer et al., 2013; Lin et al., 2019; Handley et al., 2020; O'Dea et al., 2020).

Thanks to the availability of continuous, direct measurements of species abundance, the first three points above are here treated with a quantitative, replicable approach, differently from several other studies where bioinvasion patterns and dynamics are reconstructed from qualitative or non-structured observations (e.g., Delaney et al., 2008; Azzurro et al., 2019) or from modeling approaches (Salihoglu et al., 2011; Kanary et al., 2014; Mellin et al., 2016), to compensate for the lack of continuous and direct measurements.

However, using the analysis of sediment core samples to unravel invasion histories has some stringent restrictions. The first and most obvious restriction is that this method applies only to taxa with mineralized remains that can persist in thanatocoenoses of sediments (e.g., Albano et al., 2018; Deldicq et al., 2019). Another challenging requirement is related to the nature of the sediment containing the target species: it should reflect both its habitat (in terms of substratum, grain-size, depth, etc.) and its real distribution through time, should be sufficiently sheltered from wave/current motion to avoid vertical mixing, free from bioturbation and free from reworked fossil specimens that, in the case of living species having fossil counterparts, could misrepresent the original first occurrence along the core record. This may present a problem when one of these conditions is not met. Moreover, we must keep in mind that faunal

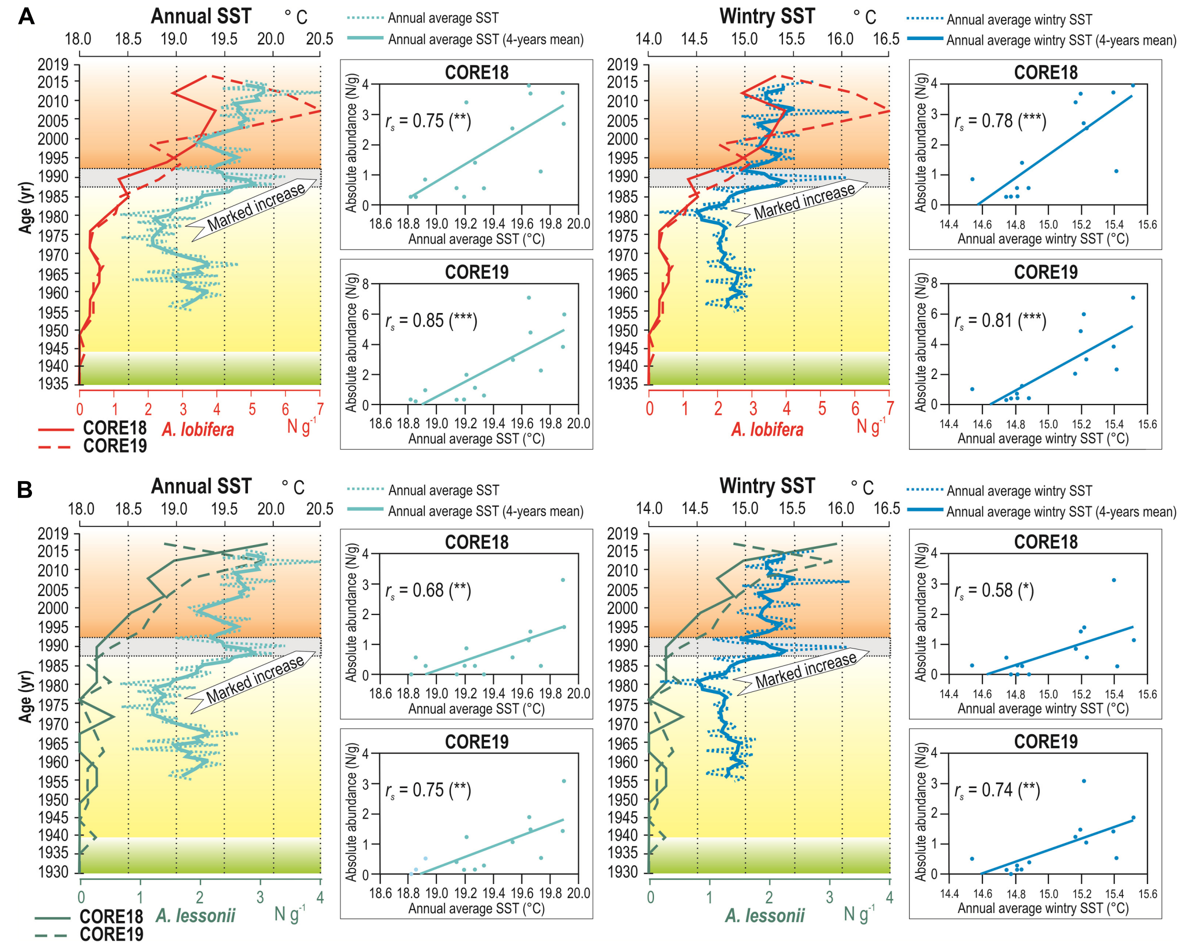

FIGURE 6 | Curves reporting from left to right: absolute abundance of *A. lobifera* **(A)** and *A. lessonii* **(B)** plotted against the annual SST and wintry SST simulated since 1955–2015. Beside: scatter-plots with Spearman's coefficients (r_S) relative to the absolute abundances of *A. lobifera* and *A. lessonii* recorded in CORE18 and CORE19 compared to the annual average and wintry SST (*p*-values of correlation indicated in brackets as follows: $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

variations observed down core strongly depend on the sediment accumulation rate (SAR): a single centimeter of sediment can correspond to multiple years, thus only in the case of very high SAR we can reconstruct annual or seasonal time series.

In spite of the restrictions mentioned above, this approach deserves to be included in the toolkit for studying invasion dynamics by these types of marine organisms, for which literature data are poorest and which are most likely to meet all the required conditions.

Sea Warming Trends and Range Expansions

Sea surface warming in the semi-enclosed Mediterranean basin has been largely documented and predicted, based on satellite measurements coupled to complex ocean models (Pastor et al., 2017; Sakalli, 2017; Macias et al., 2018). Analysis of decadal SST trends has documented a consistent warming increase starting from 1980 to 1983, which could be part of a 70-year variation linked to the Atlantic multi-decadal oscillation (Marullo et al., 2011; Pastor et al., 2017). Despite being uneven across time and space, the warming trend of the Mediterranean Sea surface has notably accelerated during the last two decades (Pastor et al., 2017; Sakalli, 2017). Since a global increase in sea temperatures has been reported to promote the redistribution of marine biodiversity, supporting the spread of thermophilic invaders (Occhipinti-Ambrogi, 2007; Marras et al., 2015; Molinos et al., 2016; Walsh et al., 2016), an accelerated warming rate should be taken into serious account when studying relationships between invasion processes and climate change, especially in the semi-enclosed Mediterranean Sea where the climatic signals can be amplified (Pastor et al., 2017). In fact, there is evidence that sea warming has been progressively shifting the horizontal and vertical distribution of Mediterranean marine species (Lejeusne et al., 2010; Marbà et al., 2015), and favoring tropical alien species to the detriment of temperate native species (Raitsos et al., 2010; Azzurro et al., 2019).

Our results, derived from historical records preserved in radiometrically-dated sediment cores, provide a unique opportunity to unravel the invasion history of cryptic invaders

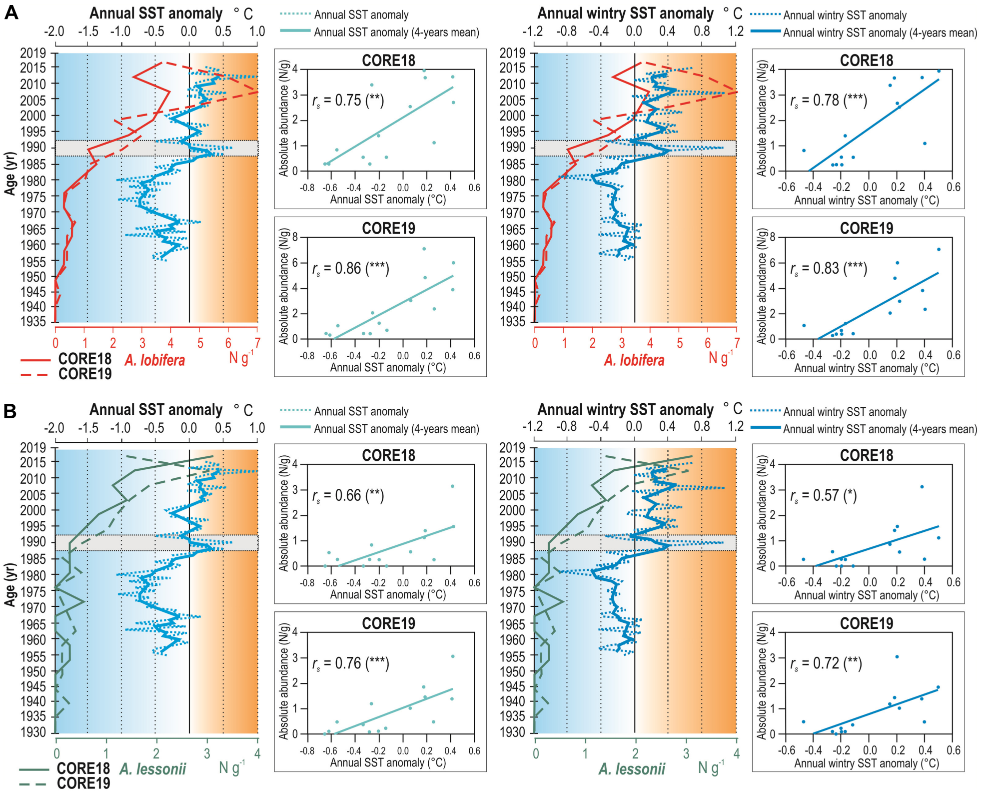

FIGURE 7 | Curves reporting from left to right: absolute abundance of *A. lobifera* **(A)** and *A. lessonii* **(B)** plotted against the annual SST anomaly and wintry SST anomaly evaluated over the period 1955–2015. Beside: scatter-plots with Spearman's coefficients (r_S) relative to the absolute abundances of *A. lobifera* and *A. lessonii* recorded in CORE18 and CORE19 compared to the annual and wintry SST anomalies (*p*-values of correlation indicated in brackets as follows: $^*p < 0.05$; $^{**}p < 0.01$l; $^{***}p < 0.001$). The beginning of the exponential growth of both populations starts around the 1990s, when the warmer phases (in orange) abruptly increase in intensity and frequency.

and to follow the 70 year-long dynamics of two tropical species that have been exposed to the progressive heating of the central Mediterranean Sea. We document that these two target species, the Indo-Pacific *A. lobifera* and the cryptogenic *A. lessonii*, reached Malta at the beginning of the 1940s, several decades earlier than their first records (Yokeş et al., 2007; Guastella et al., 2019). However, they did not develop dense populations for at least 50 years. Protracted lag times between initial introduction and population explosion have been reported for other marine alien species, such as the mussel *Brachidontes pharaonis* (Fischer, 1870) in the Mediterranean Sea (Rilov et al., 2004), the barnacle *Austrominius modestus* (Darwin, 1854) in the North Sea (Witte et al., 2010) and the cladoceran (water flea) *Bythotrephes longimanus* (Leydig, 1860) in Lake Mendoka, United States (Spear et al., 2020). Such delayed population outbreaks may be due to multiple independent introduction events occurring along time, that may enrich the population gene pool and provide genotypes more fit for the local conditions (Dlugosch and Parker, 2008; Handley et al., 2011), or to changes of the receiving environment (e.g., increased vulnerability, relaxation of biotic pressure, more favorable abiotic environment, etc.), resulting in population growth of the alien species (Sakai et al., 2001; Crooks, 2005; Walsh et al., 2016).

In the case of *A. lobifera* and *A. lessonii*, a possible explanation for the delayed population outbreak could be the altered seasonal patterns of sea temperature triggered by global climate change. For both populations, in fact, the beginning of their exponential growth started only after A.D. 1990, and more pronouncedly after A.D. 2003, when the wintry SST repeatedly exceeded 15°C (**Figure 6**) and the warmer phase abruptly increased its intensity and frequency along the timeline (**Figure 7**). Indeed, laboratory experiments and direct observations have demonstrated that 15°C represents a thermal threshold for both species, which are unable to calcify the tests at temperatures below that value (Titelboim et al., 2019). In particular, *A. lessonii* displays higher sensitivity than *A. lobifera*, and this fact could explain why the latter has so far achieved a higher invasion success in the Mediterranean basin (Titelboim et al., 2019). Additionally, *A. lobifera* collected from the eastern Mediterranean has a similar thermal response to high temperatures than *A. lobifera* from the Red Sea, showing that the thermal tolerance is retained during its invasion (Schmidt et al., 2016a). On the other hand, laboratory experiments have shown that higher temperatures (>32°C) are better tolerated by *A. lessonii* than *A. lobifera* (Schmidt et al., 2016b; Titelboim et al., 2019). This implies that in the future, according to the predicted Mediterranean warming (Sakalli, 2017; Macias et al., 2018) and the different tolerance ranges of the two species, *A. lessonii* could be favored, overcoming *A. lobifera* in relative abundance.

Historical data suggest that such switch is not unlikely. The southern portion of the Sicily Channel, an area characterized today by mean annual SST varying between 19 and 21°C (Drago et al., 2010), during Late Pliocene (3.1–2.51 Ma), was warmer on average 6–7°C than modern conditions (Herbert et al., 2015; Plancq et al., 2015; Tzanova and Herbert, 2015). In that epoch, Mediterranean amphisteginid populations were strongly dominated by *A. lessonii*, with minor abundances of the congeneric species *A. targionii* and *A. gibbosa* (Di Bella et al., 2005). Their test remains were so abundant on the sea floor after death to form biogenic deposits named *Amphistegina*-rich beds, over 1 m thick and cropping out all over the Mediterranean basin (Di Bella et al., 2005; Caruso and Cosentino, 2014; Cau et al., 2019). *Amphistegina lessonii* probably developed its acclimation ability earlier, thanks to a longer colonization history during the warmer Pliocene Mediterranean, at least since ∼ 5.3 Ma, when the species recolonized the basin after the abrupt foraminiferal extinction following the Messinian salinity crisis (Hayward et al., 2009). This ability could advantage the species even in the future, taking over from the congeneric Indo-Pacific *A. lobifera*. On the other hand, *A. lobifera* is characterized by a high dispersal capacity, and can modify its geographic range in response to global warming without requiring further adaptation (Prazeres et al., 2020).

This fact, however, might be considered realistic only if invaders spread and acclimate to new recipient environments fast enough to keep pace with climate change (Hiddink et al., 2012; Marras et al., 2015; Molinos et al., 2016). Based on a literature search and meta-analyses, Sorte et al. (2010) estimated that the poleward shift of marine species in response to climate change happens at an average rate of ∼ 19 km yr^{-1}, which is an order of magnitude faster than the estimated range shift of terrestrial species. According to a recent species distribution model (Guastella et al., 2019), *A. lobifera* is spreading in the Mediterranean Sea at a rate of 13.2 km yr^{-1}. However, this estimate was based on the known sequence of first records of the species along the eastern and central Mediterranean coasts. Here, we demonstrate that a serious bias exists in the assessment of the "first arrival" of *A. lobifera*, showing that in Malta the species went overlooked for as much as 60 years. Additionally, it could be assumed that this bias also exists in other localities around the Mediterranean Sea, because our results precede the first record of *A. lobifera* in the entire Mediterranean, making all first records of this alien species presumably incorrect, as well as questioning hypothesis of spreading due to ichtyochory by lessepsian rabbitfish (Guy-Haim et al., 2017). On the other hand, we show that the species arrived in the central Mediterranean at least in the mid-1940s, that is only 70 years after the opening of the Suez Canal occurred in 1869. Malta is about 3,200 km far from Port Said, the outlet of the Suez Canal (as calculated along the North-African coasts), a distance that both *A. lobifera* and *A. lessonii* have covered in about 70 years with a spread rate of ∼ 45 km yr^{-1}. This value is consistent with the spread rates estimated for other Erythraean invaders that move in response to climate change (referred to inter-quartile range of 25–75%: 17.0–49.8 km yr^{-1} for continental shelf dispersal and 12.6–35.1 km yr^{-1} for straight-line dispersal; Hiddink et al., 2012).

CONCLUDING REMARKS

Accurate data on the arrival and temporal dynamics of alien taxa are essential to understand invasion patterns and their drivers, and hence to design and implement effective measures. Such data are often lacking, especially for small-sized taxa that often remain undetected or are only sporadically recorded until their populations reach outstanding densities. The present work demonstrates how, in the case of taxa such as foraminifera with mineralized remains that persist in sediments after their death, micropaleontologic analysis of radiometrically-dated sediment cores can be used to reconstruct invasion histories, accurately determining the true date of introduction and subsequent temporal changes in abundance. In turn, the population abundance time-series can be linked to changes in environmental parameters. Using this approach, we unraveled the invasion history of two foraminiferal species in the Central Mediterranean Sea, reporting a considerable lag-time between their arrival and their first record, and highlighting how inadequate knowledge can lead to misleading or incorrect conclusions (e.g., expansion rates and vectors).

Finally, by relating the temporal dynamics of the two *Amphistegina* populations with trends in sea surface temperature, we also show that a link exists between sea warming and population density of both species. This suggests that temperature could represent an important driver of invasion patterns, with sea surface warming having the potential to trigger population outbreaks of these thermophilic alien species. We therefore propose the micropaleontologic approach adopted in the present work as an important addition to the toolkit for studying bioinvasions, serving to elucidate invasion histories, identify the main drivers of invasion success, and ultimately better inform management decisions.

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

NM and AM developed the idea. AC, JE, and CC collected and sampled the cores. RG and MC performed the micropaleontologic analyses. LL carried out the radiometric dating calculations. RL provided and elaborated sea surface temperature data. All authors contributed to interpretation of results and discussions and wrote parts of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.675807/full#supplementary-material

Supplementary Table 1 | Geographic coordinates and other sampling details of the study sites.

Supplementary Table 2 | Census of benthic foraminifera recorded in the study sites from the Maltese islands.

Supplementary Table 3 | Radiometric data and chronological constrain based on ^{210}Pb decay.

Supplementary Table 4 | Absolute abundances of *A. lobifera* and *A. lessonii* recorded along CORE18 and CORE19.

Supplementary Table 5 | Sea Surface Temperature (SST) in Marsamxett harbor measured at 16 m depth in the grid point of the model closest to the sampling site of sediment cores [Lat. 35°56′15″N; Long. 14°30′00″E].

Supplementary Table 6 | Spearman rank correlation coefficient (r_s) and *p*-values between sea surface temperature -SST (annual average SST, average wintry SST, annual SST anomaly, and wintry SST anomaly) and abundance of *A. lobifera* and *A. lessonii* in the two sediment cores: CORE18 and CORE19.

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Trophic, Chemo-Ecological and Sex-Specific Insights on the Relation Between *Diplodus sargus* (Linnaeus, 1758) and the Invasive *Caulerpa cylindracea* (Sonder, 1845)

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Biological invasions result in negative and unpredictable impacts on oceans worldwide. Non-indigenous macrophytes often synthesize secondary metabolites for defensive purposes and increased competition efficiency: this is the case of *Caulerpa cylindracea*, which has entered the Mediterranean Sea in 1990 and competed against local flora and fauna since. It was demonstrated that the white seabream *Diplodus sargus* (i) has included the algae into its diet, (ii) is subject to the peculiar Abnormally Tough Specimen (ATS) condition post-cooking, and (iii) suffers physiological and behavioral disturbances from caulerpin, one of the three major algal secondary metabolites. This paper confirms a feeding relationship between the fish and the algae, quantifies caulerpin accumulation in the liver, suggests a possible mollusk- and echinoderm-driven biomagnification, and highlights the fact that all ATS specimens were males. Multivariate analyses on a multi-biomarker panel reveals differential correlations to key cellular processes such as oxidative stress, metabolism, neurotoxicity, and lipid peroxidation as well as to condition indexes.

Keywords: Abnormally Tough Specimen, antioxidant enzymes, biomarker, CAR project, ecotoxicology, metabolites, multivariate analysis, white seabream

INTRODUCTION

The introduction of non-indigenous species has greatly increased in recent decades (Roques et al., 2016; Seebens et al., 2017). Despite the fact that some positive outcomes have been theorized from the colonization of coral reefs by alien species (Bellwood and Robert Goatley, 2017), biological invasions generally cause serious biodiversity losses and have been consequently regarded as one of the main anthropogenic threats in all oceans. The increasing awareness depends on the fact that such an issue reflects on multiple levels, i.e., evolutionary, ecological, economic, and social (Sax et al., 2007; Azzurro et al., 2019).

Invasive species may exert their effects through defensive secondary metabolites (Mollo et al., 2008), against which autochthonous populations lack any evolutionary adaptation (Terlizzi et al., 2011). Exotic species are provided with a higher chemical uniqueness than non-invasive/native counterparts (Cappuccino and Arnason, 2006 and references therein), and these molecules were demonstrated to bioaccumulate (Baležentienė, 2015) and biomagnificate (Costa et al., 2017) along the food web. Because bio-invaders are described once they have established close relationships with native wildlife, the assessment of their impact is unpredictable and rarely knowledgeable at an early phase (Felline et al., 2017). Also, the actual long-term extent of the damage of such metabolites on key biological/physiological features such as feeding, behavioral, and reproductive habits may be underestimated at the ecosystem level (Langkilde et al., 2017).

In the last few decades, the Mediterranean Sea has suffered from the introduction and spreading of many non-indigenous species (NIS): 265 were overall described, with Macrophyta accounting for the most numerous taxon (65 species, 84% of which established at least in one sub-region). Among them is the order Caulerpales, class Chlorophyceae. Particularly relevant are *Caulerpa taxifolia* and the *Caulerpa racemosa* complex, the latter being characterized by high morphological plasticity and therefore unclearly taxonomically classified (Klein and Verlaque, 2008). The *C. cylindracea*, formerly known as *C. racemosa* var. *cylindracea* (Sonder) Verlaque, Huisman, and Boudouresque (a new taxonomic combination proposed by Verlaque et al., 2003), was first considered a "Lessepsian migrant" (Alongi et al., 1993; Giaccone and Di Martino, 1995), but Belton et al. (2014) clarified its southwestern Australian origin. This species has spread to the entire Mediterranean basin since 1990 (Nizamuddin, 1991; Klein and Verlaque, 2008) and was reported in Italian waters for the first time in 1993 at the Island of Lampedusa and at Baia di San Panagia (Alongi et al., 1993). *C. cylindracea* colonizes photophilic algae and *Posidonia oceanica* seagrass meadows (Infantes et al., 2011) and algal turfs (Bonifazi et al., 2017), exerting profound impacts on local biological communities (Vázquez-Luis et al., 2009; Deudero et al., 2011; Rizzo et al., 2017) as it decreases α- and β-diversity (Piazzi and Balata, 2008). Its higher invasive potential compared to Mediterranean macrophytes was tentatively explained with a more efficient antioxidant capacity (Cavas and Yurdakoc, 2005). Because of such invasiveness features, *C. cylindracea* is now considered an invasive alien species (IAS) (Klein and Verlaque, 2008; Katsanevakis et al., 2014) and represents a serious threat to biodiversity and the functioning of the natural Mediterranean ecosystem.

The trophic relationship between *C. cylindracea* and the white seabream *Diplodus sargus* (Linnaeus, 1758) was brought to the notice of the scientific community in 2011 (Terlizzi et al., 2011). However, Mediterranean fishermen had witnessed a peculiar condition of the white seabream for over three decades, consisting in the hardening of the meat and the consequent decrease in edibility following the cooking of apparently healthy adult specimens. This is known as the Abnormally Tough Specimen (ATS) phenomenon, and the underlying causes were hypothesized to be pollution (organic and chemical) and a change in feeding habits (Casadevall et al., 2020). In addition to grazing on the algae, the white seabream accumulates the red pigment caulerpin in the liver, red and white muscle, and skin tissues through the diet. By using condition indices and ecotoxicological biomarkers as proxies of general health status, *D. sargus* was demonstrated to be negatively affected by the algae at the transcriptional and enzymatic levels (Terlizzi et al., 2011; Felline et al., 2012, 2017; Gorbi et al., 2014). Further controlled feeding experiments using caulerpin-enriched food demonstrated lipid metabolism and behavioral disturbances (Magliozzi et al., 2017; Del Coco et al., 2018) and elucidated the molecular pathway underneath such cellular and physiological alterations (Vitale et al., 2018; Magliozzi et al., 2019).

The present study employed a chemo-ecological approach with the aim of assessing (i) the establishment and extent of a feeding relationship between *D. sargus* and *C. cylindracea*; (ii) the incidence of the ATS phenomenon; (iii) the accumulation of the main algal secondary metabolite in a fish tissue; and (iv) potential physiological impacts thereof through a multi-biomarker panel indicative of key cellular processes. With regard to the ATS condition, we related our findings to the *ex post* fish cooking response and visually document the phenomenon. At last, the reader is supplied with the invasive seaweed phenology and percentage coverage data in a Site of Community Importance (European Commission Habitats Directive 92/43/EEC). Importantly, our approach might be applied across a broad range of marine fish species to characterize their physiological response to alike stressors.

MATERIALS AND METHODS

Fish Sampling

Diplodus sargus specimens were sampled by accredited personnel of University of Tuscia on November 1, 2019, during a FIPSAS (Federazione Italiana Pesca Sportiva e Attività Subacquee) spearfishing competition in the area of Civitavecchia (Italy) (**Figure 1**). Fish were maintained in ice to avoid tissue proteolysis until *in situ* processing. Total length (L), total weight (W), eviscerated weight (EW), gonad weight (GW), and liver weight (LW) were recorded with an electronic scale (0.01 g precision) (**Table 1**). Liver and white muscle were immediately stored in dry ice. Stomachs were removed by severing the esophagus near the buccal cavity and intestine, weighed, and preserved in 75% ethanol. All analyses were performed on a subset of 10 individuals selected for size greater than 300 g (Terlizzi et al., 2011). All individuals were maintained at a constant temperature in ice and cooked within 12 h from capture. Fish were seasoned with water, oil, white wine, tomato, and spices and oven-cooked for 40 min at 180°C. Individuals were identified as "altered" or "non-altered" based on filet appearance and organoleptic tests.

Caulerpa cylindracea Sampling

Caulerpa cylindracea was sampled following the sampling plan and technical details were reported by Piazzi and Ceccherelli

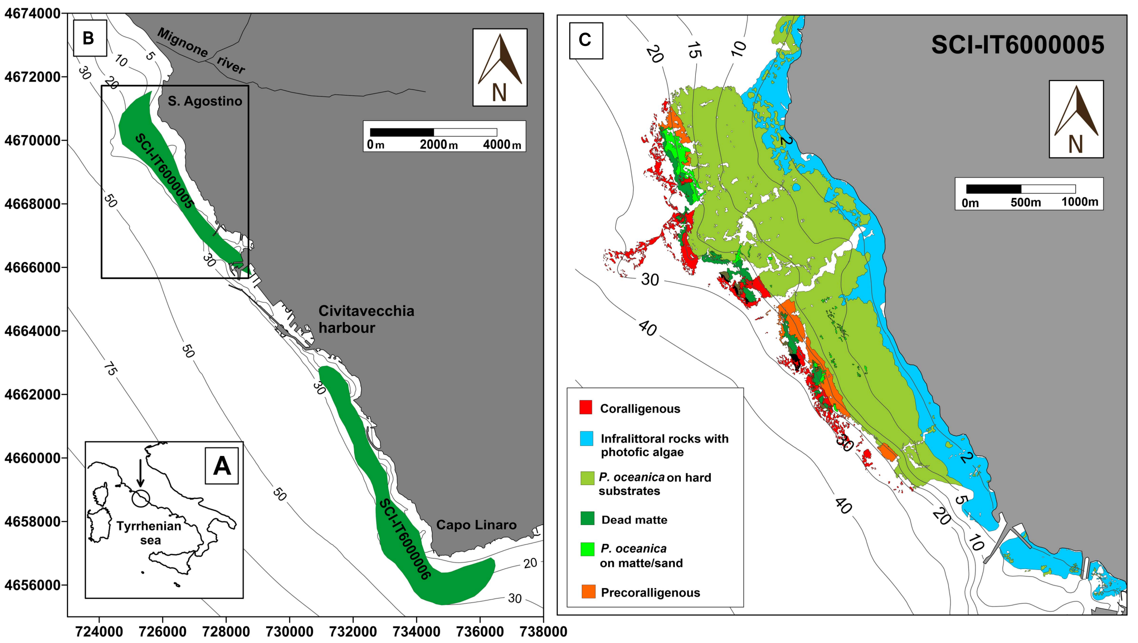

FIGURE 1 | Study area of the CAR project. Map of the sampling location at low **(A)** and high **(B)** magnification. All fish and algae sampling occurred exclusively within SCI 6000005 (black frame), which extends from Punta S. Agostino to Punta della Mattonara. SCI 6000006, because of its proximity to the area, is also indicated on the map. **(C)** Thematic map of the distribution of benthic biocenosis in the study area.

TABLE 1 | Sampling information on sex, morphometry, and bioenergetic/reproductive indicators.

Sample	Sex	L (cm)	W (g)	EW (g)	LW (g)	GW (g)	CF	HSI	GSI
FR_01	F	28.3	449.55	405.13	4.77	3.01	1.787	1.177	0.743
FR_02	F	28.1	373.32	344.17	4.09	2.23	1.551	1.188	0.648
FR_03	M	33.5	722.74	637.32	7.67	1.18	1.695	1.203	0.185
FR_04	F	29.2	415.84	378.50	3.13	2.58	1.520	0.827	0.682
FR_05	F	27.1	435.44	390.15	5.67	1.40	1.960	1.453	0.359
FR_06	F	26.7	372.23	335.08	5.69	2.55	1.760	1.698	0.761
FR_07	F	25.2	321.29	287.66	5.03	1.69	1.798	1.749	0.587
FR_08	M	31.2	542.41	491.03	8.12	0.75	1.617	1.654	0.153
FR_09	F	27.4	446.26	384.66	4.85	3.39	1.870	1.261	0.881
FR_10	M	28.2	450.91	398.04	4.07	0.34	1.775	1.023	0.085

(2006) and Ruitton et al. (2005). Monitoring and sampling operations were performed by SCUBA surveys in July and October 2019 in the same area of the spearfishing competition. An area delimited by a 400-cm^2 metal reference quadrat was scraped in triplicate after having been photographed/filmed for in-lab image analysis, and *C. cylindracea* specimens were stored in 80% ethanol-filled plastic bottles. For each replicate, the following phenological variables were measured: thickness of the horizontal stolons, length of the erect axes (fronds), length of lateral branchlets (ramuli), and diameter of the lateral pinnules (terminal vesicles) (Buia et al., 2001). Cover was expressed as percentage of seabed surface covered by vertically projected vegetative material (Boudouresque, 1971).

Condition Biomarkers

Fulton's K condition factor (CF), hepatosomatic index (HSI), and gonadosomatic index (GSI) were calculated according to Lloret et al. (2014). In particular, $CF = [(EW/L^3) \times 100]$, $HSI = [(LW/EW) \times 100]$, and $GSI = [(GW/EW) \times 100]$. EW was used in order not to account for viscera and gonad contributions.

Stomach Content Analysis

Stomach contents were analyzed under a Leica APO 8 stereomicroscope. The entire content was sorted and identified to the finest taxonomic level possible depending on the level of digestion of the prey. All prey categories were enumerated and their wet weight recorded with a 0.001 g accuracy after superficial drying with absorbing paper.

Chemical Analysis

Caulerpin accumulation was quantified in the liver samples of the subset in triplicate. Twenty-five milligrams of samples was lyophilized with a mortar in liquid nitrogen, resuspended with 0.10 ml of ice-cold ultra-pure water (18 MΩ), and sonicated five times for 10 s each. Thermal shock was applied by alternating exposure at 4°C and 37°C for 30 s. Ice-cold methanol (0.6 ml) and chloroform (0.4 ml) were added. After mixing at a 5-min interval for 30 min, samples were added with 0.10 ml of ice-cold pH-adjusted ultra-pure water and transferred to −20°C for 2–8 h. After thawing, tubes were centrifuged at 13,500 × *g* for 10 min at 4°C, supernatants were dried to visible pellets, resuspended in 0.1 ml of water containing 5% formic acid, and transferred to glass autosampler vials for LC/MS analysis. The metabolite was identified and quantified using Ultra High Performance Liquid

Chromatography system and was coupled with a Q Exactive mass spectrometer (Thermo Scientific). The system was operated in positive ion mode. The system was calibrated against positive or negative ion mode calibration mixes (Pierce, Thermo Fisher, Rockford, IL). Metabolite assignment was performed using the open source MAVEN 5.2 package[1], upon conversion of raw files into an mzXML. Caulerpin was quantified relatively to a standard calibration curve (0.0002–2 mg ml^{-1}, $R^2 = 0.9996$). The caulerpin used as standard was purified according to Magliozzi et al. (2017) and its ^{1}H-NMR spectrum was assessed against Ferramosca et al. (2016).

Biochemical Analyses

Biochemical analyses were performed on liver and white muscle homogenate supernatants. One unit (U) is defined as the amount of enzyme that catalyzes the reaction of 1 µmol of substrate per minute. Total protein content was quantified following the BCA method.

Catalase (CAT, EC 1.11.1.6) activity was investigated in terms of peroxidatic function by spectrophotometrically measuring the formaldehyde product at 540 nm with 4-amino-3-hydrazino-5-mercapto1,2,4-triazole as chromogen. Tissues were homogenized in 50 mM potassium phosphate pH 7.0 containing 1 mM EDTA (1:10, w/v) and 20 µl of supernatant was assayed with 100 µl of 100 mM potassium phosphate (pH 7.0) and 30 µl of methanol. Reactions were initiated by adding 20 µl of 35.28 mM hydrogen peroxide and incubated for 20 min at room temperature (RT); they were terminated with 30 µl of 10 M potassium hydroxide, 30 µl of chromogen, a 10-min incubation at RT, and 10 µl potassium periodate in 0.5 M potassium hydroxide. A formaldehyde standard curve was generated with seven points (0–75 µM, $R^2 = 0.995$). Bovine liver catalase was included as positive control. Standards, positive control, and unknown samples were assayed in duplicate. Activity is reported as mU mg protein^{-1}.

The level of glutathione peroxidase (GPx, EC 1.11.1.9), the biotransformation of glutathione *S*-transferase (GST, EC 2.5.1.18), and the activity of superoxide dismutase (SOD, EC 1.15.1.1) were determined on the cellular fraction S9. To extract it, a variable volume of a 50 mM phosphate buffer containing 2 mM EDTA was added to liver and muscle samples (Vidal-Liñán and Bellas, 2013). Samples were homogenized using an UltraTurrax® and centrifuged at 12,000 × *g* for 12 min at 4°C. The supernatant was aliquoted in 2-ml test tubes and stored in liquid nitrogen.

GPx was quantified following the method of Badary et al. (2005). Samples were added to a reaction mix of 10 mM GSH, 2.4 U/ml GSSG reductase and 1.5 mM NADPH. The reaction was started by adding 2 mM hydrogen peroxide and absorbance was monitored at 340 nm for 2 min. Enzyme activity was determined using the extinction coefficient of 6.22 mM^{-1} cm^{-1} and results were expressed in nmol mg protein^{-1}.

GST activity was determined following the method described by Habig et al. (1974). GSTs catalyzes the conjugation of the substrate 1-chloro-2,4-dinitrobenzene (60 mM CDNB) with 10 mM GSH. The samples were added to a reaction mix (phosphate buffer + GST + CDNB) and absorbance was measured at 340 nm for 5 min. Enzyme activity was determined using the extinction coefficient of 9.6 mM^{-1} cm^{-1} for CDNB and results expressed in mU mg protein^{-1}.

SOD activity was quantified following the method of Gao et al. (1998). This is based on the ability of the enzyme to inhibit the autoxidation of pyrogallol, which, in the presence of EDTA at the specific pH used, is 50%. Absorbance was measured at 420 nm. SOD activities were expressed as U/ml; one unit is defined as the amount of enzyme required to cause 50% inhibition of pyrogallol autoxidation.

Citrate synthase (CS, EC 2.3.3.16) activity was assessed by colorimetrically measuring the production of glutathione (GSH) at 412 nm. Homogenization occurred in ice-cold lysis buffer (1:10, w/v) and 50 µl of supernatants were analyzed with 5 µl of 0.1 mM DTNB, 2 µl of 0.3 mM acetyl-coenzyme A, and 0.5 mM oxaloacetate. Absorbance was read at a 5-min interval until the value of the most active sample exceeded the linear range of the standard curve. A GSH standard curve was generated with six points (0–40 nmol, $R^2 = 0.979$ and 0.938 for liver and muscle samples, respectively). Sample blanks were included. Standards, unknown samples, and samples blanks were assayed in duplicate. Activity is reported as mU mg protein^{-1}.

Acetylcholinesterase (AChE, EC 3.1.1.7) activity was measured according to Wilson and Henderson (2007), a variant of the Ellman assay. Briefly, tissues were homogenized on ice in 0.1 M sodium phosphate pH 8.0 containing 0.5% Triton X-100 (1:10, w/v). Supernatants were diluted 1:50 and 30 µl was used in the reaction mix together with 250 µl of 0.1 M sodium phosphate, pH 8.0, 10 µl of 10.3 mM DTNB, and 30 µl of 21.4 mM acetylthiocholine (ATCh substrate, 2 mM final concentration in total reaction volume). A tissue blank and a substrate blank lacking the substrate and the sample, respectively, were included for each sample. The optimal substrate concentration was determined by generating a concentration curve and plotting AChE activity versus the log substrate concentration. A free sulfhydryl standard curve was generated with eight points (0–50 nmol, $R^2 = 0.999$). Standard, samples, and blanks were run in triplicate. Absorbance was read at 412 nm at a 2-min interval for 12 min. Activity is reported as U mg protein^{-1}.

The extent of polyunsaturated lipid peroxidation was determined by the reaction of the natural by-product malondialdehyde (MDA) with thiobarbituric acid (TBA) at 532 nm. Briefly, tissues were homogenized in ice-cold MDA lysis buffer containing 3 µl of butylated hydroxytoluene (BHT) 100 × (1:30, w/v). Two hundred microliters of supernatant was developed by adding three volumes of TBA solution, and the mixture incubated at 95°C for 60 min. Vials were cooled to RT in an ice bath for 10 min and 200 µl of the total volume was read at 25°C. An MDA standard curve was generated with seven points (0–40 nmol, $R^2 = 0.998$). Standards and samples were run in duplicate. MDA concentration is reported as nmol mg tissue^{-1}.

The following chemicals were purchased from Merck (Darmstadt, Germany): glacial acetic acid (cat. A6283); L-Glutathione reduced > 98% (cat. G4251); 5,5′-Dithiobis(2-nitrobenzoic acid), DTNB (cat. D8130); catalase activity assay kit

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[1] http://maven.princeton.edu/

(cat. 219265); citrate synthase activity assay kit (cat. MAK193); TBARS assay kit (cat. MAK085); and BCA protein assay kit (cat. BCA1).

Univariate Statistical Analyses

Caulerpin accumulation in the liver of individual specimens was analyzed with a one-way ANOVA. The difference in caulerpin accumulation between altered/non-altered specimens was tested for significance with a Student's unpaired *t*-test assuming homoscedasticity. Both datasets were first verified to fulfill parametric conditions with a Shapiro–Wilk test. Univariate analyses were performed with GraphPad Prism v.8.0.2 (San Diego, CA, United States).

Multivariate Statistical Analyses

Dietary data were standardized by individual total sample biomass to achieve relative percentage composition and square root transformed as pre-treatment. Hierarchical cluster analysis with the group average cluster mode and a non-metric multidimensional scaling ordination (MDS) with 50 restarts were generated using a Bray–Curtis resemblance similarity matrix to display the grouping of samples and represent the samples in a low-dimensional space. The dendrogram was overlaid on the ordination by displaying the 40, 60, and 80% resemblance levels. Statistical significance was explored with a one-way analysis of similarity (ANOSIM) test and the null hypothesis of no differences in diet due to either alteration status or sex within the 10-sample subset or between size groups within the full dataset.

Condition indices and chemical/biochemical markers were first log-transformed and normalized (i.e., values were subtracted of the mean and divided by standard deviation) as pre-treatment and then entered into a principal components analysis (PCA) routine with the aim of visualizing the relatedness of samples while reducing the multidimensionality of data. The 2-d PCA eigenvector plot was superimposed with three biotic variables of the standardized dietary data as bubble plots, namely, *Caulerpa* sp., Gastropoda, and Echinodermata, to verify the contribution of individual stomach content fractions to abiotic markers.

A Euclidean distance matrix was calculated from the normalized biomarker dataset previously removed from the caulerpin accumulation variable and analyzed with a Canonical Analyses of Principal Coordinates routine to discriminate between *a priori* ATS-defined groups. To identify the contribution of each biomarker in discriminating between groups, the same matrix was employed in a Similarity Percentage routine by setting a 90% cut off for low contributions.

All datasets were analyzed using the non-parametric multivariate statistical software PRIMER v. 6.1.16 with the PERMANOVA + add-in package v. 1.0.6[2] (Clarke and Gorley, 2006).

RESULTS

Cooking Analysis

Among the cooking responses of all specimens, FR_03, FR_08, and FR_10 displayed the ATS condition, were inedible, and therefore classified as altered (**Figure 2**). During the cooking, the skin tore off from the body edges and the meat gradually detached from the skeletal structure producing a crackling sound. Once cooked, several signs of alteration were evident: dryness of the meat, complete detachment of the filet from the bones, stripped appearance of the head, skin retracted to the body center, and a rubbery texture to the touch. The fish filets were dry and chewy, with a bitter and persistent taste.

Diet Analysis

The stomach contents of *D. sargus* specimens ($n = 10$) were analyzed in terms of prey biomass and relative percentage composition (**Table 2**). The full dataset is supplied as supplementary information (**Supplementary Table 1**). The most abundant items in terms of relative prey composition were represented by gastropoda, decapoda, echinodermata, algae, and inorganic particles.

Algae on their own accounted for 78.5% (FR_05) and 94.3% (FR_10) of total stomach contents. The most abundant algae was the Rhodophyta *Ellisolandia elongata* (J. Ellis and Solander), which in some specimens (FR_08, FR_09, FR_10) represented the 95% of the total vegetal content. The most frequent vegetal species were the algae *Halopteris scoparia*, *E. elongata*, and the phanerogam *P. oceanica*, found in 80%, 70%, and 60% of fish stomach content, respectively. *Caulerpa* sp. was also identified in 60% of specimens but in extremely little amounts ranging from 0.19% (FR_02) to 1.61% (FR_05) of the total amount (g) of stomach content. Total content of animal origin widely ranged from 3.9% (FR_ 09) to 81.7% (FR_04).

The ingested animal pool was represented by a total of 320 individuals belonging to 42 species. The most abundant taxa were crustacea (53.13% of total abundance, 90% of frequency) and mollusca (35.94% of total abundance, 100% of frequency). Amphipoda, with 119 individuals and nine species, accounted for the 37.19% of total abundance and 70% frequency, and was the most abundant taxonomic group. *Ampithoe ramondi* (Audouin, 1826) resulted as the most abundant species in the animal pool, with 65 individuals overall contributing to 20.31% of total fauna. Gastropoda was the second class in terms of abundance (27.5%) and was observed in all *D. sargus* analyzed. It included 88 individuals and 13 species: the rissoid *Alvania discors* (Brown, 1818) and the cerithiidae *Bittium* spp. were the dominant ones with 51 and 15 individuals, respectively. In terms of abundance, the tanaidacean *Tanais* sp. (16 individuals), the bivalve *Musculus costulatus* (Risso, 1826) (14 ind.), and the isopod *Sphaeroma serratum* (Fabricius, 1787) (11 ind.) were also significant.

Specimens were allocated to two groups sharing a 40% resemblance level in a 2-d nMDS ordination (**Figure 3A**; stress 0.07). A 60% similarity was defined among the diets of specimens FR_01–03, 04–07, and 5–10, while those of FR_08 and FR_09 were 80% alike. Differences in nMDS sample

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[2] https://www.primer-e.com/

FIGURE 2 | The Abnormally Tough Specimen condition. Adult *D. sargus* exhibiting the ATS condition as a cooking response. For a detailed description of the filet appearance and organoleptic tests refer to Section 3.1.

allocation were better explained by the relative fractions of gastropoda, echinodermata, or photophilic algae (data not shown) than *Caulerpa* sp. (**Figure 3B**). The diets of FR_01-FR_10 did not statistically vary among sexes or altered/non-altered samples (*R* statistic: -0.071, $p < 0.667$). Altered and unaltered specimens grouped together, overall defining three sub-populations characterized by varying degrees of similarity.

On the other hand, dietary differences between individuals smaller or bigger than 300 g of BW were statistically significant, as indicated by the one-way ANOSIM ran on the full dataset with fish size as factor (*R* statistic: 0.25, $p < 0.029$, number of permutations 999) (**Supplementary Table 2**).

Caulerpa cylindracea Phenology and Percentage of Bottom Cover

The sampling area was characterized by infralittoral rocks colonized by photophilic algae, *P. oceanica* and *Cymodocea nodosa*. The percentage of surface cover of *Caulerpa cylindracea* was approximately 2.3-fold greater in July than in October, when the *D. sargus* sampling occurred, accounting to 87.5 and 38.3%, respectively (**Figure 4A**). Morphometric parameters are depicted in **Figure 4B**. All parameters showed greater values in July than October, in accordance with previous results. These data testify to the seasonal dynamics of *C. cylindracea* in SCI IT6000005.

Chemo-Ecological Panel

Results concerning caulerpin quantification are reported in **Table 3**. The metabolite was found in the liver of all specimens in the range of 2.441–3.512 $\mu g\ g^{-1}$ dry weight. Statistically significant differences in individual accumulation existed among specimens ($F_{9,20} = 16.80$, $p < 0.0001$). When averaging caulerpin accumulation based on the ATS outcome, altered specimens had a slightly higher concentration of caulerpin than unaltered fish but differences were not statistically significant ($t_8 = 0.2198$; $p = 0.8315$).

The response trends of biomarkers selected to evaluate the physiological conditions of *D. sargus* are shown in **Figure 5**. Liver data are always reported on the left *y*-axis and muscle on the right one. Data are presented for each specimen individually

TABLE 2 | Diet composition in terms of biomass (g, left) and relative percentage composition (right) for each of the 10 *Diplodus sargus* individuals analyzed.

	FR_01		FR_02		FR_03		FR_04		FR_05		FR_06		FR_07		FR_08		FR_09		FR_10	
Crustacea	0	0.00%	0.777	11.15%	0.473	3.58%	1.353	47.51%	2.330	17.64%	5.358	39.06%	4.440	37.11%	0.700	4.98%	0.585	2.25%	0.450	1.53%
Decapoda	0	0.00%	0.772	11.07%	0.473	3.58%	1.353	47.51%	1.582	11.98%	4.696	34.23%	4.401	36.78%	0	0.00%	0.170	0.65%	0.286	0.98%
Amphipoda	0	0.00%	0.005	0.07%	0	0.00%	0	0.00%	0.482	3.65%	0.662	4.83%	0.017	0.14%	0.672	4.78%	0.371	1.43%	0.085	0.29%
Tanaidacea	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0.121	0.92%	0	0.00%	0	0.00%	0.013	0.09%	0.016	0.06%	0	0.00%
Isopoda	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0.145	1.10%	0	0.00%	0.022	0.18%	0.015	0.11%	0.028	0.11%	0.079	0.27%
Mollusca	3.043	33.74%	2.461	35.30%	6.078	45.94%	0.973	34.16%	0.298	2.26%	1.055	7.69%	0.415	3.47%	0.777	5.53%	0.425	1.64%	0.996	3.40%
Bivalvia	0.085	0.94%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0.031	0.23%	0	0.00%	0.559	3.98%	0.245	0.94%	0	0.00%
Polyplacophora	0	0.00%	0	0.00%	0	0.00%	0.051	1.79%	0	0.00%	0	0.00%	0.013	0.11%	0	0.00%	0	0.00%	0	0.00%
Gastropoda	2.958	32.80%	2.461	35.30%	6.078	45.94%	0.922	32.37%	0.298	2.26%	1.024	7.46%	0.402	3.36%	0.218	1.55%	0.180	0.69%	0.996	3.40%
Echinodermata	2.201	24.40%	0.892	12.80%	1.798	13.59%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%
Teleostei	0.868	9.62%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%
Polychaeta	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0.131	0.95%	0.033	0.28%	0.583	4.15%	0.021	0.08%	0	0.00%
Photophilic algae	1.938	21.49%	0	0.00%	1.707	12.90%	0	0.00%	10.365	78.49%	4.124	30.06%	1.334	11.15%	8.560	60.90%	15.899	61.20%	27.665	94.34%
Posidonia oceanica	0	0.00%	0.082	1.18%	1.073	8.11%	0.041	1.44%	0	0.00%	0	0.00%	0.009	0.08%	0.041	0.29%	0.015	0.06%	0	0.00%
Caulerpa sp.	0.045	0.50%	0.013	0.19%	0.168	1.27%	0	0.00%	0.213	1.61%	0	0.00%	0.071	0.59%	0	0.00%	0	0.00%	0.215	0.73%
Inorganic particles	0.924	10.25%	2.511	36.02%	1.933	14.61%	0.481	16.89%	0	0.00%	0.644	4.69%	5.664	47.33%	3.314	23.58%	9.035	34.78%	0	0.00%
Plant debris	0	0.00%	0.235	3.37%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%
Animal debris	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	2.407	17.55%	0	0.00%	0.081	0.58%	0	0.00%	0	0.00%
Total animal content	6.112	67.77%	4.130	59.25%	8.349	63.11%	2.326	81.67%	2.628	19.90%	8.951	65.25%	4.888	40.85%	2.141	15.23%	1.031	3.97%	1.446	4.93%
Total plant content	1.983	21.99%	0.33	4.73%	2.948	22.28%	0.041	1.44%	10.578	80.10%	4.124	30.06%	1.414	11.82%	8.601	61.19%	15.914	61.25%	27.88	95.07%
Total gut content	9.019	100.00%	6.971	100.00%	13.230	100.00%	2.848	100.00%	13.206	100.00%	13.719	100.00%	11.966	100.00%	14.056	100.00%	25.980	100.00%	29.326	100.00%

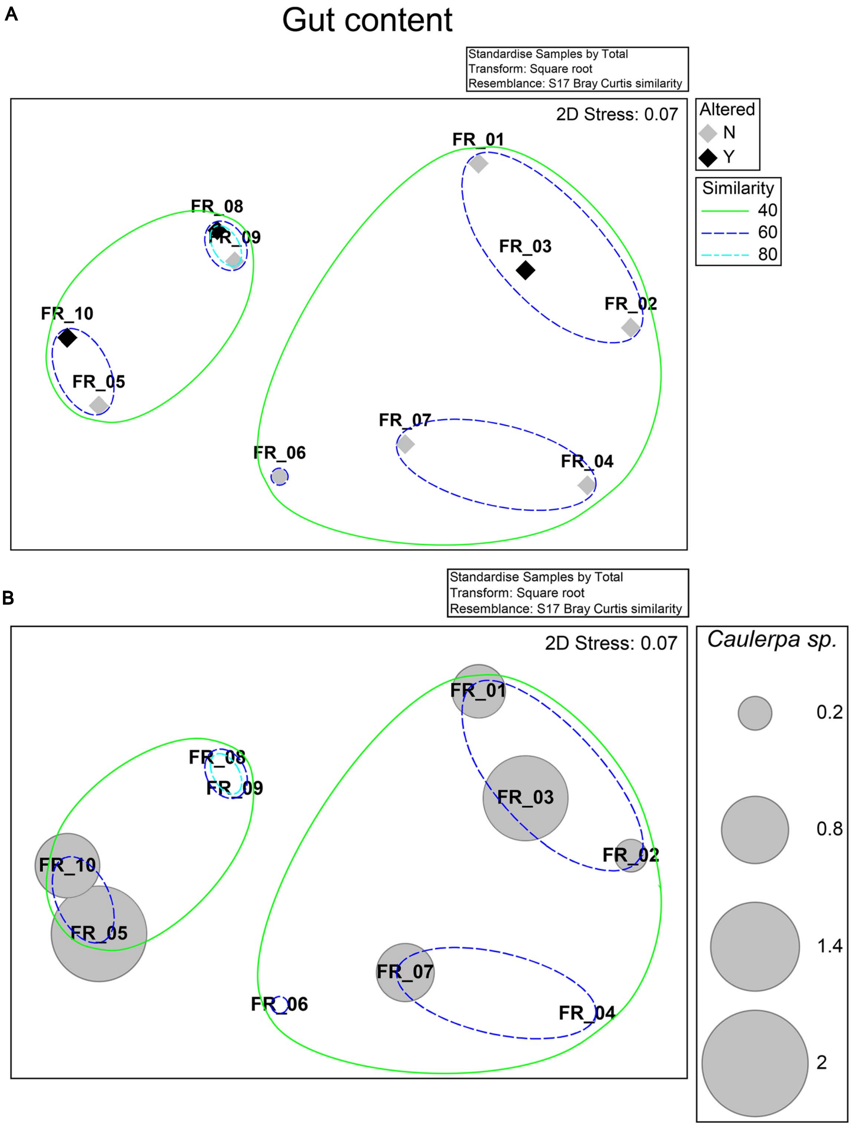

FIGURE 3 | nMDS ordination. Bray–Curtis similarity matrix from standardized and square root-transformed stomach content analysis was superimposed of the levels of the "Altered" factor and 40, 60, and 80% resemblances from a hierarchical cluster analysis **(A)** or the standardized relative stomach content percentage of *Caulerpa sp.* **(B)**.

and enzyme activity as well as MDA concentration were normalized either on protein content or weight of the tissue, respectively. The difference in the activities averaged on the basis of the ATS outcome were never significant for any of the biomarkers considered.

Catalase was approximately 100-fold more active in the liver than muscle; however, the general pattern seemed maintained between tissues, except for FR_07. The lowest activities were found in FR_08 liver (406.66 mU mg protein^{-1}) and FR_03 muscle (3.07 mU mg protein^{-1}) (**Figure 5A**).

In most samples, glutathione peroxidase (GPx) activities did not differ substantially between tissues. However, FR_02, FR_05, and FR_06 displayed approximately 10-, 6-, and 40-fold higher GPx activities in the liver than in muscle, respectively (**Figure 5B**).

A reversed situation was evident for glutathione S-transferase (GST). Activities in the liver were never elevated and the highest value accounted for 3.08 mU mg protein^{-1} (FR_05). Much higher activities were found in the muscle in six specimens, with FR_01-FR_04 exhibiting the largest fold-change differences (71, 210, 212, and 23, respectively) (**Figure 5C**).

Superoxide dismutase (SOD) activities matched almost perfectly between tissues in all samples, with liver being the most active of the two. FR_08 had the highest liver activity with 60.07 U ml^{-1} and FR_03 was one of the four most active samples with 26.66 U ml^{-1}, together with FR_05 and FR_06 (**Figure 5D**).

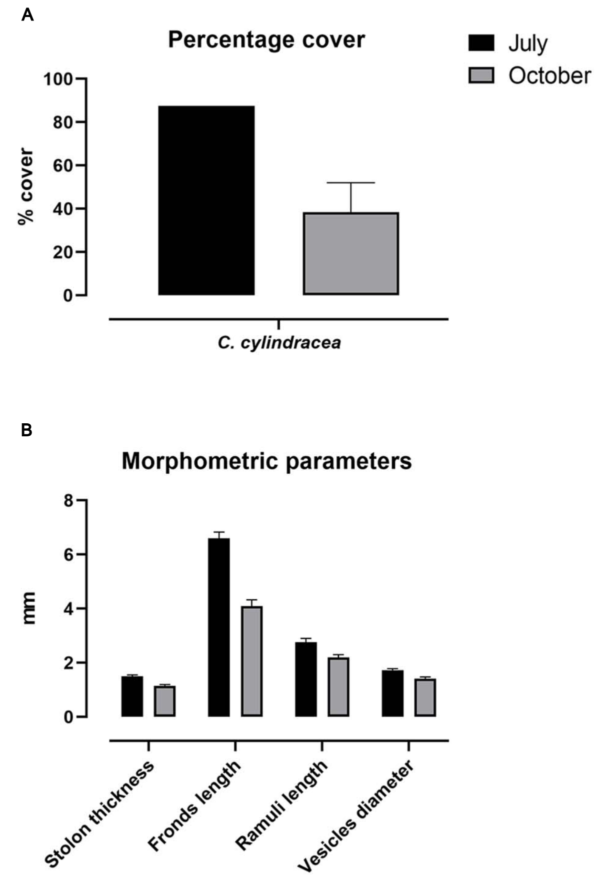

FIGURE 4 | Phenological parameters. Percentage cover **(A)** and phenology **(B)** of *C. cylindracea* sampled in July and October 2019. Morphological parameters are expressed as mean ± SEM.

Citrate synthase exhibited a higher between-tissue variability, and only samples FR_01, FR_08, and FR_10 displayed similar activities in liver and muscle; with regard to muscle, variability also occurred within sample replicates, as reflected by higher standard error of means (**Figure 5E**).

AChE trends generally matched between tissues, except for FR_04, FR_07, and FR_09. AChE activity was always greater in muscle than in liver except for FR_09 and FR_10 (**Figure 5F**).

Malondialdehyde (MDA) was appreciated in the liver, with the highest value accounting to 0.52 nmol mg tissue^{-1} in FR_03. MDA in muscle was probably below the kit detection limit (**Figure 5G**).

In the best-fitting plane, PC1 and PC2 explained 27.8 and 17.4% of variation, respectively, equaling a cumulative variability of 45.2%. An additional 14.8% was explained by PC3 (data not shown). The loading plot shows that CF, HSI, AChE_liver, LPO_liver, Caulerpin_liver, GST_liver, GST_muscle, SOD_liver, and SOD_muscle have their main contribution to the first axis, with CF, HSI, AChE_liver, GST_liver, SOD_liver, and SOD_muscle increasing from right to left. GSI, CAT_liver, CAT_muscle, AChE_muscle, CS_liver, CS_muscle, LPO_muscle, GPx_liver, and GPx_muscle are predominantly represented on PC2, with LPO_muscle increasing from top to bottom of the diagram (**Figure 6A**).

ATS-wise, FR_03 was associated with high Caulerpin_liver and LPO_liver but low CF, GPx_muscle, and GST_liver (among others). FR_10 was displayed in an almost opposite situation to FR_03. FR_08 correlated mainly with high LPO_muscle and low GSI, CAT, AChE_muscle, and CS_muscle. Although with lower concentrations, samples FR_05–FR_10 accumulated caulerpin in their liver but FR_06, FR_08, and FR_09 did not present any *C. cylindracea* content in their stomach (**Figure 6B**). Worthy of note, samples FR_05, FR_06, and FR_10 were similarly located in the 2-d space.

The spatial segregation between altered/non-altered specimens plotted by the sex factor is shown in **Figure 7**. The model had a large canonical correlation of 0.9597.

DISCUSSION

This study identified the presence of the invasive *C. cylindracea* in the northern Tyrrhenian Sea and, by means of diet analysis, phenology, as well as biological and chemo-ecological biomarkers, multidisciplinarily elucidated its impacts on the local white seabream *D. sargus* population in light of the ATS anomaly. Although much ecological research has been conducted on *C. cylindracea* in the Mediterranean Sea (Piazzi et al., 2001b; Capiomont et al., 2005; Ruitton et al., 2005; Piazzi and Ceccherelli, 2006; Klein and Verlaque, 2008; Piazzi and Balata, 2008), the biological consequences have so far been explored only in *D. sargus* individuals originating from the Adriatic Sea, Ionian Sea, and Atlantic Ocean. To the best of our knowledge and ATS-wise, this is the first study to expand the reporting and investigate the physiological status of white seabream in the Tyrrhenian Sea by compa ring the responses of altered and unaltered fish.

In the Mediterranean Sea, the opportunistic *C. cylindracea* is capable of forming continuous dense meadows in various photophilic and sciaphilic benthic assemblages such as rocky substrate, dead *P. oceanica* "matte," coralligenous, and detritic assemblages (Klein and Verlaque, 2008), causing a decrease in the total number of species and total macrophyte cover (Ceccherelli and Piazzi, 2001; Piazzi and Cinelli, 2003; Balata et al., 2004; Piazzi and Ceccherelli, 2006; Cinelli et al., 2007; Klein, 2007; Klein and Verlaque, 2007; Piazzi et al., 2001a,b, 2005, 2007). In our study area, the percent coverage and phenological parameters were greater in July than in October, contrarily to Ruitton et al. (2005). However, morpho-structural characters of the algae were reported to fluctuate pronouncedly over spatial and temporal scales (Piazzi et al., 2001a; Capiomont et al., 2005; Mezgui et al., 2007). As for the seasonal variation in our study area, the regression observed between the period of July and October is likely a phase within a larger vegetative cycle, which reaches its peak in November. Furthermore, sampling carried out in July 2020 showed a lower coverage of *C. cylindracea* compared to 2019 (our data, unpublished), in line with the typical life cycle (Ruitton et al., 2005).

The order Caulerpales synthesizes 18 secondary metabolites, the most common of which are caulerpenyne (a sesquiterpenoid),

TABLE 3 | Caulerpin bioaccumulation in *D. sargus* livers expressed as μg g^{-1} dry weight and indication of the ATS condition. Standard deviation was calculated on *n* = 3.

Samples	ATS condition	Absorbance ± SD	Caulerpin (μg g^{-1}) ± SD
FR_01	No	40,396.36 ± 4319.62	3.105 ± 0.169
FR_02	No	53,056.74 ± 13,876.86	3.512 ± 0.552
FR_03	Yes	39,323.89 ± 3579.75	3.115 ± 0.143
FR_04	No	20,925.82 ± 5957.41	2.579 ± 0.232
FR_05	No	19,647.44 ± 1846.66	2.549 ± 0.07
FR_06	No	16,263.23 ± 1021.14	2.441 ± 0.04
FR_07	No	27,211.58 ± 883.34	2.800 ± 0.036
FR_08	Yes	31,478.61 ± 3000.97	2.874 ± 0.116
FR_09	No	16,653.95 ± 789.80	2.451 ± 0.027
FR_10	Yes	18,416.70 ± 1831.49	2.490 ± 0.071

caulerpicin (a mixture of hydroxy amides), and caulerpin (a bisindolic alkaloid red pigment) (Higa and Kuniyoshi, 2000; Yang et al., 2015). Methanolic extracts of whole *C. cylindracea* were proven cytotoxic to a human breast cancer cell line (Chia et al., 2015). Crude extracts as well as purified caulerpenyne had antiproliferative, growth-inhibitory and apoptotic effects on cancerous cell lines (Fischel et al., 1995; Barbier et al., 2001; Cavas and Yurdakoc, 2005). Caulerpenyne also interfered with the sperm flagellar motility and fertilization rates as well as with the timing of first cleavage of sea urchin *Lytechinus pictus* and *Paracentrotus lividus*, respectively (Paul and Fenical, 1986; Pesando et al., 1996). Research on the effects of caulerpin has not been as thorough: a lack of acute toxicity was indicated (Vidal et al., 1984; Higa and Kuniyoshi, 2000) and antitumoral and nucleic acid fragmentation potentialities were reported *in vitro* (Schröder et al., 1998). In general terms, sesquiterpenes/sesquiterpenoids exert their toxicity by GSH and protein alkylation; DNA alkylation and epigenetic machinery deregulation leading to genetic mutations and transcriptional changes; increased oxidative stress; and disruption of mitochondrial homeostasis by inhibition of the SERCA pump (Amorim et al., 2013). Alkaloids compromise enzymatic activities, inhibit DNA synthesis, repair mechanisms and mitochondrial complex II, and affect the nervous system (Matsuura and Fett-Neto, 2015; Ferramosca et al., 2016). Recently, Vitale et al. (2018) and Del Coco et al. (2018) demonstrated that lipid metabolism is directly disrupted by caulerpin too.

Following the methods of Terlizzi et al. (2011), we selected fish larger than 300 g BW to populate our working subset (**Table 1**). Indeed, a growing body of evidence indicates that the

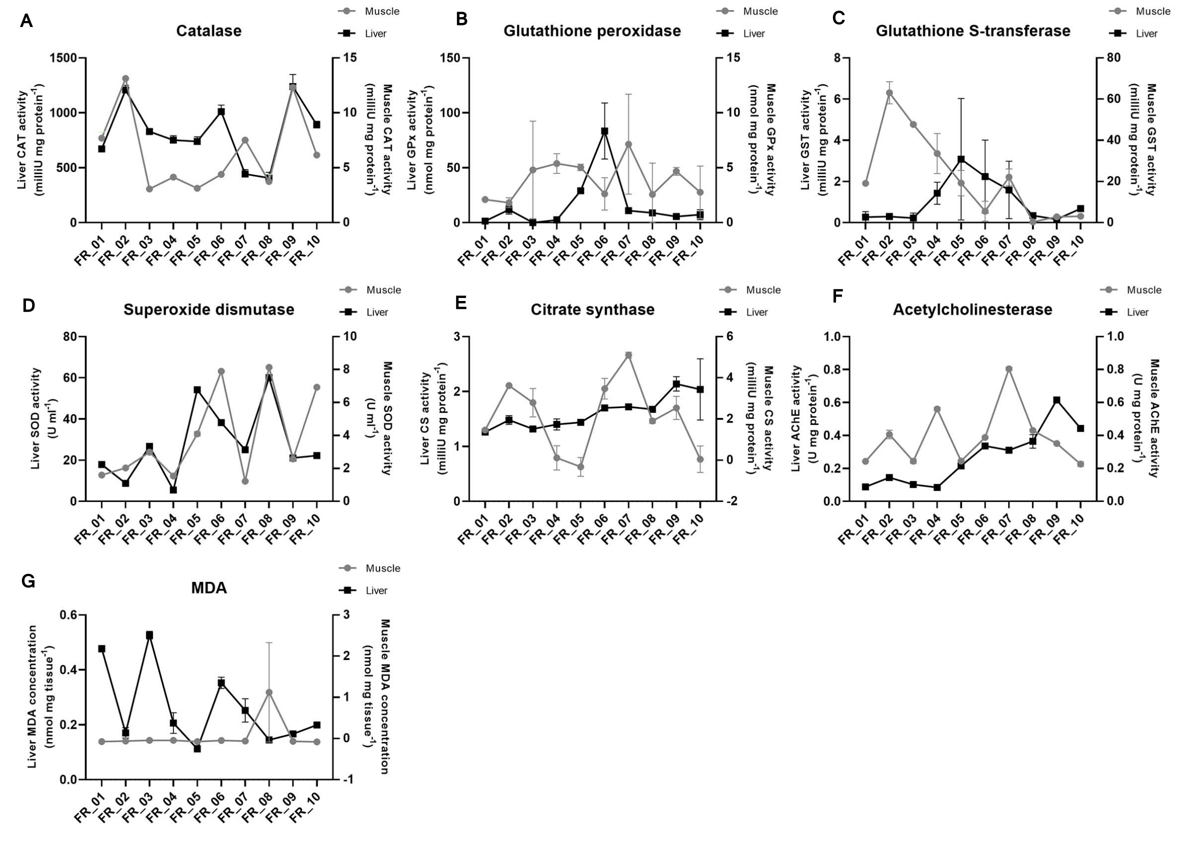

FIGURE 5 | Fish physiological responses. Catalase **(A)**, glutathione peroxidase **(B)**, glutathione *S*-transferase **(C)**, superoxide dismutase **(D)**, citrate synthase **(E)**, acetylcholinesterase **(F)**, and malondialdehyde **(G)**. Enzyme activities are expressed as either mU or U. MDA is expressed as nmol. All data were normalized to tissue protein content (mg) or weight (mg) except for superoxide dismutase. Liver and muscle data are plotted on the left and right *y*-axis, respectively, as mean ± SEM.

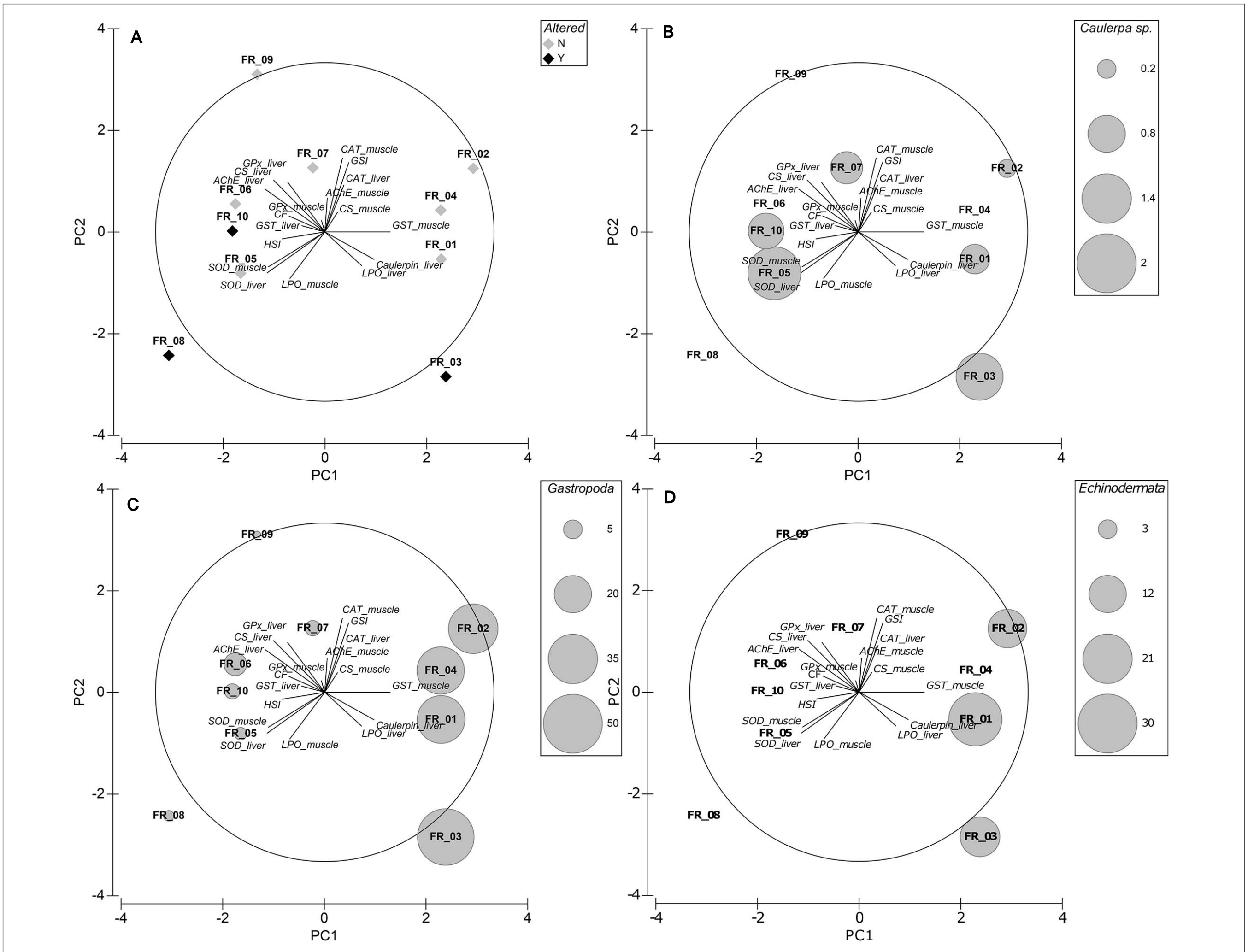

FIGURE 6 | Exploratory MVA analysis. Principal components analyses of biomarker data superimposed on the eigenvector plot **(A)**, *Caulerpa* sp. relative stomach content fraction **(B)**, Gastropoda relative stomach content fraction **(C)**, and Echinodermata relative stomach content fraction **(D)**. PC1 and PC2 explain 27.8 and 17.4% of variation, respectively.

ATS anomaly only occurs in specimens longer than 15–20 cm (Casadevall et al., 2020), a size that, according to length–weight relationships modeled from Mediterranean catches (Verdiell-Cubedo et al., 2006), corresponds to a BW of 189 g. Regardless of body size, stomach samples of the entire dataset were analyzed in their content to attest for ontogenetic diet shifts.

Caulerpa cylindracea is consumed by vertebrate and invertebrate species, either directly or indirectly and regardless of their feeding strategy: *Aplysia* sp., *Ascobulla fragilis* (Jeffreys, 1856), *Elysia tomentosa* (Jensen, 1997), *Lobiger serradifalci* (Calcara, 1840), *Oxynoe olivacea* (Rafinesque, 1814), *P. lividus* (Lamarck, 1816), *Sphaerechinus granularis* (Lamarck, 1816), *Boops boops* (Linnaeus, 1758), *Coris julis* (Linnaeus, 1758), *D. sargus* (Linnaeus, 1758), *Diplodus vulgaris* (Geoffroy Saint-Hilaire, 1817), *Pagellus acarne* (Risso, 1827), *Sarpa salpa* (Linnaeus, 1758), *Siganus luridus* (Rüppell, 1829), and *Spondyliosoma cantharus* (Linnaeus, 1758) (Klein and Verlaque, 2008; Box et al., 2009; Felline et al., 2017; and references therein; Sureda et al., 2006). Our data confirmed a trophic relationship between *D. sargus* and *C. cylindracea*, although the percentage by gut content weight was extremely low when not null, contrarily to Felline et al. (2017, 2012) and Terlizzi et al. (2011). Sixty percent of our samples prevalently displayed benthic carnivorous dietary habits (FR_01–FR_04 and FR_06–FR_07), but total plant content reached high relative percentage compositions in the remainder (FR_05 and FR_08–FR_10) (**Table 2**). Gastropoda, decapoda, echinodermata, photophilic algae, and inorganic particles were highly present in terms of gut relative prey composition in fish larger than 300 g BW. The widespread presence of *C. cylindracea* in the study area may be the reason why Crustacea and Mollusca were the most abundant stomach content taxa (89.07% of the overall total abundance): in fact, the algal-driven replacement of *P. oceanica* meadows foster changes in abundance, dominance, and diversity within local macrozoobenthic communities (Vázquez-Luis et al., 2009, 2012; Deudero et al., 2011; Pacciardi et al., 2011).

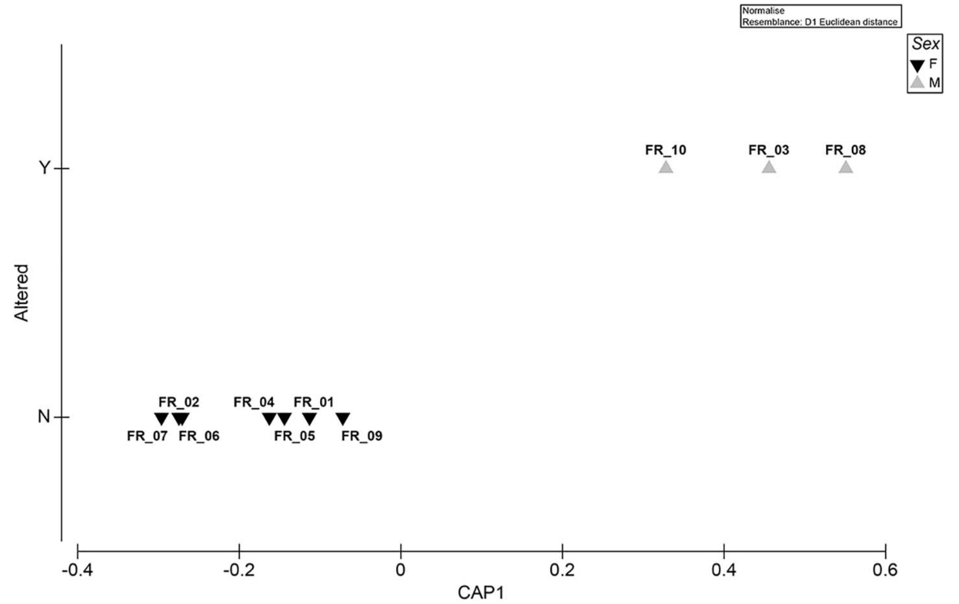

FIGURE 7 | Exploratory MVA analysis. Canonical analysis of principal coordinates run on a Euclidean distance biomarker matrix. The "Sex" factor is superimposed as plot symbols. Correlation equaled 0.9597.

Mollusks were present in all specimens but had the greatest abundance in FR_01–FR_04 and echinoderms were present exclusively in FR_01–FR_03. Direct physiological interactions between mollusks and echinoderms with *Caulerpa* spp. were reported: Tejada et al. (2013) demonstrated that *P. lividus* feeds on *C. cylindracea* and that the algae induces antioxidant adaptations in the sea urchin; Sureda et al. (2009) highlighted changes in the antioxidant responses of both the gastropod *Bittium reticulatum* and *C. taxifolia* following grazing. It must be noted that the cerithiid *B. reticulatum* was the second most abundant gastropod in our stomach content data, together with *Fissurella* spp. and *Haliotis* spp., which are all known to prey on *Caulerpa* spp. (Poore, 1972; Franz, 1990). In the PCA plot, the correlation of samples FR_01–FR_04 with the marker Caulerpin_liver was evident and sample allocation was better explained by the relative percentage of ingested Gastropoda (**Figure 6C**) and Echinodermata (**Figure 6D**) rather than the actual *Caulerpa sp.* stomach content biomass (**Figure 6B**). The present study lacks data on caulerpin bioaccumulation in local mollusks and echinoderms and further investigations are needed to clarify this relationship; however, we believe that it is highly possible that *D. sargus* bioaccumulates caulerpin in the liver due to the consumption of detritivores, which assimilate *Caulerpa*-derived compounds from the sediment, and generalist herbivores, which actively feed on *Caulerpa* sp.

According to professional and recreational fishermen, the ATS anomaly is not exclusive of *D. sargus* but also affects congeners *D. puntazzo* (Cetti, 1777), *D. cervinus* (Lowe, 1838), and *D. vulgaris* (Geoffrey Saint-Hilaire, 1817) as well as other Sparidae species (Casadevall et al., 2020). In our case, 30% of tested specimens displayed the ATS condition (**Figure 2**). We could not reject the null hypothesis of no differences among diet compositions of fish > 300 g BW on the basis of the Altered factor, meaning that dietary habits of ATS fish were not dissimilar from those of fish not displaying the ATS condition, and **Figure 3** further indicates clustering similarities independently of an ATS response. On the other hand, stomach contents of fish larger than 300 g BW differed statistically from those of fish smaller than 300 g BW, whose stomach was almost entirely occupied by Decapoda (Crustacea) and devoid of any plant content (except for FR_SI_03) (**Supplementary Tables 1, 2**). This is likely due to ontogenetic shifts in the feeding habits of the white sea bream. Similar findings were already reported in the Atlantic Ocean and Western Mediterranean Sea: Figueiredo et al. (2005) statistically demonstrated a shift in the feeding preferences of *D. sargus* at a length of approximately 25 cm; Merciai et al. (2018) found that the diet of "large" individuals (23.1–32.9 cm L) was not correlated to that of either "small" (4.9–15 cm L) or "medium" (15.1–23 cm L) ones. It is important to highlight that our samples ranged from 24.3 to 33.5 cm in length and that small fish never exhibited the ATS condition following cooking. Given the peculiar phenological parameters presented in **Figure 4** and considering past evidence (Figueiredo et al., 2005; Osman and Mahmoud, 2009), we cannot exclude the fact that *D. sargus* in the northern Tyrrhenian Sea displays seasonal dietary changes too. Our data revealed that the degree of trophic exposure of the fish to the invasive alga, and the overall diet composition of adult specimens, cannot on its own explain the occurrence of the ATS anomaly. It is also unknown whether greater *C. cylindracea* cover or fronds and ramuli lengths recorded in July contribute to aggravating the incidence of such a phenomenon. Future efforts might be directed at enlarging the sample size for increasing the power of statistical analysis and investigating fish dietary habits, ATS rates, and algal secondary metabolite content also at the peak of the seaweed proliferation period. Because (i) caulerpin accumulation in the liver of *D. sargus* fluctuates seasonally (Gorbi et al., 2014), (ii) caulerpenyne production varies temporally along the year and spatially within thallus parts (Box et al., 2010), and (iii) algae can promptly transform secondary metabolites into more toxic and deterrent compounds as a response to mechanical wounding (Jung et al., 2002), it is possible that biological responsiveness changes too, even though these aspects have not been studied yet.

The UHPLC analysis identified caulerpin in all liver samples, confirming that caulerpin, as lipophilic metabolites, can be detected even when the animal does not show any sign of direct *Caulerpa* sp. ingestion (**Table 3**). Contrarily, Felline et al. (2017) detected the metabolite only in *S. cantharus* and *S. salpa* even though the vast majority of the species they investigated had presented algal fragments in their stomachs. Rates/efficiency of detoxification pathways are probably the underlying cause of such a discrepancy. Samples FR_01–FR_03 displayed slightly higher caulerpin concentrations than FR_04–FR_10 but the overall range was 2.441–3.512 $\mu g\ g^{-1}$ dry weight. These values are much lower and less dispersed (mean ± SD: 2.792 ± 0.36 $\mu g\ g^{-1}$) than the liver contents reported by Gorbi et al. (2014) and Terlizzi et al. (2011). Such a small variance might be due to (i) highly homogenous fish sizes in the working subset, (ii) limited direct consumption of *C. cylindracea* due to reduced algal coverage and morphometric parameters (as per our data), and (iii) adaptive phenotypic plasticity driven by abiotic features and/or biotic factors of the ecological environment resulting in improved metabolic detoxification efficiency (e.g., Ghalambor et al., 2007). The third hypothesis would be the most complex to verify.

The multi-biomarker panel was built with select proxies of antioxidant defenses, metabolic activity, lipid peroxidation, neurotoxicity, and energy availability. It provided information about a possible active response to oxidative stress, the proper sustainment of metabolic processes, the impairment of cell structural integrity, disturbance of the nervous transmission, or the specimen suitability to a successful reproductive investment, respectively. Such an integrative approach is recommended for maximizing the significance of each biomarker and investigating the ecological implications of exposure to toxic metabolites at various levels of biological organization (sub-individual, individual, and population) (Moore et al., 2004). Particular attention was called on oxidative stress biomarkers because *Caulerpa* sp. bioactive compounds are considered to be a possible source of reactive oxygen species (Sureda et al., 2006, 2009; Felline et al., 2012). The comparison of the responses between altered and unaltered specimens did not uniquely or predictively explain the ATS anomaly (**Figure 6A**). FR_03, FR_08, and FR_10 was each associated with different physiological markers: those related to the liver were predominantly represented by the first axis of the PCA. Catalase did not seem to be associated with the anomaly as the eigenvector points opposite to ATS fish in the 2-d space; this may be justifiable by its typical biphasic response (Regoli et al., 2011), according to which the marker displays enhanced activity at low concentrations of the contaminant but is inhibited at higher ones. GPx activities in both tissues were associated to FR_08 and FR_10: this may represent an antioxidant compensatory mechanism, with glutathione peroxidase replacing catalase in the removal of hydrogen peroxide (Regoli et al., 2011). Specimens FR_08 and FR_10 were also correlated with high HSI, which may be explained by a liver hypertrophy condition caused by the need for increased biotransformation of lipophilic compounds (Terlizzi et al., 2011). A negative correlation between GSI and ATS fish was evident: this is in line with what was reported by Felline et al. (2012), who found higher GSI values in individuals not accumulating the algal metabolite. Lipid peroxidation of liver and muscle correlated well with FR_03 and FR_08–FR_10, respectively.

All ATS specimens were males (**Figure 7**). Despite the fact that larger sample numbers are needed to unequivocally confirm the data, to our knowledge, this is the first report of the anomaly being found exclusively in *D. sargus* of a specific sex. This species was reported to be gonochoric (Bauchot and Hureau, 1986) or have an hermaphroditic proterandry biology (Micale and Perdichizzi, 1994), with sexual inversion occurring at a length of approximately 22 cm (Mouine et al., 2007) even though the range of 25–34 cm was reported for different sub-species populations (e.g., Mann and Buxton, 1998). The constrained approach followed to discriminate among *a priori* groups was supported by a large canonical correlation and showed a clear separation of the data clouds (**Figure 7**). The SIMPER routine performed on the same matrix revealed a low within-group distance for both sexes and identified GSI as the variable that contributed the most to differentiating the groups (**Supplementary Table 3**). The fact that such differential allocation did not emerge from the unconstrained ordination was likely due to the complexity of the data, whose total variation could not be captured by the first two axes of the PCA plots (Anderson et al., 2008). In addition to lipid metabolism and behavioral disturbances (Magliozzi et al., 2017; Del Coco et al., 2018), could algal metabolites also impair fish steroidogenesis?

Taking into consideration the complex results we presented, it is not easy to detect a general trend or provide an unambiguous explanation for the occurrence of the ATS anomaly. Through a correlative study, Casadevall et al. (2020) recently suggested copper in the form of antifouling paints and aquaculture-related copper sulfate rather than *C. cylindracea* ingestion as the causative agent of the ATS anomaly. It is difficult to discriminate between the effects of caulerpin and copper because the latter does not bioaccumulate, as evidenced by the lack of any correlation between Cu concentration and fish length (Merciai et al., 2018). To clarify the potential *C. cylindracea*–ATS link, the physiology of AT individuals will be investigated with RNA-seq and metabolomics approaches.

CONCLUSION

The present paper highlights direct trophic interactions between the white seabream *D. sargus* and the invasive *C. cylindracea* in the Tyrrhenian Sea. For the first time, photographic evidence of ATS post cooking is presented. Although further studies are needed to establish whether the algae or its metabolites are directly responsible of the anomaly, the novelty of our approach was to compare altered and unaltered specimens to better elucidate possible underlying differences in their physiological responses.

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.

ETHICS STATEMENT

Ethical review and approval was not required for this study because the animals were not subject to any experimental manipulation. Fish sampling was performed in strict accordance to Directive 2010/63/EU.

AUTHOR CONTRIBUTIONS

EM, SB, and MM: funding acquisition. AM and EM: data curation. AM, EM, MB, FP, VL, and FT: investigation. EM and AT: methodology. AM: formal analysis. AM, EM, and MM: resources. MR, AT, and MM: supervision. AM, EM, and MB: visualization. AM, EM, MB, VL, and FT: writing (draft). MR, AT, and SB: writing (revision). All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.680787/full#supplementary-material

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Predation Cues Lead to Reduced Foraging of Invasive *Siganus rivulatus* in the Mediterranean

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Invasive species are one of many anthropogenic challenges to maintaining a healthy marine ecosystem. Two rabbitfish species (*Siganus rivulatus* and *Siganus luridus*) are among the more successful migrants from the Red Sea to the Mediterranean, where their intense foraging has caused damage to the algae community, thus reducing primary production and habitat complexity, and impacting nurseries for early life stages. Anecdotal evidence suggests that the impact of rabbitfish on algae is lower in Marine Protected Areas (MPAs) although rabbitfish densities are similar in protected and fished areas. One explanation could be that fear of predators, more often present inside MPAs and an important component of a healthy marine ecosystem, reduces the ecological impacts of rabbitfish. This research aimed to test if such fear effects do occur in rabbitfish. Using controlled mesocosm experiments, we tested *S. rivulatus* reactions to two chemical predation cues: chemical alarm cues released from a recently killed conspecific fish, and water-borne cues from a tank with a live grouper predator, *Epinephelus marginatus.* We found that rabbitfish significantly reduce their overall food consumption as well as their bites per minute when exposed to the alarm cue, but not when exposed to the grouper water cue. These results support the idea that MPAs, which effectively increase the density of large piscivores and hence predation, can mitigate the impact of invasive herbivorous species. If the mesocosm results can scale up to natural systems, predation cues may be artificially introduced to other target areas in order to reduce rabbitfish grazing outside reserves. Thus, this study provides information that can be used to manage the ecological impacts caused by invasive rabbitfish, both inside and outside of marine reserves.

Keywords: invasive species, predator effects, foraging, rabbitfish, MPA, siganus

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INTRODUCTION

The Mediterranean Sea is under an ongoing invasion by hundreds of exotic species, in large part due to the opening of the Suez Canal in 1869 (Belmaker et al., 2009; Katsanevakis et al., 2014; Galil et al., 2015; Castellanos-Galindo et al., 2020; Golani, 2021). Successful alien species exhibit a diversity of ecological traits that may enable them to become established in the Mediterranean Sea (Belmaker et al., 2013; Azzurro et al., 2014; Givan et al., 2017). Two exotic herbivorous fishes, *Siganus luridus* and *Siganus rivulatus* (commonly known as rabbitfish), are among the more successful migrants from the Red Sea to the Eastern basin of the Mediterranean (Coll et al., 2010; Golani, 2010; Pickholtz et al., 2018). These species of rabbitfish are among the alien species most threatening to biodiversity in the Mediterranean (Roy et al., 2019). Rabbitfish are efficient herbivores, a trait that plays a key role in supporting the health of coral reefs by controlling harmful algae growth (Fox and Bellwood, 2013; Brandl and Bellwood, 2014). In the Mediterranean, however, their grazing habits have had a detrimental effect, creating barren reefs in areas that were once abundant algal forests (Sala et al., 2011; Verges et al., 2014; Yeruham et al., 2020). Rabbitfish have also been observed to graze on both established algae and on turf which contains macroalgal recruits (Verges et al., 2014), in contrast to native Mediterranean herbivores who feed primarily on adult macrophytes. All of this disturbs the larger ecological community that depends on macroalgae and seagrass for many ecosystem functions, including primary production, nutrient recycling, habitat formation, and nurseries for many marine species (Mineur et al., 2015; Teagle et al., 2017) and leads to a shift in ecosystem functions (Rilov et al., 2020).

Mediterranean coastal ecosystems are often heavily overfished, with top predators disproportionally targeted (Jennings and Kaiser, 1998). MPAs have been established in many places around the Mediterranean basin (Claudet et al., 2020). Marine Protected Area with the highest protection level have had a strong positive impact on the density and biomass of high trophic level species (Giakoumi et al., 2017). For instance, in a series of surveys of marine nature reserves in Israel, higher numbers and larger density of groupers (*Epinephelus costae*, *Epinephelus marginatus*, and *Mycteroperca rubra*) were observed inside marine reserves (Lazarus et al., 2020). Mediterranean groupers are predators of rabbitfish (Aronov and Goren, 2008) and thus can potentially cull the population of prey fish through consumption. But, in the same MPAs surveyed, there were also high numbers of rabbitfish inside reserves, similar to the numbers outside reserves (Rilov et al., 2018; Lazarus et al., 2020).

Despite the high presence of rabbitfish, reef surfaces inside MPAs have a higher cover of marine macrophytes than in unprotected adjacent habitats (Rilov et al., 2018), suggesting reduced grazing pressure. Trophic cascades can be triggered by a reduction in prey density or by changes in prey behavior. Thus, Rilov et al. (2018) proposed that while the higher predator (grouper) density inside MPAs might not significantly affect rabbitfish density, it may reduce rabbitfish grazing activity through fear of predation, i.e., by influencing their behavior. However, this hypothesis has never been experimentally tested.

What and where fish graze can be influenced by predation risk since animals must balance the need for food with the need for safety (Hammerschlag et al., 2010). One of the ways in which predators modify prey behavior is by creating a "landscape of fear" that impacts foraging activities (Laundre et al., 2010; Catano et al., 2016). For example, prey may hide in refuges to avoid predation thereby reducing their time spent foraging in risky areas (Orrock et al., 2013). Some species of rabbitfish have developed social strategies to assist in foraging under the threat of predation, such as the formation of adult pair-bonds where one member grazes while the other remains on watch (Woodland, 1990; Brandl and Bellwood, 2015). This strategy decreases their predation risk but also reduces each individual's foraging time. Changes in foraging behavior may negatively impact the fitness of the prey by lowering their food intake or requiring them to divert resources away from reproduction. However, empirical evidence suggests that there is a high variance in whether and how strongly prey is affected by these changes (Sheriff et al., 2020).

Any change in foraging strategies would also impact the prey's resources (Peacor and Werner, 1998). These non-consumptive interactions (Abrams, 1995) have been shown to amplify through food chains, especially in the marine environment, and often have a higher impact on the resources of the prey than on the prey themselves (Werner and Peacor, 2003; Preisser et al., 2005; Rasher et al., 2017). One example of a non-consumptive trophic cascade is the impact of a generalist fish predator, the Señorita (*Oxyjulis californica)*, on habitat-forming feather boa kelp. The presence of the Señorita fish has been shown to suppress the grazing of limpets, thereby reducing kelp frond loss (Haggerty et al., 2018). Improving predator stock has been suggested as a successful strategy to aid in the restoration of key algae species by limiting the grazing pressure of herbivores (Gianni et al., 2018). However, changes in grazing patterns under threat of predation have not been shown specifically for rabbitfish.

Behavioral changes only occur when prey can detect predatory cues in their environment (Brown et al., 1999; Luttbeg and Trussell, 2013). Prey responses can be triggered by different stimuli including chemical cues, something particularly common in the marine environment (Chivers and Smith, 1998; Wisenden, 2000). Chemical cues may be released by the predator, or by other prey animals when they are attacked ("chemical alarm cues"). For example, rusty crayfish (*Faxonius rusticus*) changed their foraging behavior when exposed to water-borne odors of largemouth bass (Wood et al., 2018). In addition, reef urchins (*Echinometra viridis)* were shown to reduce grazing in response to crushed conspecific cues (Dunn et al., 2018). The ability to quickly and accurately identify predators is key to prey fitness, can be based on many different environmental cues, and may even be learned from experience (Mitchell et al., 2011).

This research aims to experimentally test how rabbitfish food acquisition activities are affected by the presence of a predator, using predator-related stimuli and predation alarm cues. We predict that in the presence of predation cues, rabbitfish will display lower overall consumption, as well as a lower bite rate. Understanding how the presence of predators changes the foraging behavior of these invasive fish can help scientists and conservationists enhance the protection and restoration of healthy ecosystems by reducing the damage caused by overgrazing.

MATERIALS AND METHODS

Experimental Design

To measure rabbitfish foraging behavior with and without predation cues, we conducted experiments using mesocosms with flow-through seawater to allow near-real sea conditions.

Mesocosms have been shown to be useful models for measuring ecological functions (Brown et al., 2011). We conducted the experiments at the Israel Oceanographic and Limnological Research (IOLR) facility in Haifa during the fall of 2019 and the summer of 2020.

To create mesocosms for the experiment, we used four large plastic containers measuring 1.12 × 1.12 × 0.8 m. For each container, we drilled a hole on the bottom of the tank for a drain, and a hole on the side of the tank for water outflow. We connected each tank to a water intake pipe and a water outflow pipe. Water in the tanks was supplied by the IOLR's constant flow-through system, allowing fresh sea water to circulate in and out of the tanks, thereby mimicking sea conditions. We also connected air inflow tubes with a diffuser in each tank to ensure an oxygen rich environment for the fish.

We collected adult *S. rivulatus* from the shallow reefs near IOLR and acclimated them to the experimental conditions before the experiment began. The fish were collected at night with hand nets by SCUBA divers and snorkelers. The acclimation period was between six (in 2019) and two (in 2020) months. We considered the fish acclimated when they began to eat regularly, quantified by the fish consuming at least 80% of the food within 5 h of feeding, when fed every other day. Once acclimated, we introduced between 4 and 5 small adult fish (rabbitfish are schooling fish, and placing one individual per tank might add stress), with an average length of 16–17 cm (± 3.4, SD), into each of four mesocosms. In the 2019 experiments, we distributed the fish between tanks in a way that resulted in a similar overall combined foraging rate between tanks. In the 2020 experiments, we measured the total length of each fish prior to introduction into the experiment tanks to ensure a similar overall biomass of fish between tanks.

The aim of the experiments was to determine if, and by how much, rabbitfish foraging behavior changes in the presence of signs of potential predation risk. To analyze this, we tested the reaction of the rabbitfish *S. rivulatus* to two different chemical cues by (1) introducing pieces of a recently killed conspecific fish into the tanks (hereafter *alarm cue*) and (2) exposing the fish to water from the holding tank of a live predator (hereafter *grouper water*). For the *alarm cue* we sacrificed one of the extra fish from a holding tank, cut the body into three equal pieces, and introduced one piece (sealed in a mesh bag) into each treatment tank. For the *grouper water* experiments, we simulated the threat of predation by using water from a tank holding a live predator (*E. marginatus*). We introduced approximately 60 liters of "grouper water" into each treatment tank as the predation risk cue.

For each experiment we used 4 tanks, with predation cues used in 3 tanks and the fourth tank left as a control (**Figure 1**). Each repetition ran for 7 days, during which we fed the fish every other day to allow time for the fish to become hungry between feedings. For the first six days the fish were allowed to forage without any predation cue. On the last day of each experiment, we added an *alarm cue* or *grouper water* cue into the treatment tanks (**Figure 1**). We measured two variables on days three, five, and seven of each experiment. The first was the amount of food foraged over specific periods of time as a measure of the fish's overall grazing impact. The second was the effort the rabbitfish spent foraging measured by bite rate per minute. See details below under section "Grazing quantification". In order to measure grazing behavior without disrupting the fish by the presence of an observer (rabbitfish are highly timid in the presence of humans), we set up online surveillance cameras inside waterproof transparent boxes in each tank.

We included two types of controls in the experimental design. The first type of control was the tanks to which we did not apply any predation risk cues (**Figure 1**). These functioned as a global control for any external impacts that might confound the results, such as the effects of surrounding conditions (e.g., weather or water supply). The second type of control was on the individual tank-level, by calculating the difference in bite rate and overall food foraged between two days of the experiment where there was no predation cue ("baseline," days 3 and 5) compared to the difference between days when we introduced the predation risk cue ("predation cue," days 5 and 7). This approach controlled for variation in fish behavior between tanks. See **Figure 1** for a graphical workflow summarizing the main steps taken within each repetition.

To increase independent replication, we repeated the *alarm cue* treatment in full three times and the *grouper water* treatment twice. For each repetition, we repeated the full set of treatments with at least one week in between experiments. We redistributed the fish between the tanks prior to each repetition so that each repetition could be treated as independent. Since we moved fish between tanks, we considered the first day an acclimation period and did not include those measurement in our analysis. We also alternated among the tanks used as controls to reduce potential tank effects. We used the same statistical approach for both experimental treatments: (a) *alarm cue* (three repetitions, $n = 9$ treatment tanks) and (b) *grouper water* (two repetitions, $n = 6$ treatment tanks). In total we had 5 global controls and 15 paired observations of "baseline" (no predation) and "predation cue." See **Supplementary Table 1** for a summary of experiment dates and repetition types.

Grazing Quantification

To create a depletable food source that can be easily quantified from a visual assay, we used sheets of nori seaweed and ice cube trays. A depletable food source, where the first few bites are more easily grazed then the remaining food, most closely resembles a natural foraging state (Brown and Kotler, 2004). We first tested the food preference of the rabbitfish and found that they were equally likely to eat packaged nori (Porphyra) and fresh Ulva, one of their natural food sources, and therefore we used packaged nori for the experiment due to ease of use and measurement.

We cut each sheet of nori into 10 equal pieces and affixed each piece into an ice cube segment using agarose. Agar is a jelly like substance, obtained from red alga and non-toxic to fish. We diluted the agar in water (2% mixture) and microwaved it for 90 s until the agar was fully dissolved. We rolled each piece of nori around small pebbles, placed each one into an ice cube tray segment, and added some warm agar-water. After the agar cooled and hardened, the top of the nori was loose, and the bottom of the nori was condensed around the pebbles and held in place by

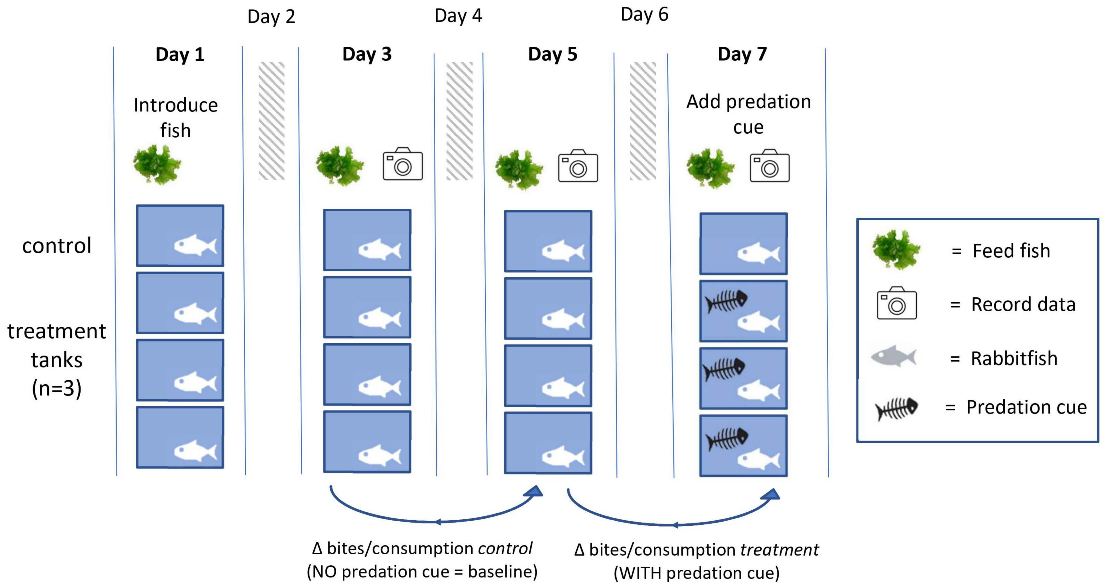

FIGURE 1 | Graphical workflow summarizing the main steps taken during each weeklong repetition of the two experiments. Fish were introduced on day 1 and fed on days 1, 3, 5, and 7. A predation cue was introduced on day 7. Data was recorded (bites per minute and overall food consumption) on days 3, 5, and 7. There were two types of controls: (1) the tanks to which we did not apply any predation risk cues and (2) the difference in bite rate and food consumption between two consecutive days of the experiment where there was no predation cue ("baseline") compared to the change in bite rate when we introduced the predation risk cue ("predation").

the agar. In this way, the initial efforts of the fish to graze were more productive than any continuing effort, which resembles their natural feeding environment.

The food trays were comprised of ten equal pieces of seaweed, one in each ice cube segments. At each interval, we further quantified the portion of seaweed remaining in each ice cube segment on a scale of 0–3. This allowed us to estimate the overall proportion of seaweed remaining in each tank at each measurement time on a scale of 0–30 (10 ice cubes per tank × 3 portions per ice cube). We assigned a value of 0/1 to each algae portion at each observation time (30 per tank, where 0 indicated a portion that was eaten, and 1 indicated a portion of food that remained). As we did not retain data on the fate of each individual ice cube segment, this was done by dividing the total number of algae portions remaining on each tray by the total original number of algae portions under the assumption that grazing was even across ice cube segments.

A single ice cube tray with 10 pieces of nori was placed within each tank. For each day of the experiment when the fish were fed, we cleaned the food trays and replenished the nori before replacing the trays into the tanks. In each repetition, on each measurement day, we recorded the fish bite rate per minute at 15, 45, and 105 min, and the overall consumption of seaweed by the fish at 15, 45, 75, 105, 135, and 165 min after introducing the food tray.

Statistical Analysis

Bite Rate

To assess the impact of the predation cue on bite rate, we ran a linear model using the following formula: Δ Bites $\sim$ Predator $+$ Time $+$ random(Tank). Here Δ Bites refers to the difference in bite rate between two consecutive measurement days in the experiment. Predator indicates whether the measurement is for the *baseline* (Predator = No; difference between first two measurement days without predation cue, days 3 and 5; see **Figure 1**) or *predation cue* (Predator = Yes, difference between measurement days 5 and 7, where day 5 has no predation cue and day 7 has a predation cue), and Tank is the unique tank number for each tank and repetition. We also added the effect of Time on the model where Time indicates the time of observation (15, 45, or 105 min after introduction of the food), and checked for a potential Predator × Time interaction. We ran this model only on the treatment tanks, and separately for the two types of treatment: *alarm cue* and *grouper water*.

We ran an additional linear model to confirm that the global controls (the non-treatment tanks) were not significantly different from the treatment tanks on days without predation cues to eliminate the possibility of external confounding effects. We used the formula: Δ Bites $\sim$ Category $+$ Time $+$ random(Tank). Here Category refers to the status of the Tank, whether it was a treatment tank or a control tank. For *alarm cue*, treatment tanks $n = 9$ and control tanks $n = 3$. For *grouper water*, treatment tanks $n = 6$ and control tanks $n = 2$.

Overall Consumption

To test whether overall consumption changed under the presence of predation risk cues, we used two statistical models, a linear model and a survival model.

For the linear model, we used the number of food portions remaining at each time in each measurement day as a continuous response variable between 0 and 30 (10 ice cubes per tank, 3 portions per ice cube). Time indicates the time of observation: 15, 45, 75, 105, 135, and 165 min after feeding. We added Predator as a categorical variable, and Time as a continuous variable. We also added Tank as a random effect. As with the bite rate, we compared the change in food remaining between two consecutive measurement days in the experiment without predation cues (days 3 and 5; see **Figure 1**) versus the change between days with predation cues (days 5 and 7, where day 5 has no predation cue and day 7 has a predation cue). Specifically, we were interested in examining if the amount of food remaining changed over time differently in the tanks with predation cues verses the tanks without predation cues and therefore focused on the interaction between predator and time as in the following formula:

$$\Delta \text{ Food remaining} \sim \text{Predator} \times \text{Time} + \text{random(Tank)}.$$

Here "Δ Food remaining" is the difference in the number of portions of food remaining in the tray between days (from 0–30 portions, 1 tray per Tank). We ran the full analysis separately for the two types of treatments: *alarm cue* and *grouper water*.

The second analysis on overall consumption was a survival model. We created survival curves showing the probability of each portion of food "surviving" a given amount of time, comparing *baseline* (no predation cue) and *predation cue* for each treatment tank. We used the survfit function in the "survivor" package in R (Therneau, 2020) to create the survival curves. The survfit function uses the Kaplan-Meier method to estimate a survival curve which predicts the probability that each patient (here: portion of food) will survive past time "t." We then used a log rank test to compare the survival curves of the *baseline* and *predation cue* to see if they differed significantly. For the log rank test, we included Tank (a unique tank number for each tank and repetition) as a random effect. Finally, we used the Cox regression to estimate an overall effect size using the hazard ratio ("HR") where HR represents the ratio of the chance a portion of food would be eaten under treatment conditions (predation cue) to the chance a portion of food would be being eaten under control conditions (the baseline period) at any specific point in time. An HR of less than 1 indicates a reduced hazard and an HR over 1 indicates an increased hazard. We ran the full analysis separately for the two types of treatments: *alarm cue* and *grouper water*. For both analyses we qualitatively examined the differences between the global control and the treatment tanks, to eliminate the possibility of external confounding effects.

RESULTS

We found that introducing an *alarm cue* led to a significant decrease in both bite rate per minute and overall food consumption in the treatment tanks. The rabbitfish in the treatment tanks had an overall lower bite rate per minute when exposed to a chemical *alarm cue* released by a recently killed conspecific fish (Predator effect: $p = 0.008$; **Table 1**). There was no significant interaction of observation time on change in bite rate ($p = 0.4834$) and therefore we report only the basic additive model. In addition, there was no significant difference between change in bite rates per day in the control tanks and in the treatment tanks on days without a predation cue (Category effect: $p = 0.981$; **Figure 2**).

The reduction in bite rate is supported by findings that overall food consumption by rabbitfish was also lower in the presence of the *alarm cue*. In the linear model, there was a higher percentage of food portions remaining over time, i.e., lower foraging impact, in treatment tanks with an *alarm cue* than in tanks with no predation cue (**Figure 3**), confirmed by the lower change in food consumption over time between consecutive days with no predation cue (Predator × Time: $p < 0.001$; **Table 2**). Survival curves produced using the Kaplan-Meier method were also significantly different from each other (log rank test $p < 0.0001$) and showed a reduction in the percent of food eaten under the *alarm cue* (**Figure 4**). The hazard ratio was 0.28, implying that around 28% of food that would have been eaten without a predation cue is eaten in the presence of the *alarm cue* (**Table 3**).

This general effect results from divergent trends between tanks. In five of the nine tanks, fish showed a significant decrease in food survival in days with the predation cue relative to days without it. In the other four tanks there was a small and non-significant effect. Results of food survival by tank can be found in the **Supplementary Figure A**.

We found no reduction in either bite rate or food consumption when using *grouper water* from the tank of a live predator to simulate predation. There was no significant change in bite rate (Predator effect: $p = 0.438$; **Table 1** and **Figure 2**). For food consumption, using a linear model we found no interaction between Predation and Time (Predator × Time: $p = 0.209$; **Table 2**) and a small *increase* in overall food grazed under predation with this predation risk cue using Kaplan-Meier survival curves. Although the difference in survival curves is significant (Grouper Water: $p = 0.001$; **Table 3**), the actual difference is negligible (**Figure 4**).

DISCUSSION

The results of the experiments indicate that *alarm cue*s significantly impact rabbitfish foraging activities, as measured by changes in the overall amount of food consumed and the bite rate per minute. Interestingly, we found that the rabbitfish reacted differently to different chemical cues of potential predation risk. There was a significant change in foraging activity when the rabbitfish were exposed to chemical alarm cues released by a recently killed conspecific fish. However, there was no noticeable change in foraging behavior when they were exposed to water from a tank holding a live predator (*Epinephelus marginatus*).

While the general pattern of predation risk impacts on foraging is well known (Brown and Kotler, 2004; Laundre et al., 2010), it had not been tested previously for rabbitfish. Rabbitfish are invasive in the Mediterranean and their intensive grazing has been shown to damage the local algae communities (Sala et al., 2011; Verges et al., 2014). The stress of alien species on

TABLE 1 | Summary of model results for the change in bite rates using the formula: Δ Bites ~ Predator + Time + (1| Tank).

Exp.	Predictors	Estimate	Std. Error	Df	t value	Pr (> |t|)	R^2
Alarm Cue	(Intercept)	2.255	7.617	27.0	0.296	0.769	R^2m = 0.176
	Predator = Yes	**(24.510)**	**8.606**	**27.0**	**(2.848)**	**0.008**	
	Time (45 min)	3.651	9.362	27.0	0.390	0.700	
	Time (105 min)	1.500	12.570	27.0	0.119	0.906	
Grouper Water	(Intercept)	13.417	8.247	27.0	1.627	0.115	R^2m = 0.018
	Predator = Yes	**(6.500)**	**8.247**	**27.0**	**(0.788)**	**0.438**	
	Time (45 min)	0.667	10.101	27.0	0.066	0.948	
	Time (105 min)	0.583	10.101	27.0	0.058	0.954	

Here R^2 refers to the marginal R^2 and reflects the variance explained by the fixed effects.

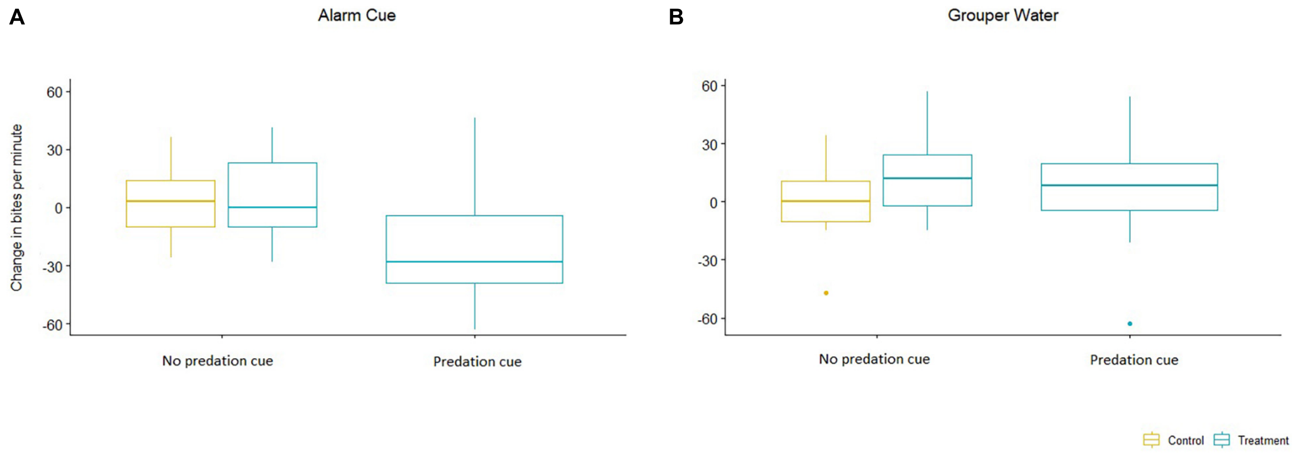

FIGURE 2 | Change in bites per minute when introducing a predator cue into the tanks. **(A)** Alarm Cue. The left graph shows the results of using a chemical alarm cue released by a recently killed conspecific fish. **(B)** Grouper Water. The right graph shows the results of using water from a predator's tank as the predation risk cue. The yellow box plot shows the global control tanks (*n* = 3 for Alarm Cue; *n* = 2 for Grouper Water). The blue box plots show the treatment tanks (*n* = 9 for Alarm Cue; *n* = 6 for Grouper Water) under two categories – "No predation cue" (difference between measurement days without predation cue) and "Predation cue" (difference between measurement days with and within a predation cue). In the plots, the boxes indicate the inter-quartile range, the horizontal lines mark the medians, the whiskers indicate values within 1.5 times the inter-quartile range beyond either end of the box, and the dots indicate outliers beyond that range.

marine ecosystems may also decrease resilience to sea warming (Corrales et al., 2018). The results presented in this study show that this detrimental overgrazing might be partially mitigated by the presence of predators whose predation activities produce an alarm cue which may reduce the foraging activity of the surviving fish. Another way of looking at this is that the Mediterranean Sea currently suffers from a synergy between having fewer predators and new grazers with high abundance.

There has been little research on how fishing protection inside MPAs affects the overall ecological impact of invasive species (Iacarella et al., 2019). The results of this study support the potential effectiveness of MPAs to indirectly mitigate the impact of invasive herbivorous species through trophic cascades, showing yet another benefit of MPAs as an effective tool to protect and restore healthy ecosystems. By limiting fishing, MPAs specifically protect commercially valuable, often larger predatory fish, the same fish that prey on rabbitfish. The higher density of predators inside MPAs (Giakoumi et al., 2017; Rilov et al., 2018; Lazarus et al., 2020) can change the foraging behavior of rabbitfish and through that mitigate their negative impact, thereby preserving the algae and seagrass that rabbitfish eat.

Our results were obtained from mesocosms experiments and hence are only the first step in understanding the ability of predators to reduce the impact of rabbitfish under natural settings. For example, the limited volume of water within mesocosms may have increased the signal of the alarm cue on rabbitfish foraging compared to that observed in nature. Indeed, there have been mixed results about the impact of MPAs on trophic cascades (Shears and Babcock, 2002, 2003; Byrnes et al., 2006; Malakhoff and Miller, 2021). It is likely we will only understand the full potential of MPAs to control invasive species once more MPAs become better enforced and more mature (Giakoumi et al., 2019a). Performing field studies to compare the foraging *in situ* and over longer temporal duration in the presence of predation cues could show the cascading impact of predation on foraging activity in the context of a more complex and realistic environment.

There are a number of possible reasons for why the fish did not react to the presence of the *grouper water* as opposed to the

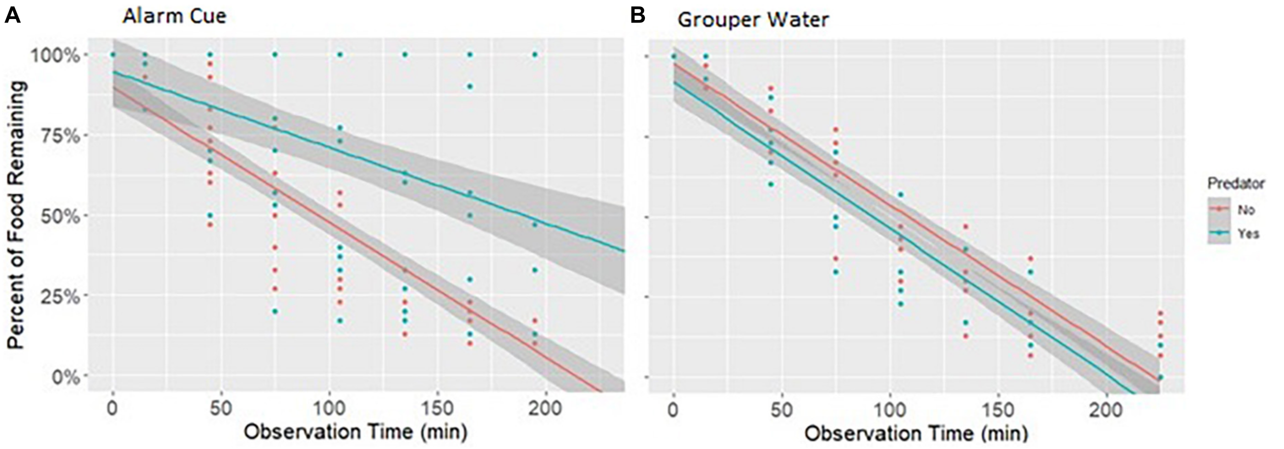

FIGURE 3 | Change in number of food portions remaining (out of 30) over time when introducing a predation cue compared to no cue. The dots represent the amount of food remaining at specific times (min) after food was introduced. Note that for illustrative purposes, the data shown here is actual food portions remaining over time, and not the change between days of food portions remaining over time used for analysis. The lines show the linear regression and the shading represents the 95% confidence intervals. Red dots and lines show the baseline food survival. Blue dots and lines show the food survival with treatment. Data shown for **(A)** Alarm Cue and **(B)** Grouper Water. Note that a higher percentage of food remaining implies a lower foraging impact. Only the Alarm Cue ("A") showed an interaction between time and predation cue ($p < 0.001$).

TABLE 2 | Summary of linear model results for change in food consumption using the formula: Δ Food remaining $\sim$ Predator $\times$ Time + (1| Tank).

Exp.		Value	Std.Error	DF	t-value	p-value	R^2
Alarm Cue	(Intercept)	0.338	1.400	132	0.241	0.810	R^2m = 0.385 R^2c = 0.504
	Predator	0.541	1.539	132	0.352	0.726	
	Time	−0.005	0.010	132	−0.500	0.618	
	Predator × Time	**0.071**	**0.014**	**132**	**5.252**	**<0.001**	
Grouper Water	(Intercept)	−2.301	1.188	87	−1.938	0.056	R^2m = 0.026 R^2c = 0.026
	Predator	−1.060	1.680	87	−0.631	0.530	
	Time	−0.004	0.011	87	−0.422	0.674	
	Predator × Time	**0.019**	**0.015**	**87**	**1.266**	**0.209**	

R^2m is the marginal R^2 and reflects the variance explained by the fixed effects. R^2c is the conditional R^2 and reflects the variance explained by the entire model, including Tank as a random effect.

alarm cue. It is possible that the chemical cues released by the predator into the water were simply not concentrated enough to elicit a response. Alternatively, the rabbitfish might react to the presence of other predators, but not specifically to *E. marginatus,* or to the presence of this predator when it is alone. A study of trophic cascades in kelp forests showed that predator diversity was key to controlling the effects of herbivores on community structure (Byrnes et al., 2006). In the Mediterranean, rabbitfish predators include fish from the grouper family as well as other native predator species such as the greater amberjack (*Seriola dumerili*), bluefish (*Pomatomus saltatrix*), Mediterranean moray (*Muraena helena*), and the Common dentex (*Dentex dentex*) (Giakoumi et al., 2019b). Other combinations of chemical cues from these predators may have elicited a behavioral response in the rabbitfish.

An additional possibility is that rabbitfish would not react to the presence of *any* specific predator or combination of predators, but only to a chemical alarm cue released from conspecific individuals during a predator attack. The ability of fish to associate a specific predator's odor with predation is likely a learned behavior associated with the proximately of predator odor to chemical alarm cues (Brown, 2003; Mitchell et al., 2015). Since these fish were collected from areas with few large groupers that posed a risk, it is likely the rabbitfish did not learn to be wary of groupers. In addition, a recent experiment showed that changes in crayfish behavior as a result of predator odors were dependent on both the prey familiarity with the predator, based on previous exposure, and the actual diet of the predator (Beattie and Moore, 2018), supporting the idea that the presence of a potential predator alone may not be enough to trigger an effect. One possible implication is that if only certain predators are present, or if the predator's main diet is composed of other prey species, their presence alone may not be sufficient to impact rabbitfish foraging behavior. Finally, it is possible that the rabbitfish would respond more strongly to visual cues than to odor.

We also found significant variation in the reaction of each tank's fish to the presence of predation cues, where some tanks did

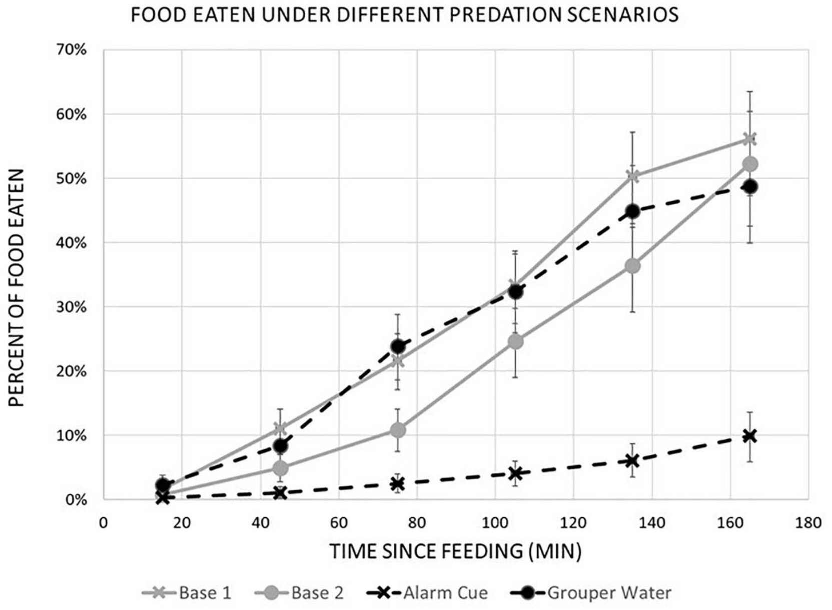

FIGURE 4 | Percentage of food eaten at each time (min) after feeding using the Kaplan-Meier method to estimate a survival curve. Gray lines show baseline results for both experiments. Dashed black lines show results of adding predation risk cues. The lines marked by **X** are the chemical alarm cue treatment and the lines marked by circles (●) are the grouper water treatment. Error lines reflect 95% confidence intervals. Baseline results where similar for both experiments. Only the Alarm Cue showed a significant (log rank test $p < 0.0001$) reduction in percent of food eaten due to predation cues.

TABLE 3 | Summary of the Cox mixed-effects model fit by maximum likelihood for the impact on food consumption using the formula: Survival ∼ Predator + (1| Tank).

Experiment	Coef	Exp(coef)	Se (coef)	Z	P
Alarm Cue	−1.263	0.2828	0.1045	−12.09	<0.0001
Grouper Water	0.4473	1.5642	0.1364	3.279	0.001

Exp(coef) is the hazard ratio of the model.

not show any reduction in food consumption under treatment conditions (**Supplementary Figure 1**). This may be partially explained by variation in signal strength. Although efforts were made to ensure that all fish tanks received the same magnitude of alarm cue, for example by dividing fish pieces equally and including some fish guts in each tank, it is possible that some tanks received a stronger signal. Alternatively, this variation among tanks may indicate that individuals react differently to predation risk. Variation in vigilance and other anti-predator behaviors have been shown to directly translate into variations in individual survival and are thus traits that can be selected for (Steinhoff et al., 2020).

These differences among individual fish and between different predation cues support previous findings that prey reactions to predator risk can vary based on many variables, including individual fitness, resource availability, and predator type (Sheriff et al., 2020). In addition, these experiments were only run on *S. rivulatus,* one of the two invasive rabbitfish species in the Mediterranean. Since *S. rivulatus* and *S. luridus* both show adaptation to different food sources (Lundberg and Golani, 1995), it is possible that the different species would react differently to predation risk.

Prey use multiple strategies to mitigate their predation risk (Sansom et al., 2009; Kotler et al., 2010). In this experiment it is difficult to tease apart the strategies of avoidance and vigilance as the fish were not able to choose between multiple foraging spots, and therefore were less able to *avoid* the predation cue. In addition, since there was a strong signal in both overall consumption and bite rate, there is no way to conclusively say if the fish spend less time foraging (avoidance) or they foraged less efficiently (vigilance). If instead, for example, the bite rate had remained unchanged, but there was lower overall food consumption under predation, it would suggest vigilance rather than avoidance.

The strong reaction of rabbitfish to chemical alarm cues suggests that the higher density of predators within MPAs may in fact mitigate the impact of these invasive species. Outside of MPAs, predator presence can also be encouraged through the use of artificial habitats and specific fishing bans. In addition, predation cues might be used directly as a management tool, that is, be artificially introduced to specific environments, even when few predators are present, in order to reduce rabbitfish grazing. Further research into the differences between types of predation cues and prey mitigation strategies could help determine how best to use these results to manage the ecological impacts caused by invasive rabbitfish under natural settings, especially in hotspots of highly impacted areas (Katsanevakis et al., 2016). Ultimately, this information can be used to manage the ecological impacts caused by invasive rabbitfish, both inside and outside of marine reserves.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by the Israel Oceanographic and Limnological Research Ethics Committee. Permit# F1933.

AUTHOR CONTRIBUTIONS

SV and JB conceived of the framework project of which these experiments were one part. DSG, JB, and GR conceived of and planned the experiments. DSG carried out the experiments, analyzed the data with support from JB, and took the lead in writing the manuscript. GR helped supervise the project. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

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SUPPLEMENTARY MATERIAL

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Monitoring Non-indigenous Species in Port Habitats: First Application of a Standardized North American Protocol in the Mediterranean Sea

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Baseline port monitoring for fouling communities is an essential tool to assess non-indigenous species (NIS) introduction and spread, but a standardized and coordinated method among Mediterranean and European countries has not yet been adopted. In this context, it is important to test monitoring protocols that allow for the collection of standardized and directly comparable data, replicated across time and space. Here, for the first time in the Mediterranean Sea, we tested a standardized protocol developed by the Smithsonian Environmental Research Center (SERC) and used now in several countries. The 3-year monitoring survey (2018–2020) was conducted in the Gulf of La Spezia (Ligurian Sea, Italy), with the deployment of a total of 50 PVC panels per year in five different sites (a commercial harbor, three marinas and a site in the proximity of a shellfish farm). A total of 79 taxa were identified, including 11 NIS, ranging from zero to seven NIS for each panel. In comparison with previous surveys, new NIS arrivals were observed in the Gulf of La Spezia: *Botrylloides* cf. *niger*, *Branchiomma* sp., *Branchiomma luctuosum*, *Paraleucilla magna,* and *Watersipora arcuata*. At the end of the 3-year monitoring, mean richness? and percent cover of NIS were measured, and both measures differed across the monitoring sites, with higher values in two marinas and in the commercial harbor. Among years, richness of NIS was relatively stable at each monitoring site. The structure of the fouling was influenced more by native and cryptogenic species than by NIS. Moreover, among the monitoring sites, the density of artificial structures was not a reliable predictor or proxy for local NIS abundance. This first application of the SERC method in the Mediterranean Sea, demonstrates both pros and cons, including the detection of new NIS reported here. Further direct comparisons with other NIS monitoring tools are recommended, and additional tests to assess its effectiveness in this biogeographical area are encouraged. A broader application of this and other standard methods across temporal and spatial scales in the Mediterranean basin should be implemented, providing critical data needed to assess changes in the structure of fouling communities.

Keywords: macrozoobenthos, sessile fauna, settlement panels, marine bioinvasions, Ligurian Sea, fouling

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INTRODUCTION

The introduction of non-indigenous species (NIS) in marine environments is considered one of the most important factors contributing to global change (Ruiz et al., 1997; Occhipinti-Ambrogi, 2007; Galil et al., 2018a), including both ecological and economic impacts (Occhipinti-Ambrogi, 2007; Katsanevakis et al., 2014a; Ojaveer et al., 2015). The Mediterranean Sea is among the most invaded marine provinces (Bailey et al., 2020), with almost 800 multicellular NIS recorded (Galil et al., 2018b), and the main vectors of introduction include: the opening and enlargement of the Suez Canal, aquaculture and shipping (Ruiz et al., 2000; Galil et al., 2014, 2015; Katsanevakis et al., 2014b; Tsiamis et al., 2020). While most species introduced through the Suez Canal and by aquaculture vectors are generally confined in relatively restricted areas, vessels can further spread NIS throughout the whole Mediterranean Basin (Galil et al., 2018a). The maritime traffic involved in the introduction and spread of NIS includes both large commercial and small recreational vessels, which can carry species inside the ballast waters or as biofouling on vessel hulls (Ruiz et al., 2000; Clarke Murray et al., 2011; Canning-Clode et al., 2013; Zabin, 2014; Ferrario et al., 2017; Galil et al., 2018b). As defined by the International Maritime Organization, biofouling is "the accumulation of aquatic organisms such as microorganisms, plants and animals, on surfaces and structures immersed in or exposed to the aquatic environment" (IMO, 2012). The macrozoobenthic component of the fouling communities is usually composed of both sessile epibionts—sponges, serpulids, bivalves, barnacles, bryozoans, tunicates—and mobile marine invertebrates, like but not limited to flatworms, amphipods, isopods, sea spiders and brittle stars (Connell and Glasby, 1999; Glasby and Connell, 1999; Connell, 2001; Lord et al., 2015; Gavira-O'Neill et al., 2018; Culver et al., 2021).

Maritime traffic plays a key role in the introduction of fouling NIS, favoring their first settlement and establishment in ports and then creating hotspot areas for NIS. It has long been considered that large commercial ports act as primary hubs for fouling NIS, due to the transit of transoceanic cargo ships, while small boats are involved in their secondary spread in marinas and neighboring areas (Minchin et al., 2006; Floerl et al., 2009; Hulme, 2009; Ros et al., 2013; Zabin, 2014; Marchini et al., 2015). On the other hand, recent studies have suggested the importance of recreational boating in the primary introduction and spread of NIS (Ashton et al., 2014; Ferrario et al., 2017; Ulman et al., 2019a,b). After introduction to a port environment, the settlement of NIS can be facilitated both by the large abundance of available artificial substrates (Glasby et al., 2007) and the tolerance of these species to different environmental conditions, including polluted waters (Piola and Johnston, 2008; Piola et al., 2009).

For decades, fouling assemblages have served as a model to study bioinvasion processes (Ruiz et al., 2009; Canning-Clode et al., 2011; Marraffini et al., 2017; Leclerc et al., 2018; Marasinghe et al., 2018; Vieira et al., 2018; Ferrario et al., 2020). Furthermore, ports and marinas can be considered ideal sites for the early detection and monitoring of NIS presence and abundance, due to their importance as hotspot areas of NIS (Olenin et al., 2011; Ojaveer et al., 2014; Marraffini et al., 2017). However, no formal protocol has yet been adopted for the standardized assessment of fouling NIS in the European and Mediterranean marine regions. Monitoring of marine fouling communities is fundamental to track the introduction and spread of NIS, as well as to evaluate the power of legislation designed for limiting further introductions (Ruiz and Hewitt, 2002; Lehtiniemi et al., 2015; Marraffini et al., 2017). The implementation of long-term monitoring programs and a preventive approach can contribute to the assessment of the potential risks of NIS that have been demonstrated to negatively affect native communities—e.g., contributing to the decrease of vulnerable species (Marraffini et al., 2017; Occhipinti-Ambrogi, 2021). For these reasons, a standardized monitoring and a coordination among countries is highly recommended, in order to obtain comparable data replicated across time and space (Hewitt and Martin, 2001; Olenin et al., 2010, 2011; Latombe et al., 2017; Bailey et al., 2020). This is also in accordance with the Marine Strategy Framework Directive (MSFD 2008/56/EC), suggesting a development and application of standardized methods to detect NIS—a descriptor included in the achievement of the Good Environmental Status in European marine waters.

Several methodologies for sampling fouling communities in port habitats have been developed over the years. Most common techniques include (i) Rapid Assessment Surveys—RAS (e.g., Cohen et al., 2005; Campbell et al., 2007; Olenin et al., 2007; Minchin, 2012; Lehtiniemi et al., 2015; Marchini et al., 2015; Gewing and Shenkar, 2017; Ulman et al., 2017); (ii) net-assisted scraping (e.g., Ferrario et al., 2017; Ulman et al., 2017; Tempesti et al., 2020), including quadrat scraping during snorkeling or scuba diving (e.g., Neves et al., 2007; Awad et al., 2014; Ulman et al., 2019b); and artificial substrate units (ASU), such as (iii) two-dimensional settlement panels (e.g., Relini, 1977; Canning-Clode et al., 2013; Dos Santos Schwan et al., 2016; Lezzi et al., 2017; Leclerc and Viard, 2018; Marasinghe et al., 2018; Carlton, 2019; Leclerc et al., 2019); and (iv) three-dimensional artificial habitat collectors (e.g., Fowler et al., 2013; Gestoso et al., 2019; Outinen et al., 2019; Holmes and Callaway, 2020; Obst et al., 2020; Ros et al., 2020). Any of the above methods involves both advantages and disadvantages, and their success or suitability depend on the aim of the study. For example, quantitative methods (e.g., quadrats, settlement panels) are useful to thoroughly assess the entire fouling community but require a lot of fieldwork and laboratory effort. Conversely, qualitative methods like RAS allow for the collection of a lot of data on species distribution in a short timeframe, but the focus is typically only on target NIS already known and easily recognizable in the field. This method can limit the early detection of new arrivals, particularly of inconspicuous NIS. Net-assisted scraping probably ensures the most comprehensive picture of the fouling community, yet it requires long laboratory analyses and does not provide quantitative outputs (although semi-quantitative abundance estimates are possible; Ferrario et al., 2017). In addition, some methods are more suitable for sessile taxa (e.g., settlement panels), while other ones are better for mobile taxa (e.g., scrapes, 3-D ASU). Light exposure and orientation of the ASU also affects the ability of collecting the photosynthetic component of fouling communities. Therefore, the best practice for comprehensive sampling fouling communities should be a simultaneous multi-method approach in the same study area, as suggested by Tait et al. (2018) and Kakkonen et al. (2019).

In recent years, several sampling efforts focused along European and Mediterranean ports were conducted to monitor NIS presence. Using RAS, Ashton et al. (2006) and Nall et al. (2015) sampled in Scotland, while Bishop et al. (2015) sampled with the same method in the English Channel. In the Normandy region, Verlaque and Breton (2019) found macroalgal NIS through a RAS by scuba diving, involving citizen scientists after a multi-year field survey. In the ports of Gdynia and Gdańsk (Baltic Sea), Brzana et al. (2019) found a non-indigenous tanaid species using both settlement panels and artificial habitat collectors. In the subtropical island of Madeira (Atlantic Ocean), Canning-Clode et al. (2013) monitored NIS presence and abundance with a 6-year fouling survey through settlement panels. Regarding the Mediterranean Sea, Ferrario et al. (2017) sampled ports in the Western sector (Sardinian Sea, Ligurian Sea, and North Tyrrhenian Sea) by scraping the artificial hard substrates with a rigid hand-held net and Tempesti et al. (2020) sampled in the same way in the port area of Leghorn. In the Taranto Sea (Ionian Sea), Lezzi et al. (2017) carried out a 2-year macrofouling monitoring program using PVC settlement panels, while along the Slovenian coasts Fortič et al. (2019) combined different methods (RAS, scraping net and scuba dives) in their monitoring. In the Aegean Sea, Bariche et al. (2020) reported for the first time a non-indigenous nudibranch, using Autonomous Reef Monitoring Structures (ARMS) protocol, proposed by Obst et al. (2020). In the Southern Mediterranean Sea, Chebaane et al. (2019) sampled through RAS fishing ports and marinas in Monastir Bay, Tunisia. Lastly, the broadest investigation of non-indigenous invertebrates in Mediterranean marinas was conducted by Ulman et al. (2017) with both modified RAS and scrapes by hand-held rigid net. During the last few years, the metabarcoding approach has also started to be employed in NIS surveys with satisfying results (see also Borrell et al., 2017; Miralles et al., 2021; Pearman et al., 2021). Metabarcoding can be considered as an efficient tool to detect cryptic species or early life stages, but one of the main disadvantages is the incompleteness of genetic sequence reference libraries (Zaiko et al., 2018; Duarte et al., 2021). For this reason, an integrative approach should be considered as the gold standard (Padial et al., 2010; Cahill et al., 2018; Obst et al., 2020; Duarte et al., 2021).

In this study we applied for the first time in the Mediterranean Sea—namely in the Gulf of La Spezia (Ligurian Sea. Italy)—an international standard protocol for sessile fouling NIS monitoring in port habitats, with the aim to assess: (i) the fouling communities and NIS colonization in different harbor sites; (ii) the development of NIS colonization in the fouling communities over time; (iii) the effectiveness of this protocol for the early detection of newly introduced NIS in the Mediterranean; and (iv) the relationship between abundance of NIS and density of artificial structures in port habitats.

MATERIALS AND METHODS

Study Area

The Gulf of La Spezia (44.09°N, 9.85°E) is one of the largest bays of the Ligurian Sea (Italy), characterized by the presence of several anthropogenic activities, including a commercial harbor and several marinas, and featuring intense anthropogenic influences such as urban runoff, naval industries, a military base, an electric power-plant and aquaculture facilities (Gasparini et al., 2009). The bay is about 5 km wide and 10 km long, and is separated in two parts by an artificial offshore dam about 2 km long (Gasparini et al., 2009).

The fouling monitoring program included five sites inside the gulf (**Figure 1**): in the marinas of Fezzano, Le Grazie and Porto Venere (FE, GR, and PV, respectively), near the commercial harbor of La Spezia (SP), and in a small marina near a mussel culture facility in Santa Teresa (ST). The trend of sea surface temperature (−0.915 m) from August 2018 to August 2020 was obtained by extrapolating data from loggers (ONSET HOBO WaterTemp Pro v2) deployed in the gulf for other experimental purposes by ENEA Marine Environment Research Centre (La Spezia).

Monitoring Protocol

The monitoring was carried out for three consecutive years (2018–2020) by applying a standardized protocol developed by the Smithsonian Environmental Research Center (Edgewater, MD, United States) that is being used to detect and track NIS in the U.S as well as Canada, Ecuador, and Panama (see also de Rivera et al., 2005; Gartner et al., 2016; Simkanin et al., 2016; Marraffini et al., 2017; Tracy et al., 2017; Chang et al., 2018; Jurgens et al., 2018; Newcomer et al., 2018, 2019; Carlton, 2019; Torchin et al., 2021). A total of 50 PVC settlement panels (14 × 14 cm) were deployed in the summer of each year in the Gulf of La Spezia, allocating 10 panels in each site. Specifically, the passive collector (substrate) is composed of a settlement panel attached to a brick, with a sanded surface facing downward to minimize algal growth and facilitate invertebrate recruitment (Crooks et al., 2011; Marraffini et al., 2017; Jimenez et al., 2018). The experimental units were secured to docks or floating pontoons using rope, suspended 1 m below the sea surface, and immersed for 3 months during the summer season, in order to maximize the larval recruitment (Freestone et al., 2011; Marraffini et al., 2017). In 2018, the panels were immersed from July to October (87 days of deployment), in 2019 from April to July (89 days), and in 2020 from May to August (101 days). Differences in community assembly time across years were due to logistical constraints, in addition to mobility restrictions related to the health emergency of Coronavirus disease (COVID-19), and were considered during data analysis. At the end of the immersion period, all panels were retrieved, photographed and observed under dissecting microscopes for taxonomic identification of sessile invertebrates.

The percent cover of the whole fouling assemblage was assessed by point count method (n = 50 points; Chang et al., 2018), using a plastic grid (7 × 7 points, with an additional 50th

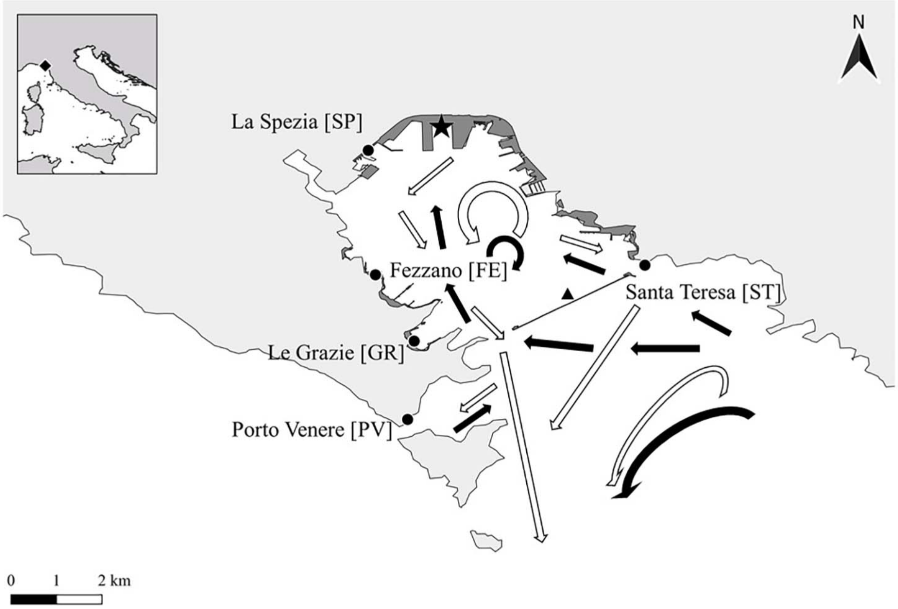

FIGURE 1 | Map of monitoring sites in the Gulf of La Spezia (Ligurian Sea, Italy). Dark gray polygons indicate main port areas; black star indicates commercial harbor of La Spezia; black triangle indicates the artificial dam; white and black arrows indicate surface and deep main currents of the Gulf, respectively (adapted from Gasparini et al., 2009).

point chosen randomly) positioned upon the plates observed under a dissecting microscope. With this method it is possible to observe more than one taxon in the same counting point, therefore percent cover for the whole community may be exceed 100%. Fouling communities on panels were retrieved and observed *in vivo* in 2018, while in 2019 and 2020 the panels were preserved in 70% ethanol upon retrieval and stored for later analysis.

Data Analysis

Species richness (S), NIS/native ratio and percent cover of each taxon were calculated for each panel ($N = 10$ per site). S and percent cover were assessed separately for all taxa, together with the assignment of biogeographic status *sensu* Chapman and Carlton (1991): NIS, native (including cryptogenic species—i.e., species that are not discernibly native or introduced, as defined by Carlton, 1996) and unresolved status (due to insufficient taxonomic resolution e.g., damaged specimens). Differences in mean values richness and percent cover of native and cryptogenic species and NIS were assessed by two-way ANOVA (Type III tests with two orthogonal fixed factors: "year," three levels: 2018, 2019, 2020; and "site," five levels: FE, GR, PV, SP, ST; unequal sample size). When pooling across sampling years, differences in mean richness and percent cover of NIS were assessed by one-way ANOVA (fixed factor "site," five levels: FE, GR, PV, SP, ST; unequal sample size). In case of non-homogeneity of the variances, data were properly transformed. A more conservative critical value ($\alpha < 0.01$) was chosen if the data transformation was not successful. Tukey's tests were used for *post hoc* comparisons of means.

Percent cover data were analyzed using principal coordinate analysis (PCO) in order to visualize the pattern of clustering among fouling communities from each site during the years of monitoring. The ordination technique was carried out on centroids (year × site), based on Bray-Curtis similarity on fourth root transformed taxa percent cover data. PCO both for the whole community, and for NIS and native species component were applied separately. Distance-based test for homogeneity of multivariate dispersions (PERMDISP) and permutational multivariate analysis of variance (PERMANOVA) were carried out to test differences in NIS percent cover values of communities among sites (Anderson et al., 2008).

Correlation between NIS percent cover and density of immersed artificial structures (e.g., docks, pontoons) of each monitoring site was tested, as suggested by Susick et al. (2020). Density of each site was measured through satellite images as the linear length of mooring structures within a 200 m diameter circle of the potential marine habitat, centered on the spatial centroid of deployed panels. Length of artificial structure visible above water was measured using the path ruler tool in Google Earth Pro v. 7.3.3.7699 (Susick et al., 2020).

Analyses were carried out with the software Microsoft Excel, R (R Core Team., 2017), QGIS (QGIS Development Team., 2020) and PRIMER 6 with PERMANOVA + add-on package (Clarke and Gorley, 2006; Anderson et al., 2008).

RESULTS

From August 2018 to August 2020, the range of surface temperature varied from 12 to 27°C (**Supplementary Figure 1**).

Mean temperatures during the monitoring periods were 25 ± 1°C in 2018, 19 ± 3°C in 2019 and 21 ± 2°C in 2020.

Overall, we collected 126 out of the 150 panels initially deployed in the 3 years; the remaining panels (nine in 2018, four in 2019, and 11 in 2020) were lost due to external causes (e.g., coastal storms or vandalism).

After the 3-year monitoring, a total of 79 sessile taxa representing seven phyla were documented from the Gulf of La Spezia (**Figure 2**), including 11 NIS: four Bryozoa, four Annelida, one Porifera, and two Tunicata (**Table 1**). Among all the NIS observed, one has not been identified at species level (i.e., *Branchiomma* sp.). This species has been firstly assigned to *Branchiomma boholense* (Grube, 1878), but an accurate analysis of the specimens revealed divergent taxonomic characters, that cannot be attributed to the other *Branchiomma* spp. known to occur in the Mediterranean Sea, including the non-indigenous *Branchiomma bairdi* (McIntosh, 1885), *B. boholense* and *Branchiomma luctuosum* (Grube, 1870). Thus, further analyses are needed to confirm its identity, which could also be assigned to a fourth NIS belonging to the genus *Branchiomma*. The majority of the NIS were detected during all three monitoring campaigns, with the exception of *Botrylloides* cf. *niger* Herdman, 1886—found only in 2018—and *B. luctuosum*, found in 2018 and 2020. NIS were detected on all panels sampled over 3 years, except for a single panel at PV in 2019. Furthermore, nine panels exhibited only one NIS, seven of which were collected in PV from 2018 to 2020. The highest values of NIS richness ($S_{NIS} = 7$) found on single panels were from FE 2018, GR 2020, and ST 2020.

For species richness, the lowest value of mean S at each site, i.e., S_{tot} (± SD), was observed in SP in 2018 (11 ± 4), while the highest in ST in the same year (22 ± 2) (**Figure 3**). Regarding the mean S_{NIS}, the lowest value was recorded in PV in 2019 (1 ± 1) and the highest in FE, GR and ST in 2018, as well as in FE and GR in 2020 (about five species on average). S of native and cryptogenic species (S_{native}) was the lowest in SP 2018 (7 ± 3) and the highest in PV 2020 (18 ± 3) (**Figure 3**). The mean NIS/(native + cryptogenic species) ratio showed a similar pattern, registering the lowest values in PV 2019 and 2020 (0.1 ± 0.04) and the highest in SP 2018 (0.6 ± 0.2). S_{native} resulted significantly different among sites, but not across years ($F = 11.3172$, $p = 9.73 \times 10^{-8}$; $F = 1.6857$, $p = 0.19003$, respectively). The site × year interaction resulted significant ($F = 3.9884$, $p = 3.462 \times 10^{-4}$) and *post hoc* test revealed that a single site (SP) contributed to this interaction (2018 vs. 2019 adjusted $p = 2.913 \times 10^{-4}$; 2018 vs. 2020 adjusted $p = 7.745 \times 10^{-4}$). Similarly, S_{NIS} showed significative differences

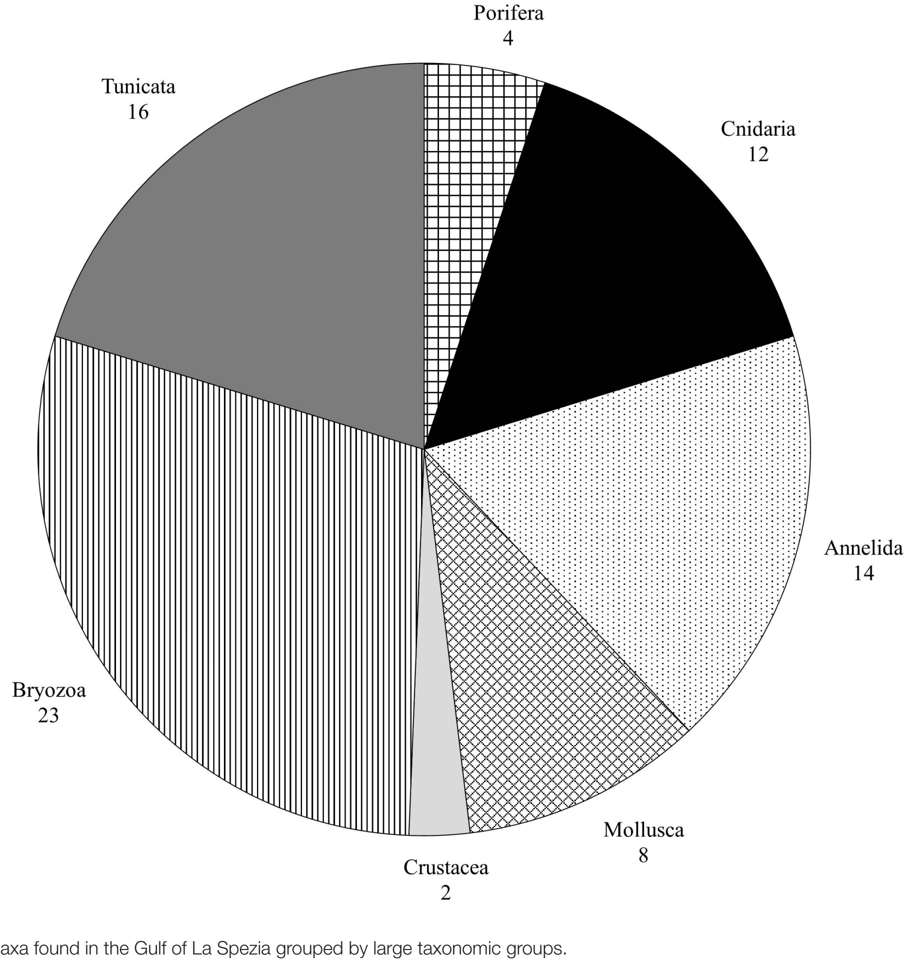

FIGURE 2 | Number of taxa found in the Gulf of La Spezia grouped by large taxonomic groups.

TABLE 1 | List of the NIS found in the investigated localities.

Phylum	Species	Fezzano			Le Grazie			Porto Venere			La Spezia			Santa Teresa		
		2018	**2019**	**2020**	**2018**	**2019**	**2020**	**2018**	**2019**	**2020**	**2018**	**2019**	**2020**	**2018**	**2019**	**2020**
Porifera	**Paraleucilla magna* Klautau, Monteiro and Borojevic, 2004	■	■	■	■	■	■	■		■	■			■	■	■
Bryozoa	*Amathia verticillata* (Delle Chiaje, 1822)	■	■	■		■					■	■		■	■	■
	Celleporaria brunnea (Hincks, 1884)	■	■	■	■	■	■	■			■	■	■		■	■
	Tricellaria inopinata d'Hondt and Occhipinti Ambrogi, 1985	■	■					■			■				■	■
	**Watersipora arcuata* Banta, 1969	■	■		■		■	■		■	■	■	■	■		
Annelida	**Branchiomma* sp.	■	■	■		■	■		■	■		■	■	■	■	■
	**Branchiomma luctuosum* (Grube, 1870)	■		■	■		■	■			■		■			■
	Hydroides dirampha Mörch, 1863	■	■	■	■		■	■			■	■	■	■		■
	Hydroides elegans (Haswell, 1883)	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
Tunicata	**Botrylloides* cf. *niger* Herdman, 1886													■		
	Styela plicata (Lesueur, 1823)	■	■	■	■	■	■			■	■	■	■		■	■

Asterisk indicates NIS found for the first time in the Gulf of La Spezia.

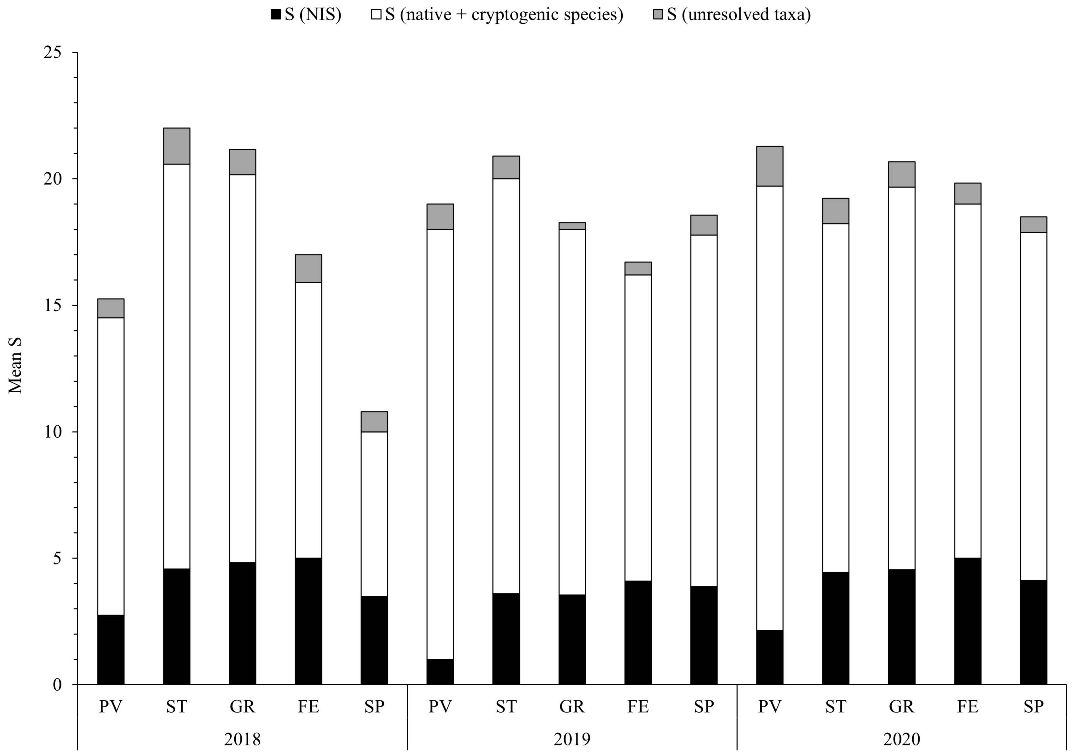

FIGURE 3 | Mean species richness (S) of each monitoring site during the years of monitoring. Sites are showed at decreasing distance from La Spezia (SP). Abbreviations of sites are indicated in **Figure 1**.

only among sites ($F = 5.5382$, $p = 4.21 \times 10^{-4}$) and not across years ($F = 1.7775$, $p = 0.1738433$), and no interaction was detected ($F = 0.988$, $p = 0.4493411$). Among the sites, PV showed significant differences with all the other four sites (adjusted $p < 0.001$).

Merging the data of the 3 years of monitoring, the sites with the highest and the lowest values of mean S_{tot} were ST (21 ± 3) and SP (16 ± 5), respectively; while considering S_{NIS}, the highest value was found in FE (5 ± 1) and the lowest in PV (2 ± 1). Most of the sites displayed higher values of mean S_{native}, namely GR (15 ± 4), PV (15 ± 4), and ST (15 ± 3); conversely, SP (11 ± 4) showed a slightly lower value (**Table 2**). During the 3 years of monitoring, mean percent cover of NIS was the lowest in PV (17 ± 23%) and the highest in SP (85 ± 50%).

TABLE 2 | Mean values of S (± SD) per site for the entire 3-year period of monitoring.

Site	S (total species)	S (NIS)	S (native + cryptogenic species)
FE	17.5 ± 4.4	4.7 ± 1.2	12.1 ± 3.7
GR	19.8 ± 4.9	4.2 ± 1.3	14.9 ± 4.1
PV	18.3 ± 4.4	2.0 ± 1.2	15.2 ± 4.0
SP	15.7 ± 4.7	3.8 ± 1.2	11.1 ± 4.2
ST	20.6 ± 3.0	4.2 ± 1.3	15.4 ± 2.9

*S (total species, including unresolved taxa), mean number per plate of taxa in each site; S (NIS), mean number per plate of NIS in each site; S (native + cryptogenic species), mean number per plate of native and cryptogenic species in each site. Abbreviations of sites are explained in **Figure 1**.*

Mean percent cover of native and cryptogenic species (**Figure 4A**) was highest value in GR 2020 (141 ± 32%) and lowest in SP 2018 (42 ± 35%). For NIS, mean percent cover (**Figure 4B**) was highest in SP 2018 (118 ± 38%) and lowest in PV 2019 (4 ± 5%). Percent cover of native species was significantly different across years ($F = 9.0727$, $p = 2.241 \times 10^{-4}$) and sites ($F = 3.1586$, $p = 0.0168232$), with no significant interaction ($F = 1.5418$, $p = 0.1508879$). Across years, the values observed in 2020 showed significant differences with 2018 and 2019 (adjusted $p < 0.001$); among sites, PV resulted significantly different from FE and SP (adjusted $p < 0.01$) (**Figure 4A**). Significant main effects were detected for NIS cover among sites ($F = 7.5473$, $p = 2.05 \times 10^{-05}$) and across years ($F = 14.5618$, $p = 2.42\text{E-}06$), but no interaction effect was detected ($F = 1.2237$, $p = 0.2918$). *Post hoc* tests revealed that percent cover of NIS was different between 2018 and the other 2 years ($p < 0.001$); between PV and the other four sites ($p < 0.01$); between ST and the other sites ($p < 0.01$) (**Figure 4B**).

The investigated sites were significantly different in terms of mean richness and percent cover of NIS, considering the 3 years of monitoring ($F = 14.691$, $p = 8.257 \times 10^{-10}$; $F = 13.494$, $p = 4.00 \times 10^{-9}$, respectively). Tukey's tests showed a significant difference in richness between PV and all the other sites ($p < 0.001$) when considering S_{NIS}. On the basis of NIS cover a significant difference came out between the sites PV-FE,

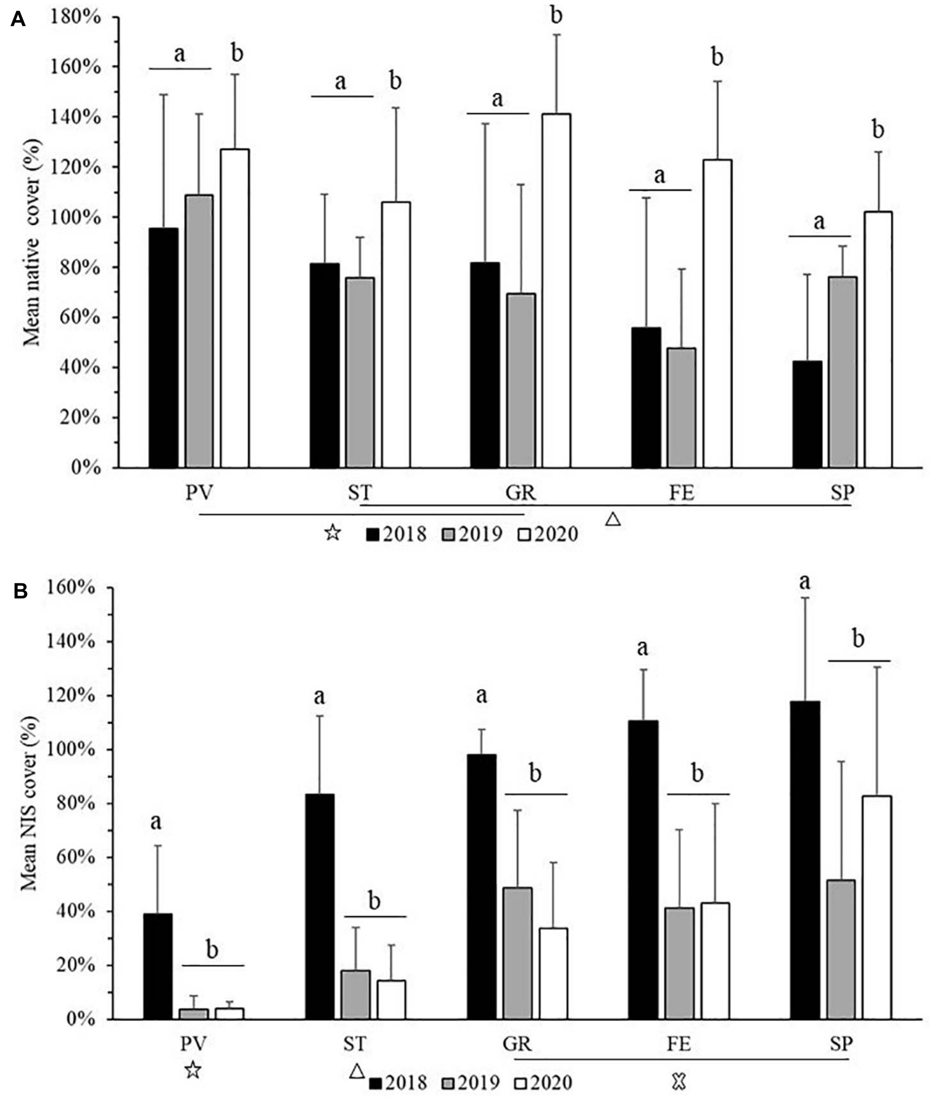

FIGURE 4 | Mean (+ SD) percent cover of native and cryptogenic species **(A)**, and NIS **(B)** in each monitoring site during the 3 years of monitoring. Letters (a, b) on the top of the two graphs indicate statistical divergence among years, while symbols on the bottom indicate statistical divergence among sites. Sites are showed at decreasing distance from La Spezia (SP) and abbreviations of sites are indicated in **Figure 1**.

PV-GR, and PV-SP ($p < 0.001$); ST-FE ($p < 0.05$) and ST-SP ($p < 0.001$). Relative abundance—i.e., percent cover data standardized to 100%—of unresolved taxa resulted very low in all sites (1–2%); while values for NIS and native species were similar in FE and SP (about 50% each); and in GR, PV, and ST the relative abundances were higher for the native species (63, 85, and 71%, respectively) in comparison with the NIS (36, 13, and 28%, respectively; **Figure 5**).

Regarding the structure of fouling communities, PCO showed slightly separated clusters per sampling year and the same distribution of sites within each year, with SP and PV being the most diversified sites (**Figure 6**). This pattern probably follows the percent cover of native species rather than NIS, which did not show a clear arrangement (**Supplementary Figure 2**). Furthermore, the values on percent cover of the non-indigenous component were more clumped than the native one (**Supplementary Figure 2**). PERMANOVA main test on NIS percent cover data of communities among monitoring sites (years merged) showed significant dissimilarity among community composition [pseudo-F = 13.138, p(perm) = 0.0001, unique permutations: 9915], while PERMDISP highlighted significant deviations of samples from centroid [$F = 3.7702$, p(perm) = 0.0116], rejecting the hypothesis of no difference in dispersion among the groups, i.e., "non-homogeneity" of samples dispersion. Moreover, pairwise PERMANOVA tests showed significant differences among sites, except for FE and GR [$t = 1.3337$, p(perm) = 0.1371, unique permutations: 9956], which therefore showed not-dissimilar NIS communities.

Density of artificial structures was the highest in SP (36.28 km $\times$ km^{-2}) and the lowest in PV (13.74 km $\times$ km^{-2}), with

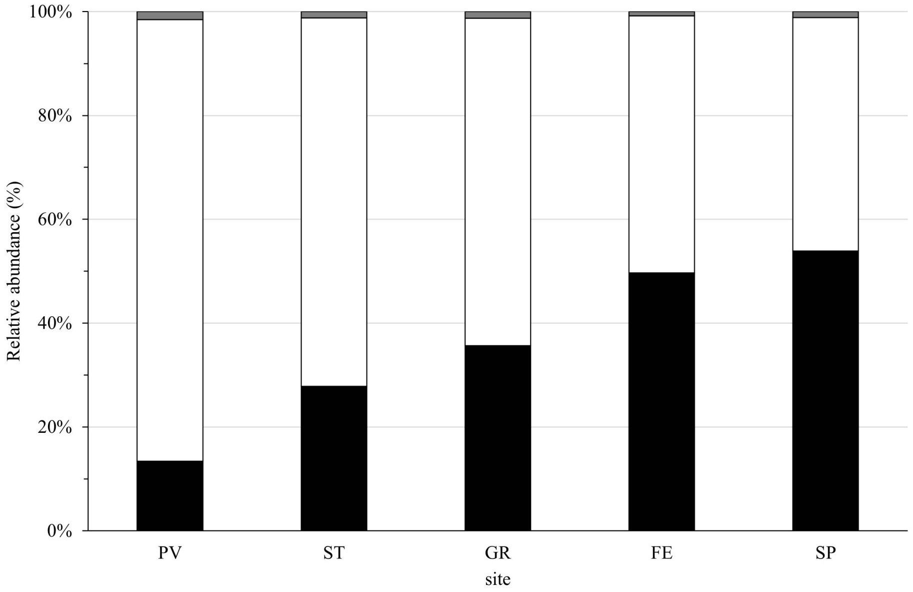

FIGURE 5 | Relative abundance (%) of NIS percent cover (black bar), percent cover of native and cryptogenic species (white bar) and unresolved taxa percent cover (gray bar) in the monitoring sites in the 3-year monitoring. Sites are showed at decreasing distance from La Spezia (SP). Abbreviations of sites are indicated in **Figure 1**.

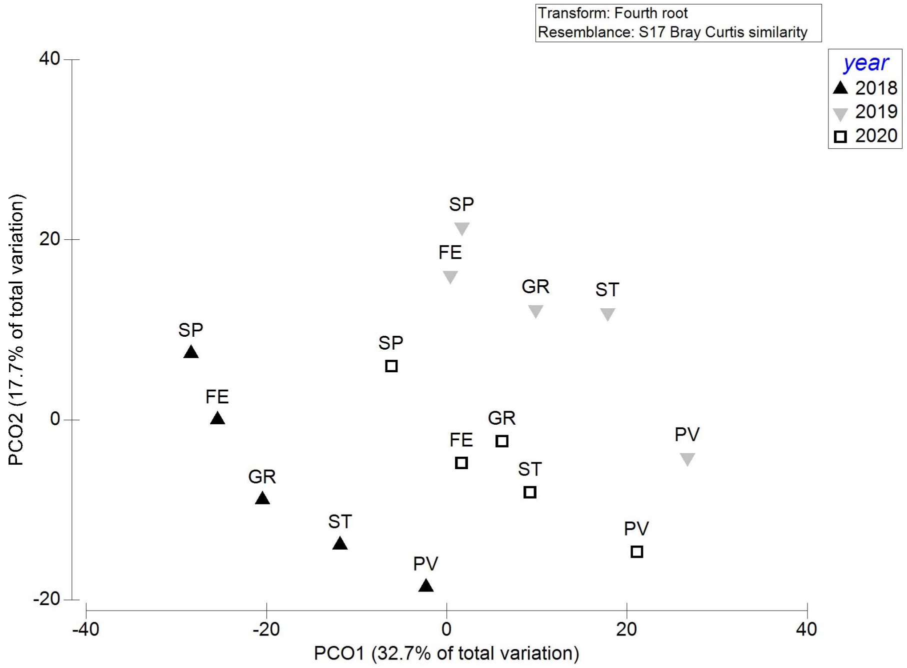

FIGURE 6 | PCO (Principal Coordinate Analysis) based on distance among centroids (year × site) of whole fouling community percent cover data. Abbreviations of sites are indicated in **Figure 1**.

intermediate values in GR (29.04 km × km^{-2}), FE (22.91 km × km^{-2}), and ST (14.51 km × km^{-2}). Correlation between NIS percent cover of samples (126) and these calculated densities resulted low (adjusted $R^2 = 0.2144$, $p = 2.876 \times 10^{-8}$; **Supplementary Figure 3**).

DISCUSSION

The 3-year monitoring on fouling communities in the Gulf of La Spezia allowed us to collect 79 taxa, half of which belong to Bryozoa and Tunicata, and to detect a total of 11 NIS. Seven

NIS were already reported in the Gulf in 2010–2013 (Lodola et al., 2012, 2015; Ferrario et al., 2017; Katsanevakis et al., 2020), while five are new records, namely the sponge *Paraleucilla magna* Klautau, Monteiro and Borojevic, 2004, the sabellid polychaetes *Branchiomma* sp. and *B. luctuosum*, the bryozoan *Watersipora arcuata* Banta, 1969 and the ascidian *Botrylloides* cf. *niger*. Interestingly, in this study three NIS from Ferrario et al. (2017), observed in the harbor of La Spezia or in the marina of Lerici (near Santa Teresa site), were not recorded during the 3 years of monitoring, namely *Amphibalanus improvisus* (Darwin, 1854), *Arcuatula senhousia* (Benson, 1842), and *Ficopomatus enigmaticus* (Fauvel, 1923).

For the NIS observed in the present study, *Paraleucilla magna* is the only sponge reported as non-indigenous in the Mediterranean Sea (Longo et al., 2007). It was first described in Brazil and its putative native origin is the Indo-Pacific region and Australia, while its current distribution in the Mediterranean includes Italy, Spain, Malta, Croatia, Greece, Turkey, and Cyprus (Ulman et al., 2017). Although *P. magna* was already reported in the Ligurian Sea, namely along the Portofino coasts (Bertolino et al., 2013; Longobardi et al., 2017), this study provides a new record for the Gulf of La Spezia. The hypothesis expressed by Bertolino et al. (2013) and Longobardi et al. (2017) on the potential expansion of this species from the mussel farms of the Gulf of La Spezia to Portofino area, is strengthened by this last finding. The highest mean cover of *P. magna* was indeed found in Santa Teresa, the monitoring site near aquaculture facilities.

Regarding *Branchiomma* sp., we think this taxon must be further investigated, as this genus showed intraspecific variability that can lead to species misidentification (Capa et al., 2013). For example, a recent taxonomic study suggested that most of the Mediterranean specimens previously identified as *B. bairdi* should be probably be considered as *B. boholense*, with the exception of data reported by Ulman et al. (2019a) in different Mediterranean countries (Del Pasqua et al., 2018; Langeneck et al., 2020).

Branchiomma luctuosum, originally described from the Red Sea (Grube, 1870), was found for the first time in the Mediterranean Sea in Italy in 1978 (Knight-Jones et al., 1991). Then, *B. luctuosum* was recorded in several localities in the whole Mediterranean Sea, where it is considered established and able to colonize both artificial and natural substrates (Giangrande et al., 2012; Fernández-Romero et al., 2021). In the Western Mediterranean, *B. luctuosum* was found along the Spanish coasts since 2004 (El Haddad et al., 2012; López and Richter, 2017), in the Gulf of Genoa in 2011–2015 (Bianchi et al., 2018), in Sardinia in 2014 (Ferrario et al., 2017; Langeneck et al., 2020), and more recently in Leghorn in 2016 (Langeneck et al., 2020; Tempesti et al., 2020); the latter locality being about 37 nautical miles away from La Spezia.

The bryozoan *Watersipora arcuata* was reported for the first time in the Mediterranean Sea in 2013 in the Ligurian Sea (Ferrario et al., 2015). This species, likely originating from the Tropical Eastern Pacific, has now been recorded in most Mediterranean regions, namely Spain, Italy, Malta, and Turkey (Ulman et al., 2017; Ferrario et al., 2018). Furthermore, a recent revision of historical samples revealed its presence along the South Atlantic coast of Spain since 1990, suggesting its pathway of introduction in the Mediterranean Sea through the Gibraltar Strait (Reverter-Gil and Souto, 2019).

Finally, *Botrylloides niger*, a colonial ascidian native from the tropical Western Atlantic, is reported in several tropical and warm water regions across the world (Sheets et al., 2016), including Madeira island (Ramalhosa et al., 2021). In the Mediterranean Sea, it was first found in 2006 in Israel (Rubinstein et al., 2013) and erroneously identified as *Botrylloides leachii* (Savigny, 1816; Griggio et al., 2014). In general, species in the genus *Botrylloides* are easily confused (Brunetti, 2009; Viard et al., 2019), and in absence of molecular analyses, we use a conservative approach by considering the identification as tentative, pending further verification.

Our monitoring showed that mean NIS richness away varied from 1 to 5 species among sites and years, with similar values across the years of monitoring. For this reason, we did not find a clear increasing pattern in new NIS detections in the different sites, but continuation of this monitoring is required to identify more robust temporal patterns. On the other hand, native species richness significantly varied only in La Spezia among years, highlighting a stable number of native species during the monitoring. It should be highlighted that *B.* cf. *niger* was recorded only in 2018 in ST, but in the following years it did not disappear from the marina. Despite not being found on panel communities, it was still observed, even if in very low abundances, on other artificial substrates (e.g., on the supporting structure of the panels and on floating pontoons; authors personal observation). For this reason, *B.* cf. *niger* may be considered as an established NIS in the study area. After 3 years of monitoring, Porto Venere was found to be the least impacted site in terms of both richness and percent cover of NIS, while the other sites showed a higher impact by NIS, with values of mean NIS percent cover ranging from 34% (Santa Teresa) to 85% (La Spezia).

In general, the different submersion periods—mid-summer in 2018 and early spring in the following years—affected the development of fouling assemblages, mainly in terms of differences in taxa percent cover. NIS richness were indeed similar across the years of monitoring, while in 2018 definitely higher mean values of NIS cover were observed in all sites, compared to the other years of monitoring. The latter result is probably a consequence of the later submersion period of 2018, which led to the dominance on the panels of one NIS, namely *Hydroides elegans* (Haswell, 1883). A similar phenomenon with the dominance of one single species in fouling communities was also observed in marinas of South-eastern Brazil (Dos Santos Schwan et al., 2016), and could be related to the seasonal reproductive peaks (Reinhardt et al., 2013; Lezzi and Giangrande, 2018; Fortiè et al., 2021). These preliminary results suggest that maintaining the monitoring program between May and July is advisable. In fact, the number of detected NIS resulted independent from the submersion period, but during the period May-July it is possible to better capture the entire spectrum of potential colonizers, avoiding the dominance of a single species.

Considering the whole data set on NIS percent cover of the 3-year monitoring, higher values were found in La Spezia, with Fezzano, Le Grazie, Santa Teresa, and Porto Venere gradually showing lower values. The same gradient from La Spezia to Porto Venere was also observed when considering the structure of the entire community, and the gradient was probably more influenced by the composition of the native species than to the non-indigenous one. These patterns may reflect the different geographic features of the monitoring sites. In fact, the sites are located at an increasing distance from the main harbor of La Spezia, one of the largest commercial ports in Italy (accessed 1 March 2021)[1]. The harbor of La Spezia hosts international cargo ships and passengers' vessels, as well as local ferries.

The distance of sites from the main harbor appears to be proportional to the mean NIS percent cover, while the proximity of Santa Teresa to the mussel farm showed higher abundances of *P. magna*, a NIS associated to aquaculture (Bertolino et al., 2013), as well as the exclusive presence of *B.* cf. *niger* in comparison with other sites (see figshare repository)[2]. In addition, the water circulation inside the Gulf is different in Porto Venere compared to the other sites, due to both the presence of the dam and the distance from the open sea (**Figure 1**; Gasparini et al., 2009). This circulation could advantage NIS larval dispersion and settlement inside the dam, where water exchange is surely lower than in Porto Venere (Gasparini et al., 2009). On the other hand, the density of artificial structures seems not to be a factor influencing NIS distribution in the area. The observed variability of NIS percent cover in the dataset can also be related to other features of sites, like the presence of floating pontoons, or the proximity to aquaculture facilities or to commercial harbors (Nall et al., 2015; Ulman et al., 2019a). All these aspects seem to affect the distribution of NIS and the composition of fouling communities in the Gulf of La Spezia. A regular data collection in the Gulf will allow us to examine in depth these results and better clarify the observed dynamics, but this first attempt should be considered in future management measures to prevent NIS introduction in the area, by implementing more rigid controls in sites closer to the harbor of La Spezia. Moreover, a longer data collection could also allow for obtaining limited statistical dispersion of samples within sites, in order to assess if difference in NIS percent cover in monitoring sites is purely due to the dissimilarity among sites and not to dispersion of samples (Anderson et al., 2008).

In conclusion, this monitoring allowed us to implement, for the first time in the Mediterranean Sea, an international standard protocol developed by the SERC to assess fouling communities and detect NIS. The number of detected NIS was in line with previous monitoring carried out in the Gulf of La Spezia and in other Ligurian port localities, but with additional new records for the area and three NIS no longer observed (Ferrario et al., 2017; Ulman et al., 2019a; Katsanevakis et al., 2020). These results are consistent with the role of commercial harbors and recreational marinas as hot-spot sites for the introduction of NIS (Clarke Murray et al., 2011; Ros et al., 2013; Ferrario et al., 2017; Verlaque and Breton, 2019), and the importance of continuous monitoring to detect changes in marine communities (Olenin et al., 2011; Ojaveer et al., 2014; Lehtiniemi et al., 2015). This monitoring protocol seems to be a strong and effective tool to control the presence and abundance of fouling NIS in the Mediterranean ports. Its main advantages are cost-effectiveness, ease of application and the capability to obtain quantitative results in a relatively short time. On the other hand, the effort for sampling and analyzing the panels is considerable. Finally, this protocol is predominantly focusing on the assessment of the sessile hard-bottom fauna, not providing complete quantitative data on the mobile component living on the panels (e.g., see Vicente et al., 2021), as well as on organisms dwelling in soft bottoms (e.g., see Chatzinikolaou et al., 2018), that should ideally be also considered for future monitoring programs on NIS in port habitats.

While this methodology provides a reliable option for sampling and monitoring fouling communities in the Mediterranean area, further analyses to test its effectiveness should be encouraged in other regional localities, together with a comparison to other fouling monitoring tools, including the use of genetic approaches (e.g., Ardura and Planes, 2017; Borrell et al., 2017; Marraffini et al., 2017; Cahill et al., 2018; Kakkonen et al., 2019; Obst et al., 2020 Duarte et al., 2021). Similarly successful implementation in other locales could lead to the proposal of this method as an adopted standard for the assessment of marine bioinvasions in the Mediterranean. In fact, the application of the SERC method across the Mediterranean Sea could provide a simple option to gain standardized and quantitative data in a key invasion-prone habitat, allowing rapid assessment and one approach to evaluate changes in the structure of fouling communities, as it is being carried out along multiple coasts and countries in the Americas.

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

JF, EK, GMR, and AO-A conceived the study. MT, JF, EK, AM, and MFR performed the field work and laboratory analyses. MT carried out the statistics and wrote the first draft of the manuscript, with the contribution of JF. AO-A and GMR acquired funds. All the authors contributed to the manuscript revision, read and approved the submitted version.

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[1] https://www.assoporti.it/en/home/

[2] https://doi.org/10.6084/m9.figshare.14758383.v2

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.700730/full#supplementary-material

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Low Pufferfish and Lionfish Predation in Their Native and Invaded Ranges Suggests Human Control Mechanisms May Be Necessary to Control Their Mediterranean Abundances

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The silver-cheeked toadfish (*Lagocephalus sceleratus*, from the pufferfish family Tetraodontidae) and the Pacific red lionfish (*Pterois miles*, family Scorpaenidae) have recently invaded the Mediterranean Sea. *Lagocephalus sceleratus* has spread throughout this entire sea with the highest concentrations in the eastern basin, while more recently, *Pterois miles* has spread from the Eastern to the Central Mediterranean Sea. Their effects on local biodiversity and fisheries are cause for management concern. Here, a comprehensive review of predators of these two species from their native Indo-Pacific and invaded Mediterranean and Western Atlantic ranges is presented. Predators of Tetraodontidae in general were reviewed for their native Indo-Pacific and Western Atlantic ranges, as no records were found specifically for *L. sceleratus* in its native range. Tetraodontidae predators in their native ranges included mantis shrimp (Stomatopoda), lizardfish (*Synodus* spp.), tiger shark (*Galeocerdo cuvier*), lemon shark (*Negaprion brevirostris*), sea snakes (*Enhydrina* spp.), catfish (*Arius* spp.),

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cobia (*Rachycentron canadum*), skipjack tuna (*Katsuwonus pelamis*), and common octopus (*Octopus vulgaris*). The only reported predator of adult *L. sceleratus* in the Mediterranean was loggerhead turtle (*Caretta caretta*), whereas juvenile *L. sceleratus* were preyed by common dolphinfish (*Coryphaena hippurus*) and garfish (*Belone belone*). Conspecific cannibalism of *L. sceleratus* juveniles was also confirmed in the Mediterranean. Pufferfish predators in the Western Atlantic included common octopus, frogfish (Antennaridae), and several marine birds. Predators of all lionfish species in their native Indo-Pacific range included humpback scorpionfish (*Scorpaenopsis* spp.), bobbit worms (*Eunice aphroditois*), moray eels (Muraenidae), and bluespotted cornetfish (*Fistularia commersonii*). Lionfish predators in the Mediterranean included dusky grouper (*Epinephelus marginatus*), white grouper (*Epinephelus aeneus*), common octopus, and *L. sceleratus*, whereas in the Western Atlantic included the spotted moray (*Gymnothorax moringa*), multiple grouper species (tiger *Mycteroperca tigris*, Nassau *Epinephelus striatus*, black *Mycteroperca bonaci*, red *Epinephelus morio*, and gag *Mycteroperca microleps*; Epinephelidae), northern red snapper (*Lutjanus campechanus*), greater amberjack (*Seriola dumerilli*), and nurse shark (*Ginglymostoma cirratum*). The sparse data found on natural predation for these species suggest that population control via predation may be limited. Their population control may require proactive, targeted human removals, as is currently practiced with lionfish in the Western Atlantic.

Keywords: cannibalism, invasive alien species, marine protected areas, predator-prey, trophic ecology, *Lagocephalus*, *Pterois*

INTRODUCTION

To date, approximately 500 of the 800 non-native or alien species detected in the Mediterranean arrived through the Suez Canal (Galil et al., 2016, 2018; Zenetos et al., 2017; Zenetos and Galanidi, 2020). The rate of introductions has further increased within the last decade, likely due to the 2015 recent widening of the Suez Canal, as well as detection and documentation by citizen scientists (Samaha et al., 2016). Invasive alien species are a global threat affecting biodiversity, tourism, recreational activities, the economy, and human health (Bax et al., 2003; Bailey et al., 2020), and, following habitat destruction, is the strongest global driver of native species extinctions (Bellard et al., 2016). Marine biological invasions are of particular concern in the Mediterranean Sea, where there are over 17,000 native species of which 20–30% are endemic (Coll et al., 2010). Despite this high biodiversity, ecosystem health is impaired by cumulative stressors (Micheli et al., 2013). The Mediterranean Sea is one of the most affected regions from overfishing, which has drastically reduced top predator populations and driven substantial changes to food web dynamics (Prato et al., 2013; Boudouresque et al., 2017). These stressors are expected to be further exacerbated by climate change and biological invasions (Bianchi and Morri, 2003; Azzurro et al., 2019). The influx of non-native species is most severe in the Eastern Mediterranean Sea (Ulman et al., 2019) where "Lessepsian migrants" (Por, 1978) enter in the Mediterranean via the Suez Canal since its creation in 1869. During 2000–2005, an average of one new Lessepsian migrant arrived per month (Streftaris et al., 2005). Native community diversity and structure appear to be dramatically altered from these introductions (D'Amen and Azzurro, 2020). For example, around the island of Rhodes (Greece), 11 out of 88 fish species recorded were found to be non-native species (Kalogirou et al., 2010). Lessepsian migrants make up 85% of total teleost abundance in southeastern Turkey in 2015 (Mavruk et al., 2017), and are likely the cause of a native mollusk population collapse in Israel (Albano et al., 2021).

Knowledge of a system's predators for an invasive species can help understand the potential direct and indirect impacts of the new species in the food web and evaluate the potential resiliency of the native community to this disturbance (Grüss et al., 2017; Chagaris et al., 2020). Such knowledge should be kept current and consider contemporary co-evolutionary and ecological processes of the ecosystem (Lee, 2002; Lambrinos, 2004). This is particularly germane for the Eastern Mediterranean as its species assemblages are undergoing rapid changes driven by warming waters, Lessepsian migrants, species tropicalization, and fishing pressure (Gücü et al., 2021). Ultimately, the application of this understanding can be used to inform management priorities for monitoring, control, and mitigation efforts.

The silver-cheeked toadfish *Lagocephalus sceleratus* (Gmelin, 1789, of the Tetraodontidae four-toothed family of pufferfishes) and the common lionfish *Pterois miles* (Bennett, 1828, of the Pteroinae subfamily of Scorpaenidae) are two piscivorous Lessepsian invaders of high concern. *Lagocephalus sceleratus* was first recorded in the Mediterranean in Turkish waters of the Aegean Sea in 2003 (**Figure 1A**; Akyol et al., 2005), followed by Israel in 2004 (Golani and Levy, 2005), and next in Rhodes and Crete in Greece (Corsini et al., 2006; Kasapidis et al., 2007). Within a few years, *L. sceleratus* were abundant throughout Aegean and Levantine coasts (Katsanevakis et al., 2020c). Their

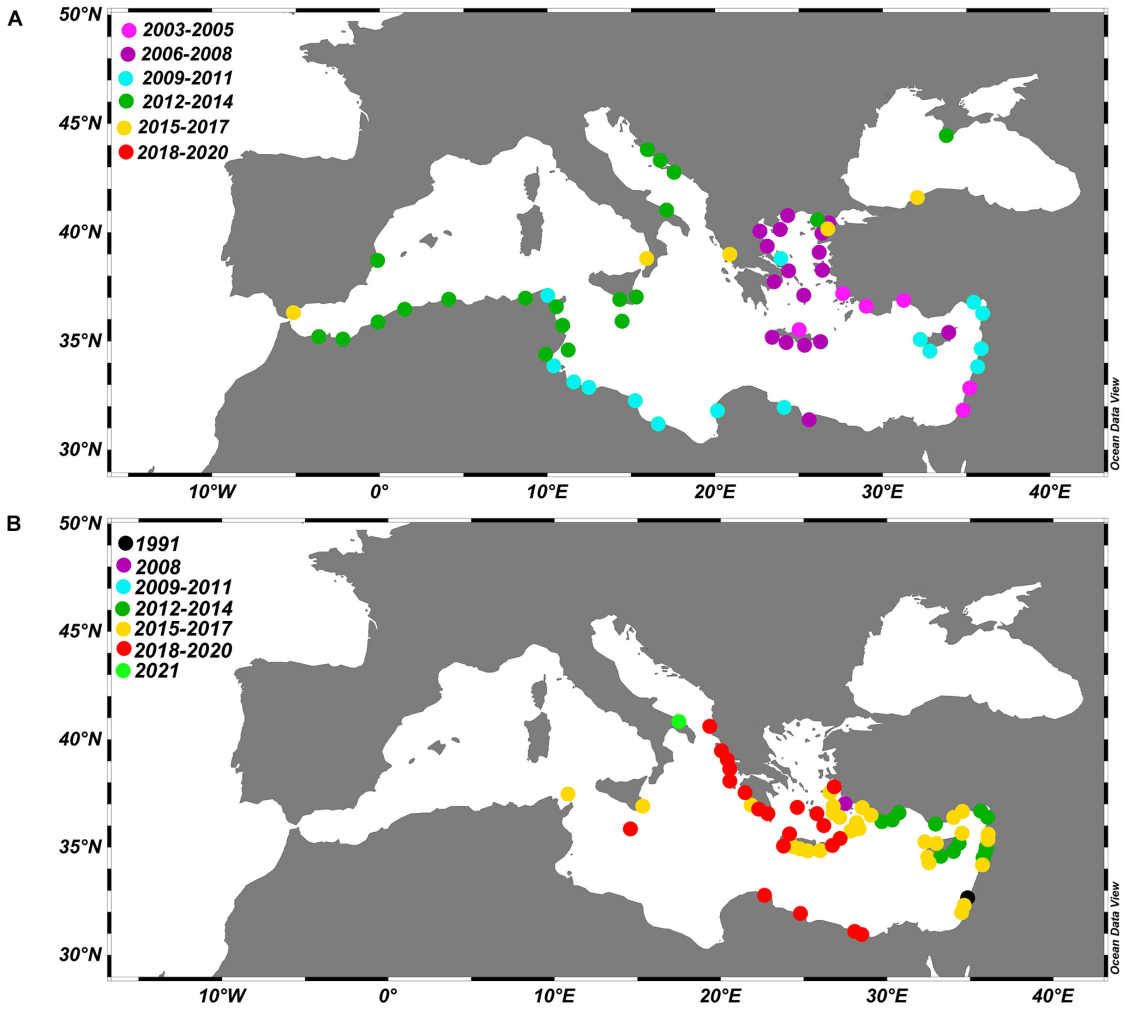

FIGURE 1 | (A) Map of *Lagocephalus sceleratus* Mediterranean distribution, 2003 to 2017; and **(B)** map of *Pterois miles* Mediterranean distribution from 1991 to 2021.

range expanded throughout the Mediterranean Sea during the following decade and are now found from the Strait of Gibraltar to the Black Sea (Akyol and Ünal, 2017; Azzurro et al., 2020; Gücü et al., 2021). Prior to 2010, *L. sceleratus* were only found at depths above 80 m, but they have been progressively expanding their depth range and, in April 2021, were recorded at their deepest depth of 220 m (Sabrah et al., 2006; Aydin, 2011; Ulman et al., in review). *Lagocephalus sceleratus* occupy a wide variety of benthic habitats, including sand, mud, rock, and seagrass meadows (Kalogirou et al., 2010, 2012; Kalogirou, 2013).

The *P. miles* invasion was first recorded in the Mediterranean in 1991 off Israel (**Figure 1B**), although was not reported again for another 20 years from Lebanon (Golani and Sonin, 1992; Bariche et al., 2013). Evidence of an expanding population were first reported from Cyprus in 2014. Their population then rapidly expanded through the entire Eastern Mediterranean to the central basin in Italy, Malta, and Tunisia (Kletou et al., 2016; Dimitriadis et al., 2020).

Lionfish in general prefer rocky substrata but *P. miles* has been found to inhabit similar habitats as *L. sceleratus* in the Mediterranean, including mud (Özbek et al., 2017) and seagrass meadows (Savva et al., 2020). *Pterois miles* has been found at 140 m depth in the Mediterranean Sea (Katsanevakis et al., 2020b; Poursanidis et al., 2020). Aggregations of over 30–35 individuals per 10 m^2 have been observed off Lebanon and Cyprus (Kleitou et al., 2021). Recently, separate reports from Turkey and Greece have recorded areas with densities as high as 30–40 *P. miles* individuals per 10 m^2 (Dimitriadis et al., 2020; Ulman et al., 2020; Kleitou et al., 2021).

Both species are highly invasive due to their potential for health impacts to humans and ecological effects in invaded communities. The *L. sceleratus* pufferfish is highly toxic due to high concentrations of tetrodotoxin (TTX) in their tissues, an extremely potent neurotoxin (Katikou et al., 2009; Kosker et al., 2016), and it is the second-highest toxic pufferfish in the Mediterranean after the yellow-spotted puffer (*Torquigener flavimaculosus*) (Kosker et al., 2018). Tetrodotoxin can be fatal to humans through paralysis even at very small doses of 1–2 mg (Madejska et al., 2019). From TTX research in *L. sceleratus*, the season, sex and tissue were found to have high variability with gonads having the highest rates, followed by liver, intestines, skin and muscle; and, in general, all-female tissues were found to be toxic throughout the year aside from female muscle in winter (Kosker et al., 2016). Consumption of *L. sceleratus* has caused dozens of human fatalities in the Mediterranean region, which is an underestimation given that many tetrodotoxin related fatalities are not officially recorded (Ben Souissi et al., 2014). *Lagocephalus sceleratus* also compete with native species and have negatively affected cephalopod populations and fisheries via predation (Kalogirou, 2013). Furthermore, they disrupt fishing operations by damaging fishing nets, severing longline and handline hooks, and depredation of catches. Costs due to damaged fishing gear per fisher have increased from 183 USD to 370 USD from 2011 to 2016 along the Turkish Levantine coast (Ünal et al., 2015; Ünal and Bodur, Ünal and Bodur; Gücü et al., 2021).

Pterois miles populations appear to be already impacting native species in the Mediterranean Sea. Their diet off Rhodes (Greece) was mainly composed of fish (78%) from the Gobiidae, Tripterygiidae, Sparidae, and Labridae families (in descending order, respectively, Zannaki et al., 2019). Similarly, off Cyprus, their diet was found to be exclusively composed of native macrofaunal species, including several commercially important species (Savva et al., 2020). The expected ecological effects from lionfish may be severe, given the widespread impacts of invasive lionfish to native fish communities and ecosystem processes in the Western Atlantic (Dahl et al., 2016; Hixon et al., 2016; Côté and Smith, 2018). Lionfish also pose risks to humans as the venom in their 18 spines can cause cardiovascular, neuromuscular, and cytolytic effects, ranging from mild reactions, including swelling, to extreme pain and paralysis in upper and lower extremities (Vetrano et al., 2002; Kiriake et al., 2013). Unlike *L. sceleratus*, no human fatalities have been reported from lionfish.

Here, a comprehensive review of predators for pufferfish and lionfish is undertaken for their native and invaded ranges, however, in the Mediterranean, predators are reviewed only for the highly invasive *L. sceleratus* and *P. miles*. Following this review, we discuss whether natural predation may offer biological control over these invasive species and then present recommendations for their Mediterranean region management.

MATERIALS AND METHODS

We compiled datasets to document predation records on: (1) pufferfish family (Tetraodontidae) and lionfish genus (*Pterois*) in their Indo-Pacific native ranges; (2) *Lagocephalus sceleratus* and *P. miles* in their Mediterranean Sea invaded ranges; and (3) Tetraodontidae and *Pterois* spp. in their Western Atlantic invaded ranges. Predation records for this review were collected from the scientific literature and unpublished sources. Unpublished sources included conference and government reports (i.e., "gray" literature), current research, author communications with fishers, and citizen reported records. These citizen records included photos and videos found online through social sharing platforms, specifically from YouTube, Facebook and Twitter. These photo and video records were validated when deemed necessary via personal communications from the authors with the citizen that recorded the predation event to ensure consumption did occur. For Twitter and Youtube, the following search terms were used: puffer, pufferfish, lionfish, pufferfish (or lionfish) eaten by, pufferfish (or lionfish) attacked by, eats pufferfish (or lionfish), attack(s) pufferfish or lionfish. For Youtube, after one record was found, other suggested similar videos were monitored for content.

RESULTS

Predation Records on Pufferfish and Lionfish in Their Indo-Pacific Native Ranges

No predation records were found specifically for *L. sceleratus* in its native range but predation records on the pufferfish

TABLE 1 | Predatory records on Tetraodontidae and *Pterois* spp. in their Indo-Pacific native ranges.

Common name	Scientific name	Date/Source
(A) Tetraodontidae predation		
Mantis shrimp	*Stomatopoda*	Lurot, 2015; https://cutt.ly/DkqVDbY
Lizardfish	*Synodus* sp.	Santhanam, 2017
Tiger shark	*Galeocerdo cuvier*	Santhanam, 2017
Moray eel	Muraenidae	Brazolov; https://cutt.ly/wkecmbC
Lemon shark	*Negaprion brevirostris*	Arthur, 2017; https://cutt.ly/ekrFP4b
Common octopus	*Octopus vulgaris*	Taylor and Miller; https://cutt.ly/gkqBpEK
Sepik beaked sea snake	*Enhydrina zweifeli*	Santhanam, 2017
Asian beaked sea snake	*Enhydrina schistosa*	Santhanam, 2017
(B) *Lagocephalus inermis* predation		
Cobia	*Rachycentron canadum*	Mohamed et al., 2013; Saha et al., 2019
Catfish	*Arius* sp.	Mohamed et al., 2013; Saha et al., 2019
Skipjack tuna	*Katsuwonus pelamis*	Mohamed et al., 2013; Saha et al., 2019
(C) Pteroinae predation		
Bluespotted cornetfish	*Fistularia commersonii*	Bernadsky and Goulet, 1991
Humpback scorpionfish	*Scorpaenopsis* spp.	Hochleithner 2008; https://cutt.ly/hly83Qf
Bobbit worm	*Eunice aphroditois*	Pistolesi, 2013; https://cutt.ly/Bly3XGM
Yellow edged moray	*Gymnothorax flavimarginatus*	Bos et al., 2017
Giant moray	*Gymnothorax javanicus*	Bos et al., 2017

family Tetraodontidae included the following (**Table 1**): Mantis shrimp (Stomatopoda), lizardfish (*Synodus* sp.), tiger shark (*Galeocerdo cuvier*), lemon shark (*Negaprion breviro*stris), sea snakes (*Enhydrina* sp.), catfish (Arius sp.), cobia (*Rachycentron canadum*), skipjack tuna (*Katsuwonus pelamis*), and common octopus (*Octopus vulgaris*).

In their Indo-Pacific native ranges, records of predation on lionfish species included bluespotted cornetfish (*Fistularia commersonii*), bobbit worms (*Eunice aphroditois*), humpback scorpionfish (*Scorpaenopsis* spp.) and moray eels (*Gymnothorax* sp.) (**Table 1**).

Predation Records on Pufferfish and Lionfish in Their Invaded Ranges

Mediterranean Records

A photograph of *C. caretta* biting an adult *L. sceleratus* was taken from Antalya, Turkey in 2019 (**Table 2A** and **Figure 2A**). This predation was later confirmed by video from the Mediterranean coast of Egypt in August 2020 (**Table 2A**). After discovery of the video, the filmmaker was contacted via email and sent photos of the existing pufferfish species in the region, and he identified *L. sceleratus* as the prey item he was filming. In support of this, the other pufferfish species do not reach the same large size as *L. sceleratus*, and from these two checks, the record was validated. We found strong evidence of *L. sceleratus* cannibalism in the Mediterranean with 16 total records of *L. sceleratus* juveniles found in adults, with six juveniles found inside one specimen from Tunisia. A dozen other Tetraodontidae species (namely the highly toxic *T. flavimaculosus*, Ulman, unpubl. data) were also found in adult *L. sceleratus* stomachs in Turkey and Tunisia (**Tables 2A,B**). Juveniles of *L. sceleratus* in the Mediterranean Sea appear to have a wider range of predators than adults. Our search indicated juveniles are preyed upon by common dolphinfish (*Coryphaena hippurus*), garfish (*Belone belone*), and larger *L. sceleratus* (**Table 2B** and **Figures 2B–I**).

Interestingly, we found four records of adult *L. sceleratus* preying on lionfish in Turkey and Cyprus (**Table 2C** and **Figure 3**). Multiple records of lionfish predation in the Eastern Mediterranean Sea were also reported for dusky grouper (*Epinephelus marginatus*) in Turkey, Greece, and Lebanon, as well as one predation record by white grouper (*Epinephelus aeneus*) in Cyprus (**Table 2C** and **Figure 3**). There is also one new record of a common octopus consuming a live lionfish (Crocetta et al., 2021).

Western Atlantic Records

As *L. sceleratus* are not present in the Western Atlantic, the Tetraodontidae predation records included octopus and frogfish (Antennaridae) and several marine birds: Yellow-footed gull (*Larus livens*), blue heron (*Ardea herodias*), tri-colored heron (*Egretta tricolor*), and osprey (*Pandion haliaetus*) (**Table 3**).

In the Western Atlantic, recorded predators of *P. volitans* and *P. miles* species included multiple species of groupers (tiger *Mycteroperca tigris*, Nassau *Epinephelus striatus*, black *Mycteroperca bonaci*, red *Epinephelus morio*, and gag *Mycteroperca microleps*; Epinephelinae), greater amberjack (*Seriola dumerilli*), moray eels (*Gymnothorax* spp.), nurse shark (*Ginglymostoma cirratum*), lemon shark (*Negaprion brevirostris*), and northern red snapper (*Lutjanus campechanus*) (**Table 3**). Cannibalism was also reported and confirmed in the Northern Gulf of Mexico (NGoM) populations with density-dependent rates (Dahl et al., 2017, 2018). Most predation records were from stomach content analyses, thus it cannot be determined whether predation took place on live or dead lionfish, except from live field observations made from SCUBA dives and studies employing tagging. Two (out of 20) lionfish installed with acoustic telemetry tags on the NGoM artificial reefs were consumed by fast-moving predators such as sharks (Dahl and Patterson, 2020). Tethering experiments on Caribbean coral reefs also show that native predators can consume live lionfish tethered to lead weights (Diller et al., 2014), although it is unknown whether these fish escaped or to what extent the tethering affected the predator-prey interactions.

DISCUSSION

This study compiled new and existing records on the predators of two highly invasive species in the Mediterranean Sea from their native and invaded ranges. For *L. sceleratus* adults in the Mediterranean, recorded predators included only loggerhead turtles and cannibalism, whereas for the less toxic juveniles, predation records included the white grouper, garfish and dolphinfish. Recorded predators of *P. miles* in the Mediterranean included *L. sceleratus,* white grouper, dusky grouper, and common octopus. Overall, we found relatively few predation records for these two species, suggesting that population control might only be possible via removal by human or natural control via disease/parasites unless we enhance and protect native predator populations (Kleitou et al., 2020). Here, the possible biological and ecological reasons for these low accounts of predation are discussed together with their implications for Mediterranean marine managers.

Both Tetraodontids and Scorpaenids have strong chemical and physical defense mechanisms that appear to deter predators. Predation on toxic Tetraodontidae species is limited to predators with TTX-resistant sodium channels in their nervous systems, capable of tolerating the uptake of the poison. It would be interesting to test if the loggerhead turtle also has these TTX-resistant sodium channels present, given that it was the only found predator thus far of an adult *L. sceleratus*. TTX resistant channels are present in other species such as the greater blue-ringed octopus (*Hapalochlaena lunulata*) and marine flatworms (Polycladida), as well as other Tetraodontidae (Saito et al., 1984, 1985), all containing TTX themselves. Scorpaenids similarly use venomous spines as a defense mechanism, and this venom contains acetylcholine and a neurotoxin affecting neuromuscular transmission (Cohen and Olek, 1989). Additionally, both species can enlarge their body size. Pufferfish can inflate with water to become 2–3 times their normal size. This inflation was fatal to a would-be predator by preventing a lemon shark from getting water to its gills (**Table 2A**)[1]. *Pterois* species also extend their

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[1] https://cutt.ly/ekrFP4b

TABLE 2 | Predatory records on *Lagocephalus sceleratus* and *Pterois miles* in their Mediterranean invaded ranges; photo credits after Figure reference.

Predator	Date	Location	Evidence type	Figure #, References, or note
(A) Predatory records of adult *L. sceleratus* in the Mediterranean				
Caretta caretta	11/2019	Antalya, Turkey	Photo	2A. *L. sceleratus* in mouth. Mayor of Antalya
Caretta caretta	08/2020	Simla, Egypt	Video	https://cutt.ly/ljSV5YH; Saad Al sharahani
L. sceleratus (500 x)	2019-2020	Datça, Turkey	Pers. comm.	Used *L. sceleratus* as bait to fish *L. sceleratus* with hook and line, S. Taşkıran
(B) Predatory records on juvenile *L. sceleratus* in the Mediterranean				
Epinephelus aeneus	04/2018	Xylofagou, Cyprus	Photo	**Figure 2B**. Stelios Yiangou
Coryphaena hippurus	07/2019	Limassol, Cyprus	Photo	**Figures 2C,D,E**. D. Papadopoulos, Kleitou et al., 2018
Belone belone	10/2019	Ierapetra, Crete	Photos	**Figures 2F,G**. Nikos Petashs. About 24 juveniles in stomach
L. sceleratus	2019-2020	Datça and Fethiye, Turkey, Tunisia	Pers. obs.	**Figures 2H,I**. 6 accounts of juvenile predation, Turkey (A. Ulman) and heavy cannibalism found in Tunisia with up to 6 juveniles found inside one adult, 1I (J. B. Souissi).
(C) Predatory records on *P. miles* in the Mediterranean				
E. aeneus	08/2019	Larnaca, Cyprus	Photo	**Figure 3A**. Stelios Yiangou
E. marginatus	05/2017	Beirut, Lebanon	Photo	**Figures 3B–D**. *P. miles* in stomach
E. marginatus	08/2020	Kasos Island, Greece	Photo	**Figures 3E,F**. Giorgos Zacharis, https://cutt.ly/jjSCSck
L. sceleratus	06/2020	Fethiye, Turkey	Photo	**Figure 3G**. A. Ulman. *P. miles* spines found inside 3 *L. sceleratus* stomachs
L. sceleratus	06/2020	Famagusta, Cyprus	Pers. obs.	*P. miles* spines found inside one *L. sceleratus* stomach, Akbora, unpubl. data
O. vulgaris	02/2021	Famagusta, Cyprus	Photos	Crocetta et al., 2021

fins and spines when threatened (Galloway and Porter, 2019). Opportunistic life-history strategies such as reproduction and recruitment, age, growth, and diet may also contribute to the success of both these species in invading the Mediterranean Sea (Sabrah et al., 2006; Fogg, 2017; Ulman et al., in review). Tetraodontidae have high TTX content in their ovaries, which is then passed onto their larvae, which may presumably inhibit larval predation. *Takifugu* spp. larvae have TTX localized on their skin and can effectively deter predation (Itoi et al., 2014). In *Pterois* species, females release eggs encased inside a mucous membrane, which may inhibit predators from sensing the scent or pheromones of the eggs and larvae (Morris et al., 2011b).

Although Tetraodontidae and lionfish are preyed to some extent in all seas, it appears unlikely that predation rates are high enough control of their populations (Arias-González et al., 2011; Barbour et al., 2011; Morris et al., 2011a; Chagaris et al., 2017). Mumby et al. (2011) reported that higher grouper biomasses in fishing-prohibited reefs resulted in a sevenfold decrease in Western Atlantic lionfish biomass, but this conclusion was made from only from 12 dive surveys. Findings from studies of high predator biomasses elsewhere in the invaded Western Atlantic reefs also challenge the hypothesis that lionfish are affected by top-down control. Surveys of 71 reefs conducted in three biogeographic regions of the Caribbean found no correlation between lionfish and native predator abundances (Hackerott et al., 2013; Valdivia et al., 2014). Generally, it appears that lionfish abundances are more strongly correlated to physical and environmental conditions than to community or native predator composition (Anton et al., 2014; Bejarano et al., 2015; Hunt et al., 2019).

It is unknown to what degree *L. sceleratus* and *P. miles* compete with each other in the Mediterranean. Their niches have some overlap, and they are both generalist predators. Fishers from Kaş (Turkey) attribute *L. sceleratus* bycatch declines in 2020 to *P. miles* abundance increases (A. K. Topuz, pers. comm.). Indeed, competition for habitat (Ellis and Faletti, 2016) and prey (Chagaris et al., 2017, 2020) may help control Western Atlantic lionfish abundances, although empirical measurements of such indirect effects are challenging (Côté and Smith, 2018). From this study, it has been revealed that *L. sceleratus* is already preying on lionfish in the Mediterranean. Further research in the Mediterranean on the extent of spatial and prey overlap of both *L. sceleratus* and its new *P. miles* potential competitor may be of interest, considering sympatry between these two species in their native Indo-Pacific. Currently, the food web and community effects of these species are largely unknown. Natural control of Western Atlantic lionfish may also be exerted by the emergence of disease or parasitism in their invasive ranges. Lionfish in the Western Atlantic initially appeared resistant to pathogens (Stevens and Olson, 2013; Stevens et al., 2016) and parasites (Sikkel et al., 2014; Loerch et al., 2015; Sellers et al., 2015; Fogg et al., 2016; Tuttle et al., 2017). However, in 2017, emergences of an ulcerative skin disease were first observed in the NGoM, with observations reported throughout the invaded Western Atlantic range (Harris et al., 2018). The NGoM lionfish populations exhibited a high prevalence of the disease and, within 18-months, their populations underwent dramatically (>50%) declines in recruitment, densities, and commercial landings (Harris et al., 2020a).

Lagocephalus sceleratus in the Mediterranean Sea and lionfish in the Western Atlantic exhibited evidence for conspecific cannibalism. Earlier reports of *L. sceleratus* diet from visual stomach analyses did not report cannibalism (Aydin, 2011; Kalogirou, 2013). However, our findings suggests that

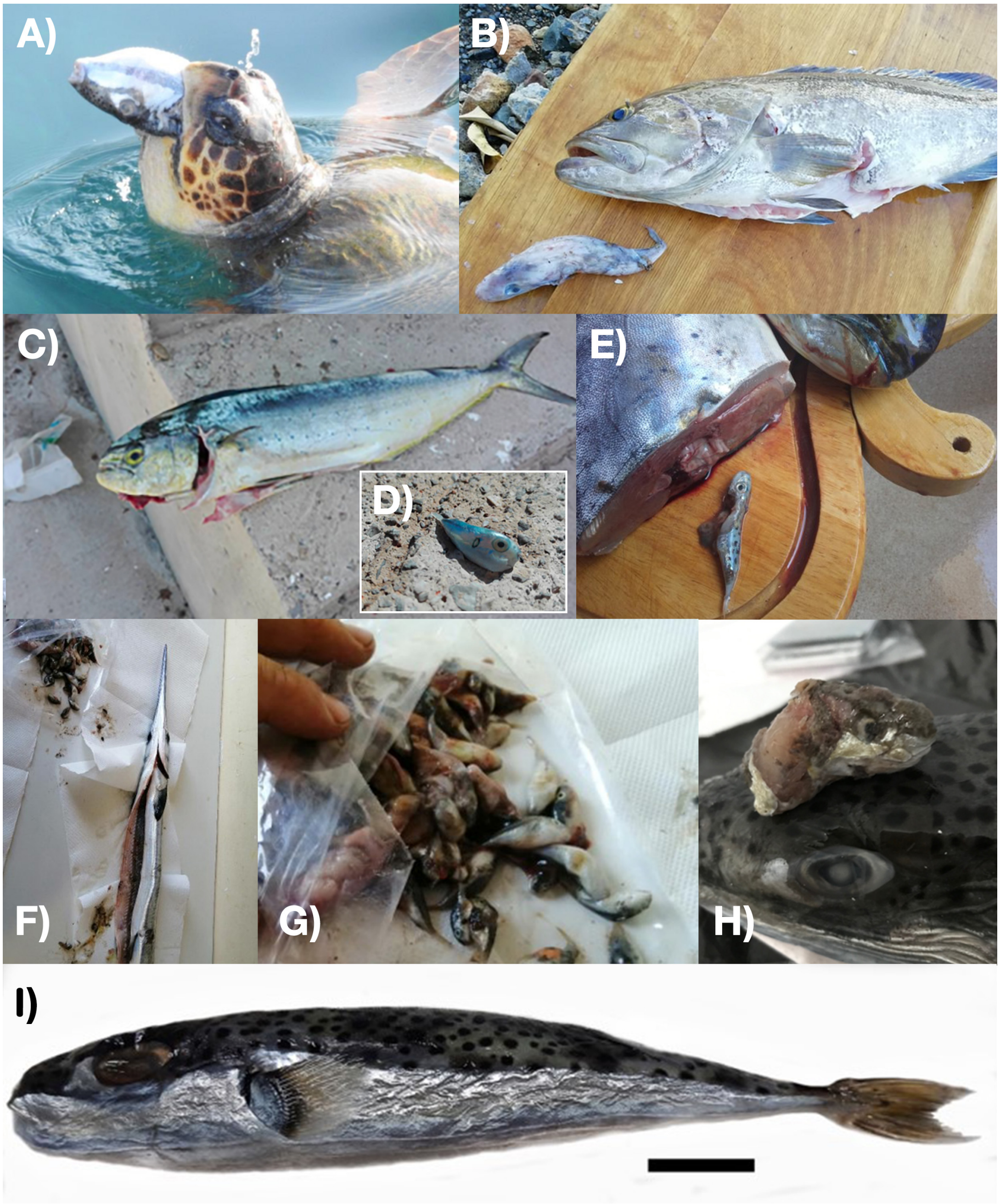

FIGURE 2 | Photographs evidencing predation on invasive *Lagocephalus sceleratus* in the Mediterranean Sea (details in **Table 2**): **(A)** *Caretta caretta;* **(B)** *Epinephelus aeneus*; **(C,D)** *Coryphaena hippurus*; **(E)** *Coryphaena hippurus*; **(F,G)** *Belone belone* with stomach full of juvenile *L. sceleratus*; **(H)** cannibalism; **(I)** well preserved specimen from cannibalism (6.4 cm total length).

density-dependent population control via cannibalism may now be occurring. Also interesting is that fishers from Datça (Turkey) conducting experimental removals of *L. sceleratus* caught hundreds of adult *L. sceleratus* using adult *L. sceleratus* as bait (Taşkıran, pers obs.). Although it is not considered a predation record, one video shows fireworms scavenging on dead pufferfish[2]. No location was provided for this record, but the worms appear to be the bearded fireworm (*Hermodice carunculata*), which are native to the Eastern Atlantic and the Mediterranean Sea.

Lionfish cannibalism has not yet been observed in the Mediterranean, but it should be expected once DNA barcoding methods are employed. Visual identification of stomach contents can produce biased diet estimates as fish rapidly lose visually identifiable characteristics within a short period of time (Schooley et al., 2008), especially in warmer waters (Legler et al.,

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[2] https://cutt.ly/5krA8mc

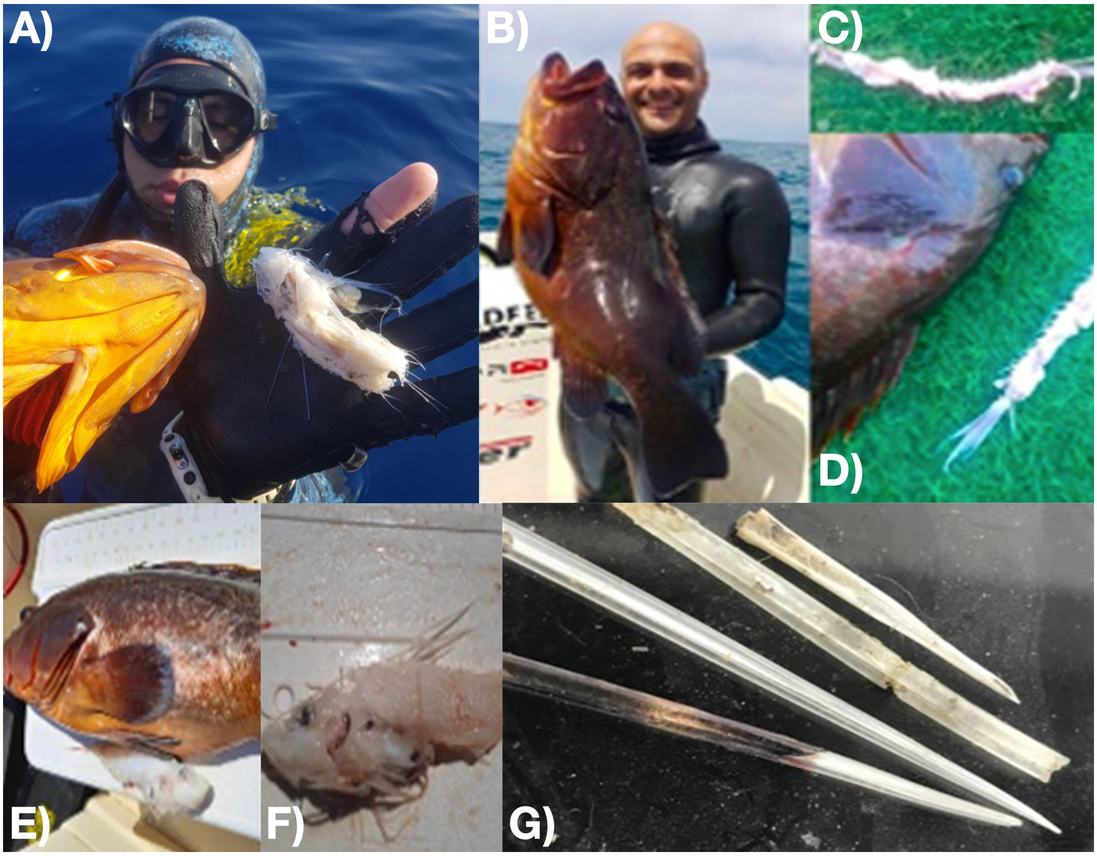

FIGURE 3 | Photographs evidencing predation on invasive *Pterois miles* in the Mediterranean (details in **Table 2**): **(A)** *Epinephelus aeneus*; **(B–D)** *Epinephelus marginatus*; **(E)** *Epinephelus marginatus*, with **(F)** regurgitated *P. miles* body; and **(G)** spines found inside *L. sceleratus* (top two spines) compared to *P. miles* dorsal spines (bottom two spines), both sets having the same tri-lobed features.

2010). Although an expansive richness of Western Atlantic lionfish diet has been well-described via thousands of visually identified stomach samples from many locations throughout the West Atlantic invasion (Peake et al., 2018), high rates of cannibalism within the same populations were only revealed when DNA barcoding technology was employed (Valdez-Moreno et al., 2012; Côté et al., 2013; Dahl et al., 2017). In fact, conspecifics comprised the most frequent identified prey taxon (14.4%) in lionfish from natural and artificial reefs located within the northern sections of the Gulf of Mexico (Dahl et al., 2017, 2018) where lionfish densities are generally 10–100 times higher than from tropical Western Atlantic reefs (Green and Côté, 2009; Darling et al., 2011; Dahl et al., 2019; Harris et al., 2019). Density-dependent competition and/or prey-switching thus appears evident in the Western Atlantic lionfish populations (Pereira et al., 2017), given the observed regime changes in prey-fish abundances and diversity on NGoM reefs (Chagaris et al., 2020; Lewis et al., 2020).

This study albeit comes with its limitations, as certainly undocumented predatory events exist. This could be improved with fishers' interviews, thus it is suggested that researchers studying these species, and whom are in contact with fishers, to ask for new records and document them. Additionally, quantitative studies could be conducted on the stomach contents of regional top predators landed in local fish markets around the invaded regions to improve predator knowledge on these new invaders. For example, a new study on the diet of the rock goby *Gobius paganellus* in the central Mediterranean showed its possible control on the invasive crab *Percnon gibbesi* (Tiralongo et al., 2021).

Records of loggerhead turtles and grouper predatory records on these invasive mesopredators should provide further support for a renewed effort toward prioritizing the rebuilding of top predators across the Eastern Mediterranean. Overfishing has driven recent declines of carnivorous fish in rocky reefs to very low levels (e.g., Sini et al., 2019), and the Mediterranean is currently considered an extinction risk hotspot for sharks and rays (Dulvy et al., 2014). There is, unfortunately, no sign of improvement over the Mediterranean International Union for Conservation of Nature (IUCN) Red List assessments of 2007 and 2016 (Dulvy et al., 2016). Marine Protected Areas (MPAs) in the Mediterranean, have proven highly effective in increasing fish biomass and restoring top predator populations (Giakoumi et al., 2017). Proposed recommendations to improve marine conservation efforts in the Mediterranean included designing effective networks of MPAs based on systematic

TABLE 3 | Predatory records on Tetraodontidae and *Pterois volitans* and *P. miles* in their Western Atlantic invaded ranges; NGoM= Northern Gulf of Mexico.

Common name	Scientific name	Date	Location	Evidence	References
(A) Predation on Tetraodontidae in the Western Atlantic					
Yellow-footed gull	*Larus livens*	2005	Florida	Video	https://cutt.ly/fkw0Skh
Frogfish	Antennariidae	2014	(Aquarium), United States	Video	https://cutt.ly/vkw0Bwr
Blue heron	*Ardea herodias*	2017	Florida	Video	https://cutt.ly/JkwVAy8
Tri-colored heron	*Egretta tricolor*	2020	United States	Video	https://cutt.ly/SkwNlE5; https://cutt.ly/tkwBNEk
Osprey	*Pandion haliaetus*	2020	Florida	Video	https://cutt.ly/9kq2to7
Common octopus	*Octopus vulgaris*	2021	Bonaire	Video	https://cutt.ly/akq2pZU
(B) Predation on *P. volitans* and *P. miles* in the Western Atlantic					
Spotted moray	*Gymnothorax moringa*	2005	NE Florida	Pers. obs.	Muñoz, 2017
Tiger grouper	*Mycteroperca tigris*	2008	Bahamas	Dissection	Maljkoviæ et al., 2008
Nassau grouper	*Epinephelus striatus* x2	2008	Bahamas	Dissection	Maljkoviæ et al., 2008
Lionfish	*Pterois volitans/miles* (cannibalism)	2009	Mexico	DNA Barcoding	Valdez-Moreno et al., 2012
Greater amberjack	*Seriola dumerilli*	2012	Gulf of Mexico	Dissection	Fogg, pers. comm.
Red grouper	*Epinephelus morio* x10	2012-2020	Gulf of Mexico	Photo	Fogg, pers. comm.
Northern red snapper	*Lutjanus campechanus* x5	2012-2020	NGoM	Photo	Fogg, pers. comm.
Nurse shark	*Ginglymostoma cirratum*	2013	Cayman Islands	Video	Diller et al., 2014
Nassau grouper	*Epinephelus striatius**	2013	Cayman Islands	Video	Diller et al., 2014
Lionfish	*P. volitans* (cannibalism)	2013-2014	NGoM	DNA Barcoding	Dahl et al., 2017, 2018
Gag grouper	*Mycteroperca microleps*	2014	Gulf of Mexico	Photo	Fogg, pers. comm.
Black grouper	*Mycteroperca bonaci*	2014	Florida	Photo	Fogg, pers. comm.
Red grouper	*Epinephelus morio*	2015	Cayman Islands	Video	youtu.be/BSn22wqJ5mA
Nurse shark	*Ginglymostoma cirratum*	2015	Cayman Islands	Acoustic tag	Candelmo, pers. comm.
Fast predator (likely shark)		2016	NGoM	Acoustic tag	Dahl and Patterson, 2020

**Predation on Pteroinae observed in Diller et al. (2014) were tethered to a lead weight but otherwise healthy.*

conservation planning principles, developing and implementing adaptive management plans, incorporating biological invasions in conservation plans, and prioritizing management actions to control invasive species (Mačić et al., 2018; Giakoumi et al., 2019; Katsanevakis et al., 2020a; Kleitou et al., 2020). Nevertheless, even if they are successful, rebuilding top predator populations will likely take decades.

Effective long-term control strategies for pufferfish and lionfish will require us to understand the factors that facilitate and control their population growth. For both species, their Mediterranean colonizations have been facilitated by environmental drivers, including ocean circulation, sea surface temperature, and the lunar cycle, which has been suggested for lionfish in the Western Atlantic invasion (Mostowy et al., 2020). Research and management will require regional coordination given their metapopulations are connected through larval dispersion (Johnston and Purkis, 2015). Therefore, the General Fisheries Commission of the Mediterranean announced in their Eastern Mediterranean Subregional Committee Meeting to establish a working group in early 2022 specifically for *L. sceleratus* and *P. miles*. This group would first be tasked with consolidating all available regional spatial and biological data on these species through an integrated monitoring platform. One output could be to present the mapping data in a current database similar to the mapping database for Western Atlantic lionfish (U.S. Geological Survey, 2016).

Finding ways to add commercial value to these species should further be explored to help the enablement of commercial fisheries to be established. In Cyprus, *L. sceleratus* removals have been incentivized with a financial bounty since 2009 (as well as in Turkey during December 2020), but regional-scale collaborated removal efforts are needed. Volunteer removals and recreational lionfish tournaments (often called "derbies") have shown to be effective at immediately controlling lionfish (de León et al., 2013; Lopez-Gómez et al., 2014). Derbies are particularly successful if the lionfish population is well-established in the area and if there is a large pool of divers (Malpica-Cruz et al., 2016). With current technologies, however, lionfish removals are limited to depths accessible to SCUBA divers (generally < 40 m), although experimental traps to expand fishing capacity to deeper populations are an active area of research, development, and testing (Harris et al., 2020b). Nevertheless, removals within depths accessible to SCUBA divers can control lionfish densities in frequently harvested areas (Frazer et al., 2012; Green et al., 2014; Dahl et al., 2016). In the NGoM, commercial diving removals have been as high as 20,000 kg per year and removal tournaments have removed over 10,000 lionfish in a single weekend (Harris et al., 2020a). In the Mediterranean, however, current regulations do not permit lionfish culling using SCUBA, with the exception of permitted culls allowed in Cyprus and Egyptian Mediterranean waters.

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.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because the research was a review of natural animal processes. No fish were collected or harmed for this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

AU and NiD contributed to the conception and design of the study. AU and TY prepared the figures. AU, NiD, HEH, and AF prepared the tables. AU, HEH, AF, TY, and NaD provided the final article revisions. All authors participated in the contribution of data and in the drafting and revising of the manuscript, contributed to the article and approved the submitted version.

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BRIEF RESEARCH REPORT
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Herbivory on the Invasive Alga *Caulerpa cylindracea*: The Role of Omnivorous Fishes

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Herbivory has long been considered an important component of biotic resistance against macroalgae invasions in marine habitats. However, most of the studies on herbivory of invasive algae refer only to consumption by strictly herbivorous organisms, whereas consumption by omnivorous species has been largely ignored and rarely quantified. In this study, we assess whether the commonest omnivorous sparid species in the Mediterranean Sea are consuming the highly invasive alga, *Caulerpa cylindracea*, and determine both, its importance in their diet and their electivity toward it as a source of food. Our results confirm that three of the four fish species studied regularly consume *C. cylindracea*, but in most cases, the importance of *C. cylindracea* in the diet is low. Indeed, the low electivity values indicate that all species avoid feeding on the invasive alga and that it is probably consumed accidentally. However, despite animals and detritus being the main food for these sparid species, several individual specimens were found to have consumed high amounts of *C. cylindracea*. This suggests a potential role that these fish species, being really abundant in shallow rocky bottoms, may play in controlling, to some extent, the abundance of the invader.

Keywords: invasive species, fish-alga interaction, grazing, biotic control, Mediterranean Sea, Sparidae, *Caulerpa cylindracea*

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INTRODUCTION

Invasive macroalgae are one of the most successful and conspicuous groups of invaders in marine systems (Schaffelke et al., 2006; Thomsen et al., 2016). These organisms, once established, contribute to the homogenization of marine habitats and affect the structure of native assemblages by reducing both native species biomass and the overall assemblage diversity (Williams and Smith, 2007; Schaffelke and Hewitt, 2008; Thomsen et al., 2009, 2016). In the receiving community, the establishment and persistence of invasive algae can be reduced and affected by biotic and abiotic factors (Dunstan and Johnson, 2007; Catford et al., 2009; Thomsen et al., 2009; Kimbro et al., 2013; Papacostas et al., 2017), thus determining the invasive success of the invader. Among biotic factors, herbivory has long been considered as a potential biotic resistance mechanism and many studies have been conducted worldwide to assess the role of this mechanism on invasive macroalgae success (see references within Kimbro et al., 2013; Papacostas et al., 2017). Until now, assessments on the role of herbivory as a limiting factor for macroalgae invasion have mainly considered strictly herbivorous species (e.g., Lyons and Scheibling, 2008;

Britton-Simmons et al., 2011; Enge et al., 2017; Noè et al., 2017; Caselle et al., 2018), reporting contrasting results depending on the assemblage and the invasive species considered (e.g., Sumi and Scheibling, 2005; Steinarsdóttir et al., 2009; Tomas et al., 2011b; Nejrup et al., 2012; Enge et al., 2017; Caselle et al., 2018). Actually, considering that omnivores have been reported to influence macroalgae and phytobenthos abundance in different ecosystems around the globe (Bellwood et al., 2006; Mendes et al., 2015, 2019; Tebbett et al., 2020), and that they can act as important functional groups, for instance, in the reversal of community phase-shifts in coral reefs (Bellwood et al., 2006; Tebbett et al., 2020), we pose that they could also potentially consume invasive alga species and influence their abundance. Therefore, to improve our understanding on the ecological role of omnivorous fishes and to assess whether they can contribute to the resistance of native habitats against invaders, it is necessary to assess invasive alga consumption by these species.

In the Mediterranean Sea, the most successful and widespread invasive macroalga is *C. cylindracea* (Klein and Verlaque, 2008; Katsanevakis et al., 2016), a green alga native to the Southwestern coast of Australia that was first detected in Mediterranean waters in Libya in 1990 (Nizamuddin, 1991). Since then, it has colonized marine communities throughout the entire Mediterranean basin (Piazzi et al., 2005; Klein and Verlaque, 2008), where it can exert strong detrimental effects on native communities (Piazzi et al., 2001; Klein and Verlaque, 2008; Bulleri et al., 2016, 2017). However, despite its rampant success, several *C. cylindracea* meadows have suffered sudden steep declines in abundance some years after the onset of the invasion (Klein and Verlaque, 2008; García et al., 2016; Santamaría et al., 2021), which may indicate the existence of certain resistance mechanisms against this invasive species. Among these, herbivory on *C. cylindracea* has been described and assessed mainly in relation to the strictly herbivorous species present in the Mediterranean Sea, such as the fishes *Sarpa salpa* and *Siganus luridus* (Azzurro et al., 2004; Ruitton et al., 2006; Tomas et al., 2011b; Santamaría et al., 2021), and the sea urchins *Paracentrotus lividus*, *Sphaerechinus granularis* and *Arbacia lixula* (Ruitton et al., 2006; Bulleri et al., 2009; Cebrian et al., 2011; Tomas et al., 2011a). Nevertheless, there are omnivorous fish species, such as *Diplodus sargus*, *Boops boops*, and *Spondyliosoma cantharus*, that have been occasionally observed feeding on *C. cylindracea* before (Ruitton et al., 2006; Box et al., 2009; Terlizzi et al., 2011). Unfortunately, information is scarce on whether *C. cylindracea* is a common food source for omnivorous fish species, or whether these fish actively elect to feed on it. Since some of these species are the dominant fish in the shallow, infralittoral rocky habitats of the Mediterranean Sea (García-Rubies, 1997; Sala and Ballesteros, 1997; García-Charton et al., 2004), information on their consumption of *C. cylindracea* is needed to assess whether they can potentially contribute to limit the abundance of the invader.

In this study, the diets of four of the most abundant omnivorous sea bream species (Sparidae) were examined in order to determine (i) whether they feed on the invasive alga *C. cylindracea*, (ii) whether *C. cylindracea* is important in their diet, and (iii) whether they actively select or avoid *C. cylindracea* as a source of food.

MATERIALS AND METHODS

Study Area

The samples for this study were collected in the Cabrera Archipelago National Park (North-Western Mediterranean Sea; 39°12′21″ N, 2°58′44″ E) (**Supplementary Figure 1**) in 2008. This marine-terrestrial protected area was established in 1991 and since then it has maintained an exceptional conservation status for its marine habitats (Sala et al., 2012; Coll et al., 2013; Guidetti et al., 2014). *C. cylindracea* was recorded for the first time in the National Park in 2003 at a depth of 30–35 m and since then its distribution has expanded to cover most of its benthic communities at depths of between 0 and 65 m (Cebrian and Ballesteros, 2009).

Analysis of *Caulerpa cylindracea* Consumption

To determine whether non-strictly herbivorous fish species consume *C. cylindracea*, specimens for this study were captured by artisanal long-lines and gillnets once a month during June and July 2008, at different sites across the Archipelago, at Ses Rates and Foradada Islets (**Supplementary Figure 1**). These two sites where chosen because they are representative areas for the Cabrera Archipelago and have similar benthic community composition and abundances of sparid fishes (Reñones et al., 1997). Fishing campaigns were performed in the summer because it corresponds to the period of the year when the activity of the targeted fishes is higher. The main fishes targeted belong to the family Sparidae: white sea bream (*D. sargus*), annular sea bream (*Diplodus annularis*), two-banded sea bream (*Diplodus vulgaris*) and black sea bream (*S. cantharus*). These species were chosen because they are common representatives of the fish assemblages found in the Western Mediterranean, they are not herbivorous but can feed on macroalgae (Sala and Ballesteros, 1997) and some of them have been observed feeding on *C. cylindracea* before (Box et al., 2009; Terlizzi et al., 2011). These four species have different abundances within the National Park, being *S. cantharus* the least abundant and *D. vulgaris* the most abundant (Reñones et al., 1997; Coll, 2020). Specifically, in a 250 m^2 area, 1.7 *S. cantharus*, 1.9 *D. annularis*, 5.3 *D. sargus*, and up to 42 *D. vulgaris* individuals, were found.

The long-lines and gillnets, two gears commonly used in artisanal fishing, were deployed in the morning at depths of between 10 and 30 m. Gillnets were 300 m long, had a mess size of 40 mm, and were left in the water for 4 h; whereas artisanal long-lines were 250 m long, had a total of 150 hooks, and were left in the water for 4 h. In total, two fishing events, one in June and the other in July, were performed at each location and with each gear. Every time a targeted fish species was hauled in, it was gutted and its stomach was stored and preserved in buffered 4% formaldehyde-seawater solution for later analysis of its content. Once in the laboratory, the species composition and abundance of the food items in each fish stomach was determined under a Stemi 2000-C stereomicroscope (Carl Zeiss, Berlin, Germany). The content of each stomach was spread onto a reticulated Petri dish and the food items were classified to the lowest taxonomic level possible. Both surface area and weight measurements can reflect the dietary contribution of food items (Hyslop, 1980; Macdonald and Green, 1983), but in this case, and to avoid biases that could be derived from the small quantities present in the stomach contents, surface area measurements were preferred over weight measurements to quantify the dietary contribution of each food item. As such, the abundance of a particular food item was estimated as the percentage cover on the reticulated fields of the Petri dish in relation to the cover of the whole stomach content. When a species had a minimal presence and its cover could not be determined, a value of 0.1% of relative coverage was assigned.

When calculating the relative measures of prey quantity (RMPQ), the stomach contents were divided into the following five food categories: *C. cylindracea*, Other algal content, Seagrasses, Animal content and Organic detritus. Subsequently, for each fish species, the percentage frequency of occurrence of each food category (FO) was calculated as:

$$FO_i = (S_i/S_t) \cdot 100$$

where S_i is the number of stomachs containing the food category and S_t is the total number of stomachs analyzed for that particular fish species. The FO_i value is a measure of the consistency with which a species selects a given food category and was used to calculate two dietary indices that allow to compare the diets between species: the Combined Index (Q) and the Geometric Index of Importance (GII).

The combined index, Q, was chosen to assess the relative importance of each food category for each fish species. This index standardizes the abundance of each category and increases the importance of frequent smaller items while reducing the importance of occasional larger items (Nilssen et al., 2019). It was calculated as:

$$Q_i = \frac{V_i \cdot FO_i}{\sum_{i=1}^{m} (V_i \cdot FO_i)}$$

where V_i refers to the percentage surface of a food category, FO_i refers to the frequency of occurrence of the given food category, and m is the total number of food categories.

On the other hand, the Geometric Index of Importance, GII, represents the degree of feeding specialization on a particular food type (Assis, 1996; Preti et al., 2001) and allows us to classify the food categories as: "Primary prey," "Secondary prey," and "Occasional prey" in relation to the larger discontinuities in a decreasing sequence of values (Assis, 1996; Tripp-Valdez et al., 2015). It was calculated as:

$$GII_j = \frac{\sum_{i=1}^{n} (V_i + FO_i)}{\sqrt{2}}$$

Finally, the degree to which the four fish species tend to elect to feed on *C. cylindracea*, was assessed by Ivlev's electivity Index (E) (Ivlev, 1961). This Index was determined by:

$$E = \frac{(d_i - a_i)}{(d_i + a_i)}$$

where d_i = % of *C. cylindracea* in the stomach content and a_i = % of *C. cylindracea* available in the environment (see following section). The values of the Ivlev's Index (E) can range from -1 (complete avoidance of the food item) to $+1$ (exclusive selection of the item), with positive values indicating that the food item is selected and eaten more than it is encountered by chance in the environment (Ivlev, 1961).

Assessment of the Abundance of *Caulerpa cylindracea* in the Community and Characterization of the Benthic Macroalgae Community

The abundance of *C. cylindracea* at the sampling sites where fish specimens were captured was assessed by means of scuba diving, also in the summer 2008. At each site, a perpendicular transect to shore was done, at depths of 10–30 m, so as to cover the same bathymetric range as that of the fishing gear used to collect fish samples. To estimate *C. cylindracea* abundance, a total of 30 quadrats measuring 25 cm × 25 cm were randomly positioned within each 10 m-depth range (total of 90 quadrats per sampling site). These quadrats were divided into 25 subquadrats of 5 cm × 5 cm and the number of subquadrats where *C. cylindracea* appeared was used as the unit of abundance (Sala and Ballesteros, 1997; Cebrian and Ballesteros, 2004; Sant et al., 2017). Subsequently, the average *C. cylindracea* abundance for the study area was calculated and this value was used in the calculation of the Ivlev's electivity Index.

Additionally, to characterize the benthic macroalgae community, the quadrat methodology was also used. In this case, at each site and at each depth stratum (10, 20, and 30 m depth), six 25 cm × 25 cm quadrats were randomly positioned and the abundance of each algal species was calculated as a function of the number of subquadrats where each species appeared. Subsequently, as there were no differences in the benthic composition between sites, the average abundance per alga species at the study area was calculated.

Statistical Analyses

To evaluate sampling sufficiency, prey accumulation curves for each of the sampled species were generated using the package *BiodiversityR* (Kindt and Coe, 2005) in the R environment (R Core Team, 2018). Two curves per species were fitted, one to the number of prey taxa and the other to the number of food categories, to assess whether the number of stomachs analyzed was adequate for the diet characterization of each species.

Differences in the specific composition of stomach contents between fish species were assessed through multivariate techniques such as non-metric multi-dimensional scaling plots (NMDS plots), analysis of similarities (ANOSIM) and similarity percentage analysis (SIMPER). All of these techniques were performed in R, within the *vegan* package (Oksanen et al., 2018). First, in order to visualize and represent stomach content composition, a NMDS (Clarke and Warwick, 1994; Cox and Cox, 2000) based on the Bray-Curtis dissimilarity matrix of the square root transformed data was plotted and the most important species that determine the least stressful ordination were detected using the envfit function within the *vegan* package. Then, the statistical differences in the food items consumed by the fish species were assessed using ANOSIM (Clarke, 1993), applied to the Bray-Curtis dissimilarity matrix, with fish species as a fixed factor. Additionally, a pairwise ANOSIM was performed by modifying the pairwise.adonis function[1] and the R-values obtained were used as an indication of diet similarity, with values near 1 indicating separation in diet composition and values near 0 indicating diet similarity (Rogers et al., 2012). Finally, a SIMPER analysis based on the Bray-Curtis dissimilarity index was used to assess the relative contribution of each food item to the overall differences between fish species diets.

RESULTS

During the sampling events, a total of 93 fishes were captured, with *D. sargus* being the most abundant ($n = 51$) followed by *S. cantharus* ($n = 22$), *D. vulgaris* ($n = 13$), and *D. annularis* ($n = 7$). All the stomachs examined contained ingested material of some kind, which, as a whole, was composed of a high diversity of taxonomic groups, with 73 different prey items identified, 32 of them to the species level (**Supplementary Table 1**).

According to the prey accumulation curves (**Supplementary Figure 2**), sampling sufficiency was a little low to quantitatively characterize the diet of the sampled species using the full resolution of the data (lowest taxonomic level), as all the curves were only beginning to plateau but did not reach the asymptote. However, asymptotes were reached for all the species when prey taxa was combined in food categories, which suggests that our data is adequate to investigate the quantitative contribution of these categories to the diet of the sampled species.

Differences in stomach content were observed between species in terms of the dominant prey categories, although organic detritus and animal content were certainly prominent in all four species (**Table 1**). In this sense, the Combined Index (Q) and the Geometric Index of Importance (GII), identified the category "Organic detritus" as the primary food item for *S. cantharus*, while the category "Animal content" was the primary prey for the other three fish species (**Figures 1**, **2**). Despite this predominance for "Animal" and "Organic detritus" items, algae and seagrasses were found in all the species, being the stomach contents of both *D. sargus* and *D. annularis* particularly rich in algae, with values of around 18 and 30%, respectively (**Table 1**).

Regarding the consumption of the invasive alga *C. cylindracea*, 81, 45, and 41% of the *D. annularis*, *D. sargus* and *S. cantharus* specimens contained *C. cylindracea* in their stomachs, respectively; whereas *D. vulgaris* was the only fish species that had not consumed the invader. However, the contribution of *C. cylindracea* to the total stomach content was generally low, except for *D. annularis*, in which 26% of the stomach content corresponded to the invasive species (**Table 1**). Actually, both dietary indexes, Q and GII, classified *C. cylindracea* as a primary food item for *D. annularis*, being the second most common food category in the stomachs and having values similar to animal content (**Figures 1**, **2**). In contrast to this, *C. cylindracea* was classified as an occasional food item for both *D. sargus* and *S. cantharus* (**Figures 1**, **2**), but most of the algae content in the stomachs of *S. cantharus* corresponded to the invasive species (≈74%; **Table 1**). However, even with the high proportion of *C. cylindracea* found in some of the studied species, the negative values obtained for the Ivlev's Electivity Index suggest that *C. cylindracea* is generally avoided by all the species (**Table 2**), despite *C. cylindracea* being the dominant species in the benthic community (**Supplementary Table 1**).

TABLE 1 | Summary of the stomach content data for each fish species.

Diplodus annularis	**Mean ± S.E. (%)**	***Diplodus sargus***	**Mean ± S.E. (%)**
Other algal content	4.69 ± 3.10	Other algal content	15.10 ± 3.67
Caulerpa cylindracea	**25.73 ± 11.80**	***Caulerpa cylindracea***	**3.56 ± 0.96**
Seagrasses	0.26 ± 0.21	Seagrasses	9.36 ± 2.84
Animal content	43.28 ± 16.00	Animal content	48.31 ± 5.53
Organic detritus	26.05 ± 15.03	Organic detritus	19.62 ± 4.72
Diplodus vulgaris	**Mean ± S.E. (%)**	***Spondyliosoma cantharus***	**Mean ± S.E. (%)**
Other algal content	8.80 ± 6.91	Other algal content	1.88 ± 0.90
Caulerpa cylindracea	**0**	***Caulerpa cylindracea***	**5.15 ± 1.94**
Seagrasses	0.29 ± 0.25	Seagrasses	0.40 ± 0.26
Animal content	54.75 ± 12.30	Animal content	14.10 ± 4.81
Organic detritus	31.54 ± 11.25	Organic detritus	78.47 ± 5.12

The ingested food items are grouped into categories and the values given are mean percentages ± S.E. for each fish species. Ingested values of Caulerpa cylindracea are highlighted in bold.

Considering the whole diet of the four sampled sea bream species, the graphical ordination suggested that there might be some overlap between diets (**Supplementary Figure 3**), yet the ANOSIM detected significant differences in the stomach content composition between all fish species (ANOSIM; $p < 0.05$; **Supplementary Table 2**), with two exceptions: *D. sargus—D. vulgaris* and *D. sargus—D. annularis* ($p > 0.05$; **Supplementary Table 2**). In this sense, the greatest dissimilarities in diet were found between *S. cantharus* and two of the *Diplodus* species, *D. annularis* and *D. vulgaris* ($R = 0.63$ and $R = 0.47$ respectively, **Supplementary Table 2**), since the diet of *S. cantharus* was more homogeneous (**Supplementary Figure 2**) and it was dominated by organic detritus rather than by animal content (**Table 1** and **Figures 1**, **2**). Actually, the SIMPER analysis identified "organic detritus" as the biggest contributor to the diet dissimilarities between the four fish species, with values ranging from 19 to 40% (**Supplementary Table 3**). Remarkably, *C. cylindracea* was one of the species most strongly influencing the graphical ordination (**Supplementary Figure 3**) and the SIMPER analysis identified the invasive alga as the second most important food item in terms of explaining the diet dissimilarities between *D. annularis* and the other fish species (**Supplementary Table 3**).

DISCUSSION

Stomach content analysis of some of the commonest species in the shallow rocky infralittoral communities in the western

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[1] https://github.com/pmartinezarbizu/pairwiseAdonis

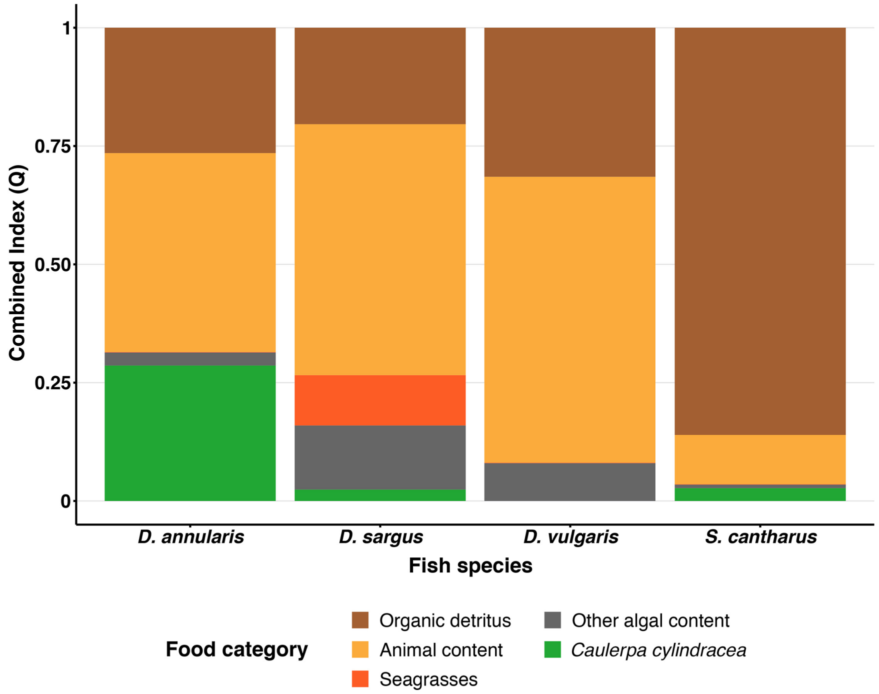

FIGURE 1 | Combined Index (Q) for each fish species. Each color represents one of five food categories (Organic detritus, Animals, Seagrasses, Other algae and *Caulerpa*).

Mediterranean Sea, revealed that three of the four studied fish species have consumed the invasive alga *C. cylindracea*. Actually, the invader was identified as the most prominent algae for two of the fish species, *D. annularis* and *S. cantharus*, and was a primary food item for *D. annularis*. On the contrary, it was only an occasional food item for both *S. cantharus* and *D. sargus*. Complementary, despite these species having a diet predominately based on animal and detritus content (Sala and Ballesteros, 1997; Gonçalves and Erzini, 1998; Pita et al., 2002; Box et al., 2009; Terlizzi et al., 2011; Felline et al., 2012, 2017), our results show that they can also feed on considerable amounts of algae, with values exceeding those previously reported for non-strictly herbivorous sea bream species (Sala and Ballesteros, 1997; Sánchez-Jerez et al., 2002; Leitão et al., 2007; Box et al., 2009).

In general, being *C. cylindracea* a widespread and regionally abundant alga (Klein and Verlaque, 2008; Katsanevakis et al., 2016), our findings clearly suggest that it can potentially become a recurrent food item for omnivorous fish species, similarly to what has happened with the herbivorous fish *S. salpa*, which has adopted the invader as a preferred food item (Tomas et al., 2011b). Actually, several studies involving lower sample sizes, had previously reported the ability of *D. sargus*, *S. cantharus*, and *D. vulgaris* to occasionally feed on *C. cylindracea* on other locations in the Mediterranean Sea (Box et al., 2009; Terlizzi et al., 2011; Felline et al., 2012, 2017; Gorbi et al., 2014). In our assessment, however, *C. cylindracea* consumption seems frequent, as a high proportion of the sampled individuals (41%) was found with the invader in the stomach. Interestingly, our findings are, to our knowledge, the first evidence of *D. annularis* feeding on *C. cylindracea* in the Mediterranean Sea, but given that almost all the fished individuals (86%) had consumed the invader and that they had high amounts of *C. cylindracea* in their stomachs, this fish species should be included in subsequent assessments to further elucidate whether this feeding behavior is common across the Mediterranean region. Unfortunately, it should be taken into account that sample sizes, mainly for *D. vulgaris* and *D. annularis*, were low (**Supplementary Figure 2**), so the findings reported here should be interpreted as a first exploration. Therefore, to fully characterize the contribution of *C. cylindracea* to the diet of the different seabream species and to thoroughly quantify *C. cylindracea* consumption, the analysis of more stomach contents is definitively needed. Despite this, however, our data clearly shows that the invader has become a recurrent food source for at least three of the four assessed species, according to *C. cylindracea* being found consistently in the stomach contents of these species.

Concurrently, despite previous studies reporting the consumption of *C. cylindracea* by omnivorous fishes, none of them reports the availability of *C. cylindracea* in the environment, making it impossible to assess whether the omnivorous fishes are actually targeting the invader. In this sense, in our study area, *C. cylindracea* was found to be the dominant species in the benthic communities where the fish species were fished, with mean coverage values close to 55% (**Supplementary Table 1**). However, electivity of the fish species for *C. cylindracea* suggests that all the species were avoiding feeding on the invader, although laboratory experiments should be considered to have a more detailed interpretation on the avoidance/preference

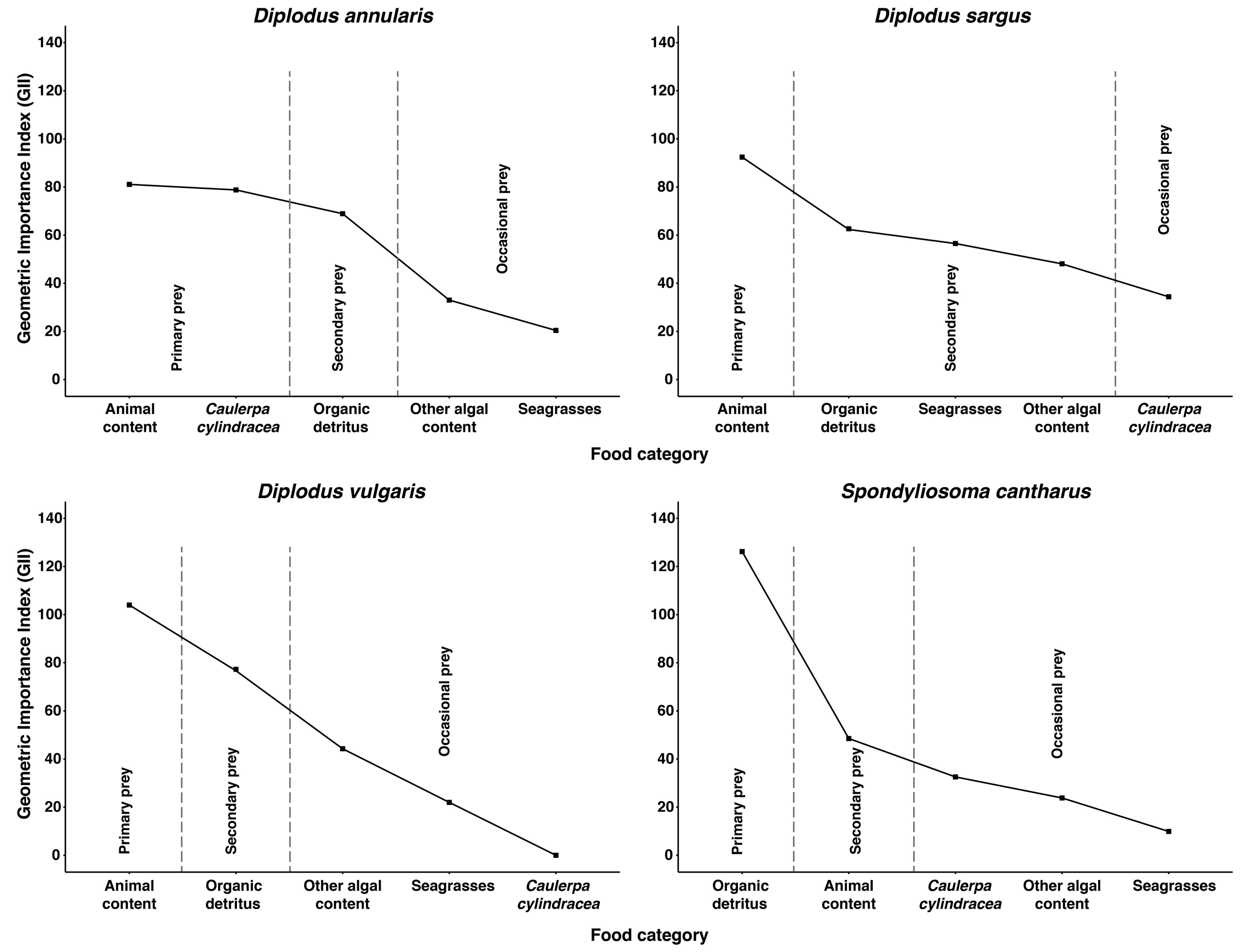

FIGURE 2 | Geometric Importance Index (GII) for each fish species. Food items are classified as: "Primary prey," "Secondary prey," or "Occasional prey" according to their relative importance to the diet of each fish species. The line connecting the points was added to help interpretation of the figure.

for the invader. Therefore, the low electivity suggests that the consumption by the omnivorous fishes was more occasional than that of the strictly herbivorous fishes, which show a positive electivity for the invader (Tomas et al., 2011b). Surprisingly, despite this general avoidance of *C. cylindracea*, high amounts of the invader were found in the stomach contents of some individuals, with values reaching over 35%. This suggests that the invasive alga is probably consumed accidentally when the fish are trying to feed on other prey living within the dense meadows of *C. cylindracea*. In fact, polychaetes, mollusks, and decapods—which are the preferential prey for most of the sea breams studied (Bauchot and Hureau, 1986; Sala and Ballesteros, 1997; Gonçalves and Erzini, 1998; Pita et al., 2002; Leitão et al., 2007)—have been found to be very abundant under the stolons of *C. cylindracea* (Carriglio et al., 2003; Galil, 2007; Box, 2008; Klein and Verlaque, 2008). Furthermore, the suggestion that consumption of the alga is accidental is also supported by the low assimilation of *C. cylindracea* in the stomach contents, as in most cases it was found intact and undigested (**Supplementary Figure 4**). However, taking into account that some of the sea breams considered here have small home ranges and show strong site fidelity (D'Anna et al., 2011; March et al., 2011; Alós et al., 2012; Di Lorenzo et al., 2014), it cannot be ruled out that they might also be forced to feed on *C. cylindracea* in heavily colonized areas, and this might have a negative impact on the physiology of the fish species. In fact, previous evidence relates *C. cylindracea* consumption to a decrease in certain essential

TABLE 2 | Mean ± S.E. values for Ivlev's electivity Index (E), as a measure of the electivity of the four sparid fish species studied towards the invasive alga *C. cylindracea*.

$E_{Caulerpa}$	
D. annularis	−0.52 ± 0.18
D. sargus	−0.90 ± 0.02
D. vulgaris	−1
S. cantharus	−0.86 ± 0.05

An E value approaching −1 indicates that the food item is avoided; whereas an E value approaching 1 indicates the species only feeds on that item.

fatty acids in fish tissues and liver (Felline et al., 2014), to an increase in the levels of antioxidants and in pro-oxidant effects (Box et al., 2009; Terlizzi et al., 2011; Felline et al., 2012), to a decrease in the condition factor (Terlizzi et al., 2011) and to a decrease in the gonadosomatic index (Felline et al., 2012), all of which may negatively affect the fish fitness. It is not yet clear what causes these physiological responses, although they could be caused by the accumulation of some of the compounds produced by *C. cylindracea*, such as caulerpenyne, a toxic, secondary metabolite that has herbivore-deterrent properties (Paul et al., 2007). However, considering that *Caulerpa prolifera*, a native species in the Mediterranean Sea, has much higher caulerpenyne concentrations than *C. cylindracea* (Box et al., 2010) and that sea breams can often consume the native *Caulerpa* species (**Supplementary Table 1**; Chaouch et al., 2013, 2014; Marco-Méndez et al., 2017), it is likely that the fish might have developed a certain tolerance and effective detoxification pathways for the toxic metabolites, as other herbivores do (Cornell and Hawkins, 2003; Sotka and Whalen, 2008; Sotka et al., 2018). In any case, more studies are needed to understand the possible long-term consequences of *C. cylindracea* consumption on the health of fish assemblages and whether these consequences could propagate throughout the food web, potentially affecting the functioning of the ecosystem through cascading effects.

Overall, our findings confirm that the invasion of *C. cylindracea* in the Mediterranean Sea has the potential to influence the feeding habits of the omnivorous fish species as it has already done with some of the strictly herbivorous organisms (Azzurro et al., 2004; Ruitton et al., 2006; Cebrian et al., 2011; Tomas et al., 2011a,b). In this sense, the assessment of *C. cylindracea* consumption by omnivorous (i.e., non-strict herbivores) fish presented here is noteworthy since most of the previous research into the effects of herbivory on invasive algae has focused only on the strict herbivores, disregarding the effect that omnivorous organisms might have (e.g., Davis et al., 2005; Sumi and Scheibling, 2005; Ruitton et al., 2006; Wikström et al., 2006; Lyons and Scheibling, 2008; Vermeij et al., 2009; Britton-Simmons et al., 2011; Cebrian et al., 2011; Tomas et al., 2011a,b; Nejrup et al., 2012; Hammann et al., 2013; Enge et al., 2017; Caselle et al., 2018). Furthermore, while some of the previous studies have highlighted the contribution of some of these herbivores to limit the abundance of invasive algae, our findings suggest that non-strict herbivores could also potentially have a similar, albeit less important contribution. Therefore, these findings provide another example on the important role that omnivorous fish species might have on marine ecosystems, where they have been reported to play a key role on the coral-algae equilibrium in coral reefs from several regions (Bellwood et al., 2006; Tebbett et al., 2020), and to become an important link between primary producers and higher trophic levels in rocky reefs (Mendes et al., 2015, 2019). In our case, given the high amounts of *C. cylindracea* found in some specimens and considering that omnivorous sparid fishes dominate the shallow rocky infralittoral habitats in the Mediterranean Sea (García-Rubies, 1997; Sala and Ballesteros, 1997; García-Charton et al., 2004; Coll et al., 2013) with abundances of up to 60 individuals per 250 m^2 and biomasses of more than 40 g/m^2 (García-Rubies and Zabala, 1990; Sala and Ballesteros, 1997; Di Franco et al., 2009; Guidetti et al., 2014; Coll, 2020), it seems that they could potentially have some impact on the abundance of *C. cylindracea*. Therefore, we suggest that the lower impact exerted by omnivorous fish could complement the higher impact exerted by strictly herbivorous organisms (Santamaría et al., 2021) and that, taken together, they might significantly reduce the abundance of *C. cylindracea* in shallow habitats. Still, considering our limited sample sizes, we pose that more in depth assessments are needed to better quantify the impact that omnivorous fish might have on the success of *C. cylindracea*. Finally, considering that sea breams are highly targeted by fisheries and have already suffered important declines in the Mediterranean basin (Sala et al., 1998; Coll et al., 2004; Sala, 2004; Morales-Nin et al., 2005; Guidetti, 2006; Lloret et al., 2008), places that promote their recovery, such as well-enforced marine protected areas (MPAs) (Mosquera et al., 2000; Micheli et al., 2005; Claudet et al., 2006; Guidetti, 2006; Guidetti and Sala, 2007; Guidetti et al., 2008, 2014; Sala et al., 2012; Coll et al., 2013), might also foster the strength of this complementary control mechanism on the abundance of *C. cylindracea*.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because the sampling was performed during 2008 in the framework of a big project carried out by the National Park and the Autonomic Government. The sampled species are commercial species usually fished in the area. For our study we received only the stomach of the fishes already sampled and we did not took part on the manipulation of the species.

AUTHOR CONTRIBUTIONS

FT and EC conceived the ideas and the experimental design. FT, EB, and EC collected the data. JS, FT, EB, and EC analyzed the data. JS and EC drafted the manuscript. All authors contributed substantially to revisions and accepted the final version before submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.702492/full#supplementary-material

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First Report of Gametogenesis and Spawning for the Invasive Alga *Caulerpa cylindracea* in the Tyrrhenian Sea: The Key Role of Water Motion and Temperature

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In this study, gamete release by *Caulerpa cylindracea* was investigated for the first time in the Tyrrhenian Sea (Sardinia, Western Mediterranean), focusing on the main environmental factors triggering gametogenesis and spawning of the species. For this purpose, a combination of field and laboratory experiments was conducted. First, a 2-year mensurative experiment was performed in the field during summer to individuate and quantify the occurrence of *C. cylindracea* gametogenesis and spawning events, linking them to environmental conditions. Then, based on the results of the field work, a laboratory experiment was performed to directly test, under controlled conditions, the effect on the above-mentioned processes of two abiotic factors that appeared to play a key role in species gamete release processes in the area: sea water temperature and water movement. During the 2 years of field research, several gametogenesis events were recorded in the study area and two spawning events also occurred. Significant differences were observed between the ratio of fertile and total thalli and between releasing thalli (RT) and fertile thalli (FT), among sites with different hydrodynamic conditions and sampling periods with different sea water temperatures. Overall, these results suggest that *C. cylindracea* sexual reproduction also occurs in the Tyrrhenian Sea, and that, therefore, new genotypes that favor the invasive profile of the species could be produced in the basin, where both its gametogenesis and spawning seem to be mainly regulated by water movement and sea water temperature.

Keywords: Tyrrhenian Sea, *Caulerpa cylindracea*, gametogenesis, spawning, environmental factors, temperature

INTRODUCTION

Over the last decades, an increasing number of studies have considered the patterns of gametogenesis, sexual reproduction, and fertilization of seaweeds by means of an integrated biological and ecological approach (e.g., Brawley and Johnson, 1992; Santelices, 2002). Several experimental and field observations allowed to characterize gametogenesis, gamete release, and subsequent fertilization of broadcasting species as a tightly synchronized and environmentally controlled process (Santelices, 2002). Certainly, a remarkable seasonality in different phases of sexual reproduction (especially gametogenesis and spawning) results from a variety of environmental factors that satisfy physiological requirements for reproduction or from the use of environmental cues to coordinate reproduction (Lüning and Tom Dieck, 1989). However, detailed information in the literature is still scarce (Holmes and Brodie, 2004). Indeed, studies that simultaneously examine the effects of several environmental factors on sexual reproduction, in particular on gametogenesis and spawning, appear to be quite difficult. Nevertheless, they are necessary to delineate the conditions that must be simultaneously met for successful sexual reproduction, especially for alien species (Brawley and Johnson, 1992). Focusing on alien invasive species, knowledge of life cycle progression transcends pure academic interest (Liu et al., 2017), as establishing the occurrence of their sexual reproduction events has important environmental management implications, both for gametes and zygote dispersal and the potential to generate more invasive genotypes (Ellstrand and Schierenbeck, 2000, 2006).

The genus *Caulerpa* (Chlorophyta: Bryopsidales) is a group of marine green siphonous algae distributed in tropical and subtropical regions (Meinesz and Boudouresque, 1996; Ratana-Arporn and Chirapart, 2006), some of which have recently seriously invaded the Mediterranean Sea (Klein and Verlaque, 2008). The *Caulerpa* genus includes about 85 species (Guiry and Guiry, 2020), and it is considered one of the most widespread, conspicuous, and abundant groups of seaweeds worldwide (Silva, 2003). Species belonging to this genus are multinucleated (coenocyte) and colonial, and they have a modular organization. They are all characterized by the lack of cellulose in their cell walls (Silva, 1992), and they present great phenotypic plasticity and morphological variability that arise in several morphological forms and variants (Belleza and Liao, 2007). In the last few decades, the genus has attracted considerable research interest, because *Caulerpa taxifolia* (Vahl) C. Agardh and *Caulerpa cylindracea* Sonder have significantly expanded their range of distribution in the basin where they are currently considered as highly invasive (Verlaque et al., 2003; Piazzi et al., 2005; Klein and Verlaque, 2008). They represent an important threat to the diversity of benthic coastal ecosystems (Boudouresque and Verlaque, 2002), interfering with native species (Piazzi et al., 2001a; Piazzi and Cinelli, 2003) and modifying benthic assemblages (Argyrou et al., 1999).

Besides vegetative dispersion (Žuljević and Antolić, 2000) that represents the main dispersal mode for these species (Ceccherelli and Cinelli, 1999; Ceccherelli and Piazzi, 2001; Ceccherelli et al., 2002), Panayotidis and Žuljević (2001) suggested that such widespread and rapid spread is also probably supported by successful sexual reproduction. Indeed, sexual reproduction is common in the life cycle of the *Caulerpa* species, and it has been documented in populations from both tropical and temperate waters (Goldstein and Morrall, 1970; Enomoto and Ohba, 1987; Ohba et al., 1992; Clifton, 1997; Clifton and Clifton, 1999). While some authors claimed that the thallus found in nature has a haplodiplobiontic life cycle with heteromorphic alternation of generations (Goldstein and Morrall, 1970; Kapraun and Nguyen, 1994), other authors suggested that a diploid or haploid life cycle with only one generation is present (Ishihara et al., 1981; Ohba et al., 1992; Benzie et al., 2000). Anyway, several articles describe the thallus as monoecious, producing and releasing simultaneously both gametes and dying after gamete release (e.g., Žuljević and Antolić, 2000).

Several observations on sexual reproduction and gamete release by the *Caulerpa* species were reported for Caribbean reefs (e.g., Clifton, 1997; Clifton and Clifton, 1999), and indicated that sexual reproduction can also play an important role in the dynamics of these algae. Nevertheless, very scarce data on sexual reproduction and gamete release for the *Caulerpa* species in the Mediterranean Sea are currently present in the literature (Varela-Alvarez et al., 2012). In particular, with regard to two of the most abundant species in the basin, *C. prolifera* and *C. taxifolia*, only male or female gametes were observed (Meinesz, 1979; Žuljević and Antolić, 2000). Finally, considering *C. cylindracea* that is currently the most invasive *Caulerpa* species in the Mediterranean, even if there is a general agreement that *C. cylindracea* also reproduces sexually (e.g., Flagella et al., 2008; Klein and Verlaque, 2008; Katsanevakis et al., 2010), the entire lifecycle has not been described, and specific data for the Thyrrenian Sea are not present in the literature yet. Indeed, only two articles describing gametogenesis and spawning in the Mediterranean strain of this species are currently available in the literature. The first referred to the Adriatic Sea and the observation of only female gametes is reported (Žuljević et al., 2012). On the contrary, both male and female gametes were observed as well as planozygotes in the Aegean Sea, but only for a particular variant of the species [*Caulerpa racemosa* var. *occidentalis* (J. Agardh) Børgesen] (Panayotidis and Žuljević, 2001).

In this article, gamete release by *C. cylindracea* in the Thyrrenian Sea (Sardinia, Western Mediterranean) was investigated for the first time, focusing on the main environmental factors triggering the gametogenesis and spawning of the species. To this purpose, a combination of field and laboratory experiments was conducted in 2018–2019. First, a 2-year mensurative experiment was performed in the field during summer to individuate for the first time and, eventually, quantify the occurrence of *C. cylindracea* gametogenesis and spawning events, linking them to environmental conditions. Furthermore, based on the results of the field work, a complementary laboratory experiment was performed to directly test, under controlled conditions, the effect of the abiotic factors that appeared to play a key role in gamete release processes of the species in the area.

MATERIALS AND METHODS

Field Experiment: Gametogenesis and Spawning in Tavolara Punta Coda Cavallo MPA

Experimental Design

The field mensurative experiment was conducted in Tavolara Punta Coda Cavallo Marine Protected Area, along the North-Eastern Sardinian coast, in the summer period of 2018 and 2019. Field observations were carried out by snorkeling in Cala Bua bay (CB: 40°52.586′ N; 9°38.173′ E) and Porto Taverna bay (PT: 40°51′55.2″N, 9°39′47.6″E) at a depth of about 2 m (**Figure 1**). Both the study sites are characterized by big patches of *C. cylindracea*, overgrowing autochthonous macroalgae, especially on sandy bottoms, and they have a similar substratum type (rocks, cobbles, and sand were present) and assemblage [composed mainly by *Acetabularia acetabulum* (L.) Silva, dark filamentous algae (DFA), *Laurencia* spp., *Liagora* spp., and *Padina pavonica* (L.) Thivy]. In each site, two different study areas of about 20 m^2 were selected where *C. cylindracea* was present (mean substratum percent cover: CB: 21.34%; PT: 19.23%); in particular, a sheltered area (SA) and an exposed area (EA) were considered in each site (**Figure 2**), considering the main winds blowing in the area in the summer months.

During 15 randomly selected days from the beginning of July until the end of September, when the maximum development of *C. cylindracea* occurs (Klein and Verlaque, 2009), its populations were sampled in the study sites. A first survey was conducted in the afternoon to detect the presence of eventual fertile thalli (FT), and to estimate their abundance (refer to Section "Laboratory Experiment: Gametogenesis and Spawning Under Controlled Conditions" for sampling methods). FT (**Figure 3**) were detected observing fronds in detail, in order to individuate the changes in thallus appearance that usually accompany gamete formation, according to Panayotidis and Žuljević (2001) and Žuljević et al. (2012). If present, FT, easily detected because of depigmentation of blades, were marked (refer to **Figure 3C**) with a large (to not damage them) rubber band, in order to check them in case of possible gamete release the following morning, basing on the protocol described by Žuljević and Antolić (2000) for *C. taxifolia*. Considering

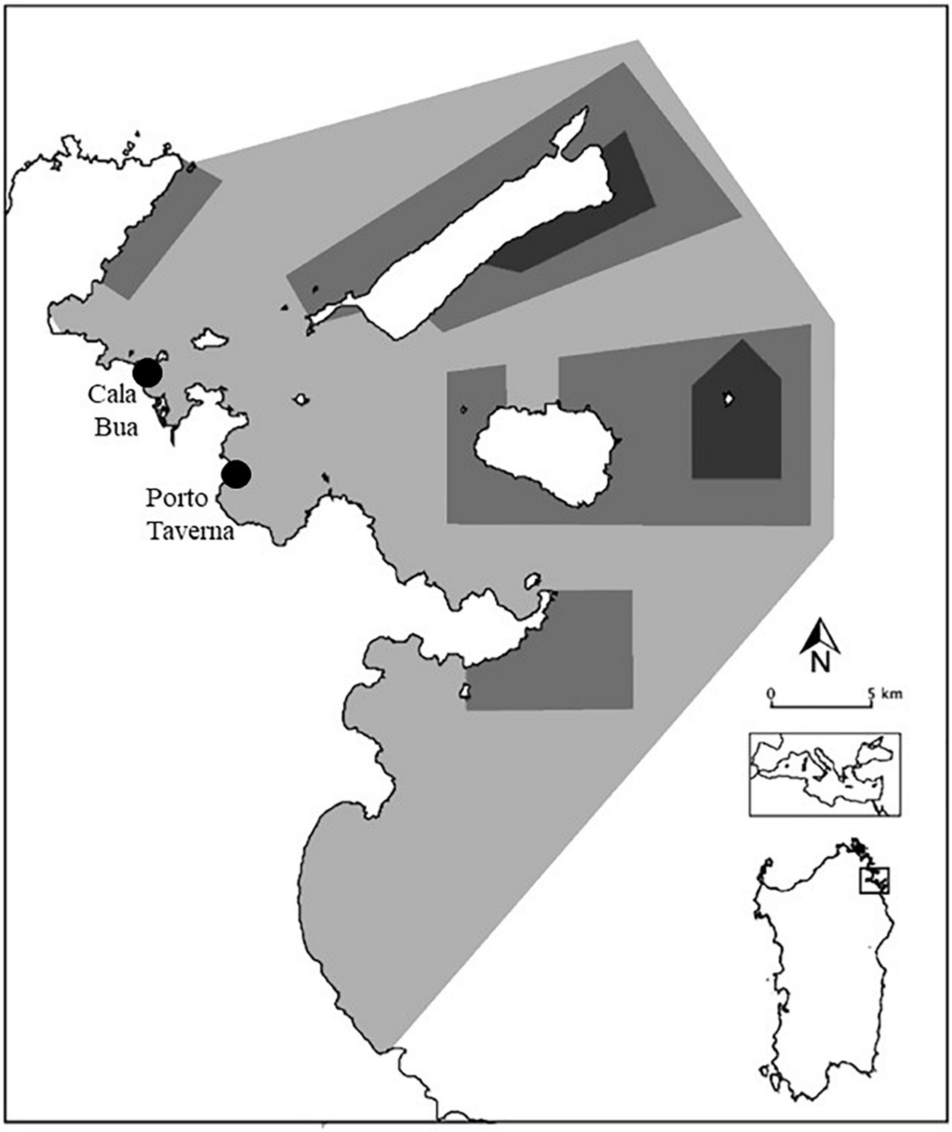

FIGURE 1 | Map of Tavolara Punta Coda Cavallo MPA with the detailed position of the two considered study sites.

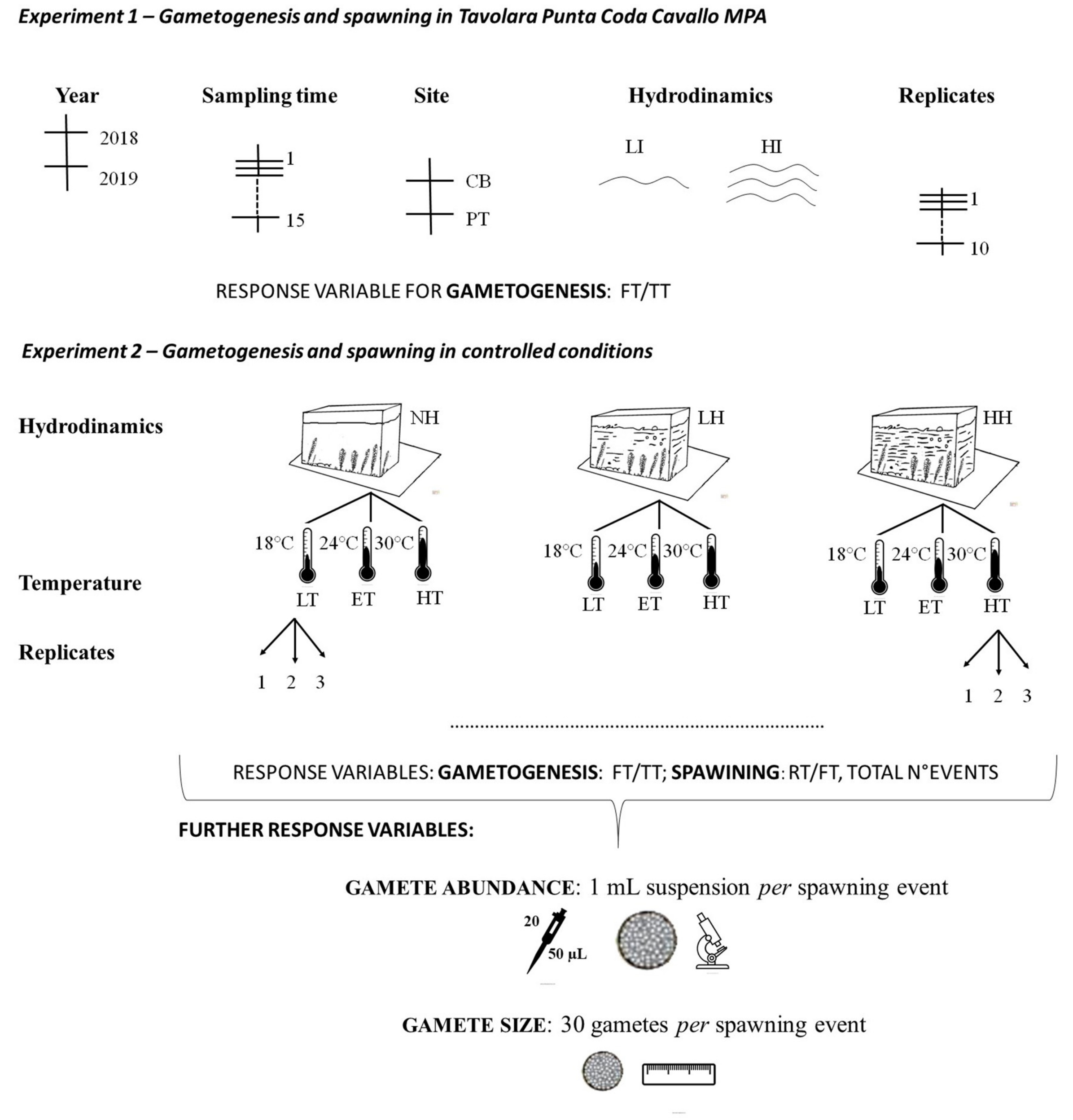

FIGURE 2 | Schematic representation of the sampling design for the two performed experiments. Sites: CB: Cala Bua; PT: Porto Taverna. Water motion: NH: no water motion; LH: low water motion; HH: high water motion. Temperatures: LT: lower temperature (18°C); ET: equal temperature (24°C); HG: higher temperature (30°C).

that gamete release by the *Caulerpa* species usually occurs 15 min before sunrise (Clifton and Clifton, 1999; Žuljević et al., 2012), morning observations started about 30 min before the sunrise. As the morphological changes interesting the thallus and the branchlets during sexual reproduction can be visible at most 2 days prior of gamete release, if no release was observed the first morning, a new survey was repeated the following day. During each morning survey, releasing thalli (RT), detectable because of the presence of a green cloud around the plant, were visually counted, and the main environmental parameters were also measured according to the methods described below that were the same used for the laboratory experiment, in order to obtain uniform data (refer to section "Laboratory Experiment: Gametogenesis and Spawning Under Controlled Conditions" for the sampling methods). According to Agrawal (2012) and Liu et al. (2017), the main environmental factors triggering gametogenesis and spawning in algae were considered: water motion, seawater temperature, light availability (in terms of irradiance), salinity, and nutrients (N and P concentration).

FIGURE 3 | (A–C) Fertile depigmented thalli with papillae in aquaria **(A,B)** and directly in the field **(C)**. **(C,D)** A particular colored branchlet is presented in **(A)**, while several discharge tubes are visible in **(B,D)**, the observed gametes with and without flagella.

Sampling Methods

Environmental Parameters

All data regarding the environmental factors were tri-replicated.

Seawater Temperature and Salinity. Data were collected using a multiparametric probe (Hanna Instruments HI991300).

Irradiance. Irradiance was estimated in terms of photosynthetically active radiation (PAR) by means of an underwater PAR meter (Apogee MQ-210).

N and P Concentration. Water samples (125 ml) in each site were randomly taken at approximately 50 cm from *C. cylindracea* patches. After collection, the water samples were shaken, filtered (0.45-mm mesh size filter), and frozen, as suggested by Balata et al. (2010). Then, in the laboratory, the concentrations of total inorganic N and P were estimated by oxidation with potassium peroxodisulphate according to the most recent method described by Cozzi et al. (2010). The same method was used also to estimate the concentration of N and P in the aquaria.

Hydrodynamics. Water movement was estimated by measuring the rate of dissolution of initially dried (60°C, 24 h) and weighed gypsum balls (diameter 3 cm) prepared following Gambi et al. (1989) and mounted on stainless steel sticks. The balls were exposed to dissolution for 24 h. After recovery, the gypsum balls were rinsed lightly with fresh water to remove salts, dried at 60°C for 24 h, and weighed. The dissolution (weight loss) of each ball was converted to an estimate of flux (cms^{-1}) according to the following function obtained by Bailey-Brock (1979):

$$\mathrm{Flux} = 3.65(\mathrm{Me}/\mathrm{Mc} - 1)$$

where Me is the weight loss of each gypsum ball during field exposure, and Mc is the weight loss of the calibration balls (left in still seawater).

Water motion estimation in the aquaria was conducted before inserting algae, using the same method.

Thalli Abundance

Within each study area, 10 quadrats of 15 cm × 15 cm were placed in random positions on the substratum, and thallus abundance was considered in each. To obtain comparable data, quadrats with less than five thalli were excluded before sampling, and thalli with a length $<$ of 5 cm and/or with less than five branches were not considered. In each quadrat, the total number of thalli (TT) and the number of eventual FT and RT were counted (Engelen et al., 2005). For each sampling day, data obtained from the 10 quadrats of each area were pooled together, and the mean value was used;

in this way, five replicates for each of the three sampling months were, on the whole, considered for each area.

Laboratory Experiment: Gametogenesis and Spawning Under Controlled Conditions

Sampling Design and Experimental Setup

Caulerpa cylindracea sterile thalli were collected along the coasts of Tavolara Punta Coda Cavallo Marine Protected Area, in Porto Taverna bay (40°51′55.2″N, 9°39′47.6″E) (**Figure 1**) in the late summer of 2019 (approximately 15–20 days before spawning events were observed in 2018). During collection, the main environmental parameters (seawater temperature, salinity, hydrodynamic stress, light availability, and N and P concentrations) were measured according to the same sampling methods used in the field in order to obtain homogeneous data (refer to Section "Field Experiment: Gametogenesis and Spawning in Tavolara Punta Coda Cavallo MPA" for the sampling methods). They all resulted in accordance with those described in the literature for the summer period in NW Mediterranean (Reñé et al., 2011; Caronni et al., 2014, 2016).

About 230 thalli (around 1 mm in diameter and 7 cm in length with at least five blades) were collected from widely separated areas at about 12 m depth. A mid-collection depth was chosen, because in the mid-depth range (5—15 m) *C. cylindracea* usually finds particularly favorable growth conditions (De Biasi et al., 1999). Moreover, such a collection depth avoided thallus exposition to significant fluctuations of the main environmental parameters characterizing shallow waters (Sarà et al., 1999) that could influence their survival and acclimation to laboratory conditions. After collection, the thalli were maintained in seawater and immediately transported to the laboratory. Once in the laboratory, the thalli were kept in small 20 L aerated aquaria with subsand filters. Artificial seawater was used, and a thin layer (1 cm) of fine sand (1 mm) was prepared as the substratum for algae settlement. All the aquaria were initially maintained in a growth chamber for 3 weeks, simulating the environmental conditions observed in the field. Water temperature was set at 24°C, and salinity and N and P concentrations were adjusted to 33 psu and 0.7 and 0.9 μM, respectively. The sea water was enriched with Provasoli's solution (Provasoli et al., 1968), as suggested by Sudatti et al. (2011). The thalli were exposed to cool-white fluorescent light with an irradiance of 100 μmol photons $m^{-2}s^{-1}$ and a 15-h:9-h light-dark cycle for an acclimation period of 3 weeks (Phillips, 2009). During this period, the *C. cylindracea* thalli remained healthy in the aquaria and did not show relevant acclimation problems (the health status of the algae was evaluated considering both the color intensity of the blades and the presence of injuries and damaged portions, according to Bennett et al., 2015).

The thalli were then exposed to different temperature and hydrodynamic conditions (the two parameters that show differences among sites during the field mensurative experiment) in an environmental test chamber for 45 days. According to Phillips (2009), indeed, this period of time is sufficient to observe, if environmental conditions are favorable, a significant number of reproductive episodes for *Caulerpa* species. In particular, six different combinations of treatments (including one simulating the values recorded on the occasion of the spawning events observed in natural conditions) were tested according to a fully orthogonal experimental design. Three replicates for each combination of treatments were considered (three different aquaria for each), and 12 different thalli with at least five branches were transferred in each of the experimental aquaria. In particular, three different temperatures [LT: lower than the mean summer Sardinian one (18°C) vs. ET: equal to the mean summer Sardinian one (24°C) vs. HT: higher than the mean summer Sardinian one (30°C)], and three different hydrodynamic conditions [NH: no water motion (0 cm s^{-1}) vs. LH: low water motion (~5 cm s^{-1}) vs. HH: high water motion (~13 cm s^{-1})] (**Figure 2**). Chamber temperature was set in order to obtain desired seawater temperatures in the aquaria, avoiding overheating. The different hydrodynamic conditions were, instead, obtained directly in the aquaria by means of adjustable aquarium stirring pumps. The other parameters (irradiance, nutrient concentration, salinity, and photoperiod) were maintained stable (refer to Section "Laboratory Experiment: Gametogenesis and Spawning Under Controlled Conditions" for the sampling methods).

At the beginning of the experiment, according to Phillips (2009), the thalli were examined daily to immediately detect fertile ones. When they showed signs of gamete formation processes (considering the morphological changes described for the species and, in particular, depigmentation and papillae development (Panayotidis and Žuljević, 2001; Žuljević et al., 2012), they were counted and separated into 1-L beakers with the aim of obtaining gamete samples for microscopic observation. For practical reasons, in the afternoon before gamete release, according to Phillips (2009), the FT were placed in total darkness to postpone gamete release for about 2–3 h. The number of RT was then determined.

Sampling Methods

Gamete Abundance

During release under controlled conditions, 20 separate suspensions of gametes were collected with a micropipette (50 μl each) near each release cloud and pipetted on a microscope graduated slide for microscope observation (×40 and ×100 magnification was used). In this way, estimation of the abundance and size of gametes produced during each event was possible (the abundances obtained in the 20 suspensions were pooled together to obtain a reliable value for each event). In particular, the length and width of at least 30 gametes obtained from each separate suspension event were measured. Data regarding the abundance and the size of gametes relative to all the events that occurred in each aquarium were pooled together, and the means were considered, in order to obtain three replicate size and abundance values for each combination of treatments.

Response Variables and Data Analysis

The ratio between the number of FT and the total TT was considered as the main response variable to evaluate gametogenesis during both experiments. Moreover, for the second experiment, the ratio between the number of RT and that of fertile ones (FT) was also considered as the response variable. Finally, three additional response variables were also taken into account for each treatment in the second experiment: the total number of spawning events, the abundance of released gametes, and their size (length and width).

The data were analyzed by means of univariate statistical analyses using the software GMAV5 (Underwood et al., 2002).

A four-way ANOVA was performed with the data collected during the first experiment. The considered factors were: year (two random levels: 2018 vs. 2019), site (two fix levels: CB vs. PT), zone (two fix levels: LH vs. HH), and month (three fix levels: July vs. August vs. September). Moreover, further, six four-way ANOVAs were performed to analyze data regarding each of the considered environmental parameters.

With regard to the second experiment, instead, five two-way ANOVAs (considered variables: FT/TT and RT/FT ratios, gamete abundance, length, and width) were performed considering as fixed factors water motion (three levels: NH vs. LH vs. HH) and temperature (three levels: LT vs. ET vs. HT).

Shapiro–Wilk and Cochran's tests were performed prior to each ANOVA to test for normality and homogeneity of variances. The Student–Newman–Keuls (SNK) test was performed for *a posteriori* comparison of the means in case of significant ANOVA results (Underwood, 1997).

Finally, Pearson correlation was used to test the relationship between gametogenesis events and environmental conditions for the first experiment (only temperature and water motion).

RESULTS

Experiment 1: Gametogenesis and Spawning in Tavolara Punta Coda Cavallo MPA

Environmental Conditions

Some significant differences were found among the six environmental parameters (**Figure 4** and **Supplementary Materials** (**Tables 1A,B**). **Figure 4** shows the mean values recorded for each environmental variable in each area of the two sites. While there were no differences among sites, years, areas, and months for salinity, irradiance, and total N and P concentrations, significant differences were observed among areas and months for both sea water temperature and water motion. With regard to seawater temperature, higher values were recorded in the sheltered areas when compared with the exposed ones (SA: 25.3°C and EA: 24.1°C). However, the most relevant temperature differences were recorded among the sampling months. Indeed, mean values appeared to be significantly higher in July and in the first part of August (25.1°C), while they were lower in the second part of August and in September (24.3°C) (**Figure 4** and **Supplementary Tables 1A,B**). On the contrary,

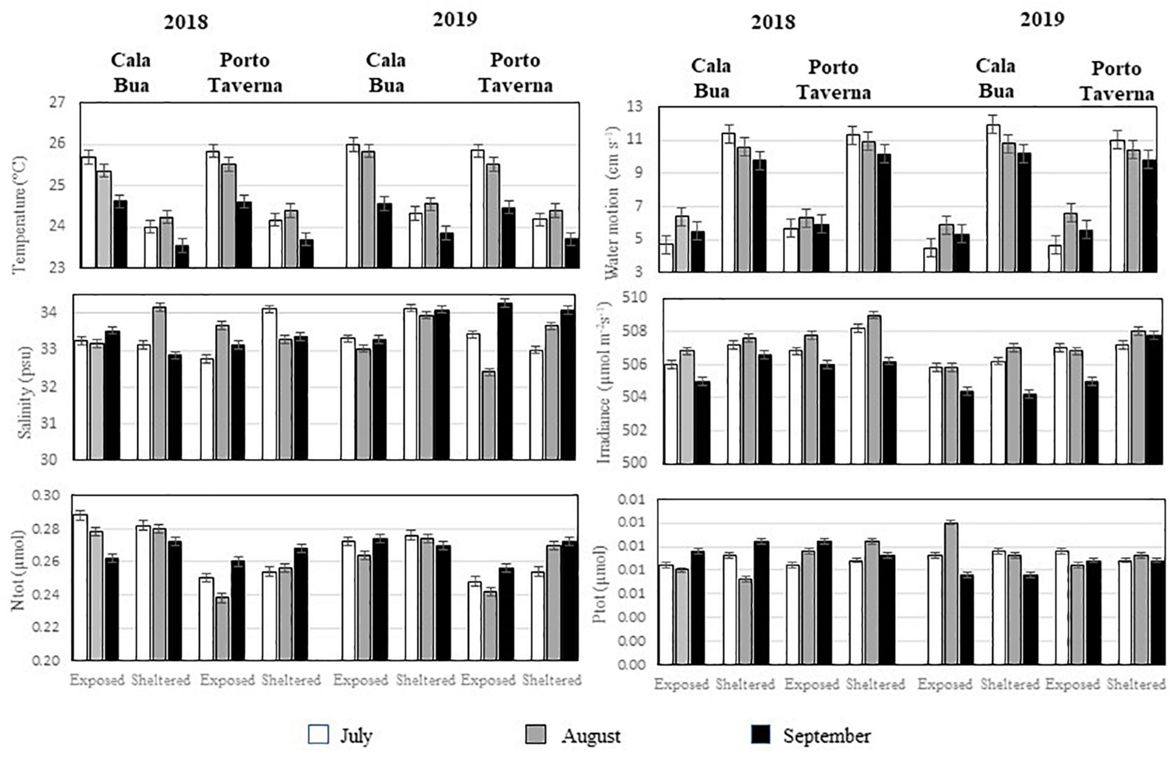

FIGURE 4 | Mean (±ES) value for each of the considered environmental factors (seawater temperature, salinity, water motion, light availability, and N and P concentrations) for each of the two areas in the two study sites, during each of the three periods considered for each study year.

TABLE 1 | (A) Results of the ANOVA on data regarding the fertile thalli (FT)/total number of thalli (TT) ratio in relation to the considered factors: Site (Si): CB vs PT; Year (Ye): 2018 vs. 2019; Area (Ar): sheltered vs. exposed; Period (Pe): July vs. August vs. September.

A

	ANOVA		
	DF	**F**	**P**
Site (Si)	1	2.71	0.1029
Year (Ye)	1	0	0.972
Area (Ar)	1	30.12	**0.0000**
Period (Pe)	2	15.15	**0.0000**
SixYe	1	0.03	0.8582
SixAr	1	2.37	0.1267
SixPe	2	1.32	0.2726
YexAr	1	2.19	0.1418
YexPe	2	0.67	0.5123
ArxPe	2	8.47	**0.0004**
SixYexAr	1	0.02	0.8968
SixYexPe	2	0.05	0.9493
SixArxPe	2	0.94	0.3928
YexArxPe	2	0.97	0.3835
SixYexArxPe	2	0.16	0.8558
RES	96		
TOT	119		
Cochran test: 0.1630 (NotSignificant)			

B

SNK test a *posteriori* (ArxPe)	
Period	
Area	**Period**
Sheltered	July < August < September
Exposed	July = August = September
Period	**Area**
July	Exposed < Sheltered
August	Exposed < Sheltered
September	Exposed < Sheltered

Significant P-values are given in bold. B: Results of the posteriori comparisons [Student–Newman–Keuls (SNK test)] for the interaction ArxPe.

with regard to water motion, the main differences were observed between the two areas of each site, as in the sheltered areas the mean values appeared to be significantly lower than in the exposed ones (SA: 5.58 cm s^{-1} vs. EA: 10.69 cm s^{-1}).

Fertile and Releasing Thalli

During the 15 sampling days of each of the 2 years of field research, about 600 gametogenesis events were, on the whole, recorded in the two study sites (at least one fertile thallus was observed during 82 of the 120 observations performed during the study) and during about 35% of the observations, half of the thalli present in the considered quadrats appeared to be fertile. Nevertheless, only two spawning events were observed throughout the whole study period (one event for each year) in Cala Bua (CB).

The FT appeared as depigmented protoplasmic masses, easily distinguishable from colored, not fertile ones; papillae and discharge tubes were well developed on the frond axes and the top of the stolon, and the upper part of the branchlets was orange-brown (**Figures 3A–C**).

Significant differences in their ratio (FT/TT) were observed between the exposed and sheltered areas and among the sampling months, while no differences were recorded among the sites and years (**Figure 4**, **Table 1**, and **Supplementary Table 1**). In **Figure 5**, the mean FT/TT ratio is presented for each study area and site in each period of the 2 years. In particular, in both sites, the FT/TT ratio was significantly higher in the sheltered areas (21%) than in the exposed ones (7.98 %). Moreover, for both years, such a ratio assumed a significantly higher value in the second part of August and in September (21%) (**Figure 5** and **Tables 1A,B**).

Also, the two spawning events that were detected thanks to the occurrence of the typical cloud, occurred in Cala Bua in September; each lasted about 9—10 min, starting 14 and 13 min before sunrise– respectively. Despite, both the spawning events were observed in quadrats in which some other FT were also present (FT/TT ratio: 55.12 and 23.16%, respectively, in the quadrat containing the first and second releasing thallus), the releasing thallus was distinctly identified, as the gamete cloud were localized only around the interested branchlets.

Finally, a negative correlation between the abundance of FT (in terms of FT/TT ratio) and water motion was found ($R^2 = -0.71$, $p < 0.05$, $n = 120$) (**Figure 6A**). With regard to temperature, only a slightly negative correlation was, instead, observed ($R^2 = -0.59$, $p < 0.05$, $n = 120$) (**Figure 6B**).

Experiment 2: Gametogenesis and Spawning Under Controlled Conditions

Fertile and Releasing Thalli

Several gametogenesis and spawning events were observed under controlled conditions with significant differences in relation to treatments. **Figure 7** shows the mean FT/TT ratio for each water motion and temperature combination. In particular, higher ratios were observed at 24°C (67.59%) while lower values were recorded both at lower (18°C: 47.22%) and highest temperatures (30°C: 17.59%). Moreover, significantly higher FT/TT ratios were found in the aquaria with no water movement (63.89%), and they remarkably decreased in the aquaria with low (41.67%) and, in particular, high water motion (29.17%) (**Figure 7A** and **Tables 2A,B**).

Gamete Abundance and Size

During the spawning events, several gametes were observed. The gametes were discharged within gametangia thanks to papillae in a stream of mucilaginous material spurting from different discharge tubes starting from the colored branchlets (**Figures 3A–C**). Gametangia are membrane-bound spherical structures that contain several gametes that, once released, form a green cloud that disperses within 10–20 min, depending on hydrodynamic conditions. In the aquaria with no water

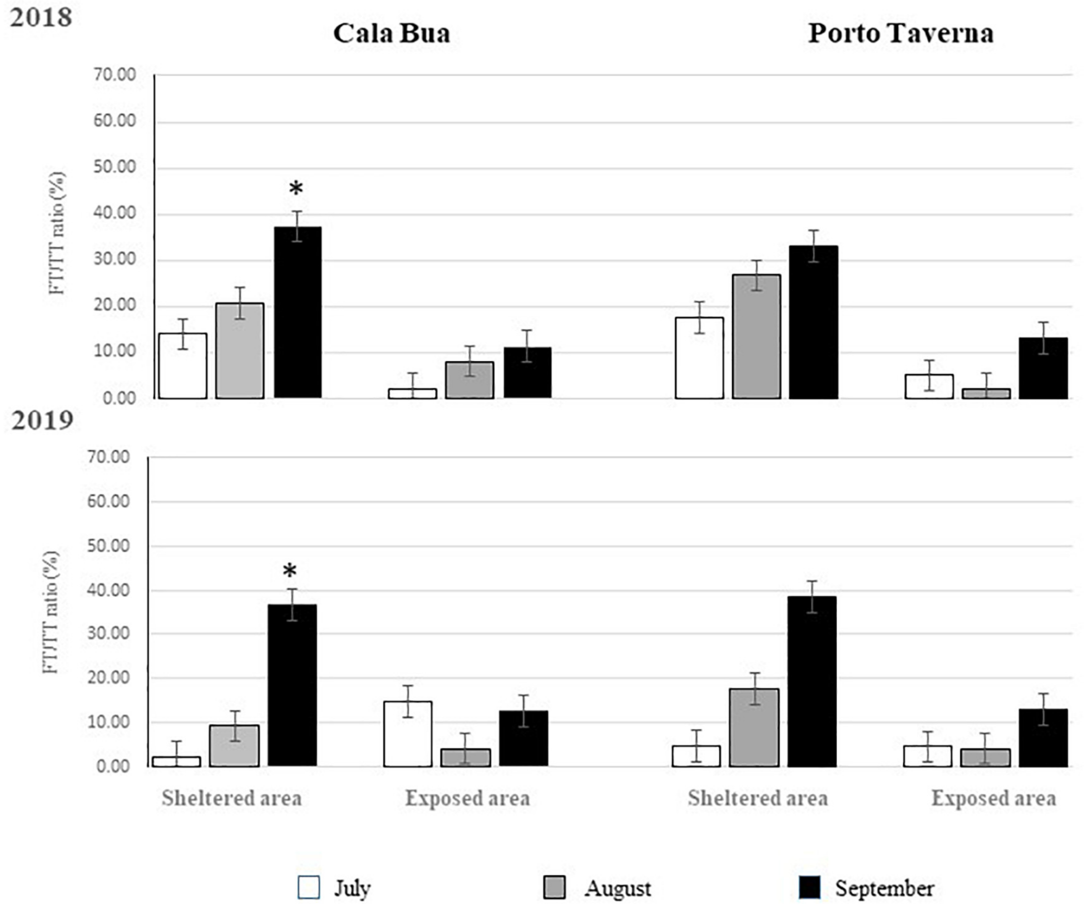

FIGURE 5 | Mean (±ES) ratio between the number of fertile thalli and the total number of thalli (FT/TT %) in the two areas of the two study sites during each of the three periods considered for each study year. Spawning events are indicated with *.

movement, the dispersion time was of about 15— 17 min, while both with the low and high water motions it ranged between 10 and 12 min. After the end of the releasing event, the parental branchlets appeared white and suddenly died; the empty thalli usually disintegrated within about 1 day (24—36 h).

Despite the spawning events, overall, no zygotes were observed during the study.

All the gametes had a length that ranged from 5.5 to 6.1 μm, while their width ranged from 1.9 to 2.5 μm (**Figure 3D**). Moreover, no eyespot was observed in the analyzed gametes while some of them (about 15% of the analyzed ones) had two visible flagella (**Figure 3D**). No differences in the size of gametes were recorded in relation to treatments (**Figures 8A,B**). On the contrary, gametes were more abundant in the aquaria with low or null water motion and with a temperature of 24°C (NH: 104 cells ml^{-1}; LH: 103 cells ml^{-1}) (**Figure 9** and **Tables 3A,B**).

DISCUSSION

This study presents the first report of gametogenesis and spawning by *C. cylindracea* in the Thyrrenian Sea and provides useful information on the environmental factors triggering such processes. First of all, several gametogenesis events were observed in Tavolara Punta Coda Cavallo MPA during the period of the study, and two gamete release events also occurred, even if no zygotes were observed, as described in few previous studies on the species (Panayotidis and Žuljević, 2001; Žuljević et al., 2012).

Despite both the observed morphology of FT and the general pattern of gametogenesis of *C. cylindracea* appearing to be quite similar to that of other conspecific species (e.g., Miyake and Kunieda, 1937; Clifton, 1997; Phillips, 2009), some relevant differences also emerged.

With regard to timing, in Tavolara Punta Coda Cavallo MPA, the spawning events appeared to be shorter (10 min) than those described by Miyake and Kunieda (1937) (1 h), while their duration was quite similar to those reported by both Panayotidis and Žuljević (2001) and Žuljević et al. (2012) for other parts of the Mediterranean basin (5—10 min). These results suggest that the duration of spawning events can be quite variable, as already observed for other algae (Pearson and Serrao, 2006).

Moreover, according to Clifton (1997), generally, algae with external fertilization, like Bryopsidales, are characterized by a highly synchronized reproductive pattern, with tens to thousands

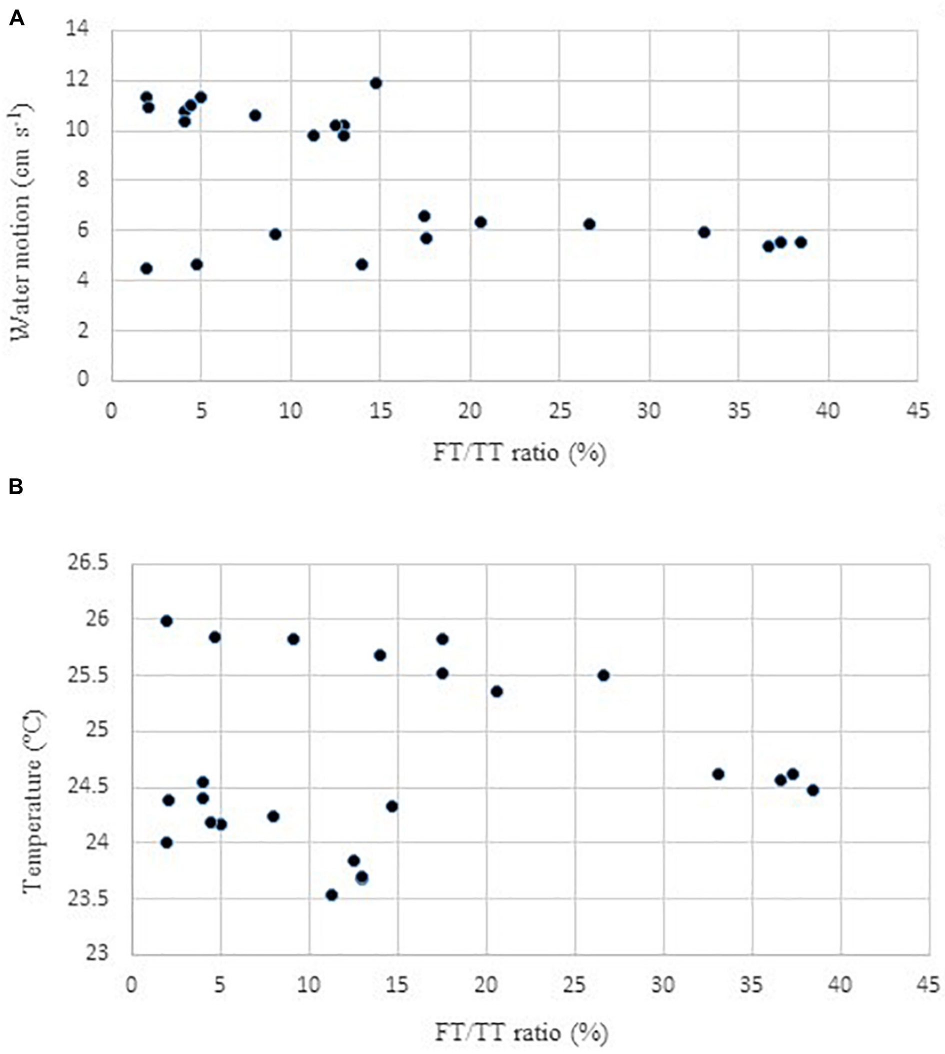

FIGURE 6 | Relationship among FT/TT ratio, **(A)** water motion, and **(B)** sea water temperature **(B)** on the pooled data (*n* = 120). R values are provided in the text.

of conspecific algal thalli (at least 3.5% of the population) becoming simultaneously fertile in the same small area overnight and several spawning events occurring together. Analyzing the data obtained during the field experiment, a different situation was instead recorded. The mean percentage of simultaneously observed FT in the 300 cm^2 considered in each site exceed 50% (max. 63.64%) during only 6 of the 120 observations and, in most cases, only one or two FT were, on the whole, counted, with less than 1% of the population fertile. Moreover, only two single spawning events were overall observed. A quite different situation was, instead, observed under controlled conditions, where in some cases FT/TT ratio exceeded 90% and RT/FT ratio exceeded 100%. The lower rates of both FT and releasing events observed during the field study, in comparison to both the laboratory ones and those recorded by Clifton (1997) for reef environments, can be explained considering that the observations of Clifton (1997) referred to the tropical waters of which *C. cylindracea* is native and not to the Mediterranean, where the environmental conditions are certainly different and so, presumably, different cues act (tidal, photoperiod, lunar phases, etc.). According to Pearson et al. (1998), indeed, environmental cues should be taken into account among the most important triggers playing a key role in coordinating gametogenesis. Considering that the most energy-consuming phase of sexual reproduction is gametogenesis (Shinkawa et al., 2019), it is plausible to suppose that gamete production and release happen when the conditions are favorable in order not to waste energy.

According to Brawley and Johnson (1992), in particular, synchronicity can act by interfering with both gametogenesis and spawning in different ways, ensuring that mature gametes are available for synchronous release when favorable conditions occur (Pearson et al., 2004). On the contrary, Yund (2000) observed that, under non-ideal conditions, environmental cues usually do not act, with the consequence that no synchronous release will occur; in this case, only few FT and spawns are recorded. Therefore, despite the remarkable capability of

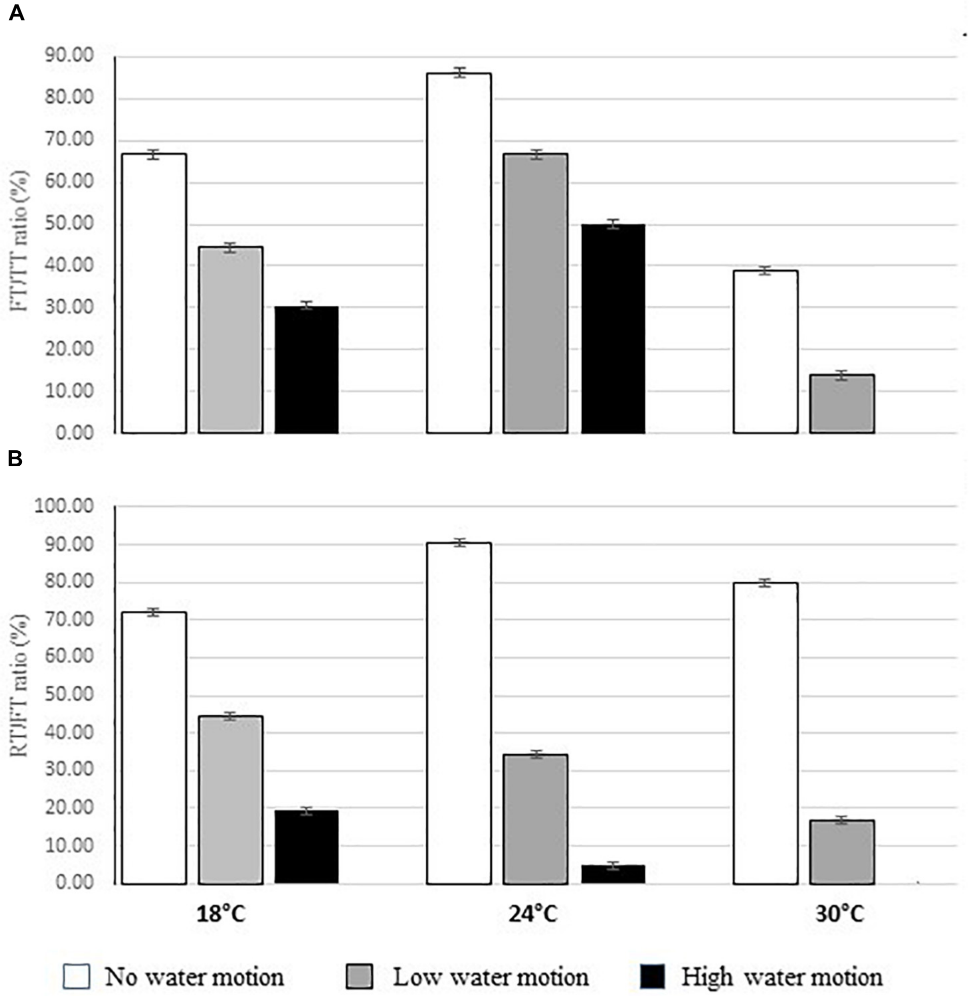

FIGURE 7 | Mean (±ES) ratio between the number of FT and the total number of thalli (FT/TT%) **(A)** and between the number of releasing thalli (RT) and that of fertile ones (RT/FT %) **(B)** in the aquaria with different temperature and water motion treatments.

C. cylindracea to adapt in response to the main ecological factors (Verlaque et al., 2000), it can be assumed that the quite low number of fertile and RT observed during the field study is due to the loss of cues regulating and coordinating sexual reproductive events.

The data from both the field and controlled condition experiments revealed remarkable differences in fertile and RT rates in relation to the environmental factors. These results suggest that also for *C. cylindracea*, gametogenesis and spawning are strictly related to environmental conditions, as already observed for several quite similar species (Clifton, 1997), and that successful reproduction usually depends on a delicate juxtaposition of different conditions (Santelices, 2002). Environmental factors directly operate to complete algal lifecycles (growth, reproduction, spore germination, and again growth) (Agrawal, 2012) and gametogenesis, and the other phases of sexual reproduction, which appeared to occur only when the main environmental factors (temperature, salinity, photoperiod, main nutrients, etc.) satisfy the physiological requirements for reproduction (Lüning and Tom Dieck, 1989).

Although during observations several environmental factors were considered as determinants for gamete production and release, no differences were observed for light and nutrient availability, as well as for salinity among the study areas and sampling periods. On the contrary, some interesting results were overall obtained considering seawater temperature and water motion. In particular, significant differences in FT/TT ratio were recorded among areas and periods with different hydrodynamics and seawater temperatures, respectively; furthermore, for the former, a clear negative correlation with the ratio of FT was highlighted from the analysis of data obtained under controlled conditions both for FT/TT and RT/FT. These results are in accordance with those of other studies that demonstrated the importance of water motion and temperature in triggering gametogenesis and spawning for other similar species (Giese and Kanatani, 1987; Clifton and Clifton, 1999), and they suggest that

TABLE 2 | (A) Results of the ANOVAs on data regarding the FT/TT and the releasing thalli (RT)/FT ratios for each treatment: Temperature (T): 18 vs. 24 vs. 30°C and Water motion (W): no water motion (NH) vs. low water motion (LH) vs. high water motion (HH).

A

		ANOVA			
		Fertile Thalli Ratio		**Releasing Thalli Ratio**	
	DF	**F**	**P**	**F**	**P**
Temperature (T)	2	157.96	**0.0000**	58.88	**0.0000**
Water motion (W)	2	86.84	**0.0000**	286.13	**0.0000**
TxW	4	0.18	0.9466	21	**0.0000**
RES	18				
TOT	26				
Cochran test:		0.2143 (NotSignificant)		0.1250 (NotSignificant)	

B

SNK test a *posteriori* fertile thalli ratio	**SNK test a *posteriori* releasing thalli ratio (TxW)**	
	Temperature	
Temperature	**Water motion**	**Temperature**
30°C < 18°C < 24°C	NH	30°C < 18°C < 24°C
	LH	30°C < 18°C < 24°C
	HH	30°C = 24°C = 18°C
Water motion	**Temperature**	**Water motion**
HH < LH < NH	18°C	HH < LH < NH
	24°C	HH < LH < NH
	30°C	HH < LH = NH
S.E. = 2.0005	S.E. = 0.3143	

Significant P-values are given in bold. B: Results of the posteriori comparisons (SNK test) for the factors temperature and water motion and the interaction (TxW), respectively, referred to FT/TT and RT/FT.

such factors must be considered when investigating *C. cylindracea* sexual reproduction.

With regard to temperature, in the field, a significantly higher FT/TT ratio was observed in the second part of August and in September, when the mean seawater temperature was about 24°C, while in the first half of summer, when the temperature was higher, a lower number of FT were observed. Furthermore, the results of the laboratory experiment highlighted a similar trend, as the FT/TT ratio was significantly higher in the aquaria with a temperature of 24°C, while remarkably lower ratios were, instead, observed at both 18 and 30°C. These results are in accordance with those of other studies on macro and microalgae (e.g., Henry, 1988; Dieck, 1992; Kalita and Tytlianov, 2003; Agrawal, 2012), and they suggest that *C. cylindracea* must be included in the list of green algae characterized by a very narrow range of temperatures favorable for sexual reproduction. For most algae, indeed, the temperature range that allows for reproduction is considerably limited compared to that for vegetative growth, and they may reproduce asexually or sexually at different, also quite small, temperature ranges (League and Greulach, 1955).

Anyway, considering the few data available in the literature for the species, some differences can be noticed. A slightly higher gametogenesis temperature was, indeed, described both by Panayotidis and Žuljević (2001), with FT primarily observed at 26°C, and Žuljević et al. (2012) with a peak of FT recorded at 25°C, even if they asserted that on the whole *C. racemosa* in the Adriatic Sea becomes fertile when the seawater temperature is between 20 and 25°C.

Some quite interesting information can also be obtained by analyzing the correlation between FT/TT and RT/FT ratios and water motion, which resulted negative. Higher ratios, indeed, were observed in the aquaria where no water motion occurred. A similar pattern was also observed in the field, where the FT/TT ratio appeared to be related to a stimulating effect of calm condition for gametogenesis. These results suggest that a stimulatory effect of calm conditions seems to exist for gametogenesis and not only for gamete release, as already observed for other species of Bropsydales (Brawley et al., 1999; Speransky et al., 2000). According to Pearson et al. (1998), *C. cylindracea* seems to be among the algae that have the ability to perceive water motion *via* boundary layer bicarbonate levels and photosynthetic metabolism (Pearson and Brawley, 1996, 1998), and to consider it as a signal coordinating the different phases of its sexual reproduction.

Stressing the remarkable direct effects of wave forces affecting intertidal organisms, Denny (1995) asserted that they primarily included damage and, especially, displacement of unattached parts that usually occur for gametes floating in water more than adult individuals attached to the substratum. The hypothesis of gamete displacement and dilution is also confirmed by the significantly lower abundance of gametes in the water near the discharge tubes observed in aquaria with high water motion. Even if, in all the aquaria, gamete abundances appeared to be quite similar to those observed by Goldstein and Morrall (1970), for several species of the *Caulerpa* genus, significantly higher abundances were observed under no water motion conditions, especially when a temperature of 24°C were simulated.

From the obtained data, the effect of temperature is evident only in the optimal condition of water motion, thus allowing us to suppose that water motion is the main factor in *C. cylindracea* gametogenesis and spawning (as also confirmed by the differences in the Pearson correlation coefficient). Temperature, instead, seems to have, especially in some cases (e.g., 30°C when water motion is remarkable) a secondary additive effect. Actually, for some other seaweeds, gamete release is strictly restricted to calm intervals (Serrão et al., 1996), thereby preventing the gamete dilution predicted to occur by turbulent flow and water exchange (Pennington, 1985; Denny and Shibata, 1989; Levitan et al., 1992). Indeed, gamete production only under low water motion conditions represents an extremely valuable adaptation for an organism inhabiting the intertidal zone, as it allows to finalize gamete release, reducing dispersion. In this way, a large number of gametes appear to be available for gamete collision and fertilization, enhancing the probability of zygote formation.

These results also provide some interesting insights into the relationship between *C. cylindracea* diffusion and the climate

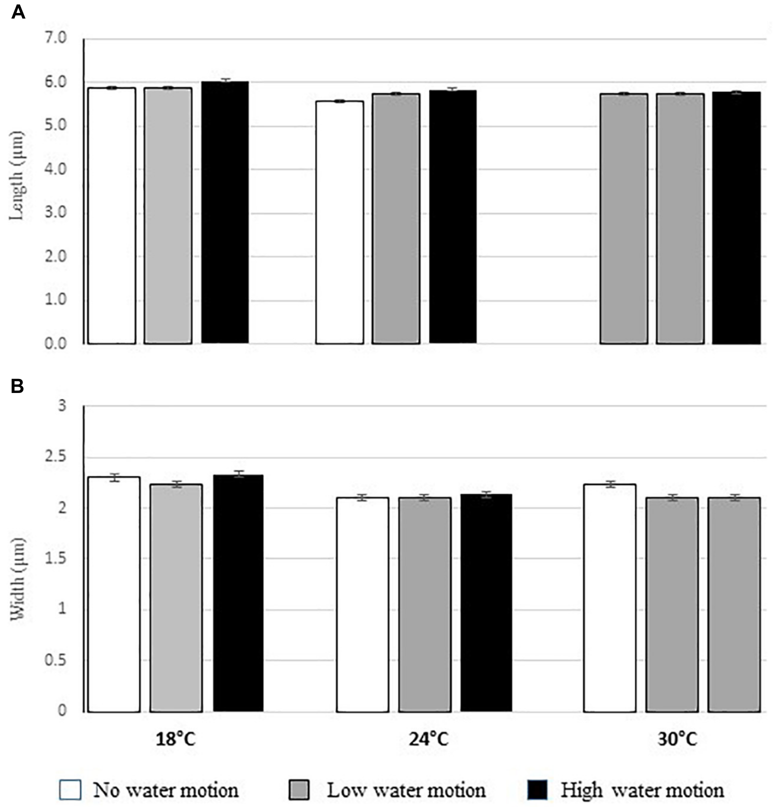

FIGURE 8 | Mean (±ES) length **(A)** and width **(B)** of the observed gametes in the aquaria with different temperature and water motion treatments.

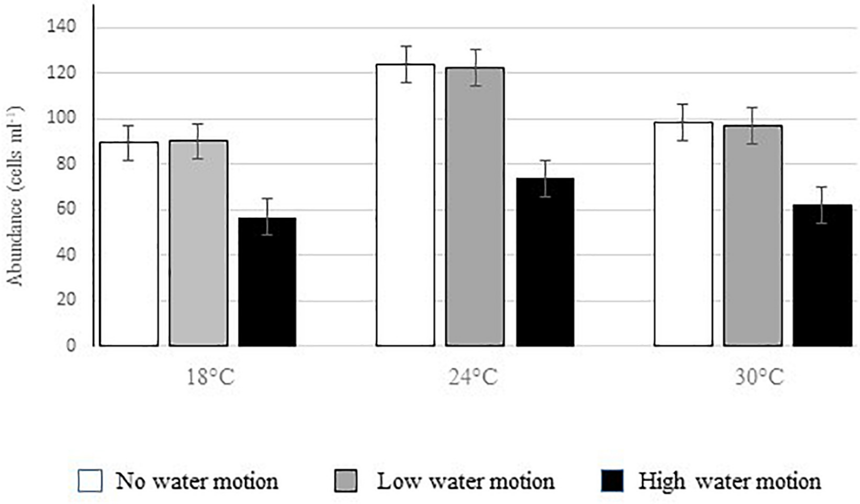

FIGURE 9 | Mean (±ES) abundance of the observed gametes in the aquaria with different temperature and water motion treatments.

changes that are interesting in the Mediterranean Sea (e.g., Adloff et al., 2015; Marbà et al., 2015). This basin is considered a miniature ocean highly affected by climate changes (Lejeusne et al., 2010). Furthermore, climatic models predict that this basin will be one of the regions that will experience the main effects of both the global warming trend and of an increase in extreme events significantly altering currents and water motion in it. Considering that the two main factors playing a key role in the gametogenesis and spawning of *C. cylindracea* appear to be precisely sea water temperature and motion, for which the

TABLE 3 | (A) Results of the ANOVA on data regarding the abundance, length, and width of gametes in relation to treatments: Temperature (T): 18 vs. 24 vs. 30°C; Water motion (W): no water motion (NH) vs. low water motion (LH) vs. high water motion (HH).

A

		ANOVA					
		Gamete Abundance		**Gamete Lenght**		**Gamete Width**	
	DF	**F**	**P**	**F**	**P**	**F**	**P**
Temperature (T)	2	156.6900	**0.0000**	4.01	0.0563	2.8600	0.0837
Water motion (W)	2	336.2900	**0.0000**	1.21	0.3210	0.3700	0.6969
TxW	4	5.4000	0.0049	0.34	0.8444	0.2900	0.8810
RES	18						
TOT	26						
Cochran test:		0.3483 (NotSignificant)		0.2111 (NotSignificant)		0.3289 (NotSignificant)	

B

SNK test a *posteriori* gamete abundance (TxW)	
Temperature	
Water motion	**Temperature**
NH	30°C < 18°C < 24°C
LH	18°C < 30°C < 24°C
HH	30°C = 18°C < 24°C
Temperature	**Water motion**
18°C	HH < LH = NH
24°C	HH < LH < NH
30°C	HH < LH = NH
S.E. = 2.0964	

Significant P-values are given in bold. B: Results of the posteriori comparisons (SNK test) for the interaction (TxW) referred to gamete abundance.

species seems to have a narrow range of tolerance, it is plausible to hypothesize that ongoing climate changes can significantly reduce the chances of successful reproduction of the species in the basin. In case of not optimal environmental conditions, indeed, reproducing asexually could be more advantageous, as recently observed for other species (Endo et al., 2021). Therefore, a situation of increased water motion and temperature similar to one of those simulated during the laboratory experiment, especially in summer, when the vegetative growth of the species reaches its maximum (Piazzi et al., 2001b), will lead to a reduction in the number of FT and, consequently, in the probability of spawning events to occur.

A further observation that a favorable combination of several interacting factors must occur for FT production and gamete release (Serrao et al., 1999; Santelices, 2002) is represented by significantly lower FT/TT ratio that was observed during field observations when compared with both of those obtained under controlled conditions. Indeed, these data suggest that in the field, other factors presumably not acting in the aquaria played a role in such processes, as already observed for the *Caulerpa* genus during other studies (Phillips, 2009; Žuljević et al., 2012). Considering the other factors triggering gametogenesis and spawning for algae (e.g., extracellular organic substances, pollution, pH of the medium, osmotic stress, etc., according to Agrawal, 2012), an active role of pheromones and other inhibiting extracellular substances can be supposed for the above-mentioned processes. Pheromones are particularly important for the chemical communication between individuals preceding the sexual reproduction of algae (Maier, 1993). While such substances can be easily diluted in the water under natural conditions, at least when thallus abundance is not particularly high, their efficiency in aquaria is expected to be higher. Indeed, in the aquaria, gametogenesis and release can also be enhanced by the absence of extracellular organic substances produced by other algae that could have an inhibitory effect on the reproduction of the considered species, as observed by Wichard and Oertel (2010) and Lüning et al. (2000) for other seaweeds.

Finally, also considering the peculiar morphological features of the observed gametes, some interesting considerations can be made. All the gametes observed during the study had the same length (5.5 to 6.1 μm) and width (1. to 2.5 μm) and lacked the eyespot (**Figure 3D**). These results are in accordance with that of Goldstein and Morrall (1970) and to Žuljević et al. (2012) that observed that anisogamous gametes for the species with female and male differ for the size and the presence/absence of the eyespot. Therefore, all the observed gametes presumably were males while no female ones were observed, contrary to what was described by Žuljević et al. (2012) for the Adriatic Sea, who observed only larger female gametes with the eyespot. A quite similar result was instead obtained by Phillips (2009) for the congeneric species *C. taxifolia*, for which, even if a few female gametes were observed, a markedly male sex ratio was noticed. These results suggest that gender expression in Tavolara Punta Coda Cavallo MPA populations of *C. cylindracea* has deviated from the 1:1 male:female allocation, expected when sex is wholly genetically determined, as already observed for *C. prolifera*, for which either only male (Meinesz, 1979) or female gametes (Dostal, 1929) were described. Even if sex ratios in macroalgal species have been poorly studied (Holmes and Brodie, 2004), and the causal factors responsible for biased sex ratios are still almost unknown, these results can be explained by considering that *Caulerpa* populations can be composed of many male plants, few bisexual plants, and no female ones.

In conclusion, the obtained data suggest that *C. cylindracea* sexual reproduction occurs in the Thyrrenian Sea and that it should be taken into account when focusing on the invasion dynamics of the species. Moreover, as the gametogenesis and spawning of *C. cylindracea* are mainly regulated by water motion and temperature, such factors must be considered for the management of the species, considering that, as sexual reproduction occurs, new more invasive genotypes for the species could be produced in the Mediterranean, even if the ongoing climate changes must also be considered in the reproduction and diffusion dynamics of the species.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

SCa, SCi, and AN conceived and designed the experiments. SCa, MD, and PP performed the study. SCa, RG, and CM analyzed the collected data. SCa and SCi wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.774274/full#supplementary-material

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frontiers in Marine Science

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Large Individual-Level Variability in Diel Activity and Depth Use for the Common Lionfish (*Pterois miles*)

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The common lionfish, *Pterois miles*, a notoriously invasive species known for its harmful effect on native fish communities in the Atlantic Ocean, has recently begun spreading across the Mediterranean Sea. The wide niche breadth of the lionfish has been hypothesized to facilitate its invasion success. However, it is unclear to what extent this wide niche-breadth is associated with individual-level variation and repeatable behavior over time. Large individual-level behavioral variations may allow individuals to adapt quickly to local conditions, increasing the species' chance of invasion success and complicating mitigation efforts. In this study, we used an acoustic telemetry system in *P. miles*' native Red Sea environment to explore individual-level variation in depth preference and diel activity. A wide depth range may indicate an ability to tolerate a variety of biotic and abiotic conditions, and variability in diel activity may indicate an ability to exploit multiple diet sources. We found large individual-level variability in *P. miles*' activity hours; although all tracked fish were active during both sunrise and sunset, certain individuals had prolonged activity hours to variable extents. Moreover, individuals often change their patterns over time, showing low repeatability. We also found that individuals had different depth preferences and commuted between shallow and deep waters over short periods of time. This study is one of the first to explore diel activity as an individual-level trait in wild fish. The variability found in depth and diel activity is likely one of the reasons *P. miles* has been so successful in invading the Mediterranean Sea. In addition, this variability may impact mitigation efforts within the Mediterranean Sea as nocturnal individuals from deeper waters might replenish diurnally culled shallow-water populations.

Keywords: repeatability, invasion, acoustic telemetry, Red Sea, native range, Gulf of Aqaba

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INTRODUCTION

The common lionfish, *Pterois miles*, is one of the most harmful marine invasive species (Morris et al., 2009; Albins and Hixon, 2013). In the western Atlantic, invasions by *P. miles* and the similar red lionfish *Pterois volitans*, have caused a severe decline in native species diversity, density, and recruitment rate (Albins and Hixon, 2008; Green et al., 2012; Albins, 2013; Tuttle, 2017). *P. miles* has also invaded the Mediterranean Sea from its native Indo-Pacific origin through the Suez Canal, and has been spreading rapidly, recently reaching the Italian coast

(Golani and Sonin, 1992; Azzurro et al., 2017; Stern and Rothman, 2018; Agostino et al., 2020). This species' impact in the Mediterranean Sea has not yet been fully established (but see Agostino et al., 2020). It has been hypothesized that wide niche-breadth is one of the traits that enable the lionfish to invade so successfully (Côté et al., 2013a; Hackerott et al., 2013). However, the contribution of individual behavior to the observed population wide niche-breadth is still unclear.

Plasticity in individual behavior may be key for understanding invasiveness. For example, behavior plasticity may enable efficient tracking of local conditions and thus increase invasiveness (Nussey et al., 2007; Sagata and Lester, 2009; Gross et al., 2010; Chapple et al., 2012; Nakayama et al., 2016). In support, invasive amphipods showed higher individual-level variation in swimming activity than native populations (Bierbach et al., 2016). In addition, distinct individual behaviors can affect invasion dynamics as, for example, aggressive and better-dispersing individuals can lead the invasion front to facilitate rapid establishment (Araújo and Gonzaga, 2007; Duckworth and Badyaev, 2007; Duckworth, 2008; Araújo et al., 2011; Bolnick et al., 2011; Fogarty et al., 2011). For lionfish, there is ample evidence of population wide niche-breadth regarding diet, movement, habitat use, depth preference, and activity time (Harmelin-Vivien and Bouchon, 1976; Jud et al., 2011; Cure et al., 2012; Lee et al., 2012; Côté et al., 2013b; Andradi-Brown et al., 2017a; Gavriel and Belmaker, 2020). However, it remains unclear to what extent (1) individuals display similar behaviors and (2) individuals show repeatable behavior over time. Documenting the extent of such behavioral plasticity is critical for understanding the role of individual-level variation in invasion success.

This study explored two traits, activity time (diel activity) and depth use. Temporal activity patterns remain relatively unexplored in marine fish (Albrecht and Gotelli, 2001; Stuber et al., 2015; Alós et al., 2017). In the context of invasion, it is assumed that variable activity hours may increase the availability of dietary sources (Kocárek, 2001; Kronfeld-Schor and Dayan, 2003; Holt et al., 2004; Valeix et al., 2007). In marine fishes, temporal niches are usually conserved as most species are exclusively diurnal, nocturnal, or crepuscular (Helfman, 1978, 1986; Reebs, 2002). However, Pearly razorfish (*Xyrithchys novacula*) showed variations in individuals' awakening and rest periods (Alós et al., 2017). A striking shift in diel activity was documented between Goldline rabbitfish (*Siganus lineatus*) populations at the Great Barrier Reef, shifting their activity from diurnal to nocturnal (Fox and Bellwood, 2011). Lionfish are traditionally described as crepuscular at a population level, but evidence of nocturnal and diurnal activity is accumulating (Harmelin-Vivien and Bouchon, 1976; Morris and Akins, 2009; Cure et al., 2012; Mctee and Grubich, 2014; McCallister et al., 2018; Gavriel and Belmaker, 2020). Individual-level variation in activity times may enable *P. miles* to exploit a broader range of prey sources and respond more quickly to a different distribution of prey throughout the day, thereby increasing their potential invasion success.

Depth represents a vital habitat axis dictating biotic (Larson, 1980; Golani, 1994; Dunn et al., 2010) and abiotic conditions (Longhurst, 1985; Harvey and Stewart, 1991; Malcolm et al., 2011). Thus, the range of depths inhabited can represent a behavioral axis (Van Snik Gray and Stauffer, 1999). Depth use can vary considerably between individuals. For instance, in Trumpet emperor (*Lethrinus miniatus*), some individuals use only shallow habitats while others exploit the entire water column (Currey et al., 2014). Lionfish show strikingly broad depth ranges from surface level to depths of 200 m (Nuttall et al., 2014; Andradi-Brown et al., 2017b; Gress et al., 2017). However, it is still not clear to what extent individuals can vary their depth and over what time scale.

Culling, i.e., targeted removal of individuals, is commonly used in the Atlantic Ocean to control lionfish populations (Morris and Creswell, 2013) and is currently being considered in multiple countries around the Mediterranean Sea (Kleitou et al., 2021; Ulman et al., 2021). Although complete eradication of lionfish is impossible, an efficient culling program can mitigate harmful effects (Côté et al., 2014a). Most culling efforts are concentrated diurnally at depths of 0–30 m (Côté et al., 2014b; Usseglio et al., 2017). However, past studies have indicated that deeper mesophotic reefs might serve as lionfish sanctuaries, replenishing shallow-water populations (Andradi-Brown et al., 2017a,b). Understanding the time scale of fish movement between shallow and deep reefs, and their nocturnal and diurnal activity, is critical for designing effective culling programs.

This study uses acoustic telemetry to explore *P. mile's* individual variability in its native environment, the Red Sea. To determine diel activity and depth profiles, we tracked free-ranging lionfish, and their acceleration and depth were recorded at minute-long resolution over a period of months. We use this data to explore the variability between *P. miles* individuals and to assess their consistency over time. Examining the invading lionfish source population in its native habitat can help us understand whether *P. miles*' behavioral variation is an inherent trait or one that only appears after invading (e.g., when the lionfish are released from their former biotic limitations (Van Valen, 1965; Bolnick et al., 2010; Tuttle et al., 2017). Moreover, this information can help scientists construct efficient management programs for invasive populations in the Mediterranean Sea.

MATERIALS AND METHODS

Study Site

The study was conducted in a sub-tropical fringing reef located along the coast-line of Eilat, within the Gulf of Aqaba (**Figure 1**). This region, located along the northern tip of the Red Sea (29.50″N, 34.92″E), is inhabited by ichthyofauna of Indo-Pacific origin (Lieske et al., 2004; Kiflawi et al., 2006). In the Gulf of Aqaba, the common members of the Pteroinae sub-family are *P. miles* and the Clearfin lionfish, *Pterois radiata* (Golani and Darom, 1997; Brokovich, 2001; Gavriel and Belmaker, 2020). Lionfish surveys conducted within the study site found that *P. miles*' mean density was 2.1 ± 1.8 (SD) individuals per 500 m^2 (Gavriel and Belmaker, 2020). The Gulf of Aqaba in general, and the study site in particular, are characterized by a narrow

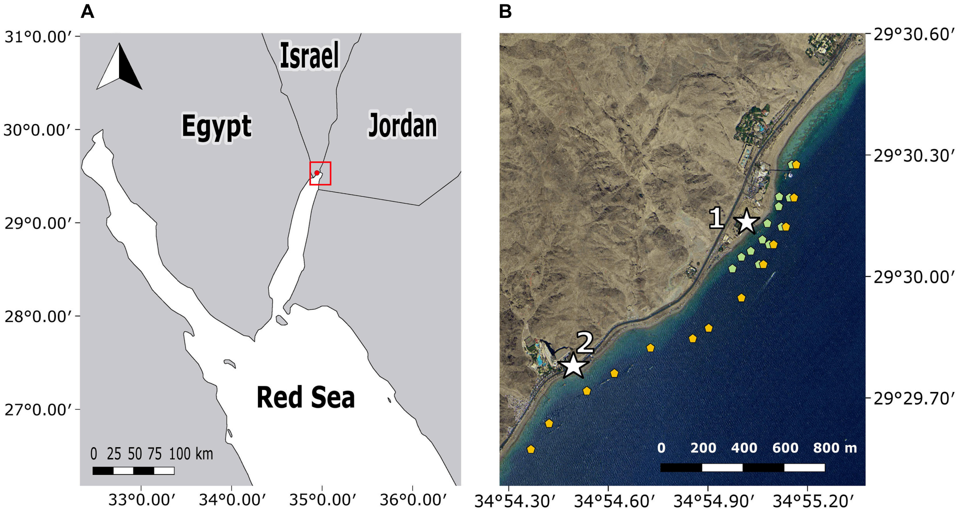

FIGURE 1 | Map of the study area located in the northern tip of the Gulf of Aqaba. **(A)** Regional map of the Red Sea with the study site marked in red. **(B)** Receiver locations, Vemco receivers marked in orange, and Thelma Biotel receivers marked in green. The two capture sites are indicated as white stars. Site 1 is The Interuniversity Institute for Marine Sciences (IUI), site 2 is the southern beach (S.B).

shelf with fringing reefs, followed by a steep slope that descends to depths of 60 m within 200 m from shore (Sade et al., 2008; Tibor et al., 2010). The selected study site was spread over 2.5 km and encompassed multiple sub-habitats such as hard-bottom substrates, soft-bottom substrates, and continuous and fragmented fringing reefs.

Acoustic Receiver Arrays

Two acoustic telemetry systems were used to track wild lionfish activity and depth patterns. The first receiver array was composed of 13 VR2W Vemco© receivers. To avoid tag collision with other studies using the same Vemco array at the same time, we constructed another array composed of 12 TBR 700 Thelma Biotel© receivers that operated on different frequencies. Together, the arrays were spread over 2.5 km, from the Eilat Coral Nature Reserve in the north down to the Taba Border Cross in the south (**Figure 1**).

The area covered included the shallow reef and extended continuously toward the deeper reef with a bottom depth of about 60 m. All receivers were oriented with the hydrophone facing down. The 13 Vemco receivers were deployed from May 2017 to February 2019, in a linear form and were attached to buoys located over the reef slope with a bottom depth of 13–40 m and affixed at 6–12 m. Receivers were tuned to a frequency of 69 kHz. In addition, 12 Thelma Biotel receivers were deployed from November 2017 until February 2019 and tuned to 71 kHz. This array was composed of two parallel lines of receivers: a seaward line with five receivers and a shoreline with seven receivers. The seaward line receivers were positioned next to the Vemco receivers, and the shoreline receivers were attached to buoys at depths of 3–10 m over bottom depth of 5–20 m. The array's configurations differed due to the lower detection range of the Thelma Biotel receivers (see section "Data Validation"), which required a denser configuration. We located the Thelma Biotel array next to the Inter-University Institution (IUI, Site 1; **Figure 1**) as this site has a high density of lionfish and could be easily accessed. Neither array provided pinpoint localizations as this kind of data necessitates overlapping reception of at least three receivers. Receivers were retrieved bi-monthly for short periods (up to 36 h) when detection data was downloaded, batteries replaced, and hydrophones cleaned.

Tag Specifications

We used two types of internal implantation transmitters from two manufacturers. Transmissions could only be recorded on the corresponding company's equipment. Thelma Biotel© AD-LP9L transmitters (9 mm diameter, 28.5 mm long, 2.5 g, operates on a frequency of 71 kHz, power output of 142 dB) were equipped with tri-axial acceleration and depth sensors. The tags were programmed to transmit every minute for the first 5 months and every 5 min for an additional 12 months. The transmission ratio was 2:1 acceleration to depth, respectively. The acceleration sensor had a range of 0–3.465 m/s^2. The depth sensor has a resolution of 0.34 cm and a range of 0–81.6 m. The second type of transmitters used were Vemco© V9AP-2x (9 mm diameter, 48 mm long, 3.6 g, operates on a frequency of 69 kHz, power output of 151 dB) equipped with tri-axial acceleration and depth sensors. The acceleration sensor values ranged between 0 and 4.9 m/s^2, and the depth sensor had a resolution of 15 cm and a range of 0–34 m. The tags were programmed to transmit every minute for the first 70 days and every 5 min for an additional 287 days. Again, the transmission ratio was 2:1 for acceleration to depth. In the "sensitivity analyses" below, we examine how the difference in maximal depth between tags may impact the result.

Fish Collecting and Tagging

During this study, 11 *P. miles* were tagged (dates and locations specified in **Table 1**). Fish were captured at night by trained

TABLE 1 | Summary information for the 11 *P. miles* tagged at Gulf of Aqaba during 2017–2018.

Fish ID	Tag type	Length (cm)	Capture site	Tagging date	Total days detected	Total number of detections	Included in the analysis
1	Vemco	22	IUI	May 23, 2017	71	7573	Yes
2	Vemco	24	S.B	May 15, 2017	43	8546	Yes
3	Vemco	28	S.B	May 15, 2017	20	6034	Yes
4	Vemco	26	S.B	May 15, 2017	272	3861	Yes
5	Thelma biotel	28	IUI	December 1, 2017	134	11,790	Yes
6	Thelma biotel	29	IUI	March 21, 2018	270	43,717	Yes
7	Thelma biotel	24	IUI	May 15, 2018	71	22,143	Yes
8	Thelma biotel	27	IUI	June 1, 2018	252	230,697	Yes
9	Thelma biotel	19	IUI	December 1, 2017	302	62,707	No
10	Thelma biotel	25	IUI	May 16, 2018	20	28	No
11	Vemco	24	S.B	May 23, 2017	6	799	No

Capture site "IUI" represents The Interuniversity Institute for Marine Sciences, and "S.B" represents the southern beach.

SCUBA divers using soft monophyletic hand nets. The fish were then transported to a lab located within 1 km from all capturing sites and kept in a 700 L water tank with fresh seawater circulation until being tagged the following morning (up to 10 h from capture). For the tag implantations, the fish were anesthetized with clove oil, and the tags were surgically implanted in the peritoneal cavity of the fish (protocol by Ghanawi et al., 2013). The tag weight to body weight ratio (mean = 1.3%, range 0.8–1.9%) was kept low to assure that tags did not alter fish activity and behavior and were consistent with ratios from previous telemetry research on this species (Bacheler et al., 2015). To facilitate recovery from the surgical procedure, fish were returned to the tank for at least 36 h after the tag implantation. After the recovery period, the fish were released to the sea at their original capture point (±50 m). Fish inserted with Vemco transmitters (five fish) were tagged during May 2017, and those with Thelma Biotel transmitters (six fish) were tagged between December 2017 and June 2018. All surgical procedures and fish handling procedures were reviewed and approved by the BGU Experimentation Ethics Review Committee, authorization number IL-18-04-2018c.

Data Validation

Fish detection was screened manually, and detections from dead fish (i.e., showing no change in location, depth, or acceleration values) were removed. Data collected in the 48 h following the fish release back to nature was removed from the analyses to avoid periods of acclimation, as were false detection attributed to tags with unknown ID numbers (ghost tags). Finally, weeks with fewer than 10 detections were omitted. Additional filtering was applied separately for each analysis (see below). We estimated the array reception areas and found that the conservative detections range was 120 m for each Vemco receiver and 50 m for each Thelma Biotel receiver (see **Supplementary Material 1**).

Analyses

Activity

To assess if individuals possess similar activity patterns, we used Generalized Additive Models (GAMs, Hastie, 2017) and tested if including individual identity increased model performance. Activity hours were estimated using the acceleration data collected from tagged *P. miles*. The underlying assumption was that a concentration of high acceleration values could accurately represent lionfish activity and compensate for the low acceleration values expected for an ambush predator (Fishelson, 1997; Albins and Lyons, 2012). We transformed the acceleration values to a binomial variable (zero indicating a non-active state and one indicating an active state) in the following way: median acceleration values (m/s^2) were calculated for bins of 30 min periods, bins with less than three detections were removed from the analysis. Then we defined the 70th percentile of the bins with higher acceleration values as active half hours and the rest as non-active half hours for each fish. This method enabled us to standardize the activity definitions and account for variation in acceleration measurements across the different manufacturers (see section "Tag Specifications").

As the response was the presence or absence of activity (1/0) in each 30-min bin, we used binomial family GAM. The primary predictor was "*time from sunrise*," modeled *via* cubic spline smoother, meaning that time was treated as cyclic [knot number (k) was defined to be 24, i.e., the day was split into 24 sections]. Other predictors used to explain some of the variations between individuals were "*depth (m)*," calculated as the median depth (m) for each 30-min bin, and applied in the model using an adaptive smoother (Wood, 2006), and "*season*" modeled as a fixed effect (December–February, March–May, June–August, and September–November). "*Fish length*" (TL in cm), and "*capture site*" (categorical variable, $n = 2$) were tested as well but excluded from the analysis in a preliminary model selection process. The variable "*time from sunset*" was also tested and produced similar results to "*time from sunrise*" and was thus omitted. We tested this model once with "*fish ID*" added as a random effect to the intercept only (similar activity pattern to all fish) and once as an interaction with the "*time from sunrise*" (creating separate activity curves for each "*fish ID*"). To identify the models best supported by the data, we used the corrected Akaike Information Criteria (Sugiura, 1978; Akaike, 1987). This criterion prioritizes models based on the maximum variation explained by the minimum variables and thus balances predictive power and overfitting (Akaike, 1987).

To assess consistency in activity hours for the same individual between days, we used repeatability scores. To compare activity across days and individuals, we calculated an index of daily activity peaks, defined as the hour of the highest density of active detections, as estimated by a kernel density smoother. Since lionfish show a general pattern of two activity peaks around sunrise and sunset, we performed the analyses separately for each period to obtain one index for the time of the highest morning activity and one index for the highest evening activity. To ensure the quality of the analysis, we only included days that had a full activity profile with at least 20 h between the first and last detections of the day and with at least one detection every 4 h.

The repeatability score was quantified using a mixed-effect model approach (Dingemanse and Dochtermann, 2013; Alós et al., 2017) as:

$$\frac{BIC}{BIC + WIC}$$

The between individual component (BIC) represents the average variance found between individuals and was calculated in the model as the variance of "*fish ID*" random intercepts. The within individual component (WIC) is the average variance found within an individual and represents the variance associated with measurement error in the model

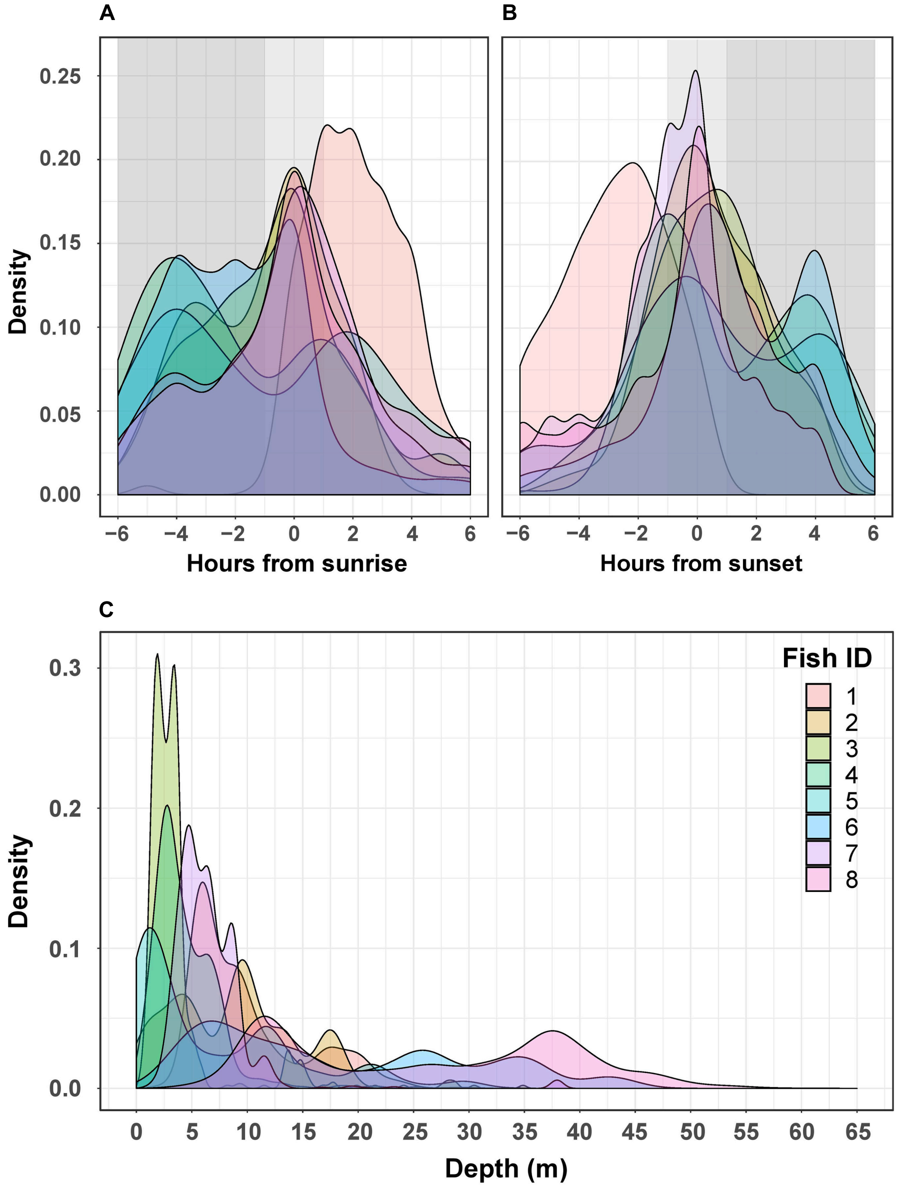

FIGURE 2 | Activity **(A,B)** and depth **(C)** for each tagged *P. miles*. The top two graphs depict the density of active detection relative to time from sunrise **(A)** and time from sunset **(B)**. Active detections were defined as the 30% most active half hours for each fish. The shaded dark gray areas represent night, and the light gray represents crepuscular hours (±1 h from sunset/sunrise). The bottom graph **(C)** shows the densities of detected depths for each individual.

(Roughgarden, 1972; Bolnick et al., 2002; Nakagawa and Schielzeth, 2010; Dingemanse and Dochtermann, 2013; Alós et al., 2017). The repeatability score ranges from 0 (low) to 1 (high) repeatability.

The models' response variables were "*morning activity peak*" or "*evening activity peak*." Predictors used to explain some of the WIC were "*season*" and "*capture site*," treated as fixed effects. We calculated adjusted repeatability scores, meaning that the variance explained by the fixed effects was not included in the denominator of the repeatability calculation (Dingemanse and Dochtermann, 2013). The confidence intervals for the adjusted repeatability score were constructed using parametric bootstraps, and likelihood ratio tests were used to calculate the *p*-value.

Depth

To estimate if individuals vary in their depth use, we tested whether including individual identity increased GAM performance. We used the median depth (m) of each 30-min bin as the response variable. The predictor variables included "*time from sunrise*" and "*season*" modeled as described above. "*Activity*" (0/1 for each 30 min bin) was used as fix effect. "*Fish length*" and "*capture site*" were also tested but were excluded in a preliminary model selection process. We tested this model compared to a similar model with "*fish ID*" added as a random effect (using AICc scores; Akaike, 1987; Hastie, 2017).

To assess individual consistency in depth preferences, we examined each individual's maximum daily depth and daily depth range and calculated the repeatability score. These indices were calculated based only on days with 18 h or more between the first and last detection and at least one detection every 6 h. These criteria are slightly less strict than those used for the daily activity indices as it is easier to characterize the daily depth profile than it is to characterize activity levels that usually have at least two peaks. Adjusted repeatability scores were quantified using mixed-effect models as described above for activity (Dingemanse and Dochtermann, 2013; Alós et al., 2017). The models' response variables were "*maximum depth*" or "*depth range*." Predictors used to explain some of the WIC were "*season*" and "*capture site*," treated as fixed effects.

Sensitivity Analyses

We performed several sensitivity analyses to assure the robustness of our results. (1) To ensure that active periods defined at a scale of 30-min bins (see section "Activity") do not represent only the periods in which fish were detected (e.g., due to low reception while hiding), we compared the density plot of the periods defined as active to the density plot of all the detections. (2) The number of detections and the tracking period were considerably higher for fish number eight (71% of all detections). Thus, activity patterns and repeatability scores might be affected by this single individual. We performed two additional analyses and compared the results to those obtained when using all the data. In the first, we excluded fish number eight entirely. In the second, we used only the first 2 months of data for this individual. This way, we retained a balanced portion of this individual's detections compared to other individuals tagged. (3) Transmitters from both manufacturers lower their transmit frequencies after a certain period (see section "Tag Specifications"). We repeated all analyses with data collected only from high transmit frequency periods to ensure this did not affect the results. (4) Three of the fish in this study were tagged with Vemco tags with a limited depth range of 34 m. As a result, the depth ranges for these fish are truncated, which can influence the perceived values of BIC and WIC (see section "Depth"). This "pseudo-repeatability" is expected to decrease the WIC, and, as a result, the repeatability score estimated might be inflated (Dingemanse and Dochtermann, 2013). To control for the possibility of biased repeatability scores, we also constructed depth models only for the four fish with the full depth range. The results of all sensitivity analyses are presented in **Supplementary Material 2**.

TABLE 2 | Summary of the GAMs fitted for the lionfish activity patterns.

Summary statistics	**Model a**		**Model b**	
Deviance explained	14.1%		17%	
AICc score	16,814		16,375	
ΔAIC	438		0	
W	0		1	
A. Parametric coefficients	**Estimate**	***p*-Value**	**Estimate**	***p*-Value**
Intercept	−2.40	<0.0001	−2.59	<0.0001
Spring	0.52	<0.0001	0.52	<0.0001
Summer	1.22	<0.0001	1.21	<0.0001
Winter	0.14	0.058	0.13	0.058
B. Smooth terms	**Edf**	***p*-Value**	**Edf**	***p*-Value**
Hours from sunrise (all fish)	19.02	<0.0001	ex	ex
Fish ID (random effect)	6.79	<0.0001	6.76	<0.0001
Depth median	20.65	<0.0001	20.45	<0.0001
Hours from sunrise by Fish 1	ex	ex	7.96	<0.0001
Hours from sunrise by Fish 2	ex	ex	7.34	<0.0001
Hours from sunrise by Fish 3	ex	ex	6.83	<0.0001
Hours from sunrise by Fish 4	ex	ex	0.01	0.754
Hours from sunrise by Fish 5	ex	ex	7.28	<0.0001
Hours from sunrise by Fish 6	ex	ex	12.29	<0.0001
Hours from sunrise by Fish 7	ex	ex	12.2	<0.0001
Hours from sunrise by Fish 8	ex	ex	19.04	<0.0001

The response variable was fish "active" versus "non-active" detections, estimated within a binomial model. Predictors include the effect of "time from sunrise" for all the individuals (model a) or separately for each individual (model b). "Season" was treated as a fixed effect, "fish ID" as a random effect, and "depth" as a smoothed covariate.

RESULTS

Fish Tagged

During this study, 11 *P. miles* were tagged, 5 were tagged with Vemco transmitters, and 6 with Thelma Biotel (**Table 1**). Out of those, eight fish survived, and their detections were analyzed. The number of detections for each fish ranged from 3,861 to 2,300,697 and the tracking period ranged between 20 and 272 days.

Activity Patterns

The activity of all *P. miles* tracked, as emerged from the telemetry data, generally peaked around dusk and dawn, with most activities ±2 h from sunrise/sunset. However, when examining individual activity plots, it became clear that there was some *P. miles* activity level almost throughout the entire day (**Figures 2A,B**), as some individuals tended to nocturnal activity (e.g., fish #5), some toward diurnal activity (e.g., fish #1), and others stayed mainly crepuscular (**Figures 2A,B**). In addition, the model fitting separate activity curves for each individual was better supported by the data (**Table 2**, ΔAICc = 438), indicating individual variation in diel activity.

When examining morning and evening activity peaks across days, we found that most activity peaks indeed occurred at sunrise or sunset (**Figures 3A,B**). However, in some cases, the maximum activity peak was as far as 6 h from sunrise or sunset. Some fish had distributions of daily activity peaks that resembled the distribution of all fish combined (**Figures 3A,B**), indicating low temporal repeatability. Accordingly, the adjusted repeatability scores for the morning and evening activity peaks were 0.21 (0.00–0.46, 95% CI) and 0.27 (0.003–0.53, 95% CI), respectively (**Table 3**). When examining the BIC and WIC, we found that the low repeatability scores were due to relatively high WIC (4.33 and 4.81 for the morning and evening activity peaks, respectively; **Table 3**) almost three times larger than the BIC.

Depth Pattern

Fish were detected at depths of 0–64 m (**Figure 2C**); however, this is likely an underestimation of the fish's full depth range as the tags' transmits were depth-limited (see section "Materials and

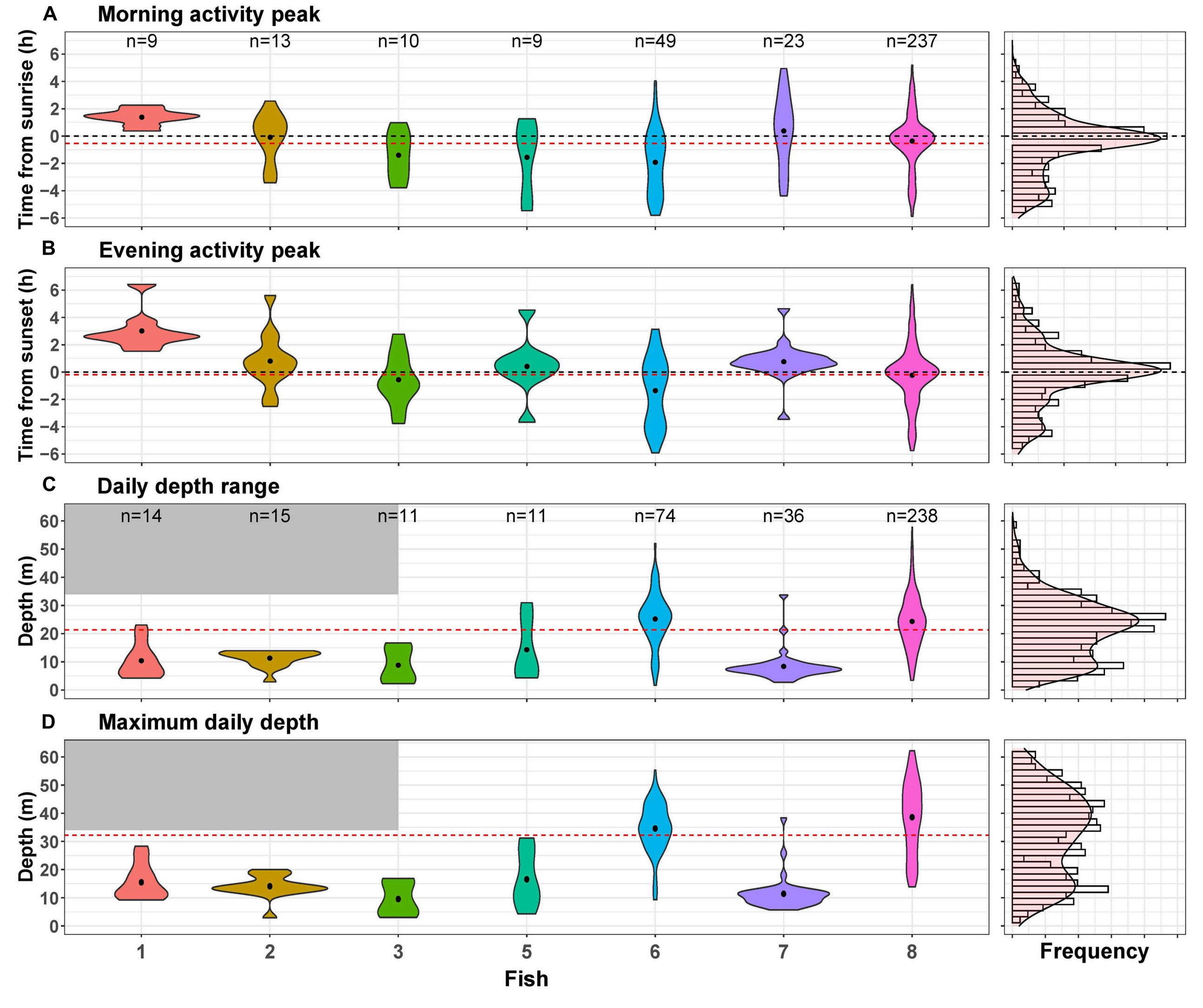

FIGURE 3 | Distribution of the four daily indices across all individuals (right) and separately for each tagged individual (left). Dots in the violin plots represent the mean value for each fish, and the red dashed lines indicate the mean value for all fish. "*n*" represents the number of days used for each violin plot. **(A)** Morning activity peak, sunrise is denoted by a dashed black line. **(B)** Evening activity peak, sunset denoted by a dashed black line. **(C)** Daily depth range and **(D)** maximum daily depth. The gray shaded areas denote the Vemco transmitter depth limitation.

TABLE 3 | Mixed effect model results including adjusted R-score, between-individual (BIC), and within-individual variation (WIC) for the daily indices (morning activity peak, evening activity peak, daily depth range, and maximum daily depth).

	Morning activity peak		Evening activity peak		Daily depth range		Maximum daily depth	
Random effect	**Variance**	**SD**	**Variance**	**SD**	**Variance**	**SD**	**Variance**	**SD**
Fish ID (BIC)	1.85	1.08	1.78	1.33	63.81	7.988	171.96	13.113
Residual (WIC)	4.33	2.08	4.81	2.19	74.76	8.646	87	9.328
Fix effect	**Estimate**	**SE**	**Estimate**	**SE**	**Estimate**	**SE**	**Estimate**	**SE**
Intercept	−0.59	0.57	0.42	0.71	14.1	3.77	15.94	6.01
Spring	−0.51	0.53	0.14	0.55	3.08	1.75	6.52	1.89
Summer	0.43	0.29	−0.01	0.3	4.67	1.18	14.17	1.27
Winter	0.29	0.33	−0.03	0.34	−1.15	1.35	1.78	1.46
Capture site	0.12	1.05	−0.43	1.3	−7.61	6.97	−12.83	11.8
Adjusted R-score	0.215 [0,0.458]		0.27 [0.003,0.528]		0.46 [0.087,0.714]		0.664 [0.23,0.83]	

The residual variance represents the WIC, and the variance associated with fish identity represents BIC. The adjusted R-scores were calculated as the ratio of the BIC/(BIC + WIC). Other fixed effects included were "capture site" and "season."

Methods"). We found that different fish had depths they tended to inhabit. Thus, the model that included "*fish ID*" as a random effect was better supported by the data than the model assuming all individuals had similar depth preferences (ΔAICc = 9234, **Table 4**). Nevertheless, individuals were not restricted to a specific depth, and individuals displayed varying depth patterns over time (**Figures 2C**, **4**). The greatest daily depth range for any individual fish was 60 m (fish #8).

We also examined depth pattern consistency and found that the daily depth range and the maximum daily depth varied considerably between individuals and within each specific fish over time (**Figures 3C,D**). Adjusted repeatability scores were 0.46 (0.08–0.71, 95% CI) for daily depth range and 0.64 (0.23–0.83, 95% CI) for maximum depth (**Table 3**). These scores represent medium to high repeatability. However, when examining the BIC and WIC, we found that the daily depth range index had similar BIC and WIC (63 and 75, respectively) and that for maximal depth, the BIC (172) was almost double the WIC (87, **Table 3**). These results imply that the relatively high repeatability scores for depth are attributed to the large differences between individuals (represented by BIC) that mask the changes in individual depth-use over time (represented by WIC).

TABLE 4 | Summary of the GAMs fitted for the lionfish depth pattern.

Summary statistics	**Model c**		**Model d**	
Deviance explained	23.8%		52.3%	
AICc score	153,744.8		144,511	
ΔAIC	9233.71		0	
W	0		1	
A. Parametric coefficients	**Estimate**	***p*-Value**	**Estimate**	***p*-Value**
Intercept	18.39	<0.0001	4.66	0.1179
Spring	−2.91	<0.0001	4.58	<0.0001
Summer	−8.36	<0.0001	12.69	<0.0001
Winter	8.61	<0.0001	2.19	<0.0001
Activity	−0.79	0.0022	−3.45	<0.0001
B. Smooth terms	**Edf**	***p*-Value**	**Edf**	***p*-Value**
Hours from sunrise	10.5755	<0.0001	10.1647	<0.0001
Fish ID (random effect)	ex	ex	6.9901	<0.0001

Predictors included "hour from sunrise" as a smoothed co-variant, "season" as a fixed effect, and "activity (0/1)" as a fixed effect. Model d also includes "fish ID" as a random effect.

DISCUSSION

We found that within their native range, *P. miles* individuals show high variability in both depth use and activity time. All *P. miles* tracked were active at both sunrise and sunset, but some individuals tended to also be active during the day or night (**Figures 2A,B**), and individuals also utilized variable depths (**Figures 2C**, **4**). Moreover, despite the small sample size ($n = 8$), we observed considerable within-individual variation between days along the day/night and depth axes. Activity peaks varied substantially across days for single individuals (**Figure 3** and **Table 3**), and individuals varied their depth unpredictably over time. This behavioral flexibility demonstrated within the species' native range may indicate a pre-adaptation allowing this species to become a successful invader in both the Atlantic and the Mediterranean.

Although lionfish as a species have been extensively studied (Morris et al., 2009; Cure et al., 2012; McCallister et al., 2018), only a few studies have specifically assessed variation between individuals. Evidence for individual variation comes from acoustic telemetry home-range studies. In two different studies, lionfish's home-range in invaded seas ranged from 360–12,812 m^2 (McCallister et al., 2018) to 158–4051 m^2 (Dahl and Patterson, 2020), suggesting high variation among individuals. In addition, a study in North Carolina found large variability in the number of receivers that detected each lionfish, indirectly implying variable home-ranges (Bacheler et al., 2015). Here, we extend the findings of the large variability in home-range between individuals to both depth use and diel activity.

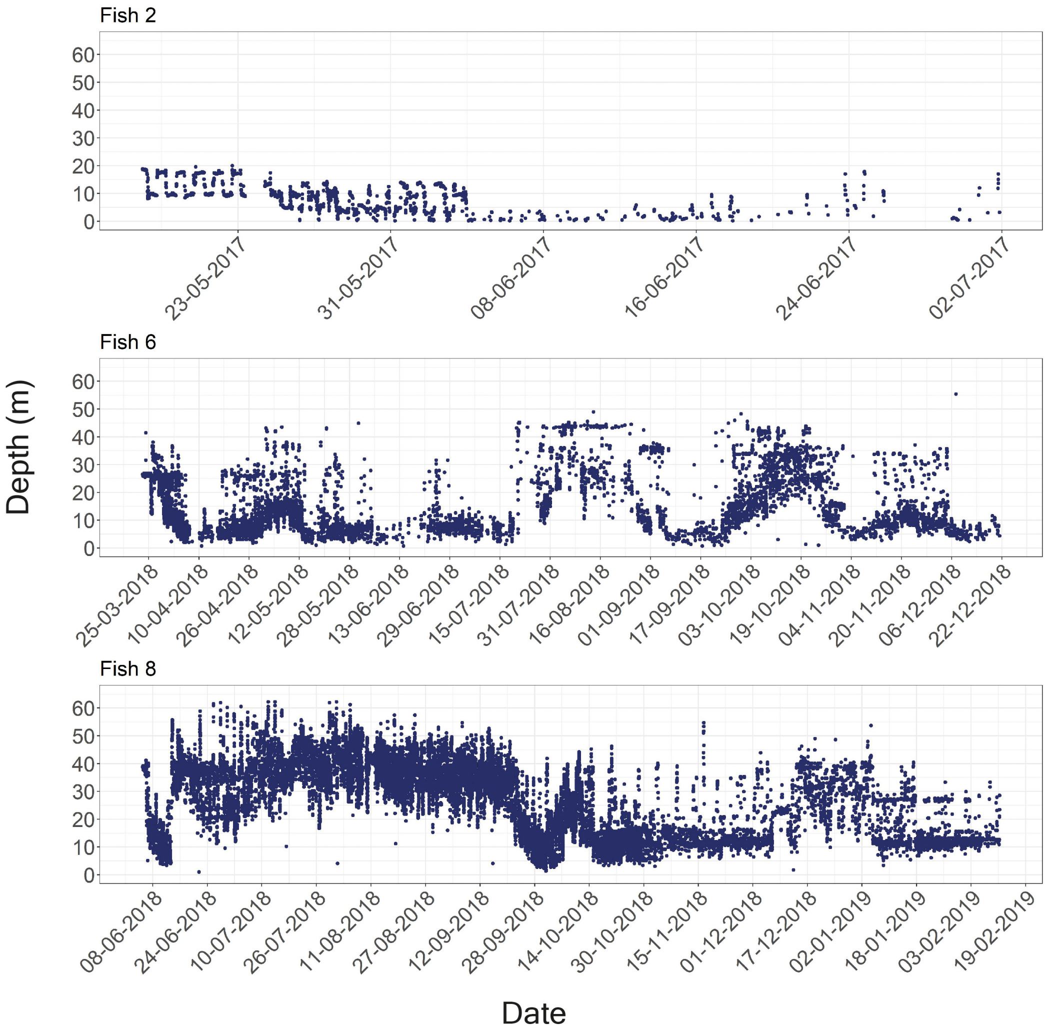

FIGURE 4 | Examples of the raw depth recorded for the entire period each fish was detected. Fish numbers two, six, and eight are shown above, and data for all fish in the study appear in **Supplementary Material 4**.

Lionfish's ability to be active at different hours throughout the day and night may increase the number of potential prey species, especially in an invaded range where the native fish are naïve (Agostino et al., 2020). Usually, marine species have highly conserved diel activity patterns (Helfman, 1978, 1986; Reebs, 2002; Pickholtz et al., 2018; but see Fox and Bellwood, 2011). Even among crepuscular species that need to adjust to dark and light conditions, individual variation is unusual (Helfman, 1986; Løkkeborg et al., 2000). For example, Ling fish (*Molva molva*) show a consistent crepuscular activity pattern with little variation among individuals (Løkkeborg et al., 2000). In this context, the diel flexibility of *P. miles* is impressive as it occurs both between individuals and within individuals over time.

Lionfish depth profiles show that some individuals occupy different depths across days (fish #6 and fish #8), while others consistently inhabit shallow areas (fish #5 and fish #7). The reason for the large variation in depth-use patterns between individuals can either be distinct personalities and depth preferences, or the depth distribution of habitats at different locations. For example, over the southern sites, where fish preferred mostly shallow depths, there is no deep reef but rather a soft bottom substrate that might be less appealing for lionfish. The depth-related repeatability scores suggest conserved individual depth use (**Table 3**). Nevertheless, these high repeatability scores originated from the high variation between individuals (BIC) that obscured the variation within individuals over time (WIC). Thus, the results also demonstrate that depth shifts within individuals are common and can occur over short time scales. For example, fish #8 crossed more than 60 m in a single day (**Figure 3C**).

The ability of individuals to move between shallow and mesophotic reefs has substantial implications for the effectiveness of shallow reef culling (Andradi-Brown et al., 2017a,b). Lionfish removal is typically restricted to shallow depths (Côté et al., 2014b; Usseglio et al., 2017), including in the Mediterranean Sea (Kleitou et al., 2021). Our findings demonstrate frequent movements of lionfish between shallow and mesophotic habitats over short time intervals. These findings raise the possibility that culled shallow-water populations can be replenished, even daily, with individuals from deeper waters. In support, a study integrating data from numerous western Atlantic locations found that shallow water culling programs did not always alter the depth abundance distribution of lionfish (Andradi-Brown et al., 2017b). At the same time, large individual-level mobility may also imply that shallow water culling can impact the lionfish population at deeper depths (similar to an edge effect in marine protected areas; Ohayon et al., 2021).

Past studies have demonstrated lionfish's ability to react to culling efforts. For example, lionfish in culled areas in the Bahamas tended to be less active during daytime compared to lionfish inhabiting non-culled reefs (Côté et al., 2014b). In the Atlantic Ocean, non-culled mesophotic lionfish had a similarly high alert rate to divers as lionfish in culled shallow areas (Andradi-Brown et al., 2017a). Understanding both the location-specific level of connectivity and the ability of individuals to change behaviors in response to fishing is critical to designing and implementing optimal culling programs.

This study has several caveats; the most prominent one is the small sample size. While larger sample sizes would have provided a more detailed understanding of the observed patterns and may lead to increased depth range and activity hour estimates, even from the current sample size, the variation in activity and depth of *P. miles* between and within individuals is clear and striking. Another caveat is that some of the study fish were tagged with transmitters with limited maximal depth (34 m). Although excluding those fish did not change the results (see **Supplementary Material 2**), the variability and range of depth estimates presented here are likely underestimated. In addition, the use of two acoustic telemetry systems possessing different detection ranges and array configuration limited our ability to conduct movement and home range assessments, preventing direct comparison to former studies exploring those traits in the invaded range. Finally, we only examined two important behavioral axes. Estimating additional traits, such as diet or space-use patterns, could provide complementary views of this species' behavior.

This study was conducted within the native environment of *P. miles* in the Red Sea. The "ecological release" theory posits that invasive species may be freed from the biotic limitations in their native range and thus appear as generalist species (Van Valen, 1965; Bolnick et al., 2002, 2010, 2011; Pickholtz et al., 2018). The observation of flexible behaviors and wide niche-breadth for *P. miles* in the native range suggests that the plasticity of lionfish in the invaded ranges is not solely the result of "ecological release." Instead, the behavioral flexibility appears to be a pre-adaptation that may have facilitated its invasion success.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee, Ben Gurion University (authorization number IL-18-04-2018c).

AUTHOR CONTRIBUTIONS

TG, RP, and JB conceived of the presented idea. TG performed the fieldwork, analyses, and wrote the manuscript. RP contributed to the design and implementation of the research and the analysis of the results. JB supervised the findings of this work. All authors discussed the results and contributed to the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.790930/full#supplementary-material

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Untargeted Metabolomics Yields Insights Into the Lipidome of *Botrylloides niger* Herdman, 1886, An Ascidian Invading the Mediterranean Sea

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Human-mediated dispersal of alien species in new biogeographic realms is one of the major drivers of biodiversity change in marine ecosystems. Among others, ascidians are invasive species spreading worldwide, thus causing ecological and economic harms in the recipient environments. An integrated taxonomic approach on selected samples allowed the identification of *Botrylloides niger* Herdman 1886 as a non-indigenous ascidian forming large aggregates and outcompeting native species in the Fusaro Lake (central-western Mediterranean Sea). This led to the opportunity to investigate in deep its metabolome for the first time. Untargeted mass spectrometry-based metabolomics unveiled *B. niger* to be a source of nutraceuticals and bioactive natural products, such as lysophospholipids, sulfonolipids, polyunsaturated fatty acids, sphingolipids, monoacylglycerols, and alkaloids. Even if causing ecosystem alterations, our results suggest that *B. niger* could be exploited for nutritional and/or pharmaceutical purposes, thereby turning a treat in a resource.

Keywords: alien ascidians, fouling communities, lipids, alkaloids, mass spectrometry, molecular networking, natural products

1 INTRODUCTION

Ascidians (Tunicata) are important members of marine benthic communities, with over 3000 species described worldwide inhabiting polar, tropical, and temperate environments, either in shallow or deeper habitats (Shenkar and Swalla, 2011). Their phylogenetic relatedness to vertebrates, coupled with a somehow fast and easy development *in vitro*, has attracted the attention of developmental biologists since centuries (Corbo et al., 2001; Dehal et al., 2002; Imai et al., 2006). In the last decades, ascidians also generally raised additional interest as potential alternative food sources in human consumption due to the presence of compounds with high nutritive content, such as proteins, amino acids, lipids, and secondary metabolites (Zhao and Li, 2016; Palanisamy et al., 2017; Watters, 2018; Dou and Dong, 2019), and examples of wide phenotypic and environmental plasticity, which is turn reflected in a high invasive potential (Bullard and Carman, 2009; Zhan et al., 2015; Rocha et al., 2019; Atalah et al., 2021). Moreover, they are source of a variety of bioactive chemical compounds, which include peptides, alkaloids, polyethers, macrolides, terpenes, and polysulfides (Palanisamy et al., 2017; Imperatore et al., 2019). Finally, ascidians are also emerging as model organisms for several studies including immunobiology, allorecognition, angiogenesis and whole-body regeneration, and their extensive regenerative capacity is developing huge interests in regenerative medicine and ageing research (Ballarin et al., 2001; Rinkevich et al., 2007; Gasparini et al., 2008; Franchi et al., 2011; Lauzon et al., 2013; Rinkevich et al., 2013; Voskoboynik and Weissman, 2015).

Noteworthy, these variegate interests of the scientific biological community also often merge in target species. Just to mention, the Korean common sea squirt *Halocynthia roretzi* (Drasche, 1884), reared and exploited as a commercial seafood in Japan and Korea (Oh et al., 1997; Hirose et al., 2009), possesses lipids that positively affected the health of diabetic/obese KK-Ay mice (Mikami et al., 2010) and ingestion of its plasmalogen enhances and keeps cognitive function through induction of neuronal growth and neuroprotective effects (Watanabe et al., 2020). The clubbed tunicate *Styela clava* Herdman, 1881, widely cultured in Asian countries (Ko et al., 2012), is in turn not only invading the Mediterranean Sea and nearby areas (Davis and Davis, 2010), but also possesses important compounds with variegate properties, including ACE (angiotensin-converting enzyme) inhibition (Ko et al., 2012) and antioxidant, immunomodulatory, and anticancer activities (Kim, 2011; Ju et al., 2014). Finally, specimens of the *Ciona intestinalis* (Linnaeus, 1767) species complex, although often reported as a sea pests in several countries worldwide, leading to conspicuous economic losses (Zhan et al., 2010; Bouchemousse et al., 2016), possess abundant phospholipids and polyunsaturated fatty acids (PUFAs) with importance to human health, thus suggesting that they could become an excellent alternative source of n-3 fatty acids (Zhao et al., 2015). Moreover, natural products (NPs) derived from ascidians have also led to the production of promising drugs already marketed for the treatment of specific cancers, such as ecteinascidine 743 or Trabectedin (Yondelis®) from *Ecteinascidia turbinata* Herdman, 1880 and the peptide dehydrodidemnin B or Plitidepsin (Aplidin®) from *Aplidium albicans* (Milne Edwards, 1841) (Ramesh et al., 2021).

Interest about ascidians is somehow eased by their ecology, as species are often abundant in shallow waters of enclosed and semi-enclosed basins such as ports, marinas, and lagoons, where they settle on artificial substrates and form high biomasses in a short time-frame (Bullard et al., 2007; Lambert, 2009; Lambert, 2019). Although this on one hand makes them a possible threat to local communities due to physical substrate dominance and release of secondary metabolites that can harm benthic assemblages (Prado et al., 2004; Minchin and Sides, 2006; Lengyel et al., 2009; Watters, 2018), on the other it guarantees easy rearing and harvesting (Davis, 1995; Manríquez and Castilla, 2007).

In this context, investigation of compounds extracted from alien ascidians may help shedding light not only in their ecological role in localities where they are introduced, but also in a possible practical use as nutritional or bioactive compounds, thus turning a treat in a resource. Charting metabolic diversity of complex natural extracts and identify novel compounds at an early stage of the research, is high challenging. Metabolomics and dereplication (i.e., early identification of already known NPs), have become more routine in the fields of NP discovery (Caso et al., 2019), environmental research (Teta et al., 2021), and ecotoxicology (De Marco et al., 2022), and may rely upon advanced and sensitive spectroscopic techniques (NMR, mass spectrometry) (Cappello, 2020). High resolution liquid chromatography coupled with tandem mass spectrometry (LC-HRMS/MS) represents a well-suited untargeted methodology to capture the entire metabolome (i.e., the chemical profile of low molecular weight metabolites) within a heterogeneous mixture, thus enabling a deep and sensitive dereplication. Indeed, high resolution metadata and acquisition of fragmentation spectra are useful in that they provide “fingerprints” of detected metabolites, thereby facilitating their identification. Many bioinformatic tools are currently available for handling such amount of metadata and provide a bird’s eye perspective of the chemical profile of a certain extract. Among these tools, to explore large MS data sets, the Feature-Based Molecular Networking represents a suitable means for fast detection, annotation and visualization of known compounds and their novel analogues as well as for discovery of completely new NPs (Nothias et al., 2020).

Herein for the first time, as far of our knowledge, we assessed the metabolome of the colonial ascidian *Botrylloides niger* Herdman, 1886, a renown tropical invader which already expanded its distributional range from tropical Western Atlantic to Eastern and Western Pacific (Sheets et al., 2016; Rocha et al., 2019; Nydam et al., 2021) and is now emerging as an invasive species also in the Mediterranean Sea.

2 MATERIAL AND METHODS

2.1 Study Area and Field Work

The Fusaro Lake (Bacoli, central Tyrrhenian Sea, central-western Mediterranean Sea) is a brackish water body situated within the Campi Flegrei Regional Park (https://www.parcodeicampiflegrei.it/) and the biggest of the four Phlegraean lagoons. The lake is connected to the Tyrrhenian Sea by three channels, although only the middle one is operative, and therefore it constitutes the only source of water exchange with the sea. During fieldwork held in June 2020 and aimed to investigate the fouling biota of the local dock wall (40.8229 N, 14.0498 E), several unidentified botryllid colonies were sampled from 0 to 2 meters. Samples were first photographed *in situ* (**Figure 1**) with a Olympus Em1 mkII camera equipped with a Zuiko 60 mm f 2.8, then scraped from hard substrates with the help of underwater knives, placed in single plastic bags filled with seawater, and brought to the Laboratory of Benthos of the Stazione Zoologica Anton Dohrn (SZN, Naples, Italy), where they were cleaned from possible contaminants (algae and other organisms adhering them) for further laboratory work.

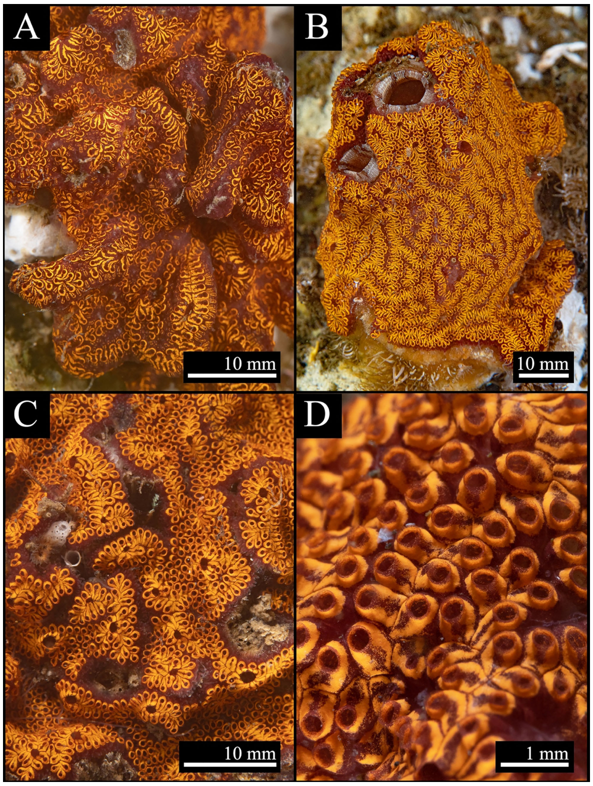

FIGURE 1 | *Botrylloides niger* Herdman, 1886 from the Fusaro Lake channel (Bacoli, central Tyrrhenian Sea, Mediterranan Sea). **(A)** Colony forming an aggregate on the dock wall. **(B)** Colony overgrowing a specimen of *Styela plicata* (Lesueur, 1823). **(C)** Colony growing amidst serpulids and other tunicates. **(D)** A close-up showing zooids placement and atrial openings.

2.2 Identification of the Colonies

The examination of the colonies external morphology with the help of a Zeiss Axio Zoom.V16 (Germany) microscope yielded an identification to genus level (see below). Then, since morphological identification of botryllid taxa could be deceiving, often resulting in high misidentification rates even when carried out by ascidian specialists (see discussions in Brunetti, 2009; Rocha et al., 2019; Viard et al., 2019), five colonies were randomly selected for DNA barcoding. After dissection, total genomic DNA was extracted from single zooids using the DNeasy® Blood & Tissue kit (Qiagen, Hilden, Germany), following the protocol as in Crocetta et al. (2020). Samples were then fixed in ethanol 99.9% and preserved in the collection of the Laboratory of Benthos, SZN (SZN-B-809ASC11A–813ASC11E). Partial sequences of the *Cytochrome c Oxidase subunit I* (COI) gene were amplified from each DNA sample using the primers designed by Folmer et al. (1994). Polymerase chain reactions (PCRs) were conducted in 25 µL volume reaction as in Tanduo et al. (2021). Amplification was performed with an initial denaturation at 95°C (5 min), followed by 39 cycles of denaturation at 95°C (1 min), annealing at 45°C (1 min), extension at 72°C (1 min), with a final extension at 72°C (5 min). The PCR products were purified and Sanger sequenced at the Molecular Biology and Sequencing Service of SZN through an Automated Capillary Electrophoresis Sequencer 3730 DNA Analyzer (Applied Biosystems, CA, USA), using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies, UK). Chromatograms for each sequence were then quality checked, assembled, and edited using Sequencher v.5.0.1 (GeneCodes, MI, USA). The identity of sequences obtained was finally checked through the Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Morgulis et al., 2008).

2.3 Phylogenetic Analyses

Since the BLAST results mostly reported high similarity (see below) with several sequences deposited as *Botrylloides niger* [or incorrectly as *B. nigrum*: see discussions in Ryland (2015) on *Botrylloides* being a masculine gender], a barely-reported alien species in the Mediterranean Sea, unpublished sequences obtained here were also included in a small phylogenetic framework as to further confirm the identification of the samples and to eventually compare them with sequences coming from other worldwide localities.

The NCBI data mining revealed the presence of twenty-eight COI partial sequences of *B. niger/nigrum*. However, the analysis of seven of them (MH367290, MH367289, KX650766, KX138503, KX138502, KT693199, and MH235543) revealed the presence of gaps producing misalignment, and thus they were excluded *a priori*. Then, by subsequently deleting identical sequences (MW858360, MT637961, MW817940, LR828514, MT232728, MT232723, KT693201, KT693200, KT693198, MW278779, KP254541, and HF548559/NC_021467), only nine sequences of *B. niger* were used for the subsequent phylogenetic analyses (**Table 1**). In addition, four sequences of *Botrylloides diegensis* Ritter & Forsyth, 1917 and one single sequence each of *Symplegma brakenhielmi* (Michaelsen, 1904) and *Symplegma viride* Herdman, 1886 were also added to our alignment (**Table 1**). *Botrylloides diegensis* is a species often misidentified in the Mediterranean as *Botrylloides leachii* (Savigny, 1816) and is known to be the sister taxon of *B. niger* (Nydam et al., 2021). *Symplegma* Herdman, 1886 taxa were chosen as outgroup of botryllids in view of recent phylogenetic studies (Perez-Portela et al., 2009; Viard et al., 2019; Nydam et al., 2021).

Sequences were aligned using ClustalW (2.1) on the CIPRES Science Gateway (Miller et al., 2010), using default parameters. The alignment was trimmed and then used to infer phylogenies through maximum likelihood (ML) and Bayesian Inference (BI) methods. The GTR+I evolutionary model was selected through the AICc (corrected Akaike Information Criterion) algorithm, implemented in JModelTest 2 v.0.1.10 (Darriba et al., 2012), as the best-fit model for the alignment produced.

ML analysis was performed using RAxML v.2.0 (Edler et al., 2021), using 1000 rapid bootstrap pseudo-replicates under the aforementioned evolutionary model. BI was instead performed using MrBayes v.3.2.5 (Huelsenbeck and Ronquist, 2001) for 10 million generations, a sampling interval every 1000 generations, and discarding 25% of the produced trees. Tracer v1.7.1 (Rambaut et al., 2018) was used to check the convergence of Markov chain Monte Carlo (MCMC) runs. The trees obtained

TABLE 1 | GenBank COI partial sequences of *Botrylloides* and *Symplegma* species used in the molecular analyses and associated accession numbers and voucher data (localities obtained from GenBank and/or relevant paper/s).

Taxon	Deposited as	Voucher/Haplotype	GenBank	Locality	Reference
Botrylloides diegensis [see Viard et al. (2019)]	*Botrylloides leachii*	BA-TR	HG931921	Taranto Gulf, Italy	Griggio et al. (2014)
Botrylloides diegensis	*Botrylloides diegensis*	IC3/BDH1	MW579604	Incheon, South Korea	Lee and Shin (2021)
Botrylloides diegensis	*Botrylloides diegensis*	YP5/BDH2	MW579605	Yangpo, South Korea	Lee and Shin (2021)
Botrylloides diegensis [see Viard et al. (2019)]	*Botrylloides leachii*	P11	LR828517	Mar Piccolo, Taranto, Italy	Salonna et al. (2021)
Botrylloides niger	*Botrylloides niger*	SZN-B-809ASC11A–813ASC11E	OM866151	Fusaro Lake, Bacoli, Naples, Italy	This study
Botrylloides niger	*Botrylloides niger*	11Mar19-2-20	MT637960	Puerto Rico, USA	Streit et al. (2021)
Botrylloides niger	*Botrylloides nigrum*	IRAR2/COI-A	KU711782	Florida, USA	Sheets et al. (2016)
Botrylloides niger	*Botrylloides nigrum*	IRC30/COI-B	KU711783	Florida, USA	Sheets et al. (2016)
Botrylloides niger	*Botrylloides nigrum*	PR12/COI-C	KU711784	San Juan, Puerto Rico	Sheets et al. (2016)
Botrylloides niger	*Botrylloides nigrum*	PR11/COI-D	KU711785	San Juan, Puerto Rico	Sheets et al. (2016)
Botrylloides niger	*Botrylloides nigrum*	MXA05/COI-E	KU711786	Veracruz, Mexico	Sheets et al. (2016)
Botrylloides niger	*Botrylloides nigrum*	BZ1/COI-F	KU711787	Twin Cayes, Belize	Sheets et al. (2016)
Botrylloides niger	*Botrylloides nigrum*	VZ6/COI-G	KU711788	Margarita, Venezuela	Sheets et al. (2016)
Botrylloides niger	*Botrylloides nigrum*	HI19/COI-H	KU711789	Hawaii, USA	Sheets et al. (2016)
Symplegma brakenhielmi	*Symplegma brakenhielmi*	MUZAC6326	LS992554	Olbia, Sardinia, Italy	Mastrototaro et al. (2019)
Symplegma viride	*Symplegma viride*	11Mar1911	MT637979	Puerto Rico	Streit et al. (2021)

Specimens sequenced in this study highlighted in bold.

were checked by eye in FigTree v.1.4.4 (Rambaut, 2018) and edited in Adobe Illustrator 2019 23.1.1 (Adobe, USA).

2.4 Preparation of Crude Extract

Approximately 30 g of sample was defrosted for the extraction, cut into small pieces, and left in water (1:20 w/v) overnight (O/N) at 20°C for removing salts. Then, the sample was extracted with methanol (1:20 w/v), O/N at 20°C. The organic phase was dried under vacuum at the rotary evaporator (R-100, BUCHI, Flawil, Switzerland) to afford about 250 mg of crude extract. The extract was fractionated by reversed-phase (RP18) column chromatography, eluted with a linear gradient of H_2O/MeOH (v/v, from 50:50 to 0:100 over 1 h) yielding three fractions (50-75-100% MeOH), which were then chemically characterized by molecular networking analysis of tandem MS data. Moreover, two additional samples of *B. niger*, collected in different areas of the Fusaro Lake channel, were subjected to methanol extraction for qualitative assessment of reproducibility in the metabolite composition. Crude extracts from the three replicates were shown to contain a similar chemical profile by LC-HRMS/MS analysis.

2.5 Liquid Chromatography - High Resolution Tandem Mass Spectrometry (LC-HRMS^2)

The RP18 eluted fractions were dissolved in methanol at a concentration of 1 mg/mL for LC-HRMS^2 analyses. MS experiments were performed using a Thermo LTQ Orbitrap XL high-resolution ESI mass spectrometer equipped with a Thermo U3000 HPLC system (Thermo Fisher Scientific, Waltham, MA, USA), which included a solvent reservoir, in-line degasser, binary pump, and refrigerated autosampler. A 5-μm Kinetex C18 column (50 × 2.10 mm), maintained at room temperature, was eluted at 200 $\mu L \cdot min^{-1}$ with H_2O (supplemented with 0.1% HCOOH) and CH_3OH, using a gradient elution. The gradient program was set as follows: 30% CH_3OH 1 min, 30%–100% CH_3OH over 30 min, 100% CH_3OH 10 min. Mass spectra were acquired in the positive ion detection mode. MS parameters were as follows: a spray voltage of 4.8 kV, a capillary temperature of 285°C, a sheath gas rate of 32 units N_2 (ca. 150 mL/min), and an auxiliary gas rate of 15 units N_2 (ca. 50 mL/min). Data were collected in the data-dependent acquisition mode, in which the five most intense ions of a full-scan mass spectrum were subjected to HRMS^2 analysis. The *m/z* range for data dependent acquisition was set between 100 and 2000 amu. HRMS^2 scans were obtained with CID fragmentation, an isolation width of 2.0, normalized collision energy of 35, activation Q of 0.250, and an activation time of 30 ms. HPLC profiles of the RP18 eluted fractions have been reported in the **Supplementary Material** (**Figures S7–S13**).

2.6 LC-HRMS^2 Data Processing and Molecular Networking

LC-HRMS^2 data from RP18 eluted fractions were processed together to generate a unique molecular network, using a previously reported method (Della Sala et al., 2020). MS raw files were imported into MZmine 2.53 (Pluskal et al., 2010). Mass detection was performed on .mzXML data and centroided masses with mass level 1 and mass level 2, by keeping the noise level at 1000 and 100, respectively. The ADAP chromatogram algorithm was used to build chromatograms setting a minimum height of 1000 and *m/z* tolerance of 0.05 (or 20 ppm). As it regards chromatogram deconvolution, the baseline cut-off algorithm was employed with the following settings: minimum height peak = 1000, peak duration range = 0.0–10.0 min, baseline level = 100, *m/z* range for MS^2 scan = 0.05, retention time range = 0.5 min. Chromatogram peaks were aligned by using the Join aligner algorithm (*m/z* tolerance at 0.05 or 20 ppm, absolute RT tolerance at 0.5 min). [M+Na–H], [M+K–H], [M+Mg–2H], [M+NH_3], [M-Na+NH_4], [M+1, ^{13}C] adducts were filtered out by setting the maximum relative height at 100%. Peaks without associated MS^2 spectra were filtered out from the peak list.

Processed mass data were exported to .mgf file for GNPS and the relevant chromatographic information (retention times and peak areas) were exported to a .csv file in order to generate the molecular network displayed in **Figure 3**, by using the Feature Based Molecular Networking (FBMN) tool (Nothias et al., 2020). FBMN parameters were set as follows: precursor ion mass tolerance = 0.02, fragment ion mass tolerances = 0.1 Da, cosine score ≥ 0.7, minimum matched fragment ions = 4. The following GNPS databases have been selected for the spectral library search: CCMS_ProteomeDatabases, CCMS_School_2019, CCMS_SpectralLibraries, RMSV000000248, and speclibs. The molecular network was visualized and analyzed in Cytoscape version 3.7.2. Chromatographic data in the .csv file were mapped to the relevant nodes in the generated network (available at https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=85d850e022d043c29ec013db633c5e91, accessed on 27/10/2021). MS tandem spectra of all compounds reported in the molecular network can be found by accessing the GNPS link.

3 RESULTS

3.1 Morphological and Molecular Analysis of the Samples

The colonies showed morphological features peculiar to the genus *Botrylloides* Milne Edwards, 1841. The zooids were closely placed to each other, jointed anteriorly by elongated dorsal lip margins which created the typical zooids succession (**Figure 1**). The cloacal openings were present at the end of each cloacal canal, which were formed by several single atrial openings of each zooids, hence lacking atrial siphons. On the contrary, in the genus *Botryllus* Gaertner, 1774, the cloacal opening is formed by the jointed atrial siphons projections of each zooids which surround the opening, giving a star-shaped morphology which is typical of the genus (see Brunetti, 2009).

A 602 base pairs (bp) partial sequence of the COI gene was obtained from the five colonies, with all sequences resulting identical each other. They were deposited in GenBank under the single accession number OM866151. The sequences shared high similarity with 28 sequences deposited as *Botrylloides niger* (96.04–100%), including one of a specimen (NC_021467: 99.83%) from the eastern Mediterranean Sea (Achziv, Israel) formerly deposited as *Botrylloides* aff. *leachii* (Rubinstein et al., 2013; Griggio et al., 2014), that was also subsequently declared by Salonna et al. (2021) (**Table 1**) to be identical to specimens from Italy (Mar Piccolo, Taranto). However, it also showed high similarity with two sequences deposited as *Botryllus schlosseri* (Pallas, 1766) from India (KT693191: 100%; MH367291: 97.01%), and a single sequence (MG009579: 99.36%) deposited as *Botrylloides* aff. *leachii*, again from the Mediterranean coast of Israel (Reem et al., 2017). High similarities also include all worldwide sequences of *B. niger* deposited by Sheets et al. (2016), including two haplotypes also found in the eastern Mediterranean coast of Israel (COI-A: KU711782; COI-B: KU711783), the former of which perfectly matches (once trimmed to the same length) both HF548559/NC_021467 and our sequences. All the other taxa deposited in GenBank showed lower similarities (≤90.53%), thus excluding conspecificity. However, concerning the two mismatches listed above, there are solid morphological and molecular evidences to discriminate the two genera *Botrylloides* and *Botryllus*, suggesting that the two Indian specimens mentioned above were misidentified (see also Brunetti et al., 2017; Reem et al., 2018), whereas the paper by Reem et al. (2017) suffers of various taxonomic uncertainties, only partially solved by other authors (Viard et al., 2019; Nydam et al., 2021).

After trimming, the final alignment used for phylogenetic analyses consisted of 16 sequences of 529 bp (**Supplementary Data Sheet**). The ML ($-lnL$ = 1587.16) and BI ($-lnL$ = 1731.83 for run 1; $-lnL$ = 1731.78 for run 2) analyses produced congruent tree topologies (**Figure 2**). Sequences obtained in this study fell (bootstrap, bs = 92, posterior probabilities, pp = 1) within all the *B. niger* haplotypes found by Sheets et al. (2016) and colleagues, and in particular clustered within the haplotypes COI-A, COI-F, and COI-H, whereas the remaining haplotypes (COI-B–COI-E, COI-G) formed an internal clade, although with a low support (bs = 56, pp = 0.98). Present results further confirm the identification of our samples as *B. niger* and are in agreement with the low haplotype divergence reported by Sheets et al. (2016).

3.2 MS-Based Molecular Networking Analysis of *Botrylloides niger* Metabolome

A sample of the marine tunicate *B. niger* was extracted with MeOH to yield the crude organic extract, which was then purified using a reversed-phase column chromatography on RP-18 silica gel.

Aiming to gain a comprehensive metabolome analysis, RP-18 eluted fractions, rather than the whole organic extract, were analysed individually by LC-HRMS^2 as this approach allows to reduce the number of co-eluting metabolites and improve the quality of MS tandem spectra when untargeted fragmentation is used (Scarpato et al., 2020). After each full MS scan, the five most intense ions in the spectrum were fragmented in subsequent MS^2 scans to generate data for the construction of a unique molecular network (**Figure 3**), representative of all RP-18 fractions, by using the FBMN tool, available on the online platform GNPS (Nothias et al., 2020). Mass spectra were acquired in the positive ion detection mode (mass accuracy ≤ 3 ppm). Based upon similarity of the MS fragmentation patterns, FBMN allows to *i*) group molecules with a similar chemical architecture into molecular families (molecular clusters), *ii*) associate these clusters with compounds reported in public available databases, and *iii*) identify substructures within a given molecule through detection of molecular fragments shared with known metabolites, thereby providing useful hints for structural elucidation of compounds.

Merging molecular networking data with an in-depth investigation of MS^2 spectra, led to the structural prediction of almost 70 metabolites from the organic extract of *B. niger*, which were assigned to nine chemical classes, namely glycero phosphocholines, glycerophosphoethanolamines, glyco

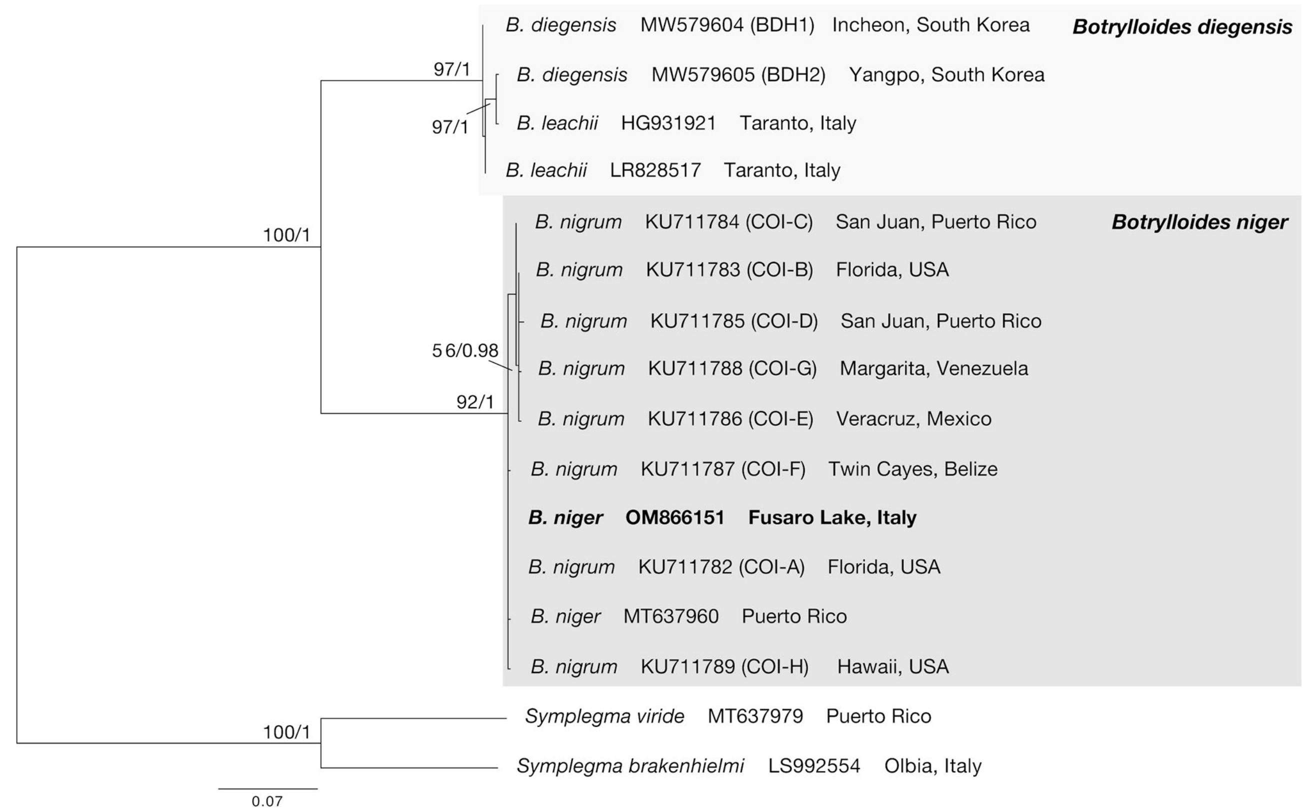

FIGURE 2 | COI phylogenetic tree obtained from the alignment of 16 terminals. Numbers above/below branches represent bootstrap values (bs) and bayesian posterior probabilities (pp). Specimens sequenced in this study highlighted in bold.

sphingolipids, sphingoid bases, sulfonolipids, fatty acids and derivatives, monoacylglycerols, indole alkaloids, and alkyl purines. In the network (**Figure 3**), the colour of each node is mapped to the relevant chemical class of the metabolite, and the size of the node is proportional to the spectral peak area of the metabolite for a visual relative quantification. Only six nodes could be annotated by comparison with the GNPS spectral database and are represented as diamonds in the network. Overall, most clusters have been shown to be related to glycerophospholipids, including lyso-glycerophosphocholines (*see* par 3.2.1.1), lyso-phosphatidylethanolamines (*see* par 3.2.1.2), and fatty acids and derivatives (*see* par 3.2.4). Nodes (highlighted in grey in the network) associated neither with known NPs nor with compounds predicted in this study, may indicate the presence of novel compounds, which deserve further studies to be isolated and structurally elucidated.

3.2.1 Glycerophospholipids

3.2.1.1 Lyso-Glycerophosphocholines (Lyso-GPCs)

Lyso-GPCs are a class of lipids featuring a glycerophosphocholine backbone, with one free hydroxyl function, at either the *sn*-1 or the *sn*-2 position. While the *sn*-2 position is usually esterified with a long chain fatty acid (monoacyl GPCs), the OH at the *sn*-1 position may bear either a long chain acyl (monoacyl GPCs) or alkyl/alkenyl group (monoalkyl/alkenyl GPCs).

The product ion spectra generated from the $[M+H]^+$ ions of lyso-glycerophosphocholines appeared to be dominated by the presence of a) the $[M+H-H_2O]^+$ fragment ion, derived from the neutral loss of a water molecule from the glycerol unit or the phosphate group and b) the diagnostic phosphocholine ion at *m/z* 184.0733 ($C_5H_{15}O_4NP^+$) (**Figures 4** and **S1A**). Additional ions giving structural information were of low abundance but still useful for structural characterization. Fragment ions arisen from trimethylamine loss $[M+H-59.0730]^+$ or sequential losses of water and trimethylamine $[M+H-H_2O-59.0730]^+$, were indicative of the choline moiety. Moreover, ions generated by fragmentation of the phosphocholine $[M+H-183.0655]^+$, revealed the acyl-, alkyl, or -alkenyl glycerol backbone (**Figure 4**).

In monoacyl GPCs, also known as lysophosphatidylcholines, fatty acyl substituents could be also indirectly inferred from the presence of the glycerylphosphorylcholine fragment ion at *m/z* 258.1101 ($C_8H_{21}O_6NP^+$) and the corresponding dehydrated ion, as resulting from losses of the fatty acid groups as ketene and carboxylic acid, respectively (**Figure 4**).

Among lyso-GPCs, putative octadecenoyl GPC and octadecenyl GPC hydroperoxides were identified, as revealed by a neutral loss of 34.0055 Da, arising from fragmentation of the hydroperoxy group (**Table 2**).

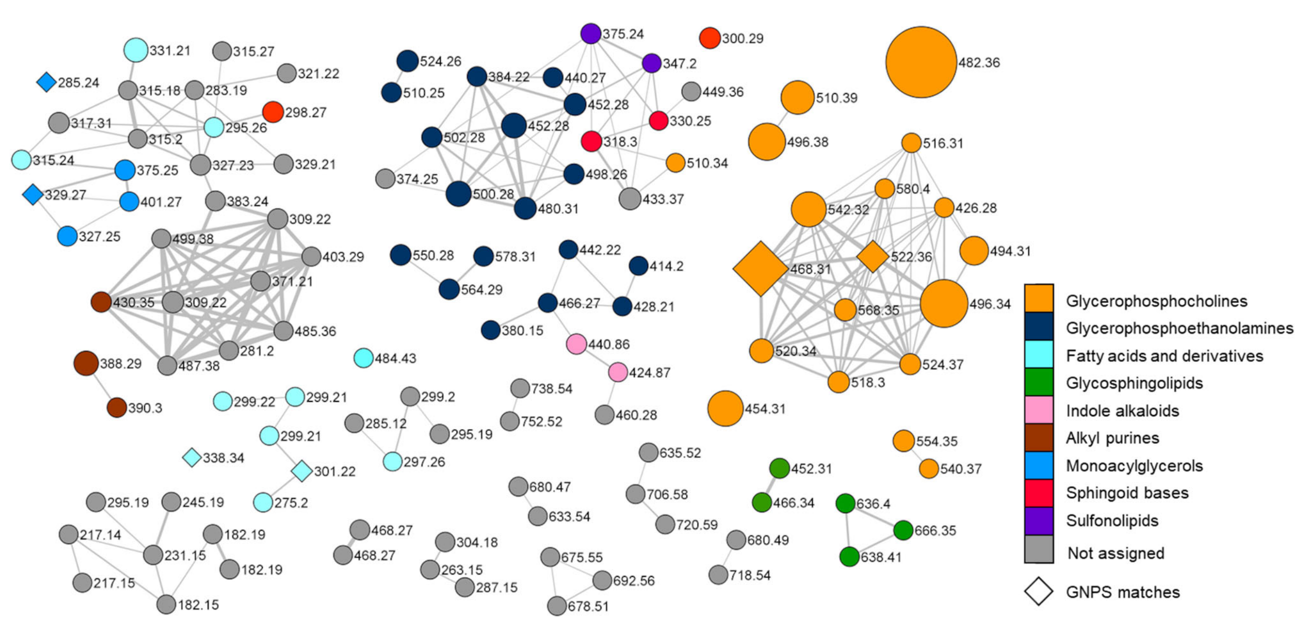

FIGURE 3 | Molecular network of the organic extract from *Botrylloides niger*. Nodes are labelled with parent mass and the colour of each node is mapped to chemical class assigned by integration of molecular networking data with extensive analysis of MS tandem spectra of the metabolites. Nodes annotated by the GNPS reference database are represented as diamonds. Edge thickness reflects cosine score similarity and node size is related to metabolite amounts (peak area).

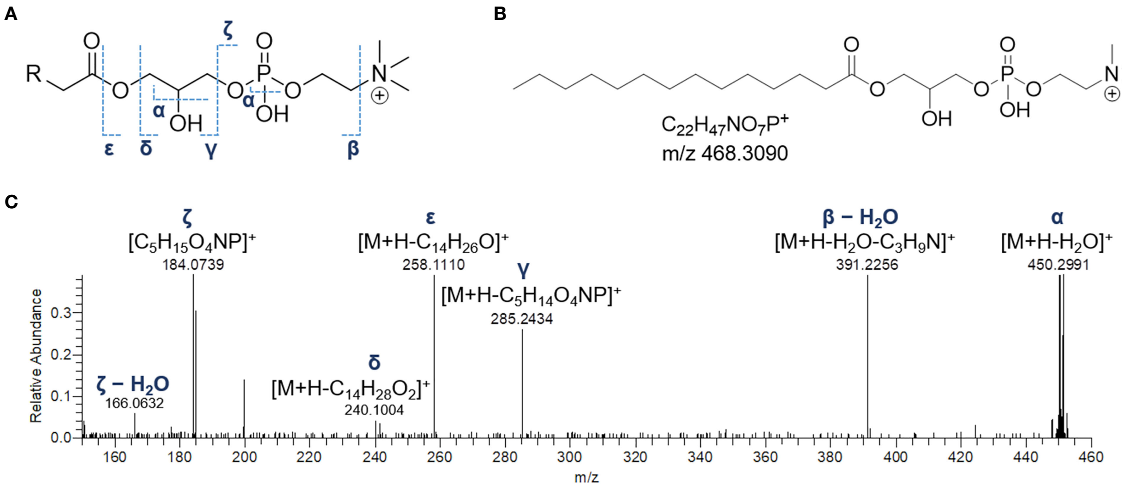

FIGURE 4 | The fragmentation patterns of lyso-glycerophosphocholines **(A)**. Chemical structure of a representative lyso-glycerophosphocholine from *Botrylloides niger*, namely 1-myristoyl-*sn*-glycero-3-phosphatidylcholine (LPC 14:0) **(B)** and HR ESI-MS^2 spectrum of the relevant $[M+H]^+$ adduct **(C)**.

3.2.1.2 Lyso-Phosphatidylethanolamines (Lyso-PEs)

Lyso-PEs, also known as monoacylglycerophosphoethanolamines (monoacyl PEs), have a glycerophosphoethanolamine moiety with a long chain fatty acid, usually located at the *sn*-1 position.

Mass tandem spectra of the $[M+H]^+$ ions of lyso-PEs displayed two abundant fragment ions, including a) the ion deriving from water loss, following the same pathway as for lyso-GPCs and b) the ion $[M+H-141.0191]^+$ generated by elimination of the phosphoethanolamine head group *via* the phosphoester bond cleavage (**Figure 5**). Rearrangement processes leading to formation of the fragment ions a) $[M+H-43.0422]^+$ following loss of aziridine (C_2H_5N), b) $[M+H-61.0528]^+$ following loss of ethanolamine (C_2H_7NO), c) $[M+H-59.0371]^+$, following loss of aminoacetaldehyde (C_2H_5NO) and d) $[M+H-97.9769]^+$ and $[M+H-172.0137]^+$ from internal losses of phosphoric acid (H_3PO_4) and glycerophosphophoric acid ($C_3H_9O_6P$), respectively,

TABLE 2 | Lyso-glycerophosphocholines identified in the organic extract from *Botrylloides niger*.

	Compound	R_t (min.)	$[M+H]^+$	m/z	Relative Abundance (%)
Monoacyl GPCs	LPC 11:0	21.1	$C_{19}H_{41}O_7NP$	426.2625	0.4
	LPC 16:1;O[a]	21.2	$C_{24}H_{49}O_8NP$	510.3199	0.8
	LPC18:4	25.5	$C_{26}H_{47}O_7NP$	516.3091	0.3
	LPC14:0	26.4	$C_{22}H_{47}O_7NP$	468.3091	16.2
	LPC 18:3	26.7	$C_{26}H_{49}O_7NP$	518.3250	1.2
	LPC 20:5	26.9	$C_{28}H_{49}O_7NP$	542.3249	7.1
	LPC 22:6	28.1	$C_{30}H_{51}O_7NP$	568.3407	1.4
	LPC 18:2	28.1	$C_{26}H_{51}O_7NP$	520.3404	2.3
	LPC 16:1	28.3	$C_{24}H_{49}O_7NP$	494.3249	4.3
	LPC 16:0	29.0	$C_{24}H_{51}O_7NP$	496.3405	12.7
	LPC 18:1	29.5	$C_{26}H_{53}O_7NP$	522.3562	6.0
	LPC 21:1;O[b]	30.4	$C_{29}H_{59}O_8NP$	580.3985	0.4
	LPC 18:0	30.9	$C_{26}H_{55}O_7NP$	524.3692	1.0
Monoalkyl GPCs	LPC O-14:0	27.8	$C_{22}H_{49}O_6NP$	454.32991	7.3
	LPC O-16:0	30.0	$C_{24}H_{53}O_6NP$	482.3610	22.9
	LPC O-17:0	31.1	$C_{25}H_{55}O_6NP$	496.3770	8.0
	LPC O-18:0	31.9	$C_{26}H_{57}O_6NP$	510.3926	6.4
Lyso-GPCs hydroperoxides	LPC 18:1;O2	24.1	$C_{26}H_{53}O_9NP$	554.3462	1.2
	LPC O-18:1;O2	25.3	$C_{26}H_{55}O_8NP$	540.3666	0.2

[a] *Monoacyl GPC with a hydroxyhexadecenoic acid as fatty acyl substituent.*

[b] *Monoacyl GPC with a putative oxidized heneicosanoic acid as fatty acyl substituent.*

LPC, lysoglicerophosphocholine.

Compounds are referred to by the LIPID MAPS abbreviations (Fahy et al., 2009).

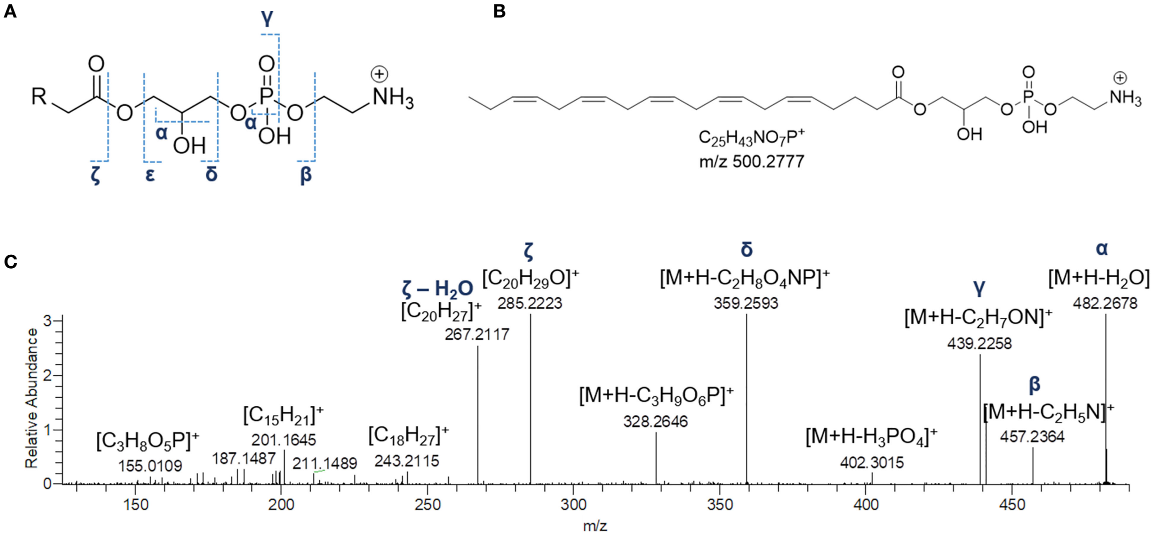

FIGURE 5 | The fragmentation patterns of lyso-phosphatidylethanolamines **(A)**. Chemical structure of a representative lyso-phosphatidylethanolamine from *Botrylloides niger*, namely 1-(5Z,8Z,11Z,14Z,17Z-eicosapentaenoyl)-glycero-3-phosphoethanolamine (LPE 20:5) **(B)** and HR ESI-MS^2 spectrum of the relevant $[M+H]^+$ adduct **(C)**.

were suggestive of the glycerophosphoethanolamine moiety and diagnostic of PEs (**Figure 5**) (Hsu and Turk, 2009). Differently from lyso-GPCs, acylium ions $[RCO]^+$, together with the relevant dehydrated ions, were clearly observed in product ion spectra of lyso-PEs and were useful to characterize the fatty acyl substituents. The fragment ion at *m/z* 198.0531 ($C_5H_{13}NO_5P^+$), arising from the elimination of the intact carboxylic acid, was another diagnostic ion in mass tandem spectra of the $[M+H]^+$ ions of lyso-PEs (**Figure S1B**). Notably, nine oxidized lyso-PEs could be detected in the organic extract of *B. niger*, featuring hydroxy and/or methoxy and/or oxo fatty acyl substituents (**Table 3**).

3.2.2 Sphingolipids

Dereplication of the organic extract of *B. niger* allowed the identification of nine compounds belonging to the sphingolipid class, including sphingoid bases (SPBs) and lyso-glycosphingolipids (**Table 4**).

Sharing similarity with previous reported ESI-MS tandem spectra (Shaner et al., 2009), the product ion spectrum of

TABLE 3 | Lyso-phosphatidylethanolamines identified in the organic extract from *Botrylloides niger*.

	Compound	R_t (min.)	$[M+H]^+$	m/z	Relative Abundance (%)
Monoacyl PEs	LPE11:0	21.2	$C_{16}H_{35}O_7NP$	384.2155	2.7
	LPE 20:6	22.7	$C_{25}H_{41}O_7NP$	498.2623	4.8
	LPE 20:5	26.9	$C_{25}H_{43}O_7NP$	500.2779	19.2
	LPE 16:1 - *Z* isomer[a]	27.2	$C_{21}H_{43}O_7NP$	452.2781	9.7
	LPE 15:0	27.4	$C_{20}H_{43}O_7NP$	440.278	3.3
	LPE 20:4	28.1	$C_{25}H_{45}O_7NP$	502.2935	4.7
	LPE 16:1 - *E* isomer[a]	28.3	$C_{21}H_{43}O_7NP$	452.2781	18.2
	LPE 18:1	29.5	$C_{23}H_{47}O_7NP$	480.3092	2.7
Oxidized Monoacyl PEs	LPE 10:3;O	1.9	$C_{15}H_{27}O_8NP$	380.1477	9.7
	LPE 11:1;O2	11.1	$C_{16}H_{33}O_9NP$	414.1896	0.4
	LPE 12:1;O2	14.5	$C_{17}H_{35}O_9NP$	428.2054	0.6
	LPE 20:4;O3	15.6	$C_{25}H_{45}O_{10}NP$	550.2785	1.0
	LPE 17:3;O3	16.3	$C_{22}H_{41}O_{10}NP$	510.2470	5.6
	LPE 13:1;O2	17.1	$C_{18}H_{37}O_9NP$	442.2208	3.3
	LPE 21:4;O3	18	$C_{26}H_{47}O_{10}NP$	564.2942	0.1
	LPE 18:3;O3	18.5	$C_{23}H_{43}O_{10}NP$	524.2624	4.8
	LPE 22:4;O3	19.8	$C_{27}H_{49}O_{10}NP$	578.3098	8.2
	LPE 16:1;O	20.3	$C_{21}H_{41}O_8NP$	466.2567	2.9

[a] Isomers were identified based upon retention times as reported by Creer and Gross, 1985.

LPE, lysophosphatidylethanolamine.

Compounds are referred to by the LIPID MAPS abbreviations (Fahy et al., 2009).

TABLE 4 | Sphingolipids identified in the organic extract from *Botrylloides niger*.

	Compound	R_t (min.)	$[M+H]^+$	m/z	Relative Abundance (%)
Sphingoid bases	SPB 18:2;O4	13.1	$C_{18}H_{36}O_4N$	330.2646	1.4
	sphingadienine or SPB 18:2;O2	25.0	$C_{18}H_{36}O_2N$	298.2747	22.7
	hydroxysphinganine or SPB 18:0;O3	25.8	$C_{18}H_{40}O_3N$	318.3009	18.0
	sphingosine or SPB 18:1;O2	26.2	$C_{18}H_{38}O_2N$	300.2904	22.8
Lyso-GSLs	dihexosyl-SPB 18:3;O4	17.8	$C_{31}H_{56}O_{14}N$	666.3708	5.8
	hexosyl-C16 hydroxysphinganine	22.0	$C_{22}H_{46}O_8N$	452.3226	12.0
	dihexosyl-sphyngadienine	23.4	$C_{31}H_{58}O_{12}N$	636.3965	3.9
	hexosyl-C17 hydroxysphinganine	23.5	$C_{23}H_{48}O_8N$	466.3382	5.9
	dihexosyl-sphyngosine	24.6	$C_{31}H_{60}O_{12}N$	638.4123	7.5

SPB, sphingoid base.

sphingosine (SPB 18:2; O2) unveiled the presence of fragments arising from losses of water, formaldehyde, water and ammonia, water and formaldehyde, and 2 water molecules and ammonia, together with minor rearrangement ions (**Figure 6A**). The fragmentation patterns of sphingadienine (SPB 18:2; O2) and hydroxysphinganine (SPB 18:0; O3) were quite similar to that of sphingosine, with the protonated hydroxysphinganine displaying an additional elimination of water, as expected. Interestingly, a putative novel sphingoid base, corresponding to the molecular formula $C_{18}H_{35}O_4N$, was detected. As sharing almost the same fragmentation pathways with SPBs and clustering together with hydroxysphinganine in the molecular network (**Figure 3**), this compound (SPB 18:2; O4), has been tentatively identified as an oxidized analogue of hydroxysphinganine, featuring two degrees of unsaturation.

Lyso-glycosphingolipids (lyso-GSLs) are SPBs with the primary OH group linked to a saccharidic head group (usually made up of glucose and/or galactose monomers) *via* a glycosidic bond, but lacking the N-acyl substituent as compared to intact glycosphingolipids (Merrill, 2011). Structural prediction of lyso-GSLs from *B. niger* led to the detection of two monohexosyl and three dihexosyl lyso-GSLs. Overall, the tandem mass spectra of lyso-GSL adducts contained fragment ions reflecting the long chain sphingoid base and the sugar constituents of the molecules, arising primarily from the glycosidic bond cleavage.

Fragmentations γ, δ, and ϵ indicated in **Figure 6B** permitted to assemble unequivocally the structures of the two monohexyl lyso-GSLs, which were shown to differ from each other as bearing a C16 and a C17 hydroxysphinganine as long chain base, respectively. In addition, the presence of the sugar unit was also suggested by fragment ions generated by a typical retro-Diels-Alder mechanism (**Figure S2**) involved in the fragmentation of the sugar moiety in glycosylated natural products (Demarque et al., 2016).

MS/MS spectra of the three dihexosyl lyso-GSLs displayed sequential losses of $C_7H_{12}O_5$ (176.0679 Da) and $C_6H_{10}O_5$ (162.0523 Da), which were consistent with a disaccharide unit composed of a putative O-methylated hexosyl starter unit linked to a hexose ring (**Figure 6C**). As shown in **Table 4**, the dihexosyl lyso-GSLs differ in degree of unsaturation and oxidation of the C18 sphingoid base.

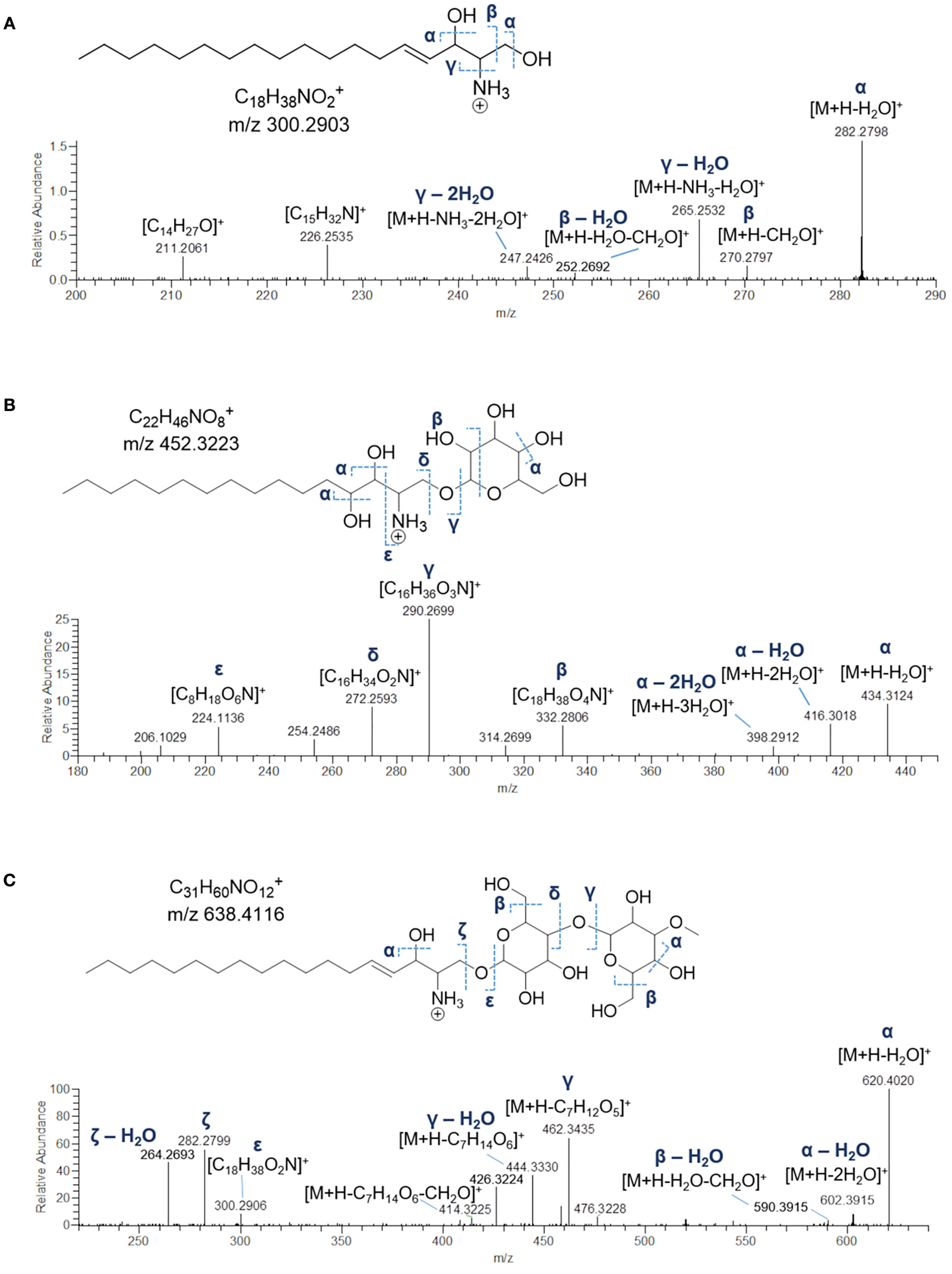

FIGURE 6 | Fragmentation patterns and HR ESI-MS^2 spectra of $[M+H]^+$ ions of sphingosine (SPB 18:1;O2) **(A)**, hexosyl-C16 hydroxysphinganine **(B)** and dihexosyl-sphyngosine **(C)** from *Botrylloides niger*.

3.2.3 Sulfonolipids

The crude extract of *B. niger* was shown to contain two sulfonolipids, as indicated by a) high-resolution (HR) ESI-MS spectra of their sodium adducts showing 4% intense M + 1.9957 isotope peaks suggestive of a sulphur atom and accounting for the molecular formula annotated in **Table 5** and b) MS tandem spectra unveiling fragments arising from neutral losses of sulfuric and sulfurous acids. Molecular formula as well as HR MS^2 spectrum of the 352-Da sulfonolipid (**Figure 7A**) were consistent with the structure of IOR-1 (Woznica et al., 2016), composed of a sulfonic acid head group and a C17 branched alkyl chain bearing two hydroxy groups. Acquisition of negative HR ESI-MS spectra provided more informative clues to confirm the identity of IOR-1, as displaying fragment ions at *m/z*

TABLE 5 | Sulfonolipids identified in the organic extract from *Botrylloides niger*.

	Compound	R_t (min.)	$[M+Na]^+$ (m/z)	$[M-H]^-$ (m/z)	Relative Abundance (%)
Sulfonolipids	C15 IOR-1	24.9	$C_{15}H_{32}O_5NaS$ (347.1870)	$C_{15}H_{31}O_5S$ (323.1865)	9.0
	IOR-1	27.2	$C_{17}H_{36}O_5NaS$ (375.2183)	$C_{17}H_{35}O_5S$ (351.2177)	91.0

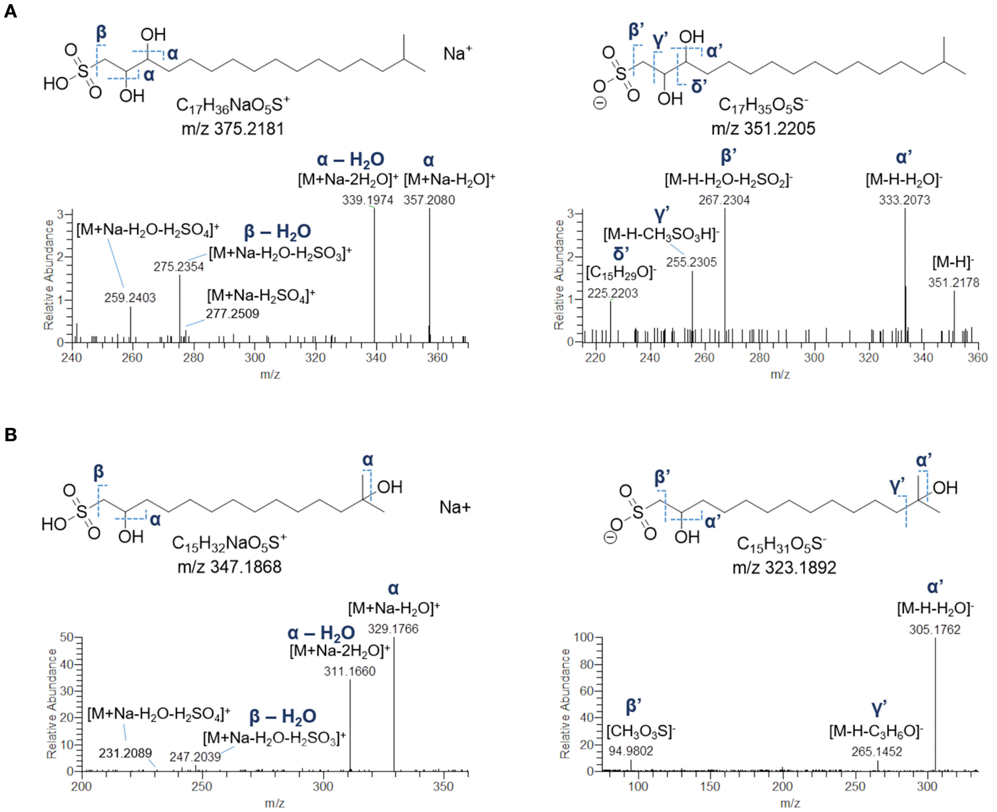

FIGURE 7 | HR ESI-MS^2 spectrum of the $[M+Na]^+$ (left panel) and $[M-H]^-$ (right panel) ions of the putative sulfonolipid from *Botrylloides niger* annotated as IOR-1 **(A)**. Predicted structure and HR ESI-MS^2 spectrum of the $[M+Na]^+$ (left panel) and $[M-H]^-$ (right panel) ions of the putative sulfonolipid C15 IOR-1 **(B)**.

255.2305 and *m/z* 225.2203 arising from the neutral losses of methanesulfonic and hydroxyethanesulfonic acids, respectively (**Figures 7A** and **S3**). In the light of these findings, the 324-Da sulfonolipid, which was detected for the first time to the best of our knowledge, was tentatively identified as an inferior homologue of IOR-1, featuring a C15 alkyl chain and, therefore, indicated as C15 IOR-1 (**Figure 7B**). While the positive MS^2 spectra were similar for both sulfonolipids, fragmentation patterns in the negative ion mode were somehow different. The ESI-MS^2 spectrum of the $[M-H]^-$ ion of C15 IOR-1 unveiled the presence of the methanesulfonate fragment anion, which was consistent with a favoured fragmentation assisted by a β-hydroxy group and, therefore, indicative of the same hydroxyethylsulfonate head group as in IOR-1. However, a) the fragmentation leading to neutral loss of acetone (**Figures 7B** and **S3**) as well as b) the lack of a fragment ion homologue to the δ' fragment in IOR-1 (**Figure 7A**), imply a different position for the second OH group in C15 IOR-1, which was predicted to be located on the terminal isopropyl group as shown in **Figure 7B**.

3.2.4 Monoacylglycerols and Fatty Acids and Derivatives

Monoacylglycerols (MGs) are glycerol esters, in which one hydroxy function is esterified with a long chain fatty acid, at the *sn*-1 (or *sn*-3) or the *sn*-2 position. However, it is widely recognised that 2-monoacylglycerols may undergo spontaneous isomerization to the corresponding 1-(or 3-) monoacyl isomers. Molecular networking analysis of MS^2 data from the extract of *B. niger* led to the annotation of 5 monoacylglycerols (**Table 6**). Fragmentation spectra of the pseudomolecular ion $[M+H]^+$of MGs was dominated by the acylium ion $[RCO]^+$ resulting from neutral loss of glycerol ($C_3H_8O_3$, 92.0473 Da), due to inductive cleavage of the ester bond assisted by the adjacent carbonyl function (**Figure 8A**). Moreover, the carbonyl group likely removes a proton from the terminal primary OH, thereby facilitating elimination of a lactone ring and formation of another diagnostic fragment, i.e. $[M+H-C_3H_6O_2]^+$ (**Figure S3**). MS/MS spectra of MGs were also characterized by the presence of the dehydrated ions of $[M+H]^+$and $[M+H-C_3H_8O_3]^+$ fragments (**Figure 8A**). Notably, two MGs from *B. niger* displayed unusual polyunsaturated fatty acyl substituents, i.e. putative docosaheptaenoic and eicosahexaenoic acids (**Table 6**).

Eleven nodes in the molecular network (**Figure 3**) were annotated as fatty acids and derivatives, including a) a small cluster of five PUFAs, b) three fatty acid methyl ester derivatives (putative artefacts originating during extraction with methanol), c) 13*Z*-docosenamide (AM 22:1), and d) docosanoylcarnitine (CAR 22:0) (**Table 6**). Among these compounds, the 11-hydroxy-5*Z*,8*Z*,12*E*,14*Z*,17*Z*-eicosapentaenoic acid (FA 20:5;O), AM 22:1, and CAR 22:0 were identified by comparing their mass spectra with the GNPS spectral database (**Figures 8B–D**). Overall, the diagnostic product ion observed in MS/MS spectra of $[M+H]^+$ adducts of PUFA (and $[M+H-H_2O]^+$for OH-PUFA) was the acylium ion, which in turn underwent water loss during fragmentation (**Figure 8B**). A series of unsaturated hydrocarbon ions with lower intensities occurred during ESI MS/MS of PUFA, due to extensive hydride shifts, thereby hampering unambiguous localization of the double bonds (**Figure 8B**). As it regards fatty acid methyl esters (FAMEs), mass fragmentation showed $[M+H-32.0262]^+$ as the base peak, corresponding to the ion arising from the loss of CH_3OH.

3.2.5 Indole and Purine Alkaloids

The HR ESI mass spectra of the $[M+H]^+$ ion peaks at *m/z* 422.8711 (R_t = 10.9 min) and *m/z* 438.8659 (R_t = 12.1 min) defined the molecular formulas of these metabolites as $C_{12}H_{13}Br_3N_2$ and $C_{12}H_{13}Br_3N_2O$, respectively, as suggested by the observed isotope pattern peculiar of tribrominated compounds, showing four peaks (each separated by two mass units) of approximate intensity 1:4:4:1. In the light of these findings, the 422 Da- and 438-Da compounds were identified as 2,5,6-tribromo-1-methylgramine and the relevant *N*-oxide derivative, as their MS/MS spectra were exactly the same and characterized by the fragment ion $[C_{10}H_7Br_3]^+$, generated by elimination of dimethylamine (45.0578 Da) and dimethylhydroxylamine (61.0528 Da), respectively (**Figure 9**). In addition, the fragment $[C_{10}H_7NBr_3]^+$ undergoes a) loss of a radical bromine and b) loss of molecular bromine, thus giving the $[C_{10}H_7NBr_2]^{\bullet+}$ radical cation at *m/z* 298.8950 and the $[C_{10}H_7NBr]^+$ ion at *m/z* 219.9763, likely due to homolytic cleavage of carbon-bromine bonds (radical fragmentation) (**Figures 9** and **S4**). Even if being unusual in CID (collision-induced dissociation)-type fragmentations, generation of odd-electron species may occur when a) unpaired electrons can occupy a delocalized antibonding orbital and b) chemical bonds are hard to be cleaved (Levsen et al., 2007), such in the case of the high conjugated indole ring in gramine alkaloids.

Finally, three nodes in the network appeared to be related to unknown alkyl purine alkaloids (**Figure S6**). Analysis of the mass tandem spectra of these molecules unveiled a diagnostic fragment at *m/z* 150.0780 ($C_6H_8N_5^+$) corresponding to the methyladenine ion, together with its related fragments deriving from NH_3 and HCN losses (**Figure S6**). Therefore, these metabolites were predicted to be methyladenine derivatives bearing different alkyl/acyl substituents. However, their structures remain unsolved and require further studies to be elucidated.

4 DISCUSSION

Human-mediated introduction of alien species in new biogeographic realms is a phenomenon that dates back centuries (Branch and Steffani, 2004; Provan et al., 2008). However, this seems to be furthermore amplified in the recent decades, with many ascidians spreading worldwide and interfering with native

TABLE 6 | Monoacylglycerols and fatty acids and derivatives identified in the organic extract from *B. niger*.

	Compound	R_t (min.)	$[M+H]^+$	m/z	Relative Abundance (%)
Monoacylglycerols	MG 16:2	23.0	$C_{19}H_{35}O_4$	327.2537	6.7
	MG 20:6	23.9	$C_{23}H_{35}O_4$	375.2539	10.3
	MG 22:7	25.3	$C_{25}H_{37}O_4$	401.2695	2.2
	MG 14:0	27.9	$C_{17}H_{33}O_3$ [a]	285.2431	5.5
	MG 16:1	28.6	$C_{19}H_{37}O_4$	329.2693	13.9
Fatty acids	FA 20:7	19.8	$C_{20}H_{27}O_2$ [b]	299.2013	<0.1
	FA 20:7	20.3	$C_{20}H_{27}O_2$ [b]	299.2013	<0.1
	FA 20:7	21.0	$C_{20}H_{27}O_2$ [b]	299.2013	<0.1
	FA18:4;O	23.3	$C_{18}H_{27}O_2$ [a]	275.2012	1.8
	FA 20:5;O	25.2	$C_{20}H_{29}O_2$ [a]	301.2169	16.9
	FA 18:2;O	26.3	$C_{18}H_{33}O_3$	297.2432	<0.1
Fatty esters	FAME 20:6;O	22.9	$C_{21}H_{31}O_3$	331.2275	33.1
	FAME 20:6	27.4	$C_{21}H_{31}O_2$	315.2325	<0.1
	FAME 18:2	28.8	$C_{19}H_{35}O_2$	295.2639	6.1
	CAR 22:0	32.1	$C_{29}H_{58}O_4N$	484.4368	2.7
Fatty amides	AM 22:1	33.3	$C_{22}H_{44}ON$	338.3428	0.7

[a] *$[M+H-H_2O]^+$.*

[b] *Putative isomers differing for unsaturation position/configuration.*

MG, monoacylglycerol; FA, fatty acid; FAME, fatty acid methyl ester; CAR, fatty acyl carnitine; AM, fatty amide.

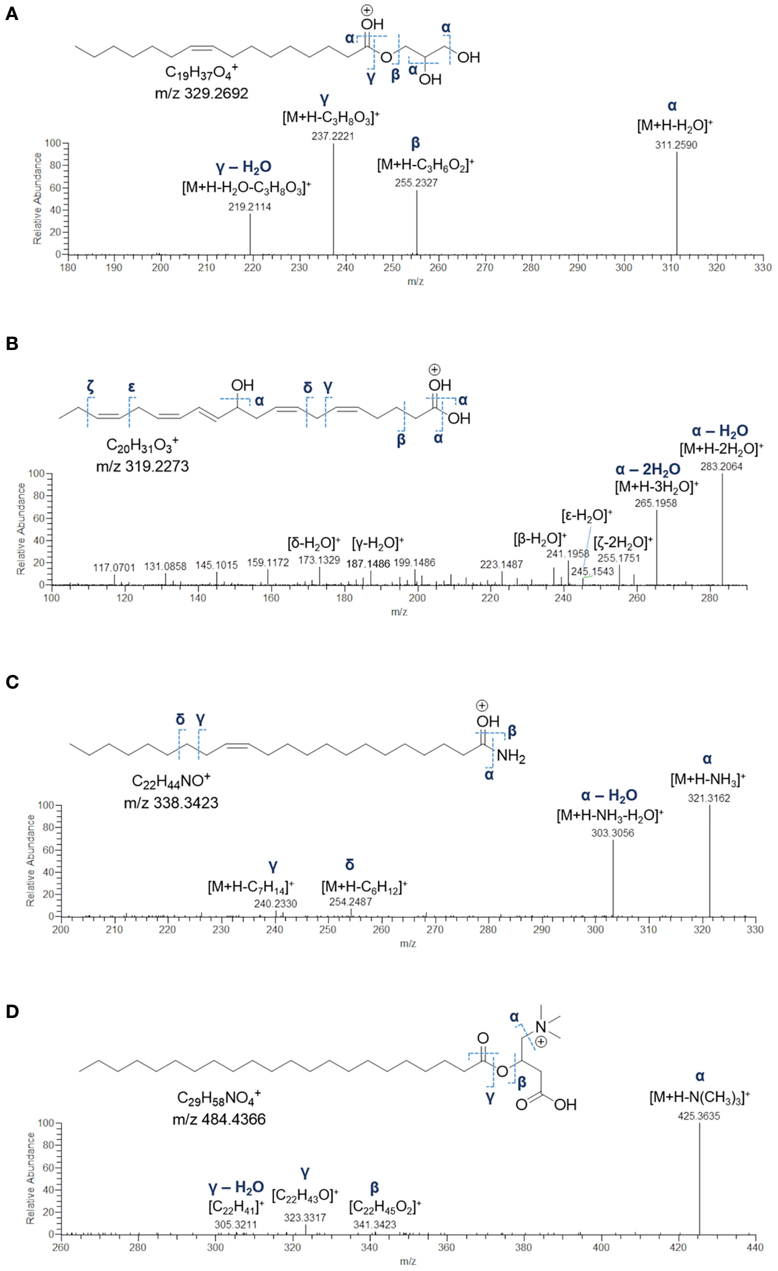

FIGURE 8 | Fragmentation patterns and HR ESI-MS^2 spectrum of the $[M+H]^+$ ion of 1-(9*Z*-octadecenoyl)-*sn*-glycerol (MG 16:1) **(A)**, the $[M+H-H_2O]^+$ ion of 11-hydroxy-5*Z*,8*Z*,12*E*,14*Z*,17*Z*-eicosapentaenoic acid (FA 20:5;O) **(B)**, the $[M+H]^+$ ion of 13*Z*-docosenamide (AM 22:1) **(C)**, and the $[M+H]^+$ ion of the docosanoylcarnitine (CAR 22:0) **(D)**.

benthic communities and habitats by creating consistent environmental and even economic damages (Lambert and Lambert, 1998; Zhan et al., 2015). In the present case, when *B. niger* was locally discovered, it already formed large aggregates in the investigated channel, and its presence was also noted in additional lagoons of the area (Virgili et al., 2022). This suggests that the early eradication phase, often highlighted to limit the spread of alien species (Willan et al., 2000; Giakoumi et al., 2019), is already far to be potentially applied, and that local communities are presumably enriched by the presence of *B. niger* since at least years. Absence of field studies but mostly taxonomic impediments may be at the basis of such a result. Indeed, contrary to other localities where the presence of *B. niger* is well acknowledged (Rocha et al., 2012; Sheets et al., 2016; Streit et al., 2021), the arrival of this colonial ascidian in the area, but in general in the Mediterranean Sea, was presumably overlooked due to rarefaction of studies in zoology and confusion with the congeneric species *B. leachii* (see Griggio et al., 2014; Virgili et al., 2022). No certainties also occur

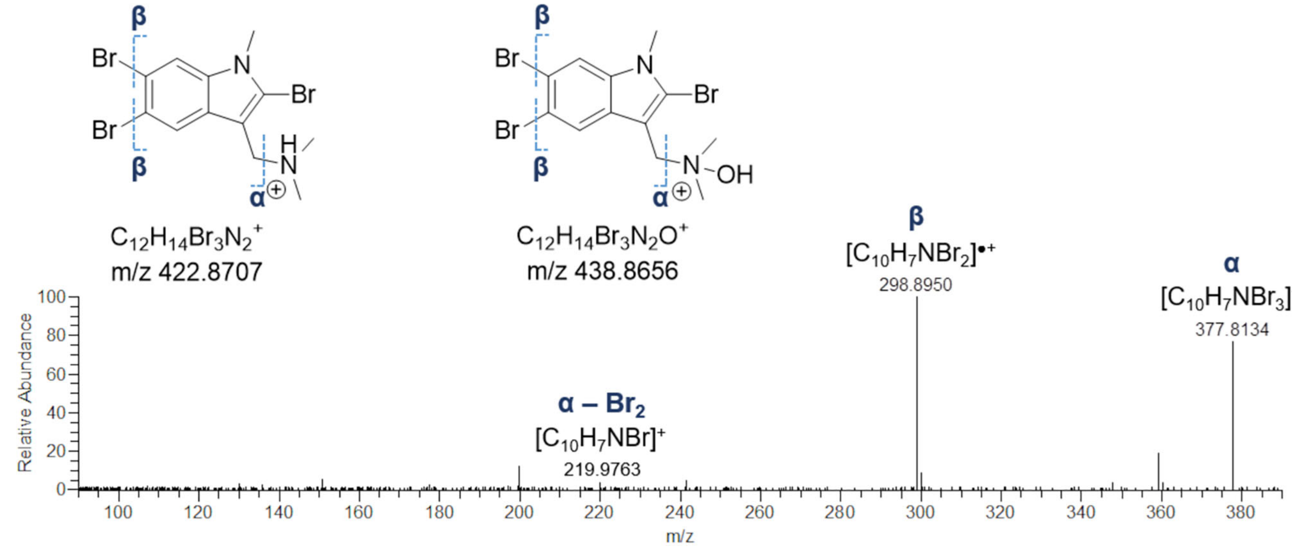

FIGURE 9 | HR ESI-MS^2 spectrum of the fragment ion at *m/z* 377.8134, generated by neutral loss of dimethylamine and dimethylhydroxylamine from 2,5,6-tribromo-1-methylgramine and 2,5,6-tribromo-1-methylgramine *N*-oxide, respectively.

regarding a possible pathway of arrival in the area. The Fusaro Lake is already known as a hub for the introduction of alien species of different phyla (e.g. Bianchi, 1983; Villani and Martinez, 1993; Crocetta et al., 2012; Crocetta et al., 2013; Hanson et al., 2013), and it also hosts a conspicuous mussel farm that may constitute the most likely source of introduction for this species in the lake. This seems to be in agreement with other studies worldwide, that suggested a species spread at a small scale based on recruitment preferences for mussel beds (Sheets et al., 2016; Rocha et al., 2019). However, another possibility is that this species is spreading locally favoured by the natural currents that exchange the lake waters through the channel, and most likely coming from the nearby Miseno Lake, an area recently acknowledged as a hotspot of non-indigenous species (NIS) ascidians, including *B. niger* (Virgili et al., 2022).

Although the dominance observed during sampling activities is worrying from an ecological point of view, our results confirm the interest of these alien species as they represent today a potential huge biological resource for nutritional or functional purposes, reducing, in this way, the economic losses caused by these invasive organisms.

Recent studies have shown that ascidians, in particular parts of their inner body tissues, are used to flavour foods intended for human consumption. In fact, all inner body tissues are rich in proteins, mainly collagens with a high essential amino acid index and high delicious amino acid (DAA) content. Moreover, they can supply the body with essential lipid components, including high contents of good-quality fatty acids (Zhao and Li, 2016). Lipids are a class of biomolecules involved in a huge number of different functions in biological systems, which make them fundamental for development and growth, and act as powerful signalling agents during metabolic disruption due to diseases, such as neurological disorders, autoimmune diseases, and cancer (Wymann and Schneiter, 2008; Cheng et al., 2016; Brown et al., 2017).

In this work, the employment of the Molecular Networking approach followed by a careful manual curation of HR ESI-MS^2 data allowed us to study the metabolome of the alien species *B. niger* for the first time, and shed light on the presence of novel NPs, several of them remaining still unknown. FBMN was shown to be an efficient tool for fast dereplication of complex organic mixtures and support LC-MS^2 data analysis for high-confidence structural prediction of detected metabolites.

Our results showed the presence of different classes of indispensable lipids, namely glycerophospholipids, glycerolipids, sphingolipids, fatty acids and derivatives, and sulfonolipids.

Among them, glycerophospholipids were the most represented lipid class, including lyso-glycerophosphocholines and lyso-phosphatidylethanolamines.

Similarly, Hou et al. (2021) recently reported the characterization of the lipid profile of *C. intestinalis*, *H. roretzi*, and *S. clava*, and, among the thirteen major lipid subclasses identified, glycerophospholipids and glycerolipids were the dominant components (66.30–90.60% of total lipids).

Lyso-GPCs and lyso-PEs are representatives of a class of mono-acylated/alkylated glycerophospholipids, commonly referred to as lysophospholipids, playing a key role as structural lipid constituents of cellular membranes and biological signalling molecules in eukaryotes and bacteria, including marine species. Lysophospholipids may regulate fundamental cellular functions such as cell growth, differentiation, survival, migration, adhesion, invasion, and morphogenesis, by interacting with their cognate receptors and/or modelling composition and fluidity of lipid rafts. Indeed, lysophospholipids have been reported to regulate cell motility in budding tunicates (Arai et al., 2004) as well as to enhance multicellular development in the choanoflagellate

Salpingoeca rosetta Dayel et al., 2011 (Woznica et al., 2016). In the latter case, lyso-PEs and the sulfonolipids RIFs and IOR-1 are produced by the choanoflagellate endosymbiont *Algoriphagus machipongonensis* Alegado et al., 2013 and proposed as actors of a metabolic interplay in which multiple bacterial cues regulate the cellular growth of *S. rosetta*. Considering that a) lysophospholipids and sulfonolipids (IOR-1 and C15 IOR-1) co-occur in the organic extract of *B. niger* and that b) bacteria live in symbiosis with several *Botrylloides* species, the notion that a network of bacterial lipids regulate multicellular development and cell motility can be extended to *B. niger*, which is expected to orchestrate a complex signalling network to control seasonal dynamics of its blooms (Rinkevich et al., 1993; Oricchio and Muniz Dias, 2020; Ramalhosa et al., 2021).

To date, there has been a growing interest in studying the biological functions and metabolism of lysophospholipids, because they could represent a good starting point for the development of new therapeutics for several diseases (Varandas et al., 2019). Indeed, these molecules can affect many biological processes, such as neurogenesis, angiogenesis, wound healing, immunity, and carcinogenesis (Ishii et al., 2004).

Noteworthy, our results showed that *B. niger* contains also lyso-GPCs and lyso-PEs bearing PUFAs [including eicosapentaenoic acid (C20:5n-3, EPA), and docosahexaenoic acid (C22:6n-3, DHA)], which accounted for the 40% of the detected lyso-glycerophospholipids. Interestingly, glycerophospholipids enriched in PUFAs were also mainly present in the inner body tissues of different ascidians, indicating that these marine organisms could potentially be used for health-promoting food for humans (Hou et al., 2021). Marine sources containing EPA/DHA-enriched glycerophospholipids are receiving increasing attention thanks to their emerging health benefit (as reviewed in Zhang et al., 2019 and Ahmmed et al., 2020). Zhou and collaborators showed that different fatty acids composition of glycerophospholipids were involved in decreasing cognitive decline and biological damage and in brain protection, and these beneficial effects were partly enhanced in presence of EPA and DHA (Zhou et al., 2016).

In addition, unusual PUFAs (**Table 6**) either linked to the glycerol backbone in monoacylglycerols or as free fatty acids and methyl ester derivatives, were also identified by molecular networking analysis of tandem MS data from *B. niger* crude extract. In tunicates unsaturated fatty acids, including PUFAs, have been shown to exert mitogenic activity and promote cell proliferation (Arai et al., 2004). Moreover, PUFAs have attracted great attention due to their enormous benefits. They can reduce or prevent the severity of several diseases, such as hyperlipidemia, diabetes, cancers, inflammation and heart and neurodegenerative diseases (reviewed in Zhang et al., 2019).

Therefore, similarly to other marine organisms, *B. niger* could potentially be used for health-promoting food for humans.

Dereplication of the organic extract from *B. niger* shed light on the presence of lyso-glycosphingolipids and free sphingoid bases. Over the years, several sphingolipids have been identified from natural sources, including microorganisms, tunicates, sponges, corals and algae. In the past, they were merely considered as components of cellular membranes, but they demonstrated to be involved in several cellular phases (Morales et al., 2007; Ponnusamy et al., 2010), and to be effective as antiproliferative drugs against different tumors (Schmelz et al., 2000; García-Barros et al., 2014; Rethna Priya et al., 2019).

Besides the presence of different lipid classes, our findings unveiled *B. niger* to be a source of indole and purine alkaloids. In fact, ascidians are prolific sources of nitrogenated metabolites, and more than 300 alkaloids have been reported from these organisms (Nathani et al., 2020) till now. Noteworthy, many of these have been shown to have important activities, including antimicrobial, anticancer, and antiviral, whereby several therapeutics have been isolated or inspired from tunicates-derived NPs.

Among the 12 marine-derived NPs approved by the FDA to date, 2 molecules are native to tunicates, including the alkaloid ecteinascidine (Yondelis®) from *E. turbinata* and its synthetic derivative Lurbinectedin (Zepsyre®) (Phase III) used to treat different types of cancer (Della Sala et al., 2018; McCauley et al., 2020; Ramesh et al., 2021).

Particularly, two brominated indole derivatives were identified in *B. niger* and predicted as 2,5,6-tribromo-1-methylgramine and its *N*-oxide analogue. Even if ascidians have been largely acknowledged as a reservoir of brominated alkaloids, these two metabolites have never been described in tunicates before, as being only isolated from the marine bryozoan *Amathia verticillata* (delle Chiaje, 1822). Particularly, 2,5,6-tribromo-1-methylgramine was found to exert antifouling activity against the barnacle *Amphibalanus amphitrite* (Darwin, 1854) and the blue mussel *Mytilus edulis* Linnaeus, 1758 (Sato and Fenical, 1983; Kon-Ya et al., 1994). In addition, three novel purine alkaloids, i.e. methyladenine derivatives, were found in the metabolome of *B. niger*, thus their structures remaining unsolved. Alkyl purines as well as brominated indoles play a key role in the antifouling mechanism of marine organisms. Detection of such bioactive molecules could at least partially explain the invasiveness of *B. niger*, which is able to prevent larvae of other marine organisms from settlement and growing on its bodies (Hiebert et al., 2019).

Indeed, by competing for space and food, a congener of *B. niger*, namely *Botrylloides violaceus* Oka, 1927, is known to displace other fouling organisms (Berman et al., 1992), including native and introduced tunicates (Dijkstra et al., 2007), bryozoans, barnacles, and mussels (Dijkstra and Harris, 2009), indicating strong competitive ability (Lambert and Lambert, 2003).

In conclusion, the alien species *B. niger* represents a prolific source of several valuable bioactive compounds, probably developed to adapt and compete with native species. It is also rich of a huge variety of lipid species that improve human health, including lipids rarely found in other foods as PUFAs. The presence of these molecules can undoubtedly transform this alien species from damage to the ecosystem into a precious bio-resource, easily available. Therefore, *B. niger* together with other invasive ascidians, although concerned from an ecological point of view, could find an important economic role to benefit society, including good opportunities in the food and pharmaceutical industry for the development of functional products based on ascidians.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

GDS, DC, and FC designed and directed the research. GDS, DC, RV, GV, VT, RT, and FC performed the experiments and analysed the data. DdP proceeded to funding acquisition. GDS, DC, and FC wrote the manuscript. All authors contributed to the editing and revision of the manuscript, and read and approved the final manuscript. All authors agree to be accountable for the content of the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.865751/full#supplementary-material

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The Miseno Lake (Central-Western Mediterranean Sea): An Overlooked Reservoir of Non-Indigenous and Cryptogenic Ascidians in a Marine Reserve

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Ascidians are the largest and most diverse class of the subphylum Tunicata, and are important components of marine benthic communities. They are also renowned worldwide invaders, of growing concern due to ease of spread and impacts on native biota. We hereby combined bathymetric and habitat mapping, extensive transect and photo-quadrat sampling, and a morphological and molecular study on selected specimens to evaluate taxonomic composition, cover, and distribution pattern of ascidians in a marine reserve of the central-western Mediterranean Sea, the Miseno Lake (Bacoli, Naples, Italy). Twenty-four taxa were found, of which 9 were non-indigenous species (NIS), 5 cryptogenic, and 10 native, delineating the Miseno Lake as a major hotspot of NIS and cryptogenic ascidians. DNA barcoding yielded 114 sequences, confirming identification for 22 taxa but also revealing single to few mismatches *per* species. Taxonomic insights were offered in the text and the Supplementary Material for several taxa, including the understudied *Ascidia colleta* and *Ascidia malaca*. The presence of a new clade was discovered in the *Distaplia bermudensis* species complex. *Didemnum pseudovexillum* and *Polyclinum constellatum* were respectively first recorded in Italy and western Mediterranean, and the same holds true for other taxa whose accurate identification was only possible through molecular evidence. The analysis of photographic archives allowed backdating and georeferencing to the Miseno Lake of the first records of *Aplidium accarense* and *Botrylloides niger* in Italy and of *P. constellatum* in the Mediterranean. Generalized Additive Models revealed that the abundance of NIS increased with availability of hard substrates (rocks or litter), decreased with depth, and was the highest in the channels connecting to other water bodies, followed by the peripheral areas of the lake. No certain evidence of competitive restriction of native ascidians was found. Finally, introduction pathways, potential impacts, and additional insights were discussed for NIS. The present study provides a baseline to evaluate shifts in the ascidian communities in the future, suggests that constant monitoring programs constitute crucial steps to achieve solid NIS management, and confirms marine reserves as widely vulnerable to biological invasions, especially when they coincide with potential hotspots of arrival or spreading such as semi-enclosed basins.

Keywords: biological invasions, Tunicata, bathymetric and habitat mapping, DNA barcoding, Phlegraean lagoons, benthic survey, *cytochrome c oxidase subunit I* (COI), generalized additive models (GAMs)

1 INTRODUCTION

The introduction of marine non-indigenous species (NIS) is a threat to native biodiversity with often severe impacts on biodiversity, economy, and human health (Vilà and Hulme, 2017; Bédry et al., 2021). The European seas are particularly affected by biological invasions, with more than 600 NIS already established in the area (Katsanevakis et al., 2014; Zenetos et al., 2017). Concerned by the issue, the European Union developed a specific Descriptor (D2) of the Marine Strategy Framework Directive (MSFD) to assess NIS pressure on ecosystems and coordinate response strategies (Directive 2008/56/CE). Nevertheless, the descriptor D2 was one of the least investigated so far, with limited dedicated monitoring programs especially in the Mediterranean Sea (European Commission, 2020a; European Commission, 2020b).

The Gulf of Naples, and generally the Campania region (Italy, central-western Mediterranean Sea), is already known as a major hub for NIS spreading in the western parts of the Mediterranean basin (Servello et al., 2019). The city of Naples and its surroundings are one of the most densely populated areas of Italy, which is reflected in a territory with a constantly growing commercial and touristic demand (De Luca and Rosciano, 2020). The area is subjected to inter-continental shipping routes and a wide local traffic, with numerous ferries that interlink Naples harbor with other Mediterranean harbors but also with the popular touristic islands of the region (Capri, Ischia, and Procida) (Appolloni et al., 2018). In addition, a number of small marinas and several aquaculture enterprises (mostly mussel farms) are distributed along its coastline (Esposito et al., 2013; Appolloni et al., 2018), and four marine coastal lagoons are present in the Phlegraean area (Miseno, Fusaro, Lucrino, and Torrefumo) (Camilletti and Lanzi, 2018). Notwithstanding these premises, the study of local NIS still lacks targeted research efforts, and previous records were most often the result of casual or opportunistic findings (Gambi, 2021 and references therein).

Among all potential hotspots for monitoring and/or early detection listed above, marine lagoons are renowned as NIS-favored environments, as filter-feeding species flourish there thanks to the combined effect of anthropogenic disturbance and general eutrophic conditions and high nutrient levels (e.g. Occhipinti-Ambrogi, 2000; Marchini et al., 2015). In the last decade, about 15 NIS have already been discovered in Italy in enclosed and semi-enclosed marine basins (e.g. Occhipinti-Ambrogi et al., 2011; Langeneck et al., 2020), and the same also holds true for the Phlegraean lagoons, which already accounted for important sightings in the recent past (e.g. Crocetta et al., 2013; Hanson et al., 2013; López-Legentil et al., 2015; Montesanto et al., 2021; Montesanto and Mastrototaro in Orfanidis et al., 2021). Within the biota living in lagoons, ascidians (Tunicata: Ascidiacea) are species that play a pivotal role among NIS due to their ease of spread and include taxa that are renowned worldwide invaders (Shenkar and Swalla, 2011; Zhan et al., 2015). They are somehow facilitated in the colonization of new areas by environmental plasticity, being well adapted to live in polluted environments (Lambert and Lambert, 1998; López-Legentil et al., 2015; Streit et al., 2021), and also easily fouling on anthropogenic substrates (Shenkar et al., 2008; Aldred and Clare, 2014; Gewing et al., 2017). Finally, in favorable conditions, ascidians also display an invasive character, resulting in sudden community shifts and dominance of the available substrates (Lambert and Lambert, 1998; Bullard et al., 2007).

Within projects aiming to investigate various aspects of the biodiversity of the Campania region, we hereby first explored the status of the Miseno Lake (Bacoli, Naples) protected area as a potential hotspot for NIS and cryptogenic species. To do so, we focused on the target phylogenetic group mentioned above (ascidians) and evaluated its local taxonomic composition, cover, and distribution pattern through a multidisciplinary approach that includes bathymetric and habitat mapping, a benthic survey carried out with transects and photoquadrats, an integrative taxonomic approach on selected specimens, and the analysis of the data obtained through Generalized Additive Models. Finally, the arrival of NIS in the area was backdated as much as possible through the analysis of photographic archives.

2 MATERIAL AND METHODS

2.1 Study Area

The Miseno Lake (~40.793111N - 14.072287E, central-western Mediterranean Sea) is a brackish-water body of around 40.25 hectares and a total perimeter of 2800 meters, located in the town of Bacoli (Naples, Italy) (Leccese and Speziale, 1967). It is included in the list of Sites of Community Importance (SCI no. IT8030017; European Commission Habitats Directive 92/43/EEC) as a Special Area of Conservation (SAC), and it also lays within the Campi Flegrei Regional Park (https://www.parcodeicampiflegrei.it/). As an area subject to protection, access and recreational activities are only allowed to authorized personnel. The lake has a regular outline, somehow resembling a right-angled triangle with the right angle cut away by a rectangular portion, and in its southern part is separated from the Tyrrhenian Sea by a 200 m-wide stretch of coast (Leccese and Speziale, 1967; ENEA, 2002; **Figure 1**). The entire lake is bordered by an artificial dock wall (~50–60 cm in height) composed of tuff, a friable volcanic rock. Water exchange with the sea is allowed by two channels, the Miliscola channel (MC), located on the western side, and the Casevecchie channel (CC), located on the eastern side. The MC is 250 m long, has a sandy bottom with scattered rocks and an average depth of ~1.5 m, and faces a stretch of open sea, the Procida Strait. The outer part is characterized by rock boulders laying on a sandy bottom, covered by littoral assemblages dominated by photophilic algae. The CC is 100 m long, has a rocky bottom and an average depth of ~1 m, and opens directly into a sheltered bay that hosts two marinas, namely the Bacoli marina and Marina Piccola, and a maritime police facility operative until 2020. Outside the marinas, two mussel farms are still active nowadays. Little is known on the lake's bathymetry and main substrates, except for preliminary characterizations of the area made about 50 and 20 years ago (Leccese and Speziale, 1967; Rigillo Troncone, 1975; ENEA, 2002; La Magna et al., 2002).

2.2 Fieldwork

2.2.1 Bathymetric Mapping

Bathymetry of the Miseno Lake was evaluated through transects held parallel to the southern shoreline (~15–20 m from each other) carried out with a 3 m-long inflatable boat equipped with a 10 cv Suzuki engine and a single beam echo sounder (SBES) (HYDROTRAC®, Odom), set at 200 KHz (accuracy 0.01 meter ± 0.1% depth). For safety reasons (boat limitations), depth measurements started from 0.6 m. Each point of measurement was real-time geo-referenced through a DGPS (Differential Global Positioning System) Vector VS330 GNSS (Hemisphere) connected to LandStar (Landstar System, Inc., USA), a satellite transmission service for GPS differential corrections, recording geographic coordinates and depths at 15 points/s. Acquired data were quality checked, filtered (set at 30 cm of distance from each other), and tidally corrected. Bathymetry was finally processed in ArcGis 9.2 (ESRI, Redlands, USA) and georeferenced according to the UTM WGS84 coordinate system (central Meridian: 15° 00'00"; false East: 500,000; scale factor: 0.9996).

2.2.2 Habitat Characterization and Ascidian Species Assessment

Fieldwork was carried out in September–November 2021 by three trained operators, two snorkeling and/or scuba diving (R.V. and V.T.) and the third (F.C.) mostly giving support on the inflatable boat. Given the particular outline, the lake was divided into a grid made by squares of ~100×100 m (**Figure 1**). Then, fieldwork used a combination of different strategies according not only to the topography of the lake but also to the investigated sections, namely the inner lake bottom (subsequently divided into inner and peripheral), the dock wall perimeter, and the two channels.

To investigate the habitat characterization of the inner bottom and the ascidian community living there, seven main latitudinal

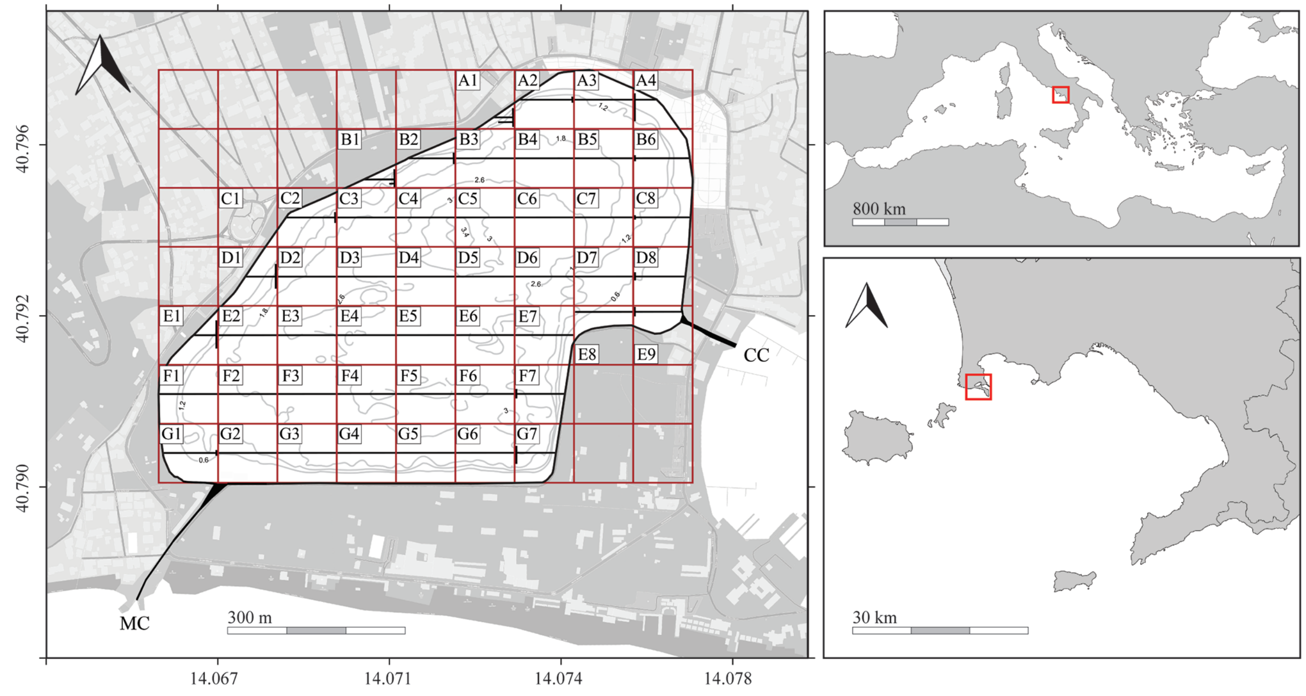

FIGURE 1 | The Miseno Lake and its position (red square) in the Mediterranean Sea (top right) and in the Gulf of Naples (bottom right). Left image highlighting sampling grid (red squares), longitudinal transects (black lines), dock wall perimeter, channels (CC, Casevecchie channel; MC, Miliscola channel), and simplified bathymetry (for a detailed bathymetric map see **Supplementary Figure 1**).

transects (A–G), which were 100 m apart, with the first (A) and the last (G) being ~50 m away from the dock walls, were investigated through snorkeling/scuba diving using a 100-m line. Each latitudinal transect was divided into 100 m-long sub-transects following the grid, for a total of 49 grid sectors. However, wherever the sector could not host a straight 100-m latitudinal transect (A1, A2, A4, B1, B2, B6, C2, C8, D1, D8, E1, E9, F7, G1, and G7), it was amended so that the total was 100 m (**Figure 1**). The two observers recorded the following information (within 1.5 m on the left/right of the line): i) main substrate encountered, namely *Caulerpa prolifera* (Forsskål) J.V. Lamouroux, gravel/dead shells, litter, mud, rocks, and sand; ii) number of specimens and/or colonies found per species; iii) type of substrate where ascidians were found, divided into four categories: rocks, litter, *C. prolifera*, and other biogenic substrates — mostly natural wood, tubes of the polychaete *Chaetopterus variopedatus* (Renier, 1804) species complex, and the bryozoan *Schizoporella errata* (Waters, 1878). For colonial species which form zooid aggregations, such as *Polyandrocarpa zorritensis* (Van Name, 1931), each cluster was counted separately.

To investigate the community living on the dock wall perimeter, each sector was surveyed through photoquadrats. In particular, three replicates were taken per sector (for a total of 72 photoquadrats, placed approximately at a fixed distance from each other according to the length of the dock wall in the sector), using a PVC frame of 49.5×49.5 cm (total area 2450.25 cm^2) fixed to a Sony RX100 mkII camera in a polycarbonate housing equipped with a INON D2000 strobe. Images were taken placing the quadrat below the surface level at the lowest tide. For each quadrat, notes on the main species found in the frame were also taken on a diving whiteboard to facilitate the subsequent laboratory work.

To investigate the communities of the two channels, a combination of both approaches was used. In particular, a single additional 100-m bottom transect was carried out in each channel, whereas the communities of the dock walls were investigated through 12 additional photoquadrats (6 photoquadrats per channel, with 3 photoquadrats at each side of each channel wall).

Finally, to investigate the proper taxonomy of the species encountered not only with visual census, but also with an integrative approach, samples were collected during transects/photoquadrats, but sometimes also opportunistically, for subsequent laboratory work (internal and external morphology and molecular analyses).

2.3 Morphological and Molecular Analyses

Ascidian samples collected were brought to the Laboratory of Benthos of Stazione Zoologica Anton Dohrn (SZN, Naples, Italy) for proper taxonomic identification. When necessary, specimens were analyzed and dissected under a Zeiss Axio Zoom.V16 (Carl Zeiss, Oberkochen, Germany) stereomicroscope. Then, single specimens (in case of solitary ascidians) or a sub-sample of each colony (in case of colonial ascidians) were isolated for molecular analyses. As a general rule (whenever possible), conspecificity between samples was tested by sequencing 5–6 samples per putative species, collected from different places of the lake.

To do so, total genomic DNA was extracted from muscular tissue of single zooids using the DNeasyBlood & Tissue® kit (Qiagen, Hilden, Germany), following the producer's protocol. Amplifications of a partial region of the *cytochrome c oxidase subunit I* (COI) gene were obtained through polymerase chain reactions (PCRs) with the following primer sets by Brunetti et al. (2017): dinF (5'-CGTTGRTTTATRTCTACWAATCATAARGA-3') and Nux1r (5'-GCAGTAAAATAWGCTCGRGARTC-3'). Polymerase chain reactions (PCRs) were carried as follows: first denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 48–50°C for 1 min, extension at 72°C for 1 min, with a final elongation at 72°C for 5 min. The PCR products were purified and sequenced at the SZN Molecular Biology and Sequencing Service through an Automated Capillary Electrophoresis Sequencer 3730 DNA Analyzer (Applied Biosystems, CA, USA), using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Renfrew, UK).

Sequences obtained were quality checked, assembled, and edited using Unipro UGene v.39 (Okonechnikov et al., 2012). Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Morgulis et al., 2008) was then used to assess the identity of each sequence against the GenBank database (last research: 30.XII.2021).

Sequenced and other collected specimens were finally fixed in 99.9% ethanol and deposited in the collection of the Laboratory of Benthos (SZN), under different accession codes (**Supplementary Table 1**).

2.4 Phylogenetic Analyses on the *Distaplia bermudensis* Complex

Since the sequences obtained for *Distaplia bermudensis* Van Name, 1902 gave unclear BLAST results (see below), we also carried out phylogenetic analyses in order to assess the phylogenetic position of our specimens within other *D. bermudensis* collected worldwide.

A total of thirty-two COI partial sequences were used in the final alignment, including the five sequences of *D. bermudensis* amplified in this study and twenty-one sequences of *D. bermudensis* retrieved from GenBank (accession numbers reported in **Supplementary Tables 1, 2**). Six additional representatives of the order Aplousobranchia Lahille, 1886, collected in this study, were used as outgroup: *Clavelina lepadiformis* (Müller, 1776) (SZN_B_1879ASC15G), *Clavelina sabbadini* Brunetti, 1987 (SZN_B_2707ASC36A), *Didemnum pseudovexillum* Turon & Viard, 2020 (SZN_B_1902ASC22A), *Lissoclinum weigelei* Lafargue, 1968 (SZN_B_3172ASC49A), *Aplidium accarense* (Millar, 1953) (SZN_B_1822ASC18B), and *Polyclinum constellatum* Savigny, 1816 (SZN_B_2884ASC43A). The selected sequences were aligned and trimmed with Unipro UGene v.39 (Okonechnikov et al., 2012), and the best-fit evolutionary model (GTR + I + G) was found with JModelTest 2 v.0.1.10 (Darriba et al., 2012), selected with the Akaike information criterion (AIC) method. The maximum likelihood (ML) analyses were run with RAxML v.8.2 (Stamatakis, 2014) using 1000 rapid bootstrap pseudo-replicates. The Bayesian Inference (BI) was performed with MrBayes v.3.2.5 (Ronquist et al., 2012) run for 10 million generations, sampled every 1000 generations, with a standard 25% burn-in. Convergence of the MCMC runs was checked with Tracer v1.7.1 (Rambaut et al., 2018).

Three species delimitation methods were then used on the same alignment to infer Operational Taxonomic Units (OTUs) boundaries within the *Distaplia* clade: Automated Barcode Gap Discovering (ABGD, Puillandre et al., 2012), Poisson Tree Processes (PTP, Zhang et al., 2013), and Generalized Mixed Yule Coalescent (GMYC, Fujisawa and Barraclough, 2013). The ABGD analysis was performed on the ABGD web platform (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html) with Jukes-Cantor (JC69) model and standard parameters. The PTP and GMYC analyses were run on the Exelixis Lab web server (The Exelixis Lab, https://species.h-its.org). The PTP was run for 500000 generations, 25% burn-in, and 100 thinning based on the rooted ML tree obtained in the aforementioned analysis. The GMYC was performed in single threshold (s-GMYC) method. As input, an ultrametric tree was obtained with BEAST v.1.10.4 (Suchard et al., 2018) based on the same alignment as above.

2.5 Species' Status in the Mediterranean Sea and Possible Backdating of NIS Arrival

Once a solid identification of the specimens was achieved, species were classified into the following categories based on the most up to date literature (see Essl et al., 2018): i) native for species with a putative Atlantic-Mediterranean origin; ii) cryptogenic for species with uncertain native range; iii) non-indigenous for species native in other biogeographic sectors and locally introduced through human activities (European Commission, 2014).

Finally, the digital photographic archives (2014–2021) of one of the authors (G.V.), who regularly surveyed and mostly photographed the lagoon biota in the past decade, was screened in order to backdate as much as possible the most probable first introduction event of selected NIS in the Miseno Lake.

2.6 Modelling Species Distribution

Images obtained (photoquadrats) were subsequently analyzed in Photoquad v1.4 (Trygonis and Sini, 2012). Each picture was calibrated according to the known frame measures. After picture calibration, abundance and cover of each species were calculated counting single specimens (for solitary ascidians) and colonies (single separated clusters) for each quadrat. Data obtained from transects and photoquadrats were then collated in a matrix in Excel (**Supplementary Data Sheet 1**). Data are reported as total number of individuals counted on the seabed and on the dock wall, as well as mean count per transect on the seabed and mean percentage cover on the dock wall ± standard error (SE).

The abundance (number of individuals per transect) of the most commonly encountered NIS ascidians, namely *Botrylloides niger* Herdman, 1886, *D. bermudensis*, *P. constellatum*, and *A. accarense*, as well as of all NIS and cryptogenic species combined together, was modeled with Generalized Additive Models (GAMs; Hastie and Tibshirani, 1990). Due to severe overdispersion (residual deviance of the fitted models much higher than the residual degrees of freedom), and thus violation of the assumption of equal mean and variance in the response variables, the negative binomial distribution was used instead of the Poisson distribution (Lindén and Mäntyniemi, 2011). The expected values of the number of individuals counted in each transect were related to spatial and environmental covariates, according to the general formulation $f(E[counts]) = LP_i = c + \Sigma_m s_m\ (z_{mi}) + \Sigma_r F_{ri}$, where f is the link function (in this case link=log), LP is the linear predictor, $s_m(\cdot)$ is the one-dimensional smooth function for covariate m, F_r are categorical predictors, and z_{mi} is the value of spatial covariate m for the ith transect. The smooth function $s_m(\cdot)$ was represented using penalized regression splines (cubic splines with basis dimension q=10), estimated by penalized iterative least squares (Wood, 2006). The optimum degree of smoothing was defined by generalized cross validation (GCV), where the amount that the effective degree of freedom of each model counts in the GCV score was increased by a factor c=1.4 according to Kim and Gu (2004) to avoid occasional overfitting. The *mgcv* package implemented in R v4.1.0 was used for model fitting (Wood, 2006; R Core Team, 2021).

Four predictor variables were used for modelling the abundance of NIS on the seafloor, namely: i) *depth*; ii) *hard*, the amount of hard substrate items counted in each transect — i.e. the sum of rocks and litter items; iii) *section*, a categorical variable with three levels representing the distinct seafloor areas investigated in the lake (channel, inner, and peripheral); iv) *Caulerpa*, a categorical variable with two values, 0 for absence and 1 for presence, indicating the presence/absence of *C. prolifera* within each transect, as the seagrass can act as substrate for epiphytic ascidians. The degree of collinearity among these variables was measured by calculating the variance inflation factor (VIF) for each covariate based on the negative binomial models. Since all VIF values were <3, there was no substantial multicolinearity among the independent variables and thus were all kept in the analyses. Overall, eight candidate models g_i were used in the analyses linked to the following hypotheses: h1 - abundance is affected by *depth*; h2 - abundance is restricted by the availability of proper substrate (*hard*+*Caulerpa*); h3 - the three different *sections* have varying conditions (other than depth and substrate) — e.g. hydrodynamism, that may affect the abundance of the species (**Supplementary Table 3**). Furthermore, the abundance of native species on the seafloor was modelled. For this, in addition to the same three hypotheses, a fourth one was investigated: h4 - abundance of native species is restricted by the abundance of NIS and cryptogenic species. An additional predictor variable was included — i.e. the abundance of NIS and cryptogenic species (*AlCr*), and the set of candidate models for native species included overall sixteen models (**Supplementary Table 4**). This hypothesis was also tested using the estimated cover of native *vs* NIS/cryptogenic species along the dock wall perimeter (photoquadrat sampling), based on only four models, the null model, one including the cover of NIS and cryptogenic species as a predictor variable, one including *section* (peripheral *vs* channels) as a predictor variable, and one including both predictors.

Akaike's information criterion was used for model selection (Akaike, 1973; Burnham and Anderson, 2002), and the AIC differences $\Delta_{i=}AIC_i$ - AIC_{min} were computed over all candidate models g_i, where AIC_{min} is the minimum AIC corresponding to the best model. To quantify the plausibility of each model, given the data and set of candidate models, the 'Akaike weight' w_i of each model was calculated, where $w_i = \exp(-0.5\Delta_i)/\Sigma_j \exp(-0.5\Delta_j)$ (Burnham and Anderson, 2002). The relative importance of each of the hypotheses made was estimated by summing the Akaike weights across all the models in the set that were linked with each hypothesis (Burnham and Anderson, 2002).

3 RESULTS

3.1 Bathymetric Mapping and Habitat Characterization

The bathymetric survey recorded a total of 228000 reliable depth measurements, interpolated to produce a detailed bathymetric map of the lake (**Supplementary Figure 1**). The average depth was ~2 m, whereas the maximum depth was ~3.5 m. The habitat characterization revealed the presence of muddy bottoms almost dominated by a dense meadow of the native alga *C. prolifera* in several grid sectors but mostly in the inner parts of the lake (A1, B2–B5, C3–C7, D2–D7, E2–E6, F2–F6, G3–G7). The meadow only becomes sparse in the majority of the peripheral parts of the lake and underneath the dock wall, nearby freshwater inputs, and in the proximity of the two channels (A2–A4, B1, B6, C1, C2, C8, D1, D8, E1, E7–E9, F1, F7, G1, G2), where the substrate is again unconsolidated but with a wider granulometric range, from mud to gravel. Worth a mention, the sector C1 was mostly characterized by a sand bank with a very low depth, and thus it was excluded from the analysis of the transects-based data, although retained for the dock wall analysis through photoquadrats. The area was also characterized by the presence of sparse rocks (n=455) and widely impacted by litter items (n=1801), mostly originating from commercial or recreational activities in the nearby town of Bacoli. Their presence and distribution was generally influenced by geographic position and conformation of the nearby substrate. Rocks were rare to absent in the inner parts of the lake and mostly found in grid sectors close to the dock wall — C2 (43), D1 (59), D8 (68), E1 (47), F1 (53), G1 (54), as they constitute waste material from the previous levee. Litter items were found in all transects, with the sectors close to the CC being the ones most impacted — D8 (296), E8 (91), and E9 (80).

3.2 Ascidian Identification and Species' Status

A total of 24 taxa were observed during our survey, belonging to the three orders of the class Ascidiacea Blainville, 1824 (nine Aplousobranchia, six Phlebobranchia Lahille, 1886, and nine Stolidobranchia Lahille, 1886) (**Table 1**, **Figures 2**, **3** and **Supplementary Table 1**). Of these, ten are native, nine are NIS, and five are cryptogenic species. Distribution in taxonomic orders varied based on the species' status in the Mediterranean Sea. Among native species, Aplousobranchia and Phlebobranchia accounted for four taxa each, whereas Stolidobranchia only accounted for two. On the contrary, NIS accounted for five Stolidobranchia, followed by three Aplousobranchia, and one Phlebobranchia. Finally, cryptogenic species accounted for two species of Aplousobranchia and Stolidobranchia each, and only one Phlebobranchia (**Table 1**, **Figures 2**, **3** and **Supplementary Table 1**).

The amplification of the COI partial region gave a total of 114 sequences, ranging from 504 to 828 base pairs (bp), with a number of specimens sequenced per species varying from one to six. The morphological identification was supported by BLASTn hits (~95–100% similarity) for 22 species (**Supplementary Table 1**). However, several ambiguities in the GenBank database were found, as follows: i) single to a few mismatches in four species — *Lissoclinum perforatum* (Giard, 1872), *Phallusia mammillata* (Cuvier, 1815), *Pyura dura* (Heller, 1877), and *B. niger* — were presumably due to misidentifications or incorrect uploads; ii) mismatches in three species — *L. weigelei*, *A. accarense*, and *Ciona robusta* Hoshino & Tokioka, 1967 — were due to identification not provided at a species level by some authors when uploading sequences in GenBank or lack of GenBank updates after the species complexes were resolved (e.g. Caputi et al., 2007); iii) mismatches in four additional taxa — *C. lepadiformis*, *D. bermudensis*, *Botryllus schlosseri* (Pallas, 1766), and *Styela canopus* (Savigny, 1816) — were due to the fact that they belong to species complexes (Turon et al., 2003; Nydam et al., 2017; Barros and Rocha, 2021; Salonna et al., 2021; Nuzzo et al., 2022), and thus the sequencing of local specimens allowed clarification of their exact taxonomic allocation and also identification of a new clade in *D. bermudensis* (see below); for the sake of text clarity, these species are still listed in the subsequent chapters as monotypic taxa; iv) mismatches in two species — *P. constellatum* and *Symplegma brakenhielmi* (Michaelsen, 1904) — have already been reported and presumably require further taxonomic work to solve the related taxonomic issues, including sequencing of specimens from the type localities of the taxa involved (Mastrototaro et al., 2019; Montesanto et al., 2022); these species are listed in the subsequent chapters following the most recently updated literature on the Mediterranean biota.

Finally, with regards the two remaining species, mismatches in *Ascidia malaca* (Traustedt, 1883) may be due to misidentifications or even uncertainties resulting from the use of the COI marker within the genus *Ascidia* Linnaeus, 1767 (see Streit et al., 2021), whereas *Ascidia colleta* Monniot & Monniot, 1970 accounted for a new GenBank entry, as it only showed 80.4–81.2% similarity with *Ascidia conchilega* Müller, 1776 (MN064596–7: Couton et al., 2019). Given taxonomic issues regarding worldwide species of this genus, these taxa are also further illustrated and discussed in **Supplementary Data Sheet 2**.

3.3 Phylogenetic Analyses of the *Distaplia bermudensis* Complex

BLAST results of *D. bermudensis* sequences from the Miseno Lake (SZN_B_1743ASC10H–1747ASC10N) gave high similarity (99.71%) to another sequence of a specimen identified as *D. bermudensis* (MT637947) from Puerto Rico marinas by Streit et al. (2021), but also significant differences (80.33–86.5%) from other *D. bermudensis* worldwide (**Supplementary Table 1**).

After sequences-editing steps, we obtained a trimmed alignment (575 bp) of COI partial sequences, with 250 parsimony-informative sites. Both the ML and BI analyses produced congruent tree topologies, hence we showed the best ML tree with bootstrap (bs) and posterior probability (pp) values reported at nodes (**Figure 4**). Both tree topologies clustered the newly collected *D. bermudensis* from Miseno with the specimen from Puerto Rico marinas by Streit et al. (2021), forming a different clade with respect to the other samples. This clade, that we first name here as clade C, was well supported (bs=99; pp=1)

TABLE 1 | Surveyed ascidian species in the Miseno Lake, the Casevecchie channel, and the Miliscola channel, with known status (S: C, cryptogenic; N, native; NIS, non-indigenous).

S	Taxa	Miseno Lake				Casevecchie Channel			Miliscola Channel		
		T n	n_m	DW n	c_m	T n	DW n	c_m	T n	DW n	c_m
	Order APLOUSOBRANCHIA Lahille, 1886										
	Family Clavelinidae Forbes & Hanley, 1848										
C	*Clavelina lepadiformis* (Müller, 1776)	–	–	–	–	–	2 (1/6)	0.04 ± 0.04	–	–	–
N	*Clavelina sabbadini* Brunetti, 1987	2 (1/48)	0.02 ± 0.20	1 (1/72)	*0.0 ± 0.0	–	–	–	–	–	–
	Family Didemnidae Giard, 1872										
C	*Didemnum pseudovexillum* Turon & Viard, 2020	–	–	–	–	–	1 (1/6)	*0.0 ± 0.0	–	–	–
N	*Lissoclinum perforatum* (Giard, 1872)	–	–	–	–	–	–	–	28	18 (4/5)	0.15 ± 0.06
N	*Lissoclinum weigelei* Lafargue, 1968	–	–	–	–	–	–	–	–	4 (2/6)	0.07 ± 0.06
N	*Trididemnum cereum* (Giard, 1872)	–	–	–	–	–	3 (1/6)	0.01 ± 0.01	–	–	–
	Family Holozoidae Berrill, 1950										
NIS	*Distaplia bermudensis* Van Name, 1902	796 (9/48)	8.30 ± 32.80	975 (25/72)	2.03 ± 0.65	1542	680 (6/6)	13.13 ± 2.33	–	–	–
	Family Polyclinidae Milne Edwards, 1841										
NIS	*Aplidium accarense* (Millar, 1953)	351 (16/48)	3.66 ± 10.56	1327 (64/72)	0.31 ± 0.06	–	1 (1/6)	0.01 ± 0.02	–	–	–
NIS	*Polyclinum constellatum* Savigny, 1816	143 (11/48)	1.49 ± 5.75	2115 (68/72)	1.13 ± 0.17	35	93 (6/6)	1.47 ± 0.61	5	48 (3/6)	0.58 ± 0.40
	Order PHLEBOBRANCHIA Lahille, 1886										
	Family Ascidiidae Müller, 1776										
N	*Ascidia colleta* Monniot & Monniot, 1970	20 (4/48)	0.21 ± 0.97	–	–	–	–	–	–	–	–
N	*Ascidia malaca* (Traustedt, 1883)	161 (10/48)	1.68 ± 5.24	36 (10/72)	0.02 ± 0.01	3	1 (1/6)	*0.0 ± 0.0	1	2 (1/6)	0.03 ± 0.03
N	*Phallusia fumigata* (Grube, 1864)	3 (3/48)	0.03 ± 0.17	–	–	–	–	–	–	–	–
N	*Phallusia mammillata* (Cuvier, 1815)	1 (1/48)	0.01 ± 0.10	–	–	1	–	–	–	–	–
	Family Cionidae Lahille, 1887										
NIS	*Ciona robusta* Hoshino & Tokioka, 1967	2 (1/48)	0.20 ± 0.20	–	–	–	–	–	–	5 (2/6)	0.06 ± 0.04
	Family Perophoridae Giard, 1872										
C	*Perophora viridis* Verrill, 1871	–	–	–	–	3	–	–	–	–	–
	Order STOLIDOBRANCHIA Lahille, 1886										
	Family Pyuridae Hartmeyer, 1908										
N	*Microcosmus polymorphus* Heller, 1877	–	–	5 (4/72)	0.02 ± 0.00	–	–	–	–	5 (2/6)	0.25 ± 0.21
NIS	*Microcosmus squamiger* Michaelsen, 1927	–	–	15 (10/72)	0.01 ± 0.00	–	–	–	–	6 (2/6)	0.02 ± 0.01

(Continued)

TABLE 1 | Continued

S	Taxa	Miseno Lake				Casevecchie Channel			Miliscola Channel		
		T n	n_m	DW n	c_m	T n	DW n	c_m	T n	DW n	c_m
N	*Pyura dura* (Heller, 1877)	105 (12/48)	1.09 ± 3.03	255 (42/72)	0.75 ± 0.14	1	7 (3/6)	0.27 ± 0.25	1	32 (6/6)	0.66 ± 0.20
Family Styelidae Herdman, 1881											
NIS	*Botrylloides niger* Herdman, 1886	2696 (28/48)	29.03 ± 62.28	5770 (72/72)	1.37 ± 0.19	794	115 (6/6)	0.24 ± 0.10	272	113 (5/6)	0.61 ± 0.31
C	*Botryllus schlosseri* (Pallas, 1766)	175 (33/48)	1.82 ± 3.10	2 (2/72)	*0.0 ± 0.0	–	–	–	4	5 (2/6)	0.01 ± 0.09
NIS	*Polyandrocarpa zorritensis* (Van Name, 1931)	47 (8/48)	0.49 ± 1.90	522 (44/72)	0.51 ± 0.97	38	128 (6/6)	3.82 ± 1.94	–	3 (1/6)	0.03 ± 0.03
C	*Styela canopus* (Savigny, 1816)	19 (4/48)	0.20 ± 1.05	533 (49/72)	0.06 ± 0.01	–	33 (6/6)	0.09 ± 0.01	–	14 (2/6)	0.03 ± 0.02
NIS	*Styela plicata* (Lesueur, 1823)	16 (4/48)	0.17 ± 0.89	14 (11/72)	0.05 ± 0.02	–	1 (1/6)	0.07 ± 0.09	–	–	–
NIS	*Symplegma brakenhielmi* (Michaelsen, 1904)	–	–	–	–	–	–	–	24	19 (1/6)	0.13 ± 0.13

*T n, total number of individuals counted on the seabed; DW n, total number of individuals counted on the dock wall; n_m, mean counts per transect on the seabed (n ± SE); c_m, mean percentage cover on the dock wall (c_m ± SE%). Data in round brackets refer to sub-transects (x/48) and photoquadrats in lake (x/72) and channels (x/6) in which the species were found. *Values lower than two digits after the decimal point are substituted by 0.0 ± 0.0.*

and the sister (bs=82; pp=0.99) of clade B *sensu* Evans et al. (2021). Results of the species delimitation analyses also well supported the three-clades grouping well (**Figure 4** and **Supplementary Data Sheet 3**). The ABGD analysis inferred three OTUs within the *D. bermudensis* sequences in the primary partition. The PTP and GMYC analyses were also consistent, reporting the three-clade grouping in all the analyses carried out, with PTP Bayesian supporting values ranging from 0.18 to 0.88 (**Supplementary Data Sheet 3**).

3.4 Backdating the NIS Arrival in the Miseno Lake Through Photographic Evidences

The digital photographic archives of the Miseno Lake biota were composed of ~400 pictures, ranging from May 2014 to October 2021. By subsequently excluding widespread NIS ascidians introduced in the entire Mediterranean Sea in historical times (*C. robusta* and *S. plicata*), and notwithstanding limitations in identifying ascidians on the basis of underwater photographs, unequivocal evidence was found regarding the presence of at least five NIS before our survey, namely *D. bermudensis*, *A. accarense*, *P. constellatum*, *B. niger*, and *P. zorritensis*. In particular, four of them were recorded since 2014, and one (*D. bermudensis*) since 2019, with three sightings that predate first-record dates and localities in Italy, one of which also accounts for the first sighting in the Mediterranean Sea (**Table 2** and **Figure 5**). It is noteworthy that four of these species were the most common NIS encountered during our survey (see below). Finally, photographic evidence of the impact of *D. bermudensis* on dock wall communities were also found, and illustrated in **Figures 5F, G**.

3.5 Community Assessment and Special Focus on NIS

Overall, a total of 20197 ascidian individuals/colonies were counted in the Miseno Lake. Of these, 4537 were censused during transects on the seabed of the lake, 11570 were found in the dock wall, and the remaining 4090 in transects and dock walls in the Casevecchie and Miliscola channels (CC=3482; MC=608) (**Table 1** and **Supplementary Data Sheet 1**).

Fifteen species were found in the seabed transects, among which *B. niger* (n=2696, n_m=29 ± 62.3) and *D. bermudensis* (n=796, n_m=8.3 ± 32.8) were the most common (**Table 1** and **Supplementary Data Sheet 1**). The distribution of the four most abundant NIS is depicted in **Figure 6**. However, the peripheral parts of the lake hosted all species mentioned above, whereas the ascidian diversity was highly reduced in the inner parts of the lake, with only seven species found, among which only *B. schlosseri* and *B. niger* ranked high in numbers, which was also reflected in their presence in the transects (33/48 and 28/48, respectively) (**Supplementary Data Sheet 1**). With regard to substrates: 13 species were found on/below rocks, with *B. niger* and *A. accarense* being among the most commonly found, 12 species were found associated with litter, with *B. niger* and *D. bermudensis* almost completely dominating the group, five species were found associated with other biogenic substrates, among which *D. bermudensis* and *B. niger* were again the most commonly encountered, and two species only were found

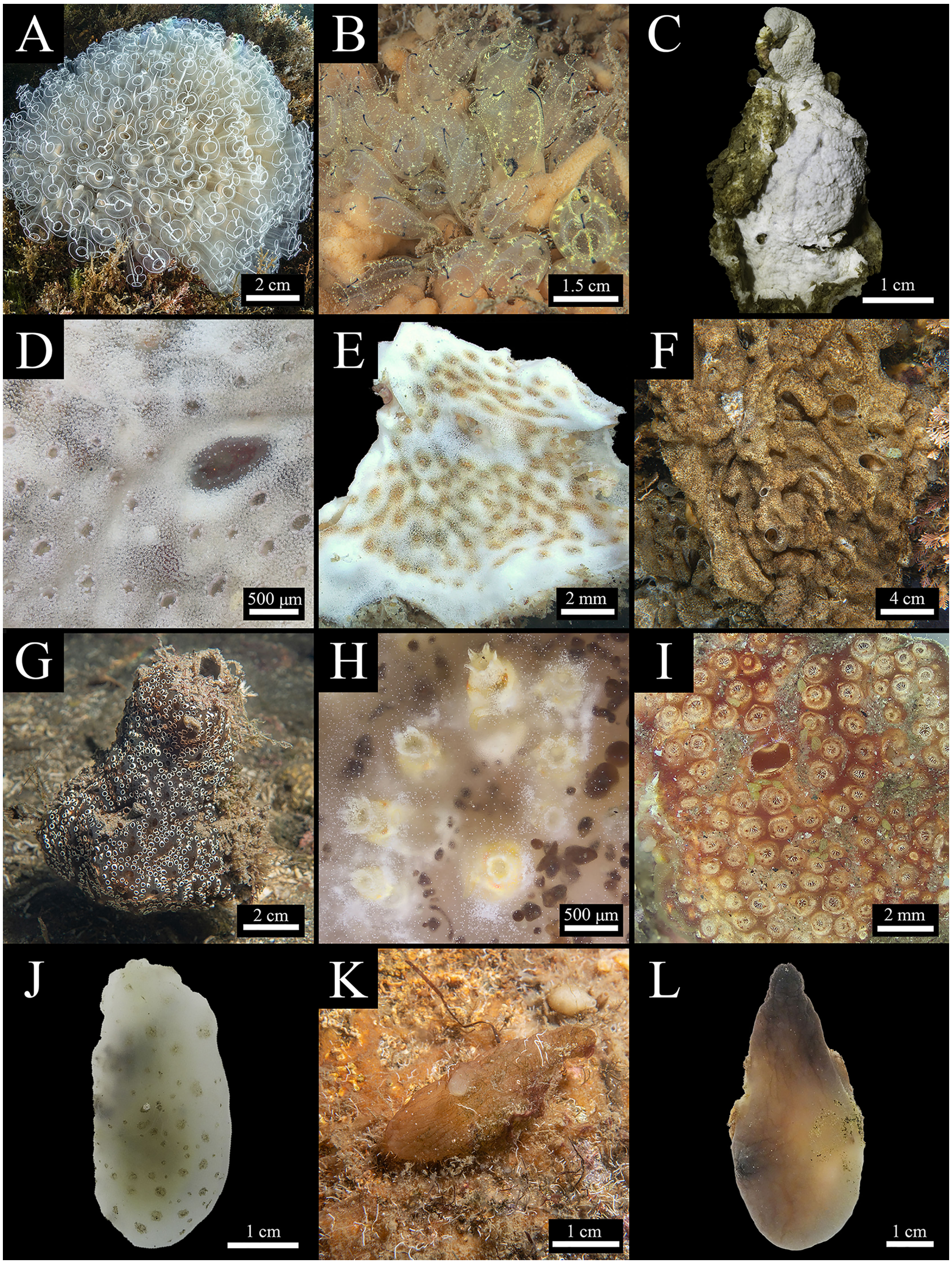

FIGURE 2 | Ascidian species from the Miseno Lake (part 1) (see **Table 1**). Abbreviations used: C, cryptogenic; N, native; NIS, non-indigenous. Order Aplousobranchia. Family Clavelinidae: **(A)** *Clavelina lepadiformis* - C (picture not taken during surveying activities). **(B)** *Clavelina sabbadini* - N. Family Didemnidae: **(C)** *Didemnum pseudovexillum* - C (alcohol preserved). **(D)** *Lissoclinum perforatum* - N. **(E)** *Lissoclinum weigelei* - N. **(F)** *Trididemnum cereum* - N (picture not taken during surveying activities). Family Holozoidae: **(G)** *Distaplia bermudensis* - NIS (overgrowing a tube of the polychaete *C. variopedatus*). Family Polyclinidae: **(H)** *Aplidium accarense* - NIS. **(I)**. *Polyclinum constellatum* - NIS. Order Phlebobranchia. Family Ascidiidae: **(J)** *Ascidia colleta* - N. **(K)** *Ascidia malaca* - N. **(L)** *Phallusia fumigata* - N.

as epiphytes on *C. prolifera* leaves, namely *B. niger* and *B. schlosseri* (**Supplementary Data Sheet 1**).

Thirteen species were found in the photoquadrats along the dock wall perimeter, among which the NIS *B. niger* (n=5770, c_m= 1.4% ± 0.2%), *P. constellatum* (n=2115, c_m=1.1% ± 0.2%), *A. accarense* (n=1327, c_m=0.3%), and *D. bermudensis* (n=975, c_m=2% ± 0.6%) were the most widespread (**Table 1** and **Supplementary Data Sheet 1**). The distribution of these four species is depicted in **Figure 7**. Such abundances were also somewhat reflected in the overall presence of the species in the

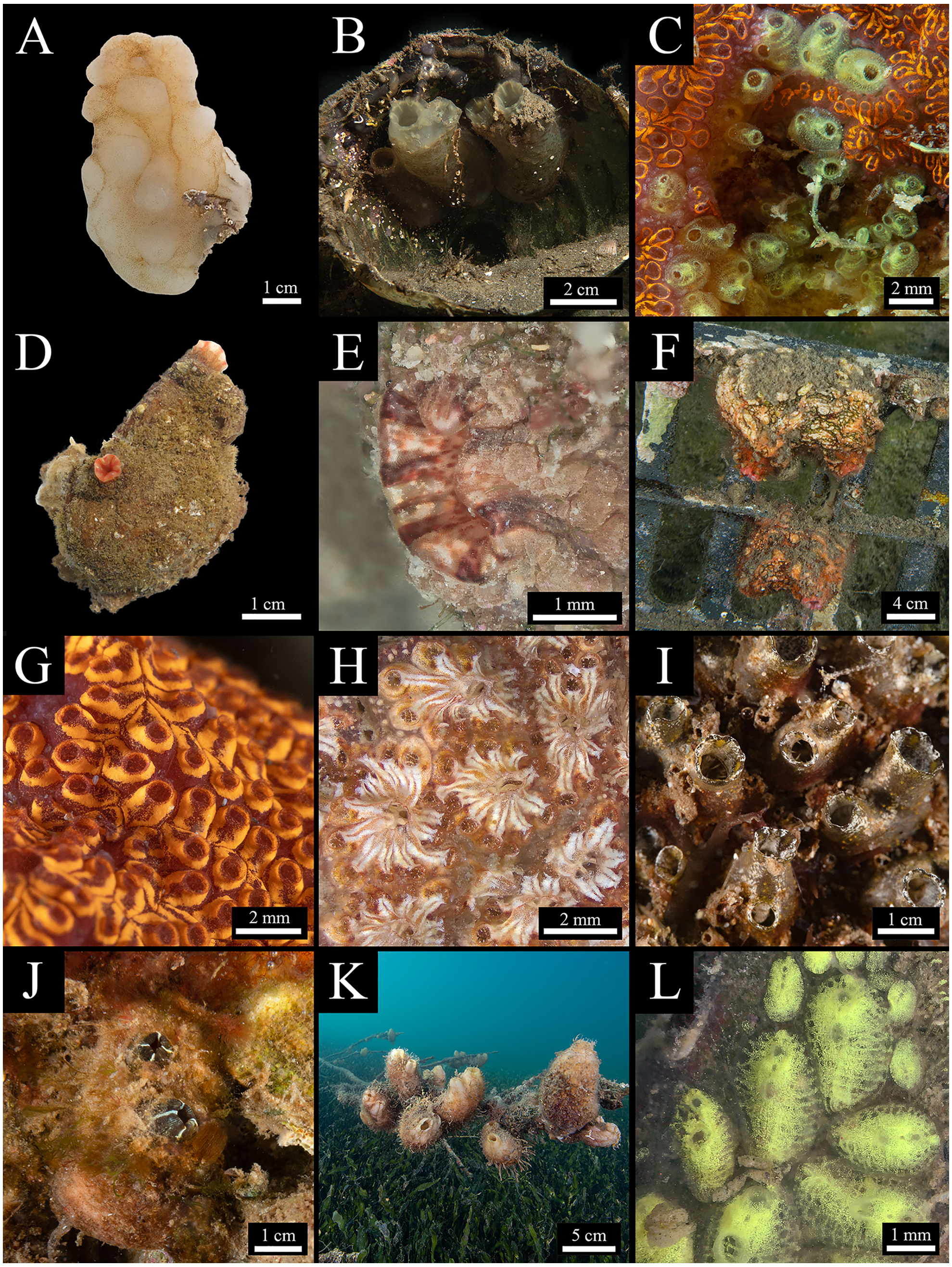

FIGURE 3 | Ascidian species from the Miseno Lake (part 2) (see **Table 1**). C, cryptogenic; N, native; NIS, non-indigenous. Order Phlebobranchia. Family Ascidiidae: **(A)** *Phallusia mammillata* - N. Family Cionidae: **(B)** Two specimens of *Ciona robusta* - NIS, flanked on the left by *A. malaca* (on litter). Family Perophoridae: **(C)** *Perophora viridis* - C, nested within *B. niger*. Order Stolidobranchia. Family Pyuridae: **(D)** *Microcosmus polymorphus* - N. **(E)** *Microcosmus squamiger* - NIS, detail of the oral siphon. **(F)** *Pyura dura* - N (on litter). Family Styelidae: **(G)** *Botrylloides niger* - NIS. **(H)** *Botryllus schlosseri* - C. **(I)** *Polyandrocarpa zorritensis* - NIS. **(J)** *Styela canopus* - C. **(K)** *Styela plicata* - NIS (on wood) (picture not taken during surveying activities). **(L)** *Symplegma brakenhielmi* - NIS.

photoquadrats, with *B. niger* appearing in all photoquadrats (72/72), followed by *P. constellatum* (68/72), and *A. accarense* (64/72). On the other hand, *D. bermudensis* was locally abundant in only few sectors (25/72) (**Table 1**, **Figure 7** and **Supplementary Data Sheet 1**). The dock wall sectors with the highest mean total cover were E8 (c_m=17.0% ± 7.2%), mostly dominated by *D. bermudensis* (n=214, c_m=10.4% ± 3.8%), *B. niger* (n=855; c_m=1.1% ± 0.1%), and *P. constellatum* (n=132, c_m=0.7% ± 0.2%), followed by C8 (c_m=14.7% ± 9.2%) and A3 (c_m=8.7% ± 4.4%) (**Supplementary Data Sheet 1**).

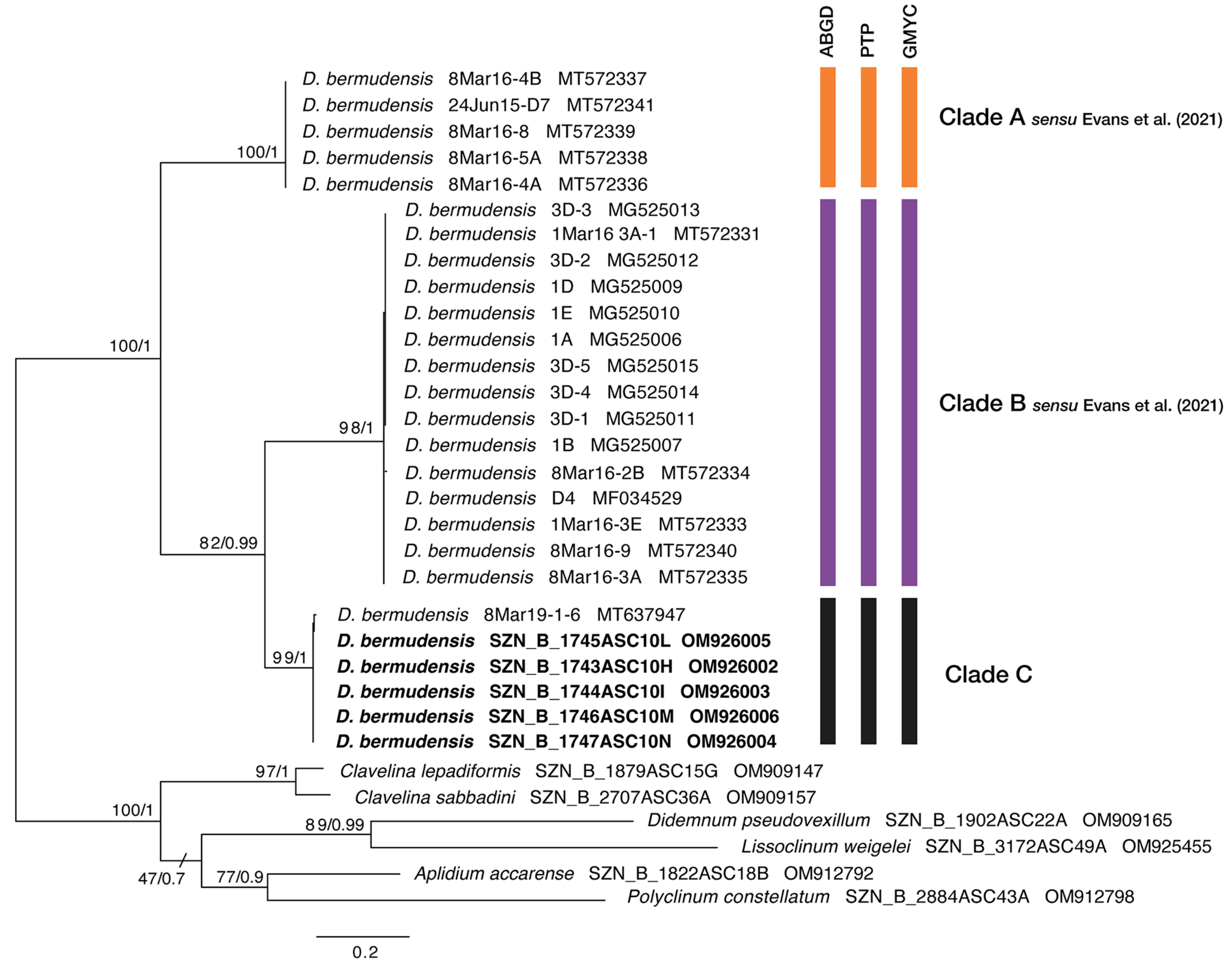

FIGURE 4 | Best ML phylogenetic tree obtained from selected COI sequences. Numbers above/below branches represent bootstrap (bs) and bayesian posterior probability (pp) values. Species delimitation outputs are graphically represented through bars: ABGD (Automatic Barcode Gap Discovery), PTP (Poisson Tree Process), and GMYC (Generalized Mixed Yule Coalescent). Colors of clades A and B as in Evans et al. (2021).

TABLE 2 | First finding date of NIS ascidians in the Miseno Lake (ML) (see **Figure 5**), with date and locality of first published records from the Mediterranean Sea and Italy (when different).

Species	ML	Figure	Mediterranean			Italy		
			Date	Locality	Reference	Date	Locality	Reference
Distaplia bermudensis	10/ 2019	**5A**	1953	Ibiza (Spain)	Pérès (1957)	2000	Taranto (Italy)	Mastrototaro and Brunetti (2006)
Aplidium accarense	**09/ 2014**	**5B**	2012–13	Catalonia (Spain)	López-Legentil et al. (2015)	≤2015	Fusaro Lake, Napoli (Italy)	X. Turon (pers. observ.) in Montesanto et al. (2021)
Polyclinum constellatum	**06/ 2014**	**5C**	2016	Port-Said (Egypt)	Halim and Abdel Messeih (2016)	2018	Taranto harbour (Italy)	Montesanto et al. (2022)
Botrylloides niger	**06/ 2014**	**5D**	2013	Israel	Rubinstein et al. (2013)	2018	Mar Piccolo, Taranto (Italy)	Salonna et al. (2021)
Polyandrocarpa zorritensis	05/ 2014	**5E**	1974	La Spezia (Italy)	Brunetti (1978)			=

Records (ML) backdating NIS arrival in Italy and/or in the Mediterranean Sea highlighted in bold.

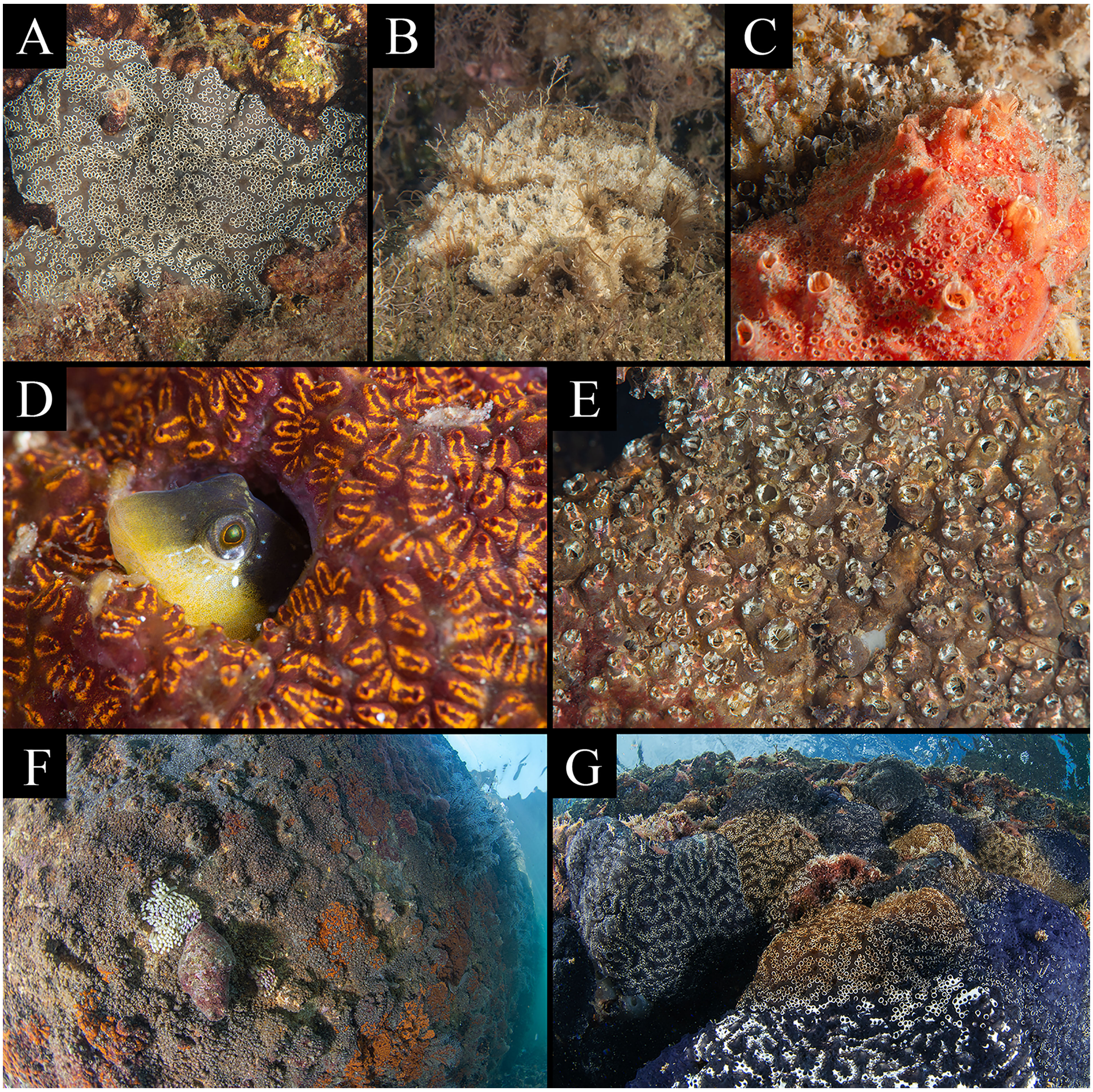

FIGURE 5 | Photographic evidence backdating NIS arrival in the Miseno Lake and impact of *Distaplia bermudensis* on dock wall communities (see **Table 2**). **(A)** *Distaplia bermudensis*, October 2019. **(B)** *Aplidium accarense*, September 2014. **(C)** *Polyclinum constellatum* (with *P. zorritensis*), June 2014. **(D)** *Botrylloides niger*, June 2014. **(E)** *Polyandrocarpa zorritensis*, May 2014. **(F, G)** The Casevecchie channel before and after the arrival of *D. bermudensis*, with abundant colonies of *P. zorritensis* and *B. niger* (left, August 2015) and colonies of *D. bermudensis* (of different colours) colonizing the same dock wall area (right, March 2021).

Fourteen and thirteen species were respectively found in CC and MC, with assemblages differing between them and both channels hosting exclusive taxa. With regards CC, eight species were found in the transect, with *D. bermudensis* (n=1542) and *B. niger* (n=794) being the most common, whereas 12 species were found in the photoquadrats, with *D. bermudensis* (n=680, c_m=13.1% ± 2.3%) dominating the assemblages (**Table 1** and **Supplementary Data Sheet 1**). The CC also accounted for the highest cover in a quadrat (c=44.3%) and for the highest mean cover per sector (c_m=22.3% ± 4.6%) (**Supplementary Data Sheet 1**). With regards MC, seven species were found in the transect, whereas 13 species were found in the photoquadrats; *B. niger* was the most common species in both of them (n=272; n=113, c_m=0.6% ± 0.3%) (**Table 1** and **Supplementary Data Sheet 1**). *Microcosmus polymorphus* Heller, 1877 ranked low in number of specimens found (n=5), but high in cover (c_m=0.2% ± 0.2%) due to the big size of the specimens found. The overall mean cover (c_m=2.6% ± 0.8%) was much lower than in CC (**Supplementary Data Sheet 1**).

Through the GAM analyses it was found that, when NIS and cryptogenic species were combined, the best model for their overall abundance on the seafloor was the full model (g_7), including all predictor variables (**Figure 8** and **Supplementary Table 3**). Their abundance declined with depth, increased with the availability of hard substrates, and was the highest in the

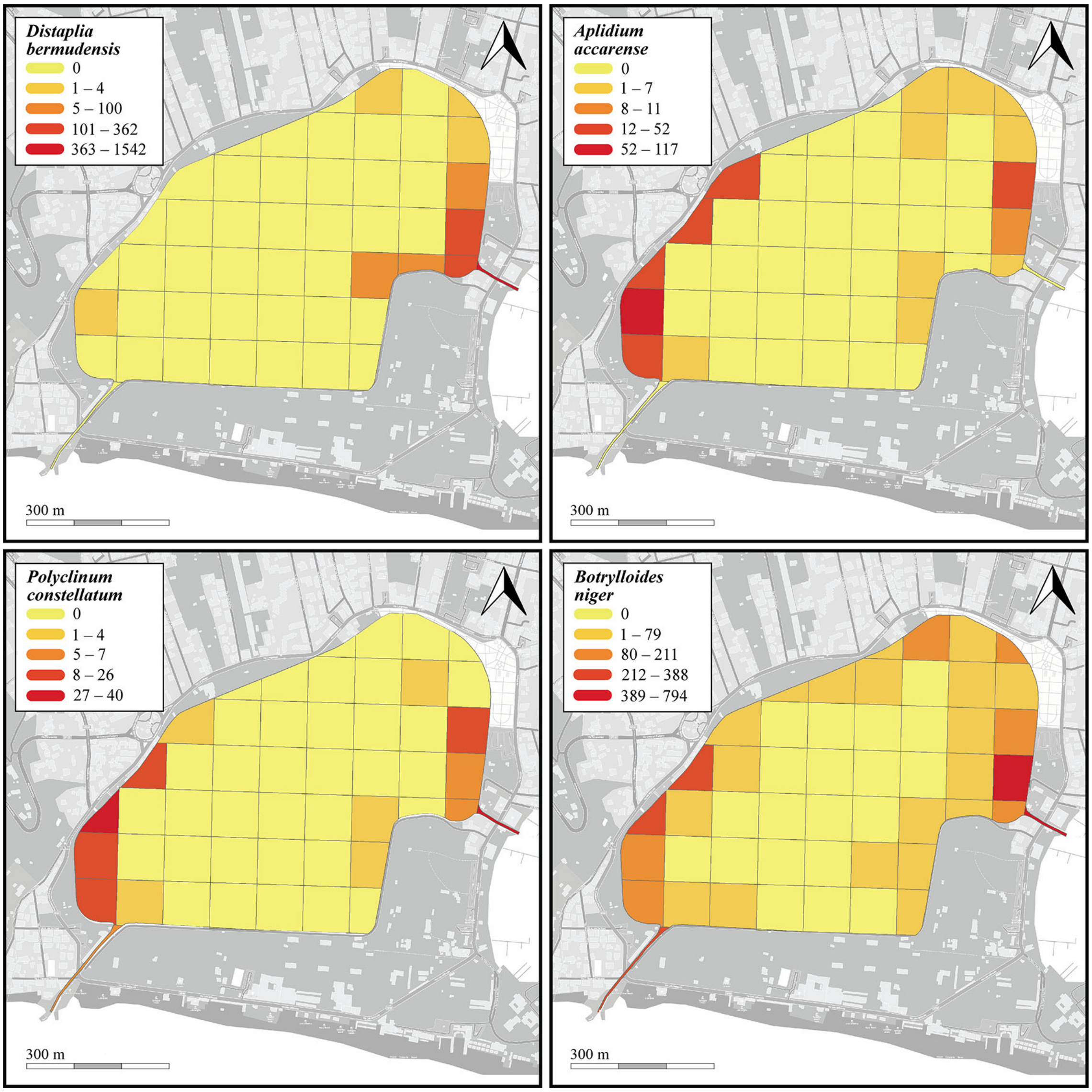

FIGURE 6 | Heatmaps showing abundance (counts per 100-m transect) of the four most common NIS (*Distaplia bermudensis*, *Aplidium accarense*, *Polyclinum constellatum*, and *Botrylloides niger*) found on the seabed of the Miseno Lake. For a detailed account of the sectors see **Figure 1**.

channels, particularly in the CC, and the lowest in the inner zone (**Figure 8**). The best model for the abundance on the seafloor of all native species combined together was g_1 (**Supplementary Table 4**), with only *section* as a predictor variable, having substantially higher support than the null model. For native species only h3 had high support, with no strong apparent effect of NIS or cryptogenic species (low support for h4, although it cannot be totally discarded) (**Table 3** and **Supplementary Figure 2**). With regards the dock wall perimeter, the model using the abundance of NIS and cryptogenic species as predictor variable was the best, although its Akaike difference with the null model was only 0.6, and thus h4 does not have substantially higher support than the null hypothesis; hypothesis h3 had even less support (**Supplementary Table 4**). Hence, although the cover of the native ascidians was negatively correlated to the cover of NIS and cryptogenic species (**Supplementary Figure 3**), no solid conclusion can be made at this stage.

With regards the four dominant NIS encountered in the Miseno Lake, namely *D. bermudensis*, *A. accarense*, *P. constellatum*, and *B. niger*, when analyzing the seabed, the best models had significantly higher support by the data in comparison to the null model (with Akaike differences >20), indicating that the assumption of completely random distribution of the species in Miseno Lake has no support (**Supplementary Table 3**). In all four cases, *section* was included in the best model, which indicates that the conditions among the two channels and the peripheral and the inner zones of

FIGURE 7 | Heatmaps showing percent cover per sector of the four most common NIS (*Distaplia bermudensis*, *Aplidium accarense*, *Polyclinum constellatum*, and *Botrylloides niger*) found on the dock walls of the Miseno Lake. For a detailed account of the sectors see **Figure 1**.

the lake were sufficiently different to affect the abundance of NIS ascidians; hypothesis h3 had high support (**Table 3**). Three of the four species (*D. bermudensis*, *P. constellatum*, and *B. niger*) had the lowest abundance in the inner part of the lake (zero in the case of *D. bermudensis*), and the highest in the channels and in particular in the CC (**Supplementary Figures 4**, **6**, **7**), whereas *A. accarense* was absent from the channels but still had higher abundance in the peripheral than in the inner zone (**Supplementary Figures 5**). *Depth* (hypothesis h1) was negatively correlated with abundance in the cases of *B. niger* and *P. constellatum* (**Supplementary Figures 6**, **7** and **Supplementary Table 3**); in the case of *B. niger*, h1 had high support (**Table 3**). In particular, *B. niger* and *A. accarense* seemed to be favored by the existence of appropriate substrates, with higher local abundances with increased availability of rocks and litter items; however, the hypothesis h2 had only medium support for these species (**Table 3**).

4 DISCUSSION

4.1 A Protected Area That Is an (Overlooked) Hotspot of NIS and Cryptogenic Species

Marine reserves are protected areas aiming to achieve conservation of nature with its associated ecosystem services and cultural values. Notwithstanding legal limitations that are in

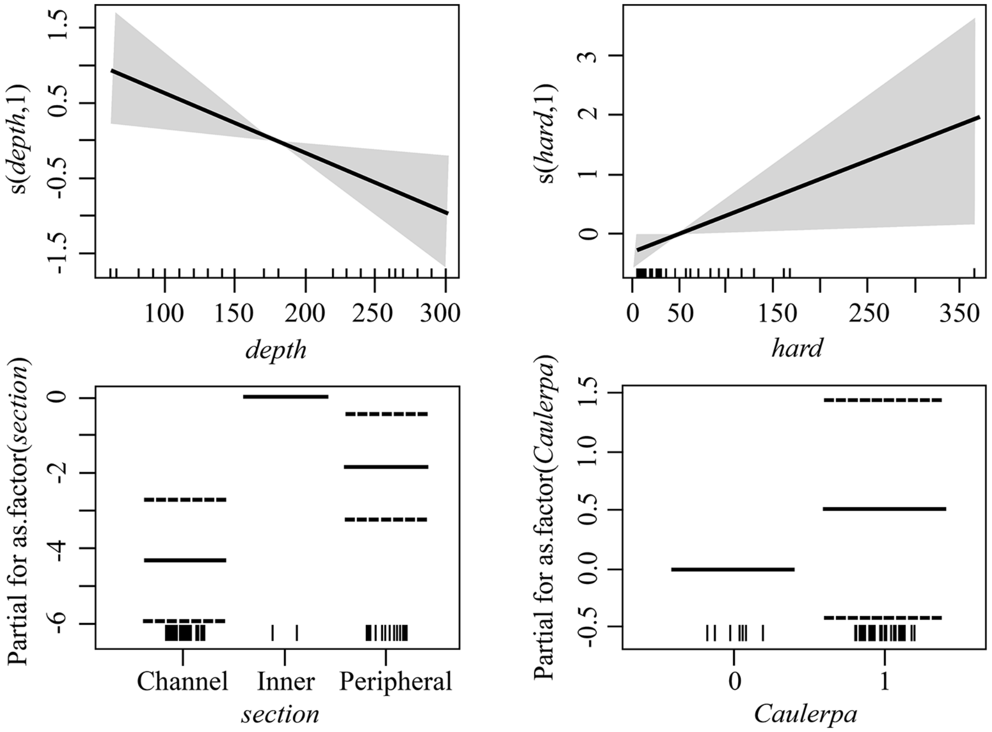

FIGURE 8 | Estimated terms in the best model for all alien and cryptogenic species abundance on the seafloor. The smooth terms or estimated levels of categorical predictors (unbroken black lines) are given in the linear predictor scale. The corresponding 95% confidence intervals are given as gray shadows/dotted lines. Rug plot on the horizontal axis represents density of data points.

TABLE 3 | Support by the data of the various hypothesis on the factors that affect ascidian abundance, based on Akaike weights (%).

Hypotheses	*Botrylloides niger*	*Distaplia bermudensis*	*Polyclinum constellatum*	*Aplidium accarense*	All alien and cryptogenic	All native
h1	**100.0**	43.3	57.7	8.7	**94.3**	29.1
h2	55.0	17.0	24.7	61.0	**72.8**	20.1
h3	**100.0**	**99.7**	**95.2**	**100.0**	**100.0**	**97.5**
h4	–	–	–	–	–	24.9

Hypotheses with high support (>70%) highlighted in bold.

place in these areas, a growing amount of literature concerns their vulnerability to biological invasions, in particular when they host, coincide with, or are close to potential hotspots of NIS arrival or spreading (Otero et al., 2013; Giakoumi et al., 2019; Bilecenoğlu and Çınar, 2021). Both qualitative and quantitative results of the present study match this statement. The benthic survey carried out here revealed that the Miseno Lake hosts a previously undetected thriving community of ascidians, with native taxa that still rank high in species richness (10 taxa: ~41.5%), but that are fewer than NIS (9 taxa: 37.5%) plus cryptogenic species (5 taxa: ~21%) combined.

Moreover, among NIS, only two (*C. robusta* and *S. plicata*) are relatively old introductions and thus almost ubiquitous in the Mediterranean Sea (Caputi et al., 2007; Pineda et al., 2016a), whereas the majority of them are only known from a few records at a regional level, with some taxa that were never found in the study area (*P. constellatum*, *P. zorritensis*, and *S. brakenhielmi*), others that were only known from single opportunistic sightings (*D. bermudensis*, *A. accarense*, and *B. niger*: Montesanto and Mastrototaro in Orfanidis et al., 2021; Montesanto et al., 2021; Della Sala et al., 2022), and *Microcosmus squamiger* Michaelsen, 1927 which was the only species widely recorded in the Gulf of Naples, but has not been reported again in grey or published literature for more than 20 years (since 1996: Turon et al., 2007). However, the absence of records also holds true at Mediterranean level and is particularly surprising for the four NIS dominating the assemblage. Indeed, the most abundant NIS in the Miseno Lake was *B. niger*, only known from a few specimens sequenced from Israel (Rubinstein et al., 2013; Griggio et al., 2014) and Italy (Taranto: Salonna et al., 2021; Fusaro Lake, Naples: Della Sala et al., 2022), followed closely by *P. constellatum*, only known by few localities in the eastern Mediterranean (Halim and Abdel Messeih, 2016; Aydin-Onen, 2018; Montesanto et al., 2022), and *A. accarense*, the only species apparently more widespread, but only in some areas of the western Mediterranean (López-Legentil et al., 2015; Montesanto et al., 2021). Finally, *D. bermudensis* is again only known from a few records in Spain (Balearic Islands: Pérès, 1957) and Italy (Taranto: Mastrototaro and Brunetti, 2006; Miseno Lake: Montesanto and Mastrototaro in Orfanidis et al., 2021). The present paper highlights a high local invasiveness for all these species, report *P. constellatum* for the first time in the western Mediterranean, document the presence of another species (hereby named clade C) in the complex of *D. bermudensis*, and finally backdate and georeference to the Miseno Lake the first finding of *A. accarense* and *B. niger* in Italy and of *P. constellatum* in the Mediterranean Sea.

Cryptogenic species also accounted for a significant part of the ascidian biota of the lake. In particular, *S. canopus* and *B. schlosseri* were widespread, while *C. lepadiformis*, *D. pseudovexillum*, and *Perophora viridis* Verrill, 1871 were rare. The first two taxa are common in shallow sheltered waters and account for at least five different clades each in the Mediterranean (Barros and Rocha, 2021; Salonna et al., 2021). Specimens of *S. canopus* from the Miseno Lake well match the lineage G10 *sensu* Barros and Rocha (2021), already known from India, Angola, and Mediterranean Spain, and first recorded here from Italy, whereas specimens of *B. schlosseri* belong to the subclade A1 *sensu* Salonna et al. (2021), widely recorded worldwide including in Italy. *Clavelina lepadiformis* is represented by two clades in the Mediterranean (Turon et al., 2003; Nuzzo et al., 2022), and specimens from the Miseno Lake belong to *C. lepadiformis* sp. B *sensu* Nuzzo et al. (2022), commonly found in semi-enclosed basins and only known with certainty in Italy from the nearby Fusaro Lake (Nuzzo et al., 2022). Finally, with regards the two remaining species, *P. viridis* was described from the northwestern Atlantic (Buzzard's Bay, Massachusetts, USA) (Verrill, 1871) but is very similar to several congeneric species, thus suggesting that at least part of the Mediterranean historical records may be based on misidentifications and making it difficult to trace a putative colonization history of the basin (Zenetos et al., 2017). This species was already recorded from the area by Neppi (1921), Salfi (1931), and Chimenz et al. (1985), and the Gulf of Naples is also the locality of description of its junior synonym *Perophora dellavallei* Neppi, 1921. Sequences of specimens from the Miseno Lake are the first based on Mediterranean specimens and proved conspecific with those from the USA deposited in GenBank, thus strengthening the synonymy between *P. viridis* and *P. dellavallei* and suggesting that historical records from the Gulf of Naples may be true. Finally, *D. pseudovexillum* was only known so far from Roscoff (Atlantic France) and Catalonia (Mediterranean Spain) and the present record widens its distribution to Italy. Prior to its formal description, this species was often mixed up with other didemnids, such as *Didemnum vexillum* Kott, 2002 (see discussions in Turon et al., 2020), and even the present finding was incidental, as the only colony found in the Miseno Lake was initially misidentified in the field as the native didemnid *Trididemnum cereum* (Giard, 1872).

Finally, some native species found here deserve attention. Among them, four — *Phallusia fumigata* (Grube, 1864), *P. mammillata*, *M. polymorphus*, and *P. dura* — are almost ubiquitous in Mediterranean coastal areas (e.g. Brunetti and Mastrototaro, 2017), whereas those of the family Didemnidae Giard, 1872 (*L. perforatum*, *L. weigelei*, and *T. cereum*) usually live in fouling communities of harbors and semi-enclosed basins (e.g. Chimenz et al., 1985; Casso et al., 2018), and even their presence in the Miseno Lake may be due to intra-Mediterranean transfers, as already speculated for the occurrence of both species of the genus *Lissoclinum* Verrill, 1871 on ship hulls in the Gulf of Taranto (Lafargue and Tursi, 1975). Integrative approaches, which include literature reviews and population genetic studies, may shed further light on that. It is noteworthy that the three remaining species (*C. sabbadini*, *A. colleta*, and *A. malaca*) are apparently quite rare in the Mediterranean Sea. The two former species were never recorded in Italy outside their area of description, namely the Adriatic Sea (Brunetti and Mastrototaro, 2017; Mastrototaro and Montesanto, 2021): for *A. colleta* this may be due to misidentifications with the very similar *Ascidia muricata* Heller, 1874, while *C. sabbadini* is almost unmistakable even in the field, suggesting that it may have ecological requirements that limit its distribution. On the other hand, *A. malaca* was originally described by Traustedt (1883) based on specimens collected in the Gulf of Naples, and considered conspecific with *Ascidia depressa sensu* Heller (1874) from the Lesina Lagoon (Adriatic Sea), an environment similar to the one investigated here. We figure topotypical specimens for the first time in more than a century. Although taxonomic uncertainties in the genus *Ascidia* prevented us from reaching any sort of taxonomic conclusion, the implementation of the genetic information on this genus, with two species newly sequenced here, may represent an advance toward resolving its taxonomy.

The present revealing results from an investigated area that is less than 1 km^2 in extent are somewhat unexpected, but are likely due to two main previous limitations, namely taxonomic impediments (or general absence of zoologists and taxonomic specialists in the area) and lack of specific monitoring programs. Although the ascidian fauna of the central-western Mediterranean Sea is considered as the most studied in the entire basin (Koukouras et al., 1995), the rich literature published on the Gulf of Naples and nearby areas mostly dates back to a century ago, when early pioneers focusing on Mediterranean biodiversity investigated the local biota (e.g. Della Valle, 1877; Della Valle, 1881; Traustedt, 1883; Salfi, 1929; Salfi, 1931; Parenzan, 1959). Then, in the subsequent historical periods, faunistic and taxonomic work has been often considered old-fashioned, resulting in a lack of proper studies but for a single one assessing seasonal diversity and shifts of ascidian species in Ischia harbor (Chimenz et al., 1985) and few NIS records generated opportunistically by specialists (López-Legentil et al., 2015; Montesanto and Mastrototaro in Orfanidis et al., 2021; Montesanto et al., 2021). All this somehow makes it difficult to discuss the ascidian assemblage found in the Miseno Lake in a local perspective. However, Chimenz et al. (1985) reported 12 species in the investigated area, seven of which — *C. lepadiformis*, *T. cereum*, *C. intestinalis* (now *C. robusta*), *P. viridis*, *B. schlosseri*, *S. partita* (synonym of *S. canopus*), and *S. plicata* — were shared with the Miseno Lake. The absence of the NIS now found dominating the Miseno Lake suggests that they may have arrived only recently in the Gulf of Naples, although no genetic support was available for the 1985 study, and the knowledge of the Mediterranean ascidian biota was more limited, and thus we cannot exclude that putative discrepancies in taxonomically difficult families (e.g. Didemnidae, Ascidiidae Müller, 1776, Styelidae Herdman, 1881, and Pyuridae Hartmeyer, 1908) may be the result of misidentifications. On the other hand, studies at the Mediterranean level are indeed more widespread. Knowledge of ascidian biota in selected areas was gained either through targeted studies (e.g. Gewing et al., 2017; Casso et al., 2018; Arroyo et al., 2021), data gathering for the creation of checklists (e.g. Lafargue et al., 1986; Koukouras et al., 1995; Moreno et al., 2014), or scattered records in surveys of fouling communities (e.g. Leclerc and Viard, 2018; Lezzi et al., 2018; Giangrande et al., 2021). Studies investigating port environments also often focused on NIS, including ascidians (e.g. López-Legentil et al., 2015; Marchini et al., 2015; Tempesti et al., 2020), whereas specific surveys in enclosed and semi-enclosed basins were carried out in several Mediterranean localities (e.g. Pérez-Ruzafa, 1989; Mastrototaro et al., 2008; Chebbi et al., 2010; Davis and Davis, 2010). In general, the native ascidian assemblage found in the Miseno Lake is substantially coherent with the ones reported in the aforementioned studies, whereas no studies reported such a high and contemporary presence of NIS and cryptogenic species in a single and somewhat restricted study site. All this identifies the Miseno Lake as a major hotspot of NIS and cryptogenic ascidian species in the Mediterranean basin, highlights that the spreading of at least some of these taxa went partially overlooked for about a decade, and suggests that the Miseno Lake protected area deserves the establishment of appropriate monitoring programs for new NIS detections at early stages of arrival.

4.2 General Characteristics of the Assemblage and Species-Specific Traits

The spatial analyses of the ascidian biota of the Miseno Lake revealed a general homogeneity of assemblages in terms of species composition, with dominant species occurring in almost all investigated transects and areas of the lake, although their cover and abundance often varied on the basis of the topography of the lake as well as of the main benthic substrate and presence/absence of additional substrates available for settlement (whether natural or anthropogenic). Finally, both channels also hosted exclusive taxa, notably different between them.

The dock wall represented the main pool of ascidians in the basin, although showing species-specific distributional patterns and abundances. As an example, *A. accarense* and *B. niger* were somewhat widespread in terms of mean cover and number of colonies in the entire lake, whereas *P. constellatum* was mostly found in the western side of the lake, where colonies also seemed to be bigger in average size, and *D. bermudensis* displayed a marked segregation, being mainly restricted to sectors close to the CC except for a cluster of colonies found in the southern side of the lake. Other species did not show a clear distribution pattern but were widely scattered, namely *P. dura*, *P. zorritensis*, and *S. canopus*, whereas the natives *C. sabbadini* and *M. polymorphus* and the NIS *M. squamiger* were rarely encountered.

The inner sectors hosting the dense *C. prolifera* meadow were not a preferred habitat for almost any of the ascidian species found in the Miseno Lake. In particular, although 15 species were censused in the transects and seven in the *Caulerpa* meadows, only *B. niger* and *B. schlosseri* were found as epiphytes on the seaweed, with *B. schlosseri* being widespread in small scattered colonies evenly distributed within the meadow, while *B. niger* was limited to the outermost parts of the meadow, thus close to the dock wall where it is dominant. However, where present, *B. niger* was apparently more aggressive in its colonization, fouling a large part of the leaves. On the other hand, in the peripheral parts of the lake, where the seaweed meadow becomes sparse or ends abruptly, the substrate was mainly sandy/muddy and ascidians were mostly found on/under scattered rocks, litter, or other biogenic substrates, with a community similar to the one living in the dock wall, but with the addition of *A. colleta* and *P. fumigata*. As revealed by GAMs, the presence of hard substrates such as rocks and litter also influenced ascidians distribution in the lake. Indeed, although litter was found in all the lake, the sectors near the dock wall were mostly characterized by heavy-weight sunken objects (e.g. tyres, cans, tarpaulins, and hard plastic), whereas the inner part was mostly littered by lower-density drifting objects (e.g. tissues and soft plastic), still often detached from the substrate. Therefore, litter colonization by ascidians mostly occurred in the peripheral parts of the lake, where litter objects soon turn into additional anthropogenic substrates available for the resident fauna. Finally, when present, other kinds of natural substrates were also often overgrown by tunicates, such as sunken woods or polychaete tubes.

Although we did not investigate here chemico-physical parameters such as water salinity or turbidity, the observed differences in ascidians' distribution are presumably related to the species-specific traits of the species found. Unfortunately, little is known of species' autoecology for the majority of the taxa found in the Miseno Lake, although several of them, namely *A. accarense*, *P. constellatum*, *B. niger*, and *P. zorritensis*, are well known to adapt to different environmental conditions (Van Name, 1945; Mastrototaro and Brunetti, 2006; Mastrototaro et al., 2008) and that is presumably why they thrived in almost all the lake from the calm inner side, under rocks or inside crevices, to the unsheltered areas of the channels. Other species, such as *P. mammillata*, *C. robusta*, and *S. plicata*, are known to tolerate calm waters and poor environmental conditions (Chimenz et al., 1985; Mastrototaro et al., 2008; Caputi et al., 2013), and thus their distribution in the lake is likely to be influenced by additional factors. However, the renowned invasiveness of several NIS indeed implies wide environmental tolerances, with species able to adapt to various environments and being able to resist temperature shifts (Shenkar and Loya, 2008; Grey, 2011) and sunlight exposure (Forward et al., 2000), or even to cope with the local environmental metabolome (Palanisamy et al., 2018). With regards to this, antifouling properties due to secondary metabolites are widely reported from the alga *Caulerpa prolifera* and congeneric species (Smyrniotopoulos et al., 2003; Dobretsov et al., 2006), and thus they may also act locally as inhibitors for larval settlement of ascidians, being thus at the basis of the absence of the majority of the species in the inner parts of the lake. Interestingly, the two only species fouling the leaves (*B. niger* and *B. schlosseri*) are phylogenetically closely related, which may led to the speculation that these species possess antagonist strategies against the antifouling molecules produced by the alga. In addition, litter material has already been shown to be a collector for sessile species on unconsolidated bottoms, enhancing the exploitable substrate (Katsanevakis et al., 2007; Crocetta et al., 2020) and influencing the overall biomass of benthic assemblages (Ramirez-Llodra et al., 2013). In our case, litter was mostly fouled by NIS (e.g. *D. bermudensis*, *A. accarense*, *P. constellatum*, and *B. niger*), thus possibly enhancing their invasiveness in the lake, as also supported by the GAM analyses.

Finally, the two lake channels differed markedly in terms of species diversity. The CC showed an ascidian diversity which is comparable to the rest of the lake, although with a significantly higher NIS cover and dominance. It also hosted four exclusive taxa, namely *C. lepadiformis*, *D. pseudovexillum*, *T. cereum*, and *P. viridis*. In contrast, the MC showed an overall lower ascidian cover, as well as a significantly different community, with *L. perforatum*, *L. weigelei*, and *S. brakenhielmi* only found there. The importance of the channels as species and biomass enhancers in lagoon habitats was already acknowledged by several studies (e.g. Sordino et al., 1989; Procaccini and Scipione, 1992; Macali et al., 2013), and this is mostly the result of the peculiar hydro-dynamic conditions occurring in these areas and of the dynamic balance between the massive arrival of larvae and the interference that currents may cause to larval settlement (Bingham and Young, 1991; Valentine et al., 2009). The higher ascidian cover, and particularly of NIS, found in the CC dock wall is presumably influenced by the general similarity of conditions between the eastern side of the Miseno Lake, the CC, and the nearby Bacoli Bay, an additional enclosed bay connected to the Miseno Lake through the CC itself. On the other hand, the MC is characterized in its outer part by an open marine environment dominated by photophilic algae, thus suggesting that only this channel has a true role as an ecotone, with a somewhat impoverished presence of brackish-water ascidians colonizing the dock wall. The marked difference observed in transects is, instead, likely due to the different substrate composition (rocky in the CC and sandy with scattered rocks in the MC).

4.3 Introduction Pathways, Potential Impacts, and Insights on NIS

The Mediterranean Sea is widely acknowledged as hotspot of native biodiversity, but at the same time it also hosts a high number of NIS (Coll et al., 2010; Zenetos et al., 2017), arriving in the basin through different pathways and with varying patterns by ecoregion (Galil, 2009; Zenetos et al., 2012; Katsanevakis et al., 2013; Katsanevakis et al., 2014; Convention on Biological Diversity, 2014). However, once NIS arrive in a biogeographic sector, the high functional connectivity in the Mediterranean Sea favors their secondary spread, often aided by small-scale (e.g. fishing and recreational boats) to medium- and large-scale (e.g. shipping) human transport (Katsanevakis et al., 2014; Ferrario et al., 2017), thus obscuring their routes and complicating the tracing of putative introduction pathways unless at early stages of arrival.

The conspicuous community of NIS ascidians found here in syntopy in the Miseno Lake immediately raises the question about their origin. The assemblage was composed of species native to different localities worldwide (from Western Atlantic to Indo-Pacific), thus suggesting that such co-occurrences are the result of different introduction events. However, the majority of them are renowned worldwide invaders that also already colonized other Mediterranean areas, and also sometimes co-occur there (e.g. Mastrototaro et al., 2008; López-Legentil et al., 2015; Casso et al., 2018). In addition, they are widely known to colonize new areas through shipping, whether in ballast water or as fouling communities of ship hulls (Gewing and Shenkar, 2017; Lambert, 2019), or through aquaculture (Mastrototaro et al., 2019; Ramos-Esplá et al., 2020), with the sole exception of *P. constellatum*, commonly considered to be a Lessepsian migrant (Halim and Abdel Messeih, 2016; Aydin-Onen, 2018; Montesanto et al., 2022). However, even for the latter species, the present record from the Miseno Lake, as well its presence in the area since 2014, would dismiss such hypothesis and rather suggest that transport-stowaway may have played a primary role also here, or that at least a combination of both pathways may have occurred in invading the Mediterranean Sea. Unfortunately, with few exceptions (e.g. Pineda et al., 2011; Pineda et al., 2016a; Pineda et al., 2016b for *S. plicata* and Rius et al., 2008 for *M. squamiger*), no detailed genetic information is known at a population level for all these species in the Mediterranean Sea and even worldwide, which makes it difficult to trace putative sources and pathways. This is also particularly relevant in the case of *D. bermudensis*, whose clade C is also present at least in Puerto Rico (see Streit et al., 2021), but for which no additional information is available from other Mediterranean localities, thus making it unknown whether other lineages of the complex are invading the basin yet. Finally, the general absence of genetic data in support of faunal studies worldwide is also why at least two of the species found here are considered as cryptogenic in the Mediterranean Sea, namely *P. viridis* and *D. pseudovexillum*. The former species was already discussed above, whereas, in the case of the latter species, further integrative taxonomic approaches focused on "whitish/creamish didemnids" could easily reveal that its distribution is not only overlooked in the Mediterranean, but even worldwide. Notwithstanding these premises, the known auto-ecology of these species and the presence of mussel farms and two active marinas in the vicinity of the Miseno Lake point towards the speculation that these areas may be the first sites of impact and thus the true reservoir of these species. Further field studies to be carried out in these localities will confirm or dismiss such a hypothesis.

Moreover, although this benthic survey did not focus on species-specific impacts of NIS, and no certain evidence of competitive restriction of native ascidians was found, several additional considerations can be made. As already mentioned above, lack of knowledge and mostly of field studies presumably hindered so far the recognition of true invasiveness of these species in the Mediterranean Sea. However, among these, *B. niger* is widely known to dominate fouling communities in native and invaded areas (Sheets et al., 2016; Nydam et al., 2021; Ramalhosa et al., 2021), and this also holds true for *A. accarense* (López-Legentil et al., 2015; Montesanto et al., 2021), *M. squamiger* (Turon et al., 2007; Rius et al., 2008), *P. zorritensis* (López-Legentil et al., 2015; Casso et al., 2018; Ramalhosa et al., 2021), and *S. brakenhielmi* (Mastrototaro et al., 2019; Ramos-Esplá et al., 2020), which are worryingly expanding in the Mediterranean and becoming stable presences in littoral environments. In addition, the eminent invader *P. constellatum* is also considered a serious potential threat to native communities (Tovar-Hernández et al., 2010; Govindharaj et al., 2022). All these statements well match the high abundances of NIS observed here. Two of these species, namely *D. bermudensis* and *B. niger*, were also found to aggressively overgrowing tubes of the polychaete *Chaetopterus variopedatus* in the sectors close to CC (C8, D8, E7, E8, E9), sometimes totally covering the aperture of the tubes and thus providing evident stress to the native polychaete (see **Figure 2G**). Such interaction was never reported before in the Mediterranean Sea, but aggressive overgrow on benthic organisms by other members of the genus *Distaplia* Della Valle, 1881 was already reported for *Distaplia viridis* Kott, 1957 and *Distaplia* cf. *stylifera* (Kowalevsky, 1874), and may possibly lead to mass mortality events of the local biota (Russ, 1982; Moreno-Dávila et al., 2021). Although true impacts of these NIS on *C. variopedatus* have still to be properly evaluated, the observed occurrence was worrying since most of the tubes observed were affected, and *D. bermudensis* already showed aggressive behaviour, as also highlighted by comparison of CC dock wall communities before and after its arrival. Finally, the finding of *S. brakenhielmi* in a typically marine environment in the outermost part of the MC, and its absence in other areas of the lake widely colonized by NIS, somewhat disagrees with previous Mediterranean records of this species, generally confined to mussel farms and port environments (Mastrototaro et al., 2019; Ramos-Esplá et al., 2020), and suggests that this species may easily thrive even outside the areas usually targeted for NIS detection.

5 CONCLUSIONS

The presence of NIS is a constantly growing phenomenon in the Mediterranean Sea, which can only be tackled through coordinated and pan-Mediterranean efforts to control their main pathways and identify priority areas for constant-surveillance for a wide range of taxa. Constant monitoring programs should be linked to an early warning-rapid eradication mechanisms, as eradication in the marine environment can only be feasible at the very early stage of introduction.

The present study, carried out through a multidisciplinary approach, provides the first snapshot of the ascidian biota of the Miseno Lake and in general of the wider region about 40 years after the last investigation, revealing a massive presence of NIS, several of which are only known from a few records throughout the entire Mediterranean basin. Thus, these species are presumably more widespread than suspected, suggesting that it is already too late for a successful eradication and that local introduction pathways of NIS have been acting undisturbed for at least a decade.

Finally, this also constitutes the first study on a previously understudied environment, and provides a strong baseline to evaluate eventual shifts in the ascidian communities in future and to document the arrival of new NIS in the area. It also confirms semi-enclosed basins as hotspots of introduced species and shows that even marine reserves may be widely vulnerable to biological invasions. Further studies aiming to explore this and other phyla are therefore awaited from the Miseno Lake, the Phlegraean area, and more generally the Gulf of Naples, to evaluate the true magnitude of local NIS invasions.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

AF and FC proceeded to funding acquisition. FC designed and directed the research. RV, VT, GV, and FC performed fieldwork. RV and VT performed molecular analyses. FT performed bathymetric mapping. SK performed the statistical analyses. RV and FC wrote the main manuscript. All authors reviewed and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.866906/full#supplementary-material

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Lessons From the Western Atlantic Lionfish Invasion to Inform Management in the Mediterranean

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Major invasions of Indo-Pacific lionfish (*Pterois volitans* and *P. miles*) are underway in the Western Atlantic Ocean and the Mediterranean Sea. While the establishment of lionfish in the Western Atlantic is perhaps the most well-studied marine fish invasion to date, the rapidly expanding invasion in the Mediterranean is more recent and has received less attention. Here we review and synthesize successes and failures from two decades of lionfish management in the Western Atlantic to give policy recommendations for their management in the Mediterranean. Two failed approaches that were attempted multiple times in the Western Atlantic and that we advise against are (1) feeding lionfish to native fish to promote predation and (2) implementing bounty programs to incentivize lionfish harvest. Broadly, the most important management lessons that we recommend include (1) conducting routine removals by spearfishing with scuba, which can effectively suppress local abundances of lionfish; (2) encouraging the development of recreational and commercial lionfish fisheries, which can promote long-term, sustainable lionfish population control; and, (3) engaging local communities and resource users (e.g., with lionfish removal tournaments), which can concurrently achieve multiple objectives of promoting lionfish removals, market-development, research, and public education.

Managers in the Western Atlantic often needed to adapt current conservation policies to enable lionfish removals in areas where spearfishing with scuba was otherwise prohibited for conservation purposes. The risk of abusing these policies was mitigated through the use of gear restrictions, diver trainings, and through participatory approaches that integrated scuba divers and stakeholder organizations in lionfish research and management. Our review of policies and practices in the Mediterranean Sea found that many of our recommended lionfish management approaches are not being done and indicate potential opportunities to implement these. We expect and fully recommend that work continues towards multinational cooperation to facilitate regional coordination of research, control, and management efforts with respect to the Mediterranean lionfish invasion. As with other major biological invasions, lionfish are unconstrained by political borders and their control will require rapid and strategic management approaches with broad cooperation among and between governments and stakeholders.

Keywords: fisheries management, invasive species, marine policy, Mediterranean sea, *Pterois sp*

INTRODUCTION

Indo-Pacific lionfish (collectively *Pterois volitans* (Linnaeus, 1758) and the visually indistinguishable *Pterois miles* (Bennett, 1828) represent some of the most successful marine fish invasions on record (Albins and Hixon, 2008; Savva et al., 2020). Their expansion, establishment, and densities have been facilitated by their broad diet (Dahl and Patterson, 2014; Peake et al., 2018), foraging techniques novel to naïve prey (Albins and Lyons, 2012; Akins et al., 2014; Green et al., 2019), a relative lack of biotic resistance to their establishment (Valdivia et al., 2014); and an opportunistic life history strategy (Fogg et al., 2017). Lionfish are also defended against predators by venomous spines and have few natural predators (Ulman et al., 2021), and natural control of lionfish appears limited due to resistance to parasites (Sikkel et al., 2014; Fogg et al., 2016; Tuttle et al., 2017; but see Harris et al., 2018; Harris et al., 2020a). Within their invaded range, lionfish have reached densities up to ten times that of their native range and this has caused demonstrable negative impacts on native species and ecological processes (Côté and Smith, 2018; Savva et al., 2020). They remain a key concern for marine conservation and management (Morris et al., 2012; Sutherland et al., 2017).

Two lionfish invasions are currently underway. In the Western Atlantic, lionfish (*P. volitans* and *P. miles*) were likely introduced *via* aquarium releases in SE Florida and were first detected in 1985 (Morris and Akins, 2009), and their range expansion began in the early 2000s (Schofield, 2009). Populations are now established throughout the Gulf of Mexico, Caribbean Sea, and southeastern seaboard of the United States of America (**Figure 1A**). Their year-round northern range reaches North Carolina, and their southern range is actively expanding along the South American seaboard (Kimball et al., 2004; Luiz et al., 2021). Given the species' thermal tolerances, continued southward expansion is expected along the coast of Brazil (Kimball et al., 2004; Biggs, 2009; Luiz et al., 2021). The first recorded lionfish in the Eastern Mediterranean Sea was in 1991, but their initial establishment in the region in 2012 was in Lebanese waters (Bariche et al., 2013). Their introduction into the Eastern Mediterranean was *via* the Suez Canal (Bariche et al., 2017), which accounts for over half of over 600 established non-native species in the Mediterranean (Galil et al., 2018; see also Galil et al., 2015; Samaha et al., 2016; Mavruk et al., 2017; Stern et al., 2019; Zenetos and Galanidi, 2020). In the last decade, the range of lionfish has expanded to the Central Mediterranean and is progressing towards the Western Mediterranean region (**Figure 1B**) (Kletou et al., 2016; Azzurro et al., 2017; Ulman et al., 2020; Ulman et al., 2021; Dragičević et al., 2021).

This study aims to help inform lionfish management in regions where the species recently invaded or are expected to invade. Given the longer invasion, much of the current biological and ecological knowledge of lionfish comes from research in the Western Atlantic (Fishelson, 1975; Darling et al., 2011; Cure et al., 2012; Kulbicki et al., 2012; Pusack et al., 2016; Savva et al., 2020; Watkins et al., 2021). We synthesize key lessons learned from the Western Atlantic for the management approaches that were successful—or failed—to help control lionfish. The information we collected about existing policies and adaptive management in the Western Atlantic and Mediterranean are provided in full *via* our companion data repository published online (Candelmo et al., 2022). While our synthesis and recommendations were developed with the objective of guiding marine invasive species management in the Mediterranean Sea, we expect that these can also be applied to the expanding range of lionfish in South America.

LESSONS FROM THE WESTERN ATLANTIC

Unsuccessful Approaches

Training Native Fish to Become Lionfish Predators

In the early phases of the lionfish invasion, well-intentioned divers attempted to train native predators to prey on lionfish by

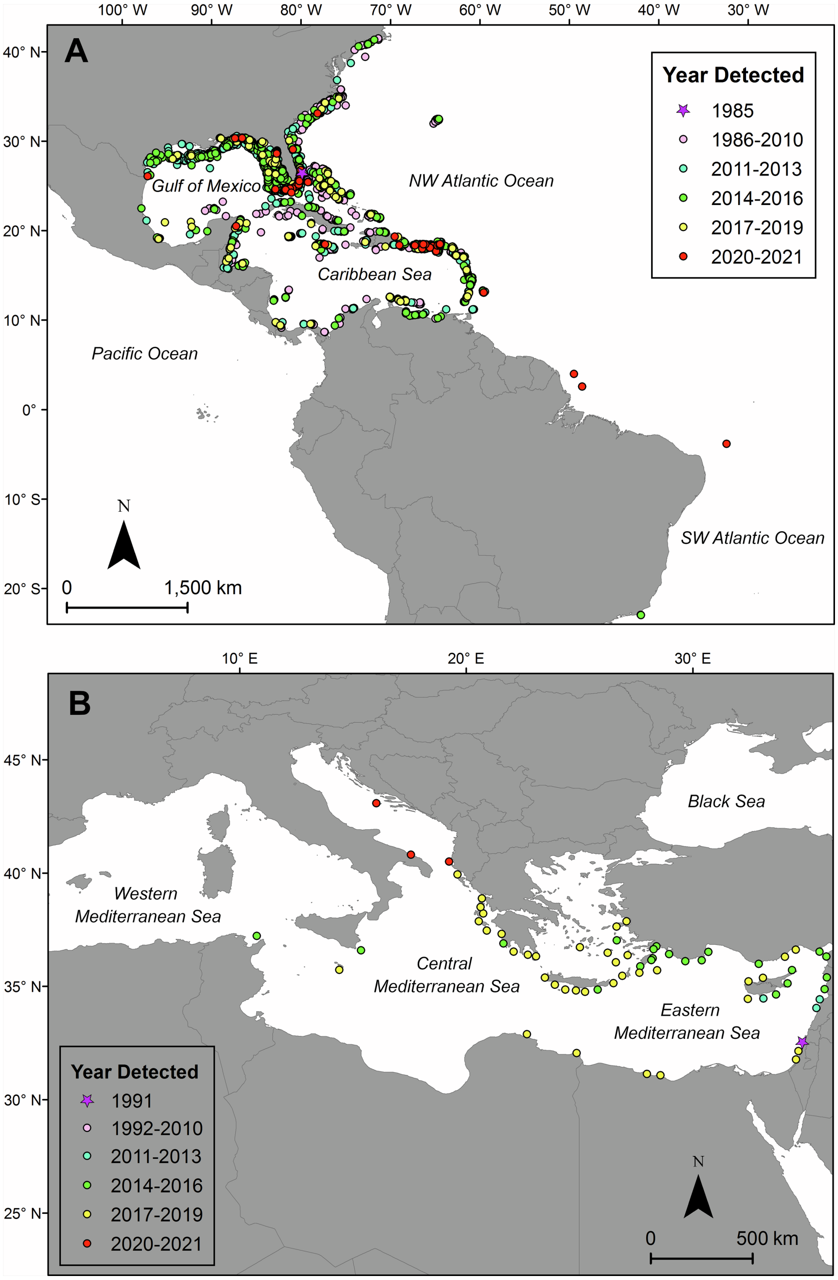

FIGURE 1 | Recorded sightings and chronology documenting the expansion of non-native lionfish (*Pterios volitans* and *P. miles*) in the **(A)** Western Atlantic Ocean and **(B)** Mediterranean Sea to 2021.

feeding them speared fish (Kilgo, 2014). For example, there are accounts of resident Nassau groupers that learned to lead divers to lionfish, stopping and turning when they reached one (pers. Obs., S. Gittings). Although there were instances of many native fish willingly ingesting speared lionfish, there is little evidence that native fish have become independent predators on lionfish as a result of this training. Moreover, dangerous interactions with marine predators increased due to this feeding practice. For example, nurse sharks (*Ginglymostoma cirratum*), Caribbean reef sharks (*Carcharhinus perezi*), bull sharks (*Carcharhinus leucas*) and moray eels (*Gymnothorax funebris*) began to associate divers with food (pers. Obs., authors). These predators will follow divers closely and even attempt to take them from containment devices (pers. Obs., A. Fogg). Lionfish removal activities were halted in Saba due to dive staff being attacked by a shark while spearing lionfish (Carballo-Cárdenas and Tobi, 2016). The issue has led some authorities to restrict lionfish removals to trained personnel (Candelmo et al., 2022). In some places, divers now dedicate one to watch for and repel aggressive sharks or eels, while the other diver or divers search for and remove lionfish. The degree to which this problem occurs varies from place to place, but presenting lionfish to native fish in an attempt to promote predator control has made lionfish removal efforts more dangerous and difficult. We advise that all culled lionfish be contained and removed.

Bounty Programs to Motivate Lionfish Hunters

Bounty programs incentivize lionfish removal by paying individuals for each lionfish collected. The government of Belize initially offered $25 BZD per lionfish. However, the program exhausted its funds in less than six months (Chapman et al., 2016). A $5 USD bounty per lionfish was similarly tried early in the invasion of the northern Gulf of Mexico by the state of Mississippi, but funds for this program were also rapidly depleted. Additionally bounty programs may also fail to engender stewardship ethics that would promote sustained harvest (Akins, 2012). With the current recognition that lionfish cannot be eradicated due to refuge and metapopulation dynamics (Johnston and Purkis, 2014; Johnston and Purkis, 2015; Andradi-Brown, 2019), we advise the use of economically self-sustaining control measures (discussed below).

Successful Approaches

Organized Removals Can Control Lionfish Densities

Since lionfish eradication is not possible, functional eradication can mitigate their impacts on invaded fish communities and the fisheries they support (Bogdanoff et al., 2021; Green and Grosholz, 2021). Human removals are currently considered the most effective means of controlling densities and impacts (Morris et al., 2011; Chapman et al., 2019). These removals are accomplished primarily *via* spearfishing with short pole spears on scuba (Frazer et al., 2012; de León et al., 2013; Côté et al., 2014; Dahl et al., 2016; Malpica-Cruz et al., 2016; Green et al., 2017; Côté and Smith, 2018; Harms-Tuohy et al., 2018; Harris et al., 2019; Kleitou et al., 2021a). Most jurisdictions within the Western Atlantic invaded range allow for removal of lionfish, either as a general policy or by special permission (Candelmo et al., 2022). Many jurisdictions allow for lionfish removal *via* spearfishing within MPAs (ibid). For example, in Honduras, Roatan Marine Park distributes pole spears and provides training specifically for removing lionfish (Peiffer et al., 2017). The dive sites where these removals took place resulted in lower lionfish densities compared to non-removal sites (ibid). Culling frequency is an important factor in minimizing negative ecological effects from the invasion (Dahl et al., 2016). Implementing control programs early in the invasion is also recommended (Côté et al., 2014). In Bonaire, a spearfishing removal program led by volunteers was immediately initiated after the first lionfish sighting in 2009 (Ali et al., 2013) and a similar program began on Curaçao two years later (de León et al., 2013). More than a decade later, lionfish densities within recreational dive limits at marked dive sites in Bonaire have remained low relative to similar areas elsewhere (pers. Obs., F. Ali).

Even dive destinations visited less frequently (e.g., due to remoteness, lack of dive infrastructure, access restrictions, or seasonally unfavorable weather) can benefit from organized removal efforts. At the Flower Gardens Banks National Marine Sanctuary, 160 km offshore in the northwest Gulf of Mexico, culling events have been organized since 2015 by specifically licensed dive operators. These "lionfish invitationals" have only been able to be conducted once or twice per year; however, the events remove hundreds of lionfish within the sanctuary. Evaluation of these removal efforts by Davis et al. (2021) showed that the efficiency of removals can be maximized by using experienced lionfish hunters and when removals take place during dawn and dusk (due to higher foraging rates of lionfish and detection by divers during these crepuscular periods). The densities of lionfish in the sanctuary have remained low compared to Western Atlantic reefs, although additional removal effort may be needed to reverse upward trends in lionfish abundances (M. Johnston, pers. Comm.).

Lionfish Tournaments Offer Concurrent Benefits

Lionfish tournament events (also called "derbies," "roundups," and "rodeos") coordinate concentrated organized removals. Tournaments consist of planned, competitive removal events, whereby divers compete to remove lionfish within a set time period. The top prize is typically awarded to the dive team that harvests the most lionfish during the tournament. There are usually categories for the largest and smallest lionfish as well, which may be won by less experienced spearfishers and encourage novices to participate in the competition. Based on our review, derbies have been organized in at least 25 Western Atlantic jurisdictions (Candelmo et al., 2022). Tournaments conducted in The Bahamas and Florida Keys showed reduced lionfish densities by >50% in an approximately 200 km^2 (Green et al., 2017). The largest tournament to date is the annual Emerald Coast Open tournament held in Destin, Florida. In 2019, 189 scuba divers competed for over $70,000 in prizes and removed over 14,000 lionfish in just two days.

Importantly, lionfish tournaments can concurrently achieve multiple objectives beyond the removals to help support research, education, and conservation awareness (Anderson et al., 2017). Researchers use these mass removal events to collect data on population structure, life history, and diet (e.g., Fogg et al., 2017). Indexes of abundance can be assessed by examining participants' catch-per-unit-effort (Harris et al., 2020b). To do so, tournament organizers need to collect data on fishing effort (e.g., number of divers and dives, total dive time) as well as harvester catches. Tournaments also attract out-of-town participants who contribute to the local economy. Trotta (2014) found that 47% of their participants were not residents of the community where the tournament was held. The tournaments and their associated public events (e.g., lionfish tastings, chef cook-offs, festivals) can help raise awareness and provide education about lionfish, as shown in Florida (Trotta, 2014) and Belize (Chapman et al., 2019). Priority public outreach messaging about lionfish consists of communicating that lionfish are (1) non-native, (2) environmentally destructive, and (3) safe and desirable to eat. Finally tournaments can serve as a platform for marine and environmental conservation education about other priority issues, such as invasive species more generally, overfishing, marine litter, and climate change.

Sustaining organized lionfish removal events year after year can be challenging. In some cases, historic, high profile annual events have been discontinued (e.g. Guy Harvey Lionfish Safari, NE Florida Lionfish Blast). This can be the result of lack of interest, dilution of participation due to excessive frequency (e.g., weekly), loss of financial support, and difficulties with planning around unpredictable weather. In the state of Florida, the Florida Fish and Wildlife Conservation Commission (FWC) has provided financial and logistical support for tournaments since 2014 (FWC, 2018). The number of lionfish tournaments in the state peaked in 2015 with 40 FWC-supported events in 2015 (ibid). This has gradually declined to only eight in 2021. Part of the decline may be attributed to the consolidation of tournaments into less frequent but larger events. For example, the aforementioned Emerald Coast Event, which offers considerable prizes as well as novel events and additional ways to participate beyond the two-day event that are less dependent on favorable weather conditions.

Community and Stakeholders Should Be Engaged to Facilitate Lionfish Management

Stakeholder participation involves the inclusion of diverse resource user groups in the management and planning process. For lionfish management, this has included volunteers, citizen scientists, fishers, seafood wholesalers and retailers, chefs, students and local citizens. Stakeholder participation in lionfish research has been particularly effective for facilitating monitoring and control efforts for collecting specimens, reporting observations, and the dissemination of information (Clements et al., 2021). For example, in Bonaire, culled lionfish were used to study their invasion ecology, and partnerships were formed between the management agency (Stichting Nationale Parken Bonaire), a research facility (CIEE Research Station, Bonaire), dive operators, and volunteer divers (Ali, 2017). Stakeholder engagement can range from the use of informal partnerships, such as allowing or promoting culling and derbies, to formal strategic planning. Belize's 2019-2023 National Lionfish Management Strategy used a formal planning process built on the concept of coupled human and natural systems (Chapman et al., 2019) to develop recommendations for lionfish management. Social and ecological indicators were assessed and reviewed during community meetings, and monitoring these indicators enables adaptive management (Allen and Garmestani, 2015). To mitigate potential risks of noncompliance and poaching, Belize's Lionfish Working Group also implemented specialized training programs for tourist divers (Chapman et al., 2019). Additionally, local divers and diving organizations enable consistent monitoring and removal efforts. For example, the Caribbean Oceanic Restoration and Education (CORE) Foundation in the U.S. Virgin Islands helped to develop the territory's Caribbean Lionfish Response Program and has conducted regular (generally weekly) lionfish removals since 2009.

Opportunities Exist to Develop Commercial Lionfish Fisheries

Commercial lionfish fisheries offer a potential market-based solution to control densities (Morris et al., 2012; Noll and Davis, 2020), improve food security, and diversify fisher catches and livelihoods (Chapman et al., 2016). These removals may be of greatest value in regions where governments have not pursued control efforts (Graham and Fanning, 2017). Lionfish meat is considered high-quality (Morris et al., 2011; Blakeway et al., 2019; Noll and Davis, 2020), and annual commercial spearfishing landings of lionfish for food in the U.S. northern Gulf of Mexico have been as high as 20,000 kg (Harris et al., 2020a). Current ex-vessel prices of lionfish in the United States are approximately $10–$14 USD per kg, which compares to high-end reef fish prices (Simnitt et al., 2020). Prices in Belize and the Cayman Islands are also similar to high-end reef fish. In these areas, prices have increased over time, mostly due to public awareness campaigns, which communicated that lionfish are a safe-to-eat, tasty, and an environmentally-friendly seafood product (Morris et al., 2011; Chapman et al., 2016; Blakeway et al., 2019).

Lionfish byproducts, e.g., leather from lionfish skin or jewelry made from lionfish fins and spines (Karp et al., 2015; Mulgrew, 2020), could increase their dock-side price and incentivize their harvest. For example, a lionfish jewelry women's group supported income generation, social wellbeing, and skill acquisition among women in Belize's artisanal fishing communities (Karp et al., 2015; Guerrero, 2020). Live captured lionfish can be sold in the aquarium industry and some jurisdictions have implemented moratoriums on the import of live lionfish to encourage local harvest and to reduce the introduction of new genetic material (e.g., Florida, USA). Innovative harvest technologies may also offer opportunities to maximize fisheries resources (Harris et al., 2021). Lionfish have been observed as deep as 300 m (Gress et al., 2017) and experimental harvest gear such as lionfish traps could enable commercial harvest of these populations when their catch efficiencies make them economically viable (Harris et al., 2020b).

There are also reasonable concerns for developing markets for an invasive species,. First, short-term economic dependence could result from a burgeoning lionfish harvest (Nuñez et al., 2012; Pasko & Goldberg, 2014). For spearfishing lionfish, however, their harvest may simply augment current fishing activity. Levels of removal are comparatively small, but so is the capital investment in harvest gear (i.e., short pole spears and a lionfish container), making continued harvest pressure likely. Strong communication between managers and fishers is recommended to prevent overcapitalization and ensure that objectives are shared (i.e., mitigating lionfish impacts and improving fisheries resources). Although harvest control rules (e.g., bag limits, size limits, or seasons) are clearly not recommended for lionfish given the goal to reduce their impacts, an ideal fishery would achieve an "optimum lionfish yield," with quantitative targets that allow managers to adapt policy strategies to achieve functional control of lionfish without losing the supplemental economic and socio-ecological benefits of harvesting them (Chagaris et al., 2017; Chagaris et al., 2020; Bogdanoff et al., 2021; Green and Grosholz, 2021).

Policy Support for Management

Policy Adaptation Enables Effective Lionfish Management

Lionfish management plans have been developed throughout the Western Atlantic region across a variety of scales (Graham and Fanning, 2017). In the U.S., for example, response specific plans exist for national parks (McCreedy et al., 2012) and national marine sanctuaries (Johnston et al., 2015), in addition to the overall U.S. National Plan (Invasive Lionfish Control Ad-hoc Committee of the Aquatic Nuisance Species Task Force, 2014). Forecasting potential undesirable consequences is essential to effective policy development (Levin et al., 2012). In the case of permitting spearing while using scuba in areas, policymakers must consider non-compliance with resource regulations and the opportunistic take of protected species (Solomon et al., 2015). Policy changes to allow scuba divers to spear lionfish with special permits have been adopted in Bermuda, Cayman Islands, the Turks and Caicos Islands, Saba, St. Eustatius, St. Lucia, Aruba and Bonaire, as well as Cyprus, Israel and the Egyptian Mediterranean (Candelmo et al., 2022).

Invasive Species Policy and Management Should Be Regionally Coordinated

The vast and connected nature of oceans makes managing marine biological invasions exceptionally challenging (Simberloff, 2000; Bax et al., 2001; Thresher and Kuris, 2004). Regional strategies should include measures to detect, monitor, and control populations (Mehta et al., 2007; Burgiel, 2014). The 24th General Meeting of the International Coral Reef Initiative (ICRI) created an *Ad Hoc* Committee to develop a strategic plan for the control of lionfish (Gómez Lozano et al., 2013). The resulting Regional Lionfish Management Strategy served as the framework for governments and other stakeholders to use when creating their national lionfish management plans (Gómez Lozano et al., 2013). The objectives were to: (1) facilitate collaboration between stakeholders for coordinated efforts across political and geographic boundaries; (2) encourage coordinated research and monitoring; (3) encourage governments to review and amend relevant legislation and, if necessary, develop new regulations and policies to control lionfish; (4) control invasive lionfish populations where possible, and; (5) provide education and outreach mechanisms to generate public support and foster stewardship in invasive lionfish programs (Morris et al., 2012; Gómez Lozano et al., 2013). Similarly, the Florida FWC sponsored a "Lionfish Summit" in 2013 as well as a follow-up summit five years later in 2018. The goals of these summits were to: (1) assess the efficacy of current research, management, control, and outreach efforts; (2) prioritize areas for future lionfish control, and; (3) improve collaborations amongst multiple agencies and geographic locations (FWC, 2018). The results of these summits were used by the agency to help to address regulatory barriers for lionfish fisheries; inform resource allocations by the state of Florida for research, education and outreach; and direct future research and technology development (e.g., lionfish-specific traps and other novel gear types).

CURRENT EASTERN MEDITERRANEAN LIONFISH MANAGEMENT

Some areas have begun limited management efforts to control lionfish. For example, in Turkey permission has so far been provided to allow single-day scuba removal events. In Israel, a permit was issued as a pilot study to a single diving center for culling lionfish with scuba diving and pole spears. In the Egyptian Mediterranean, 70 scuba divers were issued spearfishing permits. Overall, however, targeted control efforts in the Mediterranean remain limited. Currently, national and European laws prohibit spearfishing with scuba in much of the Eastern Mediterranean. Although such regulations were developed for conservation purposes, they also inhibit removals of invasive species including lionfish. Lionfish removals by scuba divers are currently prohibited in Greece, Turkey, Lebanon, Tunisia, Algeria, and Israel (Candelmo et al., 2022).

Cyprus has initiated official policy changes for lionfish management and is the only locality currently undertaking substantial targeted removal efforts (Candelmo et al., 2022). On its northern coasts, permits to spear lionfish were granted in 2017 from regional authorities to conduct a pilot program allowing scuba divers to spear lionfish under co-management of the Underwater Research and Imaging Center and Deep Dive Diving Center. Culling has occurred at least once a week, changes in lionfish abundance are being monitored, and the program has removed over 35,000 lionfish between June 2018 and August 2021 (Çiçek, unpubl. Data). In 2017, authorities also permitted a hunters federation to organize lionfish derbies for freedivers and scuba divers and over 3,800 lionfish were removed from the four tournaments to date. Additionally, a recently proposed by-law to authorize individuals and other dive operators to apply for permits to harvest lionfish with scuba is awaiting parliamentary approval. On the southern coasts of Cyprus, governmental authorities issued a permit in 2018 to the RELIONMED EU LIFE project to train scuba divers to remove lionfish for the purposes of research and control. The permit allows project researchers and 100 members of "Removal Action Teams" to remove lionfish with scuba and pole spears. These divers were trained on hunting and handling safety and first aid for stings, taught the ecology and history of the lionfish invasion, and received lionfish spearfishing equipment—including pole spears, containment units, puncture-resistant gloves, and heat packs to treat stings. The removal events were supervised and monitored by scientists, and have provided data for assessing the efficiency of controlling lionfish in Cyprus (Kleitou et al., 2019; Kleitou et al., 2021a; Kleitou et al., 2021b). To date, over 5,500 lionfish have been removed in about 40 targeted removal events hosted by RELIONMED, including eight tournaments.

SYNTHESIS OF MANAGEMENT RECOMMENDATIONS

Biological invasions are notoriously difficult to manage. Critical examination of current management may be needed following an unprecedented event like a biological invasion (Kleitou et al., 2021b); however, delayed management response can result in protracted ecological and economic impacts the invasion (Hewitt et al., 2009; Kleitou et al., 2021c). With invasive lionfish in the Mediterranean, for example, policy examination required three years before new policies were implemented in Israel (Stern and Rothman, 2019a; Stern and Rothman, 2019b). The situation is complicated by the fact that nations like Israel and Egypt border two seas—the Mediterranean where lionfish are invasive and the Red Sea where lionfish and other *Pterois* species are native protected species that cannot be fished or traded. Lessons from Western Atlantic indicate lionfish abundances increase rapidly following detection and can reach peak levels two years (Linardich et al., 2021), highlighting the need for rapid action.

This review synthesizes lessons from several decades of lionfish management in the Western Atlantic to provide actionable, policy-relevant management recommendations. Broadly, our review suggests that invasive lionfish managers should support sustained lionfish removals with participatory approaches and cooperative management. Crucially, these strategies require strong communication between government managers, stakeholder organizations, and individuals. To facilitate this, we summarize our recommendations with an illustrated infographic (**Figure 2**). Illustrative examples of these lessons and key considerations for these recommendations are provided in **Table 1**.

Policy Changes to Permit Lionfish Removal

Policy makers are better able to manage the impacts of lionfish *via* evidence-based decisions and coordinated responses. Given limited resources, it is important to identify and/or prioritize removal efforts spatially and temporally. These may include highly visited tourist areas, marine protected areas, and nursery and spawning aggregation sites for commercially and ecologically important fishes that are impacted by lionfish (Akins, 2012; Linardich et al., 2021).

Effective adaptive management must consider the unintended consequences of policy changes (Levin et al., 2012). In the case of permitting lionfish removals with the use of scuba and spear gear, policymakers must balance the potential for non-compliance and the take of protected species (Solomon et al., 2015). In the Mediterranean, groupers are largely overfished and thus there is reasonable apprehension for permitting spearfishing using scuba. At the same time, lionfish primarily consume native taxa (D'Agostino et al., 2020) and compete with native Mediterranean groupers, so providing lionfish control would likely benefit the groupers and ecosystem health. This issue of potential non-compliance was similarly faced by Western Atlantic states that enacted policies permitting lionfish removals with scuba in areas where spearfishing on scuba was otherwise prohibited. The risk of abuse was mitigated by (1) gear restrictions that mandated use of short pole spears (e.g., less than 1 m in length), which are largely ineffective in harvesting most other fishes, and (2) pursuing a participatory approach by working with scuba divers, local stakeholders and organizations. These participatory management approaches for lionfish removals, monitoring, and research appear to have largely resulted in cumulative positive social, economic, and ecological effects (Reed, 2008; Clements et al., 2021). In the Mediterranean, lionfish management in Cyprus [e.g., The Underwater Research and Imaging Center (URIC) and RELIONMED projects] has demonstrated similar successes for awareness programs, control, monitoring and data collection (Kleitou et al., 2019; Kleitou et al., 2021a).

Support for Commercial and Recreational Lionfish Fisheries to Sustain Removal

Opportunities exist to develop commercial and recreational lionfish fisheries throughout their invaded ranges (Simnitt et al., 2020). Commercial lionfish fisheries can help diversify fishing livelihoods and control lionfish populations (Chapman et al., 2019; Bogdanoff et al., 2021). The development of these fisheries will require a high enough ex-vessel price to incentivize commercial fishing effort. Innovation for lionfish by-products (e.g., extraction of fish oils, collagen, jewelry, leather, etc.) and harvest gear offers additional means to add value to their market price, which further support economic harvest. The development of recreational lionfish fisheries has been largely successful. To promote these, managers can leverage social incentives (which may vary based on culture) including recognition and camaraderie with other divers; competition between fishers; inspiring marine conservation; consumption of lionfish; and, where appropriate, incentives and awards for competitive removal tournaments. Indeed, many dedicated lionfish harvesters are not motivated by profit, but rather by conservation concerns, enjoyment of the activity, and finding purpose in their hobby (Frey and Jegen, 2002).

Participatory and Regional Coordination to Align and Evaluate Removal Efforts

Regional collaboration to address the Mediterranean lionfish invasion should include synthesis of research and action plans among the marine managers working on the issue. These efforts can be assisted with organized summits (in person, online, or hybrid) for lionfish researchers and managers to share experiences, set and prioritize research and management objectives (FWC, 2018), and develop regional management plans (Anderson et al., 2017; Graham & Fanning, 2017; FWC, 2018). Scientific workshops are also beginning to emerge in the Mediterranean. For example, Turkey's UNDP MARIAS Project organized an East-West lionfish knowledge exchange workshop in late 2020, Cyprus's RELIONMED Project hosted a lionfish workshop in early 2022, and a joint pufferfish-lionfish international conference is planned in Turkey in May 2022. Such workshops provide opportunities to communicate and collaborate on research initiatives, and strengthen regional ties which can benefit future regional management directives. We see opportunities for continuing and expanding regional management coordination in the Mediterranean Sea. For example, the General Fisheries Commission for the Mediterranean (GFCM) can make binding recommendations and enforce compliance in its 23 contracting parties from the Mediterranean, EU, and Black Sea. In the most recent Eastern Mediterranean Working group meeting (May 2021), the GFCM

DO'S AND DON'TS OF LIONFISH MANAGEMENT

PROMOTE LIONFISH HUNTING TOURISM
Helps support dive operators and tourism; divers need training and proper equipment.

ALLOW HARVEST VIA SCUBA AND POLE SPEARS
Noncompliance can be mitigated by gear use restrictions and working with stakeholder groups.

ENCOURAGE RECREATIONAL TOURNAMENTS
Derbies provide a participatory approach to conduct removals, research, and public education.

ENCOURAGE COMMERCIAL MARKETS
Develop market-based solutions to control lionfish densities and diversify fishers' catches.

ENCOURAGE PARTICIPATORY MANAGEMENT
Stakeholder engagement can support removals, market building, and strategic planning.

DON'T RELY ON "BOUNTY" PROGRAMS
Funds can be quickly exhausted. Sustainable control is better achieved by improving education, stakeholder engagement, and lionfish fisheries.

COORDINATE REGIONAL MANAGEMENT
Biological invasions require rapid and strategic management approaches with multinational cooperation.

DON'T "TRAIN" NATIVE PREDATORS
Feeding speared lionfish to predators results in aggressive behavior by them towards divers.

FIGURE 2 | Infographic summary of recommended and failed approaches for invasive lionfish management.

determined that lionfish will be listed as a priority invasive species to study. Also in 2021, a regional online GFCM repository was developed to help coordinate fisheries and aquaculture legislation in the Mediterranean and facilitate access to the multi-lingual data for fishers, policy makers, researchers, and the general public. Such work provides the foundation for multi-national coordination in fisheries and invasive species management.

TABLE 1 | A summary of recommended actions for lionfish control in the Mediterranean Sea using examples and key considerations from management in the Western Atlantic Ocean.

Recommended action		Illustrative example(s)	Key considerations that were managed
Do	Allow harvest *via* scuba and pole spears	Culling managed by stakeholder groups.	Potential for noncompliance and abuse mitigated by gear use restrictions and working with stakeholder groups
Do	Recreational tournaments	Emerald Coast Open Lionfish tournament has removed 10-20k lionfish per year	Resources are needed to conduct event
Do	Lionfish hunting tourism	Florida Keys; Bahamas; Belize; Lionfish tournaments	Novice divers need training
Do	Participatory management	Belize Lionfish Working Group; REEF tournaments and surveys; Cyprus Underwater Research and Imaging Centre; Cyprus RELIONMED	Managers and scientists must communicate and work with stakeholders and organizations
Do	Commercial fishery removals	Markets for lionfish in Belize and Florida, jewelry small businesses in Belize, Aruba.	Price must be high enough to incentivize harvest; Overfishing will decrease catch efficiencies; Potential for economic dependencies
Do	Coordinate regional management	GCFI lionfish working group efforts; GFCM fisheries management	Logistical complexities and possible tensions across geopolitical boundaries
Don't	Rely on "bounty" programs	Belize; Mississippi (USA)	Insufficient resources inhibit long-term solution; Better return on public investment can be achieved through developing self-sustaining incentives and programs
Don't	"Train" native predators	Sharks and moray eels in the U.S. Virgin Islands	Increases aggressive behavior towards divers

AUTHOR CONTRIBUTIONS

AU and HH contributed to the conception and design of the study. AU, FA, HH, SRG, and AF led the writing of the manuscript. AF prepared the map figure. All authors participated in the contribution of data, the drafting and revising of the manuscript, the final article revisions and approved the submitted version.

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Tracking Jellyfish Swarm Origins Using a Combined Oceanographic-Genetic-Citizen Science Approach

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Biological invasions of jellyfish may critically affect ecosystems and ecosystem services, yet their complex life cycle makes tracking their origins and dispersal vectors a challenging task. Here we combine citizen science observations, oceanographic modeling, and population genetics to track swarms of the invasive nomad jellyfish, *Rhopilema nomadica*, across the Eastern Mediterranean Sea. Jellyfish observations were recorded by citizens from two Israeli beaches in two consecutive years. A Lagrangian model coupled with a high-resolution 3D hydrodynamic model (SINMOD) was then used to simulate drift of ephyrae from probable polyp bed locations. Finally, mitochondrial DNA (mtDNA) sequence was constructed to examine swarm connectivity. Temporal (both seasonal and interannual) variation in observed swarms generally exceeded spatial differences between the two surveyed beaches. Early detection of swarms by citizens in offshore waters and the higher offshore particle distribution shown by the drift model, point to considerable offshore transport of the swarms. However, a higher probability was found for a nearshore location of the polyp beds, as nearshore origins were more closely correlated to hits on target beaches. *R. nomadica* released as ephyrae in early spring were likely to reach target beaches 200-300 km down current within two to three months as swarms of young adults in the early summer bathing season. *R. nomadica* populations exhibited little temporal or spatial genetic differentiation, a typical feature of a species that has recently undergone rapid population expansion. The offshore transport, the lack of genetic structure, and the interannual differences in both hydrodynamics and citizen scientist observations, all indicate decentralized swarm origins. This type of interdisciplinary approach can thus provide viable tools to track bloom formations. Understanding the complexity of jellyfish swarm dynamics supports future management strategies such as forecasting, preparedness and public education.

Keywords: biological invasion, jellyfish tracking, citizen science (CS), oceanographic modeling, Mediterranean Sea-Eastern, population genetics-empirical

INTRODUCTION

Jellyfish blooms are a global natural phenomenon that is sometimes considered to be exacerbated by human activities (Purcell et al., 2007). Jellyfish are typically perceived as a nuisance, negatively affecting human interests such as coastal leisure, tourism, fisheries, aquaculture, power, and desalination plants (Purcell et al., 2007; Edelist et al., 2021). They also play an important role in marine ecosystems and can be considered as a boon for fishers, a valuable source of food and other products when harvested sustainably (Edelist et al., 2021). For all these reasons, understanding jellyfish swarm dynamics and tracking their origins are important. *Rhopilema nomadica* is the largest, most venomous, and most prominent jellyfish species in the Eastern Mediterranean (Galil et al., 1990; Edelist et al., 2020). This species may have entered the Mediterranean Sea *via* the Suez Canal (Lessepsian migration), though it was rarely recorded outside the Mediterranean. *R. nomadica* was first recorded in the Mediterranean Sea in 1977 (Lotan et al., 1994), and its population has increased considerably since the early 1980s. Appearing in Israel first (Galil et al., 1990), and to date Israeli beaches are the only ones where polyps of *R. nomadica* were found *in situ* (albeit only on settlement plates, see Nakar, 2011; Dror, unpublished), large swarms were then recorded in Lebanon and Syria (Lotan et al., 1994), Turkey, Greece, Malta (Brotz et al., 2012) Egypt (Abu El-Regal and Temraz, 2016; Madkour et al., 2019) and most recently Tunisia, Sardinia and Sicily (Balistreri et al., 2017). *R. nomadica* swarms affect tourism by stinging bathers, thwart fishing operations by clogging nets and power and desalination plants by blocking seawater intake gear (Edelist et al., 2020). This species is therefore regarded as one of the worst invasive species in the Mediterranean Sea (Streftaris and Zenetos, 2006).

Like most coastal scyphozoan jellyfish species, *R. nomadica* has a metagenic life cycle, alternating between a free-swimming, sexually reproducing medusa stage, and an asexual benthic polyp stage. Jellyfish polyps may survive over many years (Purcell et al., 2007) and it is thus plausible that swarms will display some degree of spatial consistency (Fleming et al., 2013). *R. nomadica* polyps were shown to strobilate mostly in mild temperatures of 20-26°C when reared in laboratory conditions (Lotan et al., 1994), they may produce ephyrae almost year-round, and their activity in nature remains unknown. The life cycle of medusae from sessile polyps to sexually mature jellyfish and on to new polyps relies on circulation patterns that provide them with a suitable environment and disperses them over vast expanses of ocean (Johnson et al., 2001; Moon et al., 2010). However, our knowledge of polyp bed locations and the distances travelled by adult medusae are still very limited (Schnedler-Meyer et al., 2018).

Oceanographic models have played a key role in facilitating our understanding of oceanic processes, including jellyfish swarms (e.g. Kawahara et al., 2006; Moon et al., 2010). Shelf currents were among the main factors determining jellyfish distribution and swarm dynamics in the case of *Nemopilema nomurai* in the East China Sea and East/Japan Sea (Moon et al., 2010). In the eastern Mediterranean the Libyo-Egyptian Current is a major vector spreading jellyfish from the North African coast toward Turkey, *via* Israeli coastal waters (Edelist et al., 2020). Anticyclonic eddies may develop along this route, to the west of the Israeli coast (Rosentraub and Brenner, 2007), impacting the along and cross-slope transport routes. South of Cyprus, an anticyclonic eddy (Cyprus Eddy, **Figure 1**) is a fairly persistent feature that significantly impacts Levantine water transport (Mauri et al., 2019). In addition, the surface currents outside the Israeli coastal zone include cyclonic and anticyclonic eddies associated with the Shikmona Gyre system (**Figure 1**). South-East of Cyprus, there is an eddy system (Latakya Eddy) that can be either cyclonic or anticyclonic. Offshore transport by eddies may thus help explain the large seasonal and interannual fluctuations and complex dynamics in *R. nomadica* swarming (Edelist et al., 2020).

In the absence of frequent oceanographic surveys of gelatinous zooplankton in both coastal and offshore waters, our ability to follow the jellyfish swarm dynamics *in situ* is often highly limited. Here, reports by the public are essential to complement scientific knowledge. Citizen science initiatives are flourishing in recent years (Guerrini et al., 2018; Johansen et al., 2021). Today, smartphone apps and social networks facilitate collection of local ecological knowledge from the public in unprecedented quantity and quality (Newman et al., 2012). Such endeavors have been in use for observing species distribution and invasive species in general (Giovos et al., 2019) and jellyfish swarms particularly in the Mediterranean for more than a decade (Fleming et al., 2013; Edelist et al., 2020; Marambio et al., 2021).

To be able to understand jellyfish swarm dynamics, it is crucial to focus on the origin of the bloom and the connectivity of the populations in time and space; whether the individuals we observe in a swarm are from common local origins, have drifted from another region, or have accumulated in a specific region from multiple sources (Dawson & Hamner, 2008). *R. nomadica* adults were recently shown to have a considerable swimming ability (Malul et al., 2019). Adult jellyfish do not only passively drift (horizontally) but may travel up and down in the water column or, to a limited extent, with or even against the current (Fossette et al., 2015), while juvenile stages exhibit a more passive drift behavior. Therefore, oceanographic current models can be useful in studying swarms especially for ephyrae and juvenile stages, even though they predominately assume a passive drift. Similarly, when locations of the polyp populations are not known, as is the case with *R. nomadica* in the Mediterranean, the population genetic approach using adult specimens forming the swarm can provide valuable information on the scale and origin of the swarm. Population genetics, using either mitochondrial DNA cytochrome oxidase I (mtCOI) as a marker or a microsatellite approach, have been used to clarify the connectivity or distinctness of jellyfish aggregations for several jellyfish taxa (e.g., Stopar et al., 2010; Lee et al., 2013). For some taxa, the population genetic approach enabled clear population subdivisions defining locations where swarms occur among geographically isolated populations (Lee et al., 2013). Conversely, some taxa have shown low genetic diversity and lack of genetic structure (Stopar et al., 2010). For *R. nomadica*, several

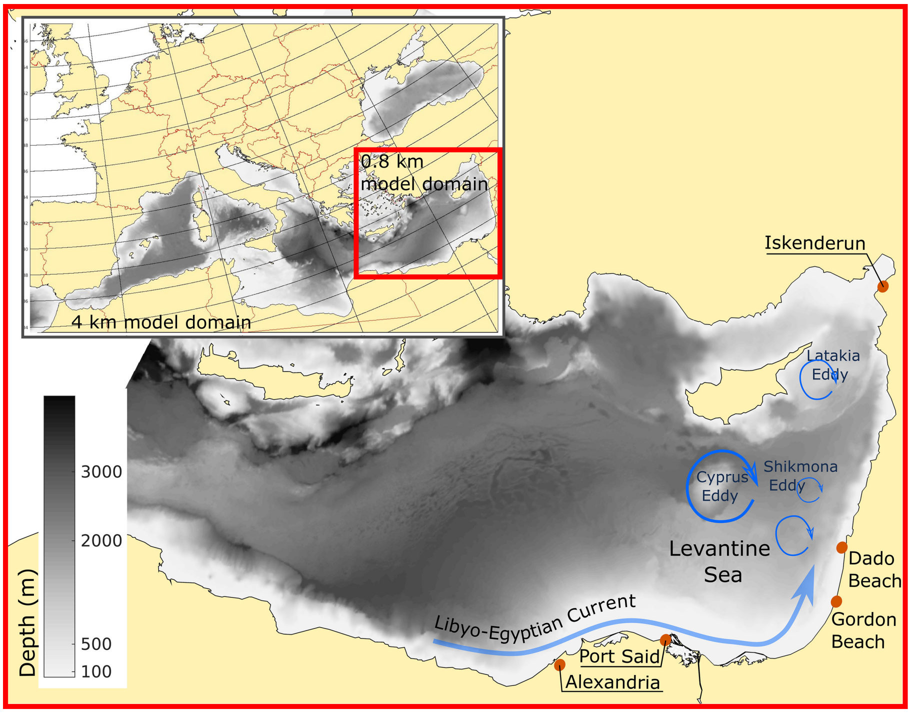

FIGURE 1 | Study area: Bathymetric map of the Mediterranean Sea, with main circulation patterns (blue arrows) and release and target beaches explored in the study (red circles).

microsatellite loci have been identified by a whole genome sequencing approach (Douek et al., 2020). Long-term changes in population genetic features of *R. nomadica* were recently studied by looking at the mtCOI haplotype diversity in the Eastern Mediterranean (Giallongo et al., 2021). The Giallongo et al. (2021) dataset concluded that all specimens of *R. nomadica* collected in the area belong to a single population, lacking clear genetic structure and there was no significant difference in the four seasons along a 12-year sampling period.

In the present study, we applied a high-resolution numerical model of Eastern Mediterranean current vectors to estimate distribution time and track distance scales from probable polyp habitats to areas in which mature *R. nomadica* swarms were observed by a citizen science project. In addition, we used a complementary population genetic approach, to determine the homogeneity of these *R. nomadica* swarms observed in the coast of Israel.

METHODS

In the following we describe methodology for: a) distribution of jellyfish using data from the Israeli citizen science project *Meduzot Ba'am* ("Jellyfish Inc."), b) hydrodynamic tracking used to simulate dispersal from ephyra release to adult swarming along the Levantine coast and c) population genetics of medusae collected along the coast.

Jellyfish Sightings Based on Observations From the Citizen Science Project, *Meduzot Ba'am*

Jellyfish sightings have been recorded by volunteer reporters (citizens) along the entire Israeli coastline since 2011, highlighting massive swarms in the June-July bathing season (Edelist et al., 2020). A subset of *R. nomadica* observations was thus extracted to focus on the two popular urban bathing beaches of Dado (Haifa) and Gordon (Tel Aviv) for the bathing season (calendar weeks 18-40) of 2017 and 2018. Observations were recorded *via* a dedicated internet website (www.meduzot.co.il) and the subset was extracted from Pangaea - an Open Access data archiving and publication repository (https://doi.pangaea.de/10.1594/PANGAEA.897389). For the present study, *R. nomadica* was chosen since it is the most common, conspicuous, stinging, and recognizable species in the Levant. Across the entire eight-year database, out of 6,033 sightings of eight scyphozoan jellyfish species, *R. nomadica* was by far the most common with 4,031 sightings (66.8%). Moreover, other scyphomedusa species in the region, mainly *Rhizostoma pulmo, Phyllorhiza punctata, Aurelia* sp., *Marivagia stellata and Cotylorhiza* spp., typically co-occur and intermix with *R. nomadica* during summer swarms (Edelist et al., 2020). The locations were chosen to represent main urban bathing beaches with sufficient spatial and temporal reporting coverage. Detailed information facilitating species identification was provided on the website. The distance from shore, type of activity, jellyfish species encountered, their quantity and size, whether stranded jellyfish were observed on the beach and if volunteer reporters were stung by jellyfish were also recorded (See Edelist et al., 2020).

The relative abundances of jellyfish observed by volunteers was ranked according to categorical values of "0", "2", "10" and "50", denoting "zero", "few", "medium" and "swarm" situations respectively. The average number of jellyfish per report per week [Eq. (1)] was used to create JSI - a Jellyfish Swarm Index (Edelist et al., 2020), defined as:

$$JSI = \frac{\sum_{i=1}^{n} A_i}{n}$$

where Ai is the binned quantity (0, 2, 10 or 50) of *R. nomadica* per report *i*, and n is the number of reports per week. The main modification in the JSI calculated here (in comparison to the JSI defined in Edelist et al., 2020) was that the JSI was calculated per week rather than per month, in order to match the higher resolution of the oceanographic model. Differences between mean ranked abundances were further tested by Kruskal-Wallis test – a nonparametric one-way ANOVA on ranks.

Hydrodynamic Model for Jellyfish Swarm Tracking

The present study examines how currents along nearshore areas in the Eastern Mediterranean Sea can affect horizontal drift of particles with a 40 m depth threshold, targeting beaches located down current from the release sites (**Figure 1**).

Jellyfish may modulate their swimming behavior in relation to currents, thereby increasing swarm formation or reducing the probability of stranding (Fossette et al., 2015). Despite the fact that large scyphozoan jellyfish like *R. nomadica* show a certain degree of active mobility (Malul et al., 2019), a passive drift in relation to Levantine currents was assumed here, as summer swarms of *R. nomadica* are comprised of more juvenile stages than winter swarms (Edelist et al., 2020). Using passive drifting as a proxy to track jellyfish blooms is a likely assumption especially for ephyrae and young medusae, since drift is the principal dispersal mechanism for early medusae life stages (Moon et al., 2010).

Ocean Model Configuration

In the present study, the SINMOD model framework has been used to track the movement of passive tracers to the Israeli shores. SINMOD is a coupled 3D model developed over the past 30 years to simulate physical (hydrodynamic) and biological processes (see Wassmann et al., 2010). The model's hydrodynamic component is based on the primitive equations that are solved using a z*-coordinate regular grid with square cells.

A model domain covering the Mediterranean Sea with 4 km horizontal resolution and 43 vertical layers was run from 2010 to 2018. Tides from TPXO (www.txpo.net/global), freshwater supply from various sources and atmospheric forcing (ERA5 from European Centre for Medium-Range Weather Forecasts, ECMWF) were applied. A high-resolution model with 0.8 km resolution was nested in the 4 km model and run for 2017 and 2018. The high-resolution model is computationally expensive to run but gives a good representation of ocean dynamics including gyres, coastal currents, and eddies. The spatial scale of the dynamic mesoscale field can be calculated from the internal Rossby radius of deformation and was previously found to be 5-12 km in the Mediterranean Sea (Pinardi and Masetti, 2000), hence the high resolution model will significantly improve representation of mesoscale variability.

Jellyfish Tracking Module

A Lagrangian particle tracking approach allows examination of multiple release points for simulated *R. nomadica* ephyrae. The particles (ephyrae) drift passively with currents horizontally but are allowed to migrate upwards if they were mixed or advected below 40 m depth. Particles were released at both 8 m and 15 m depths at 269 positions (in total 538 positions) along the Egyptian coast between Alexandria and Port Said. The position of the particles was updated every timestep (80 s) and saved every hour for later analysis. It has been shown that *R. nomadica* accelerates strobilation rates when Levantine waters begin to warm, generally around mid-April (Lotan et al., 1994). We therefore chose April 18th as the start date for release of the particles both in 2017 and 2018, and the simulation was allowed to run until August 31st. The simulations were run for 2017 and 2018, releasing 538 new particles every hour. The particles were not allowed to grow or die during the simulations and were tracked from release to the end of the simulations.

To date Israeli beaches are the only ones where polyps of *R. nomadica* were found *in situ* (Nakar, 2011; Dror, unpublished). We therefore ran a second release from Israel. Particles were released from 55 locations (at 8m and at 15m for each, in total 110 particles released per hour) near the coast off Dado beach in Israel starting on April 18th to the end of August. The aim was to test further spread of the particles to Eastern Turkey - an area where *R. nomadica* is long known to swarm (Avsar et al., 1996). In addition, we calculated the drift time from the Dado release sites, until the particles return to Dado by recirculation in an eddy offshore Dado. While Iskenderun Bay only receives a fraction of the longshore counterclockwise current, *R. nomadica* has long been known to swarm beaches in this region (Avsar et al., 1996; Duysak et al., 2013).

Molecular Analysis for Jellyfish Swarm Identification

Rhopilema nomadica were sampled in the Eastern Mediterranean Sea in different seasons in 2020-2021 and from three locations along the Israeli coast. For the analysis we defined those areas as South and Centre (relating to the Tel-Aviv region), and North (relating to the Haifa region). We focus on two time periods: summer (June-July 2020) and winter (January-March 2021 (for details see **Supplementary Material**). Most medusae were hand-collected from a small boat, 0.5-1.5 nautical miles offshore where bottom depth ranged between 5 to 35m by means of a dip net and 20 L buckets. Tissue samples were also taken from several freshly stranded medusae that had washed to shore. Whenever possible, bell diameter, weight, sex and sea surface temperature were documented. Pieces of gonads or oral arms were removed and immediately fixed in 70% ethanol. DNA from specimens was extracted using a modified Chelex rapid-boiling procedure as explained in Granhag et al. (2012). The mtCOI gene was amplified using primers FFDL and FRDL (Armani et al., 2013). The polymerase chain reaction (PCR) was conducted in a total volume of 20 μl, containing 1.0 μl of DNA template, 0.4 μL Phire® Hot Start DNA polymerase, 4 μL of Phire ® reaction buffer, 1μL of each primer (final concentration 0.2 mmol), 0.4 μL of DNTP, 0.6 μL of 3% DMSO, and 1.6 μL nuclease-free water. PCR conditions consisted of an initial cycle at 95°C for 5 min, 40 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 90, and a final extension at 72°C for 10 min. PCR products that yielded a clear band on 1.5% agarose gel by electrophoresis were sent to Macrogen Europe for purification and sequencing. All samples were sequenced in the forward and reverse direction to assure the accuracy of description for each polymorphic site and nucleotide haplotype. The resulting nucleotide sequence electropherograms were checked by eye for poor base calls using Chromas Lite 2.1 (Technelysium Pty Ltd). During the process misreads were corrected, and poorly resolved terminal portions of sequences were discarded. High-quality sequences were assembled using BioEdit software (Hall, 1999), and identities of sequences were confirmed by BLAST search in GenBank (>98% identity). Individual sequences were aligned with the MAFFT online service (Katoh et al., 2019). Genetic diversity within populations was estimated by computing haplotype diversity (H) and nucleotide diversity (π) (Nei, 1987) using DnaSP v5 (Librado and Rozas, 2009). A median-joining network showing the relationships between the mtDNA haplotypes was constructed using the PopART (http://popart.otago.ac.nz/howtocite.shtml; Bandelt et al., 2000). Genetic differentiation was calculated by means of pairwise FST values using 10,000 permutations in ARLEQUIN 3.1 (Excoffier et al., 1992) within the analysis of molecular variance (AMOVA) framework (Excoffier et al., 1992). The neutrality statistics Tajima's D and Fu's FS were used to test constant population size versus population growth (Ramos-Onsins and Rozas, 2002) using DnaSP v5. Also, mismatch distributions (Rogers and Harpending, 1992) were generated in DnaSP. Although there is a mismatch between the years used for the simulation of jellyfish distribution (2017-2018) and the years when jellyfish were collected for genetic analyses (2020-2021), we assume the current regimes do not change on an annual basis and that the genetic makeup of *R. nomadica* in this region is stable and does not change rapidly, as also noted in Giallongo et al. (2021). The sequences reported in the study have been deposited in the European Nucleotide Archive repository with accession numbers: OW055719-OW055735.

RESULTS

Jellyfish Observations

Jellyfish reports in the two study beaches for the study period yielded 701 observations of either "*R. nomadica*" (n=198) or "zero jellyfish" (n=503). Most reports were submitted for the inshore zone, from the coastline to a distance of 200 m from shore, with Haifa beaches characterized by a higher quantity and proportion of offshore reports in both study years (**Table 1**).

Both Haifa and Tel Aviv beaches were impacted by jellyfish swarms in both study years. However, a longer and more intense early summer swarm was recorded in 2017; while 2018 was characterized by a shorter early summer swarm followed by a later autumn swarm in mid-September (**Figure 2**). Juveniles (0-10 cm) were only observed in the late summer 2018 swarm and jellyfish typically arrived as young adults (11-30cm) to full adult (30-60) sizes, with a few rare observations of larger specimens in 2017 (**Figure 2**, although large specimens >60 were viewed in the summer and other seasons as well – see Edelist et al., 2020).

Swarms were detected at the northern Dado Beach (Haifa) before they were observed in the southern (Tel Aviv) Gordon Beach (**Figure 2A**). In 2017, swarms arrived earlier and were larger and more diverse in size composition, including extra-large specimens >60cm, while the 2018 autumn swarm consisted of predominately smaller individuals (**Figure 2B**). On average, more *R. nomadica* individuals per report were sighted offshore (>200 m from the beach) than inshore at both beaches, in both study years (**Figure 3**), and distance from shore was found to be a statistically significant factor in determining swarm probability (Kruskal-Wallis H(2) = 70.46, P = 5.551e-16).

Ocean Model Results

Over the two study periods, significant differences in current patterns were simulated. The dynamic structure of Levantine eddies/gyres and currents are known to vary seasonally and interannually (Özsoy et al., 1993). The weak longshore current recorded in May from Egypt towards Israel intensified considerably in June in 2018 and less so in 2017 (**Figure 4**).

The Levantine longshore currents either entered the Shikmona eddy, meandering clockwise south of Cyprus, or flowed northwards closer to shore carrying particles (and jellyfish) towards Turkey (**Figure 4**). Looking into current maps for surface, 8m and 15m depth from May to August, we generally found that the current is a bit weaker at 15m than at shallower depths for the release area and eastwards. This is mainly valid for the shallow shelf and less so for deeper parts of the study area. When we compared the particle drift patterns for particles released at 8m and 15m, only few differences were discernible in the model results, and we used an averaging of results from both depths in further analysis.

Release From the Egyptian Coast

A hindcasting approach was used here, based on citizen reports of adult medusae. These led to postulated ephyra release

TABLE 1 | Number of inshore and offshore observations of *R. nomadica* in 2017 and 2018 in weeks 18-40 (May to September) in Haifa and Tel Aviv beaches.

Location	Distance from shore	2017	2018	Total
Dado Beach – Haifa	Inshore (<200 m)	192	105	297
	Offshore (>200 m)	34	28	62
Gordon Beach – Tel Aviv	Inshore (<200 m)	126	189	315
	Offshore (>200 m)	13	14	27

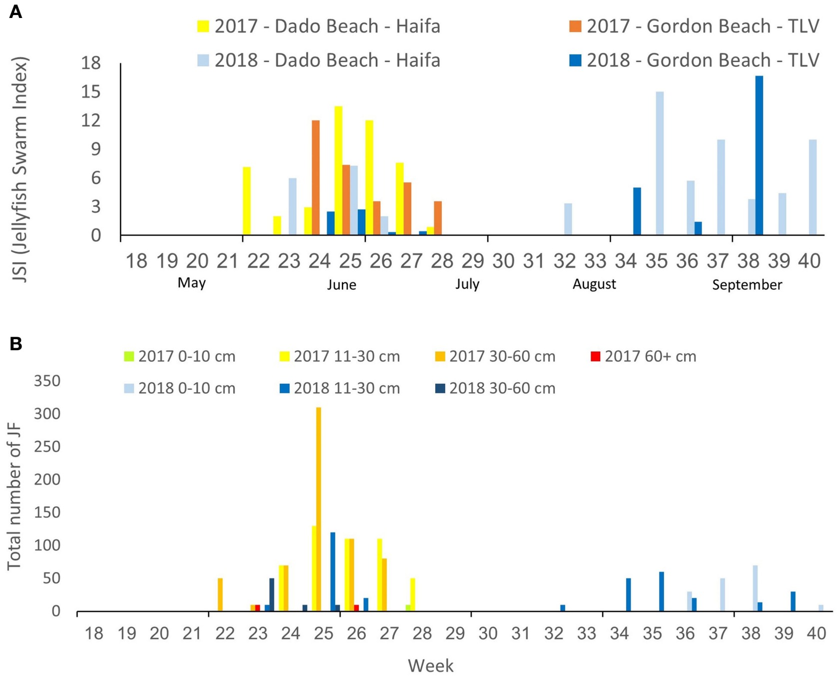

FIGURE 2 | Weekly Jellyfish Swarm Index (JSI) in Tel Aviv and Haifa beaches in the summers of 2017 and 2018 **(A)**, and total number of *R. nomadica* reported per week, ranked by size (bell diameter) distribution **(B)**.

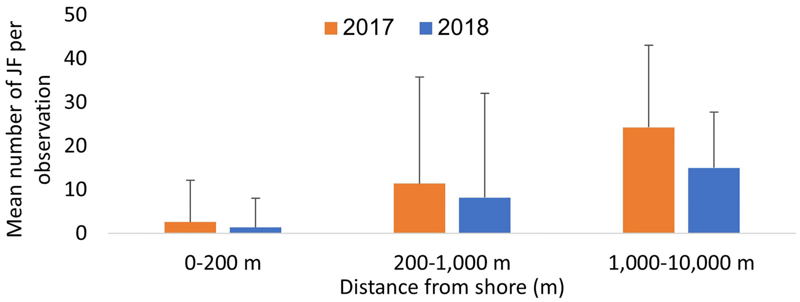

FIGURE 3 | Mean number of jellyfish per observation (JSI) in inshore (0-200 m, n=612) mid-range (200 m – 1,000 m, n=71) and offshore (1,000 m – 10,000 m, n=17) observations in the two study years.

locations, from which the drift of particles was simulated to produce "hits" on the target beaches. Nearshore particle released from the continental shelf (<200m depth) in the Eastern region of Bardawil and Port-Said to Damietta (Egypt) were shown to have the highest correlation with hits on both target beaches in both study years (**Figure 5**).

Interannual differences in the current regime may affect results somewhat (in 2018 stronger currents could have widened hindcasting range westwards), but overall offshore release points and release from points further west in the Baltim-Alexandria region west of the Nile delta showed a significantly lower hit correlation with the two target beaches than the Bardawil-Port Said-Damietta area, (**Figure 5**).

The final (August 18th) distribution of released particles is shown in **Figure 6**. The highest number of hits was found in the shelf area along the coasts of Egypt and Israel, while some particles meandered offshore and into the gyres. Fewer particles drifted with the longshore currents to the coasts of Lebanon, Syria and Turkey (**Figure 6**).

Overall, the inshore areas east of Alexandria, including the nutrient rich Nile delta were tracked as the probable origins of Israeli summer swarms (**Figure 5**). The early summer swarms

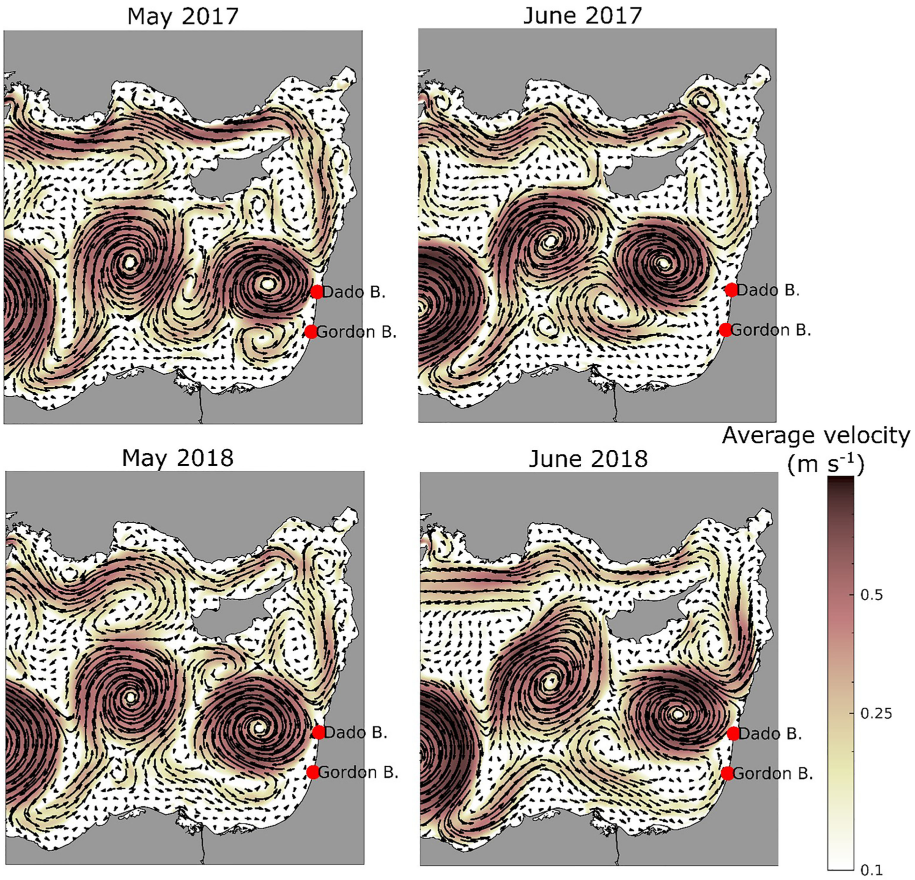

FIGURE 4 | Monthly average surface current velocity and direction used in the particle model for May (left) and June (right) in 2017 (top) and 2018 (bottom).

probably emanate from enhanced strobilation and release as water begins to warm in April, reaching Tel Aviv and Haifa in 47.1 and 58.3 days respectively (**Figure 7**).

Release From the Israeli Coast

Most particles released from the Israeli coast hit the beaches of Lebanon and Syria, and lower numbers reached Turkey. There was also considerable meandering of particles westwards towards the gyres and the Cypriot coast (**Figure 8**).

In 2018, there was a significantly lower hit correlation to Iskenderun than in 2017, and a longer mean duration of 93.6 days of particle drift time from Haifa to Iskenderun (**Figure 9** and **Table 2**).

In the simulation from April 2017 and 2018, particles were released from 55 points in northern Israel (**Figure 8**). In 2017, there were high hit rates of particles reaching Iskenderun (**Figure 8**) and the mean transportation time was 69.1 days (**Table 2** and **Figure 9**).

Some particles released near Haifa did not follow the coast northwards as **Figure 8** indicates, but instead went into the Shikmona eddy or southwestwards before returning to Haifa. Counting returned particles older than average drifttime from offshore Egypt (57 days), gave mean/standard deviation of 69.4 d/14.4 d drift time for 2017 and 72.1 d/16.9 d for 2018, indicating the probability of cyclic swarming dynamics. In 2017 the number of paticles from Haifa to Iskenderun was higher than particles from Haifa to Haifa, but for 2018 the situation was reversed.

Sequence Diversity and Population Structure

To assess the genetic diversity of *R. nomadica*, a sequence length of 433 bp of the mtCOI gene was analysed from a total of 42 *R. nomadica* individuals sampled from the three areas during the summer of 2020 and winter of 2021). Over the whole data set collected in this study, 22 polymorphic sites, of which 9 were parsimony informative, were identified leading to the definition of 18 haplotypes. Haplotype richness was high ($h = 0.90 \pm 0.03$ on average), but differentiation among haplotypes was modest (π (%) $= 0.49 \pm 0.5$). All geographical regions showed similar haplotype and nucleotide diversity (**Table 3**).

A haplotype network based on mtCOI supports the non-differentiation of *R. nomadica* in the various regions along the Mediterranean coast of Israel. Analysis of molecular variance

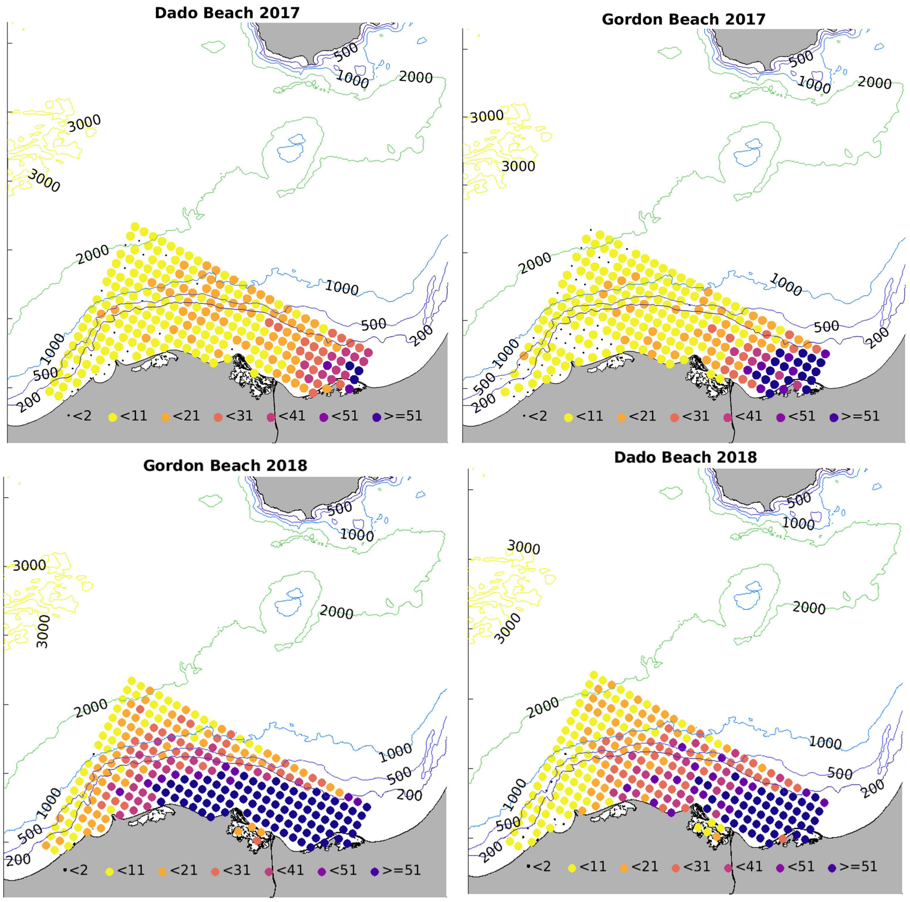

FIGURE 5 | Colour-coded release points off the Egyptian coast according to particle hit distribution on Israeli target beaches in 2017 and 2018, averaged across both release depths of 8 m and 15 m. The numbers refer to hits at Gordon (Tel Aviv) and Dado (Haifa) for every release point.

(AMOVA) showed that maximum variance was obtained within geographic regions (100%) while the variation among populations displayed negligible variance. This picture was concordant with pairwise F_{ST} values (**Table 4**).

Similarly, when comparing specimens collected from the coast of Israel in summer 2020 and the same region in winter 2021 (n=39), 22 polymorphic sites, of which 8 were parsimony informative, were identified leading to the definition of 18 haplotypes (**Figure 10**) and no significant inter-population genetic divergence was observed (F_{ST}=-0.01, $p> 0.05$). Moreover, all measures of genetic diversity and population structure calculated across populations pointed to low genetic structure regarding seasonal variations.

Despite the low sample number, the overall mismatch distributions of mtCOI haplotypes from the coast of Israel displayed unimodal distributions similar to the curve expected when a population has undergone rapid population growth in the past (Roman and Darling, 2007). Moreover, tests of neutrality also showed low and significant values of Fu's FS (−11.93, $p < 0.05$) and Tajima's D (−1.099, $p < 0.05$), further pointing to a recent demographic expansion. The low subdivision observed across space and time was also recorded across sizes, further strengthening the notion of a single unstructured population.

DISCUSSION

Currents are an important factor that can alter the formation of jellyfish swarms, in addition to productivity, storms, light, moon phase, substrate availability, salinity, and temperature (Gibbons et al., 2016). It was suggested that in the Levant, swarming synchronicity of *R. nomadica* is triggered by temperature (Lotan et al., 1994). However, the presence of large swarms both in winter (Edelist et al., 2020) and/or autumn, as shown in this study (**Figure 2**), suggests that the process is more complex. Here, our model suggests that timing of *R. nomadica* swarm arrival is congruent with ephyrae releases hundreds of km up current, and our observations suggest that the typical expected drifting period of early life-stages of *R. nomadica* is two to three months, before swarming is recorded at the target beaches. The growth rates of *R. nomadica* in the wild are unknown, and as

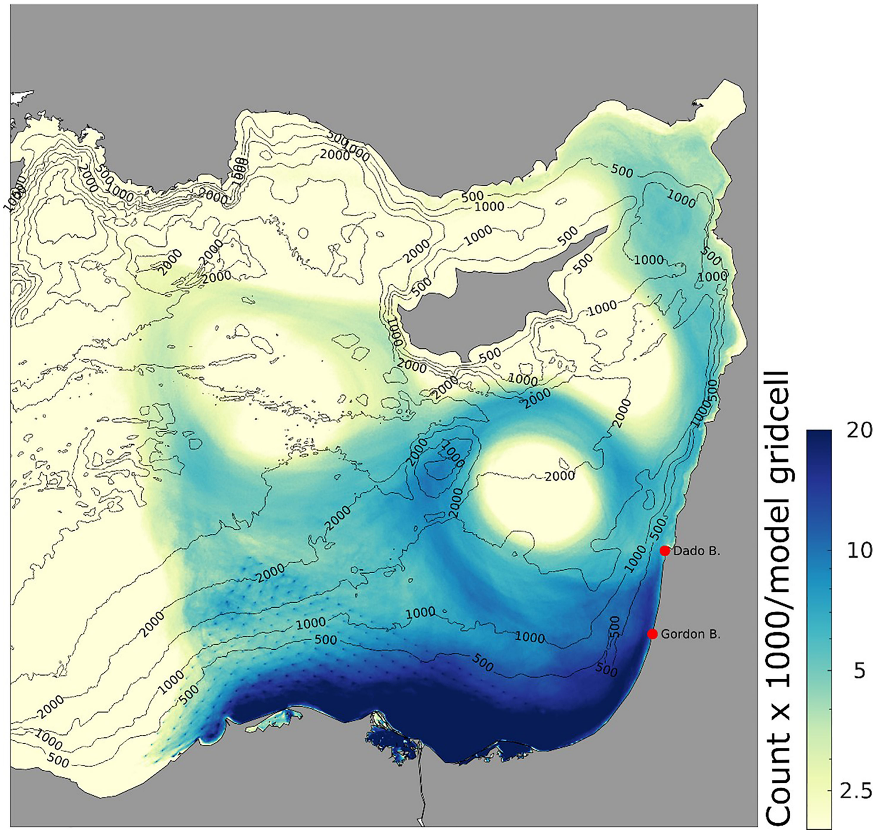

FIGURE 6 | Total number of particle hits within each model gridpoint, released from April 18th to August 31st 2018. Release locations along the Egyptian coast are shown in **Figure 5**.

ephyrae of metagenic jellyfish species may spend their early life period below the benthic boundary layer (Higgins et al., 2008), an earlier release and delayed transport may also occur. In the 2017 bathing season, a long, dense swarm of *R. nomadica* of mixed sized specimens was recorded in both Israeli beaches from mid-June to late July, while in 2018 a low-density swarm was recorded in early July followed by another low-density autumn swarm in September (**Figure 2**). Such autumn swarms are relatively rare and have only been recorded twice (2013 and 2018) over the past decade, while winter swarms comprised of large adult specimens are much more common (Edelist et al., 2020). In 2017, currents were strong in April and May and weakened in June while 2018 showed an opposite trend. In this study, we tackled the question - did the weaker longshore current in May 2018 lead to a smaller and later swarm? Temperatures in the Levant were relatively high throughout the winter of 2018 (Herut et al., 2019), so is there a logical explanation for the smaller, later swarm? Usually, a single factor is too simplistic to explain interannual variations in jellyfish phenology and swarming, and other factors like productivity, salinity, and storm conditions need to be taken into consideration to understand the full complexity of bloom dynamics (Boero et al., 2008). Here, we propose that such complexity may involve the entry of the jellyfish into offshore eddies (**Figures 6**, **8**) or alternative factors affecting strobilation in the polyp beds. While no observations of *R. nomadica* in the offshore Levantine gyres are known from literature, their presence in offshore pelagic and mesopelagic realms should not be excluded *a priori* and further studies investigating the presence of *R. nomadica* in open offshore waters are needed.

The present study suggests that ephyrae of *R. nomadica* travel hundreds of kilometers over a period of 2-3 months to reach beaches down-current as young adults during the bathing season. The simulations of current-induced particle drift provide scenarios for the nearshore jellyfish observations in the Eastern Mediterranean Sea, as well as some offshore meandering, triggered by a complex system of eddies and gyres (**Figures 4**, **6** and **8**). A recent study (Kuplik et al., 2021) has shown that *R. nomadica* is able to successfully utilize scarce resources found in hyper-oligotrophic environments, such as the Levantine offshore waters. This property of *R. nomadica* may allow survival during extended offshore migrations, e.g. in the Shikmona-Cyprus eddies, or the counterclockwise transport from the Levant longshore to Turkey and then back through the gyre system. The arrival of large specimens in winter (Edelist et al., 2020) and the genetic connectivity between summer and winter specimens

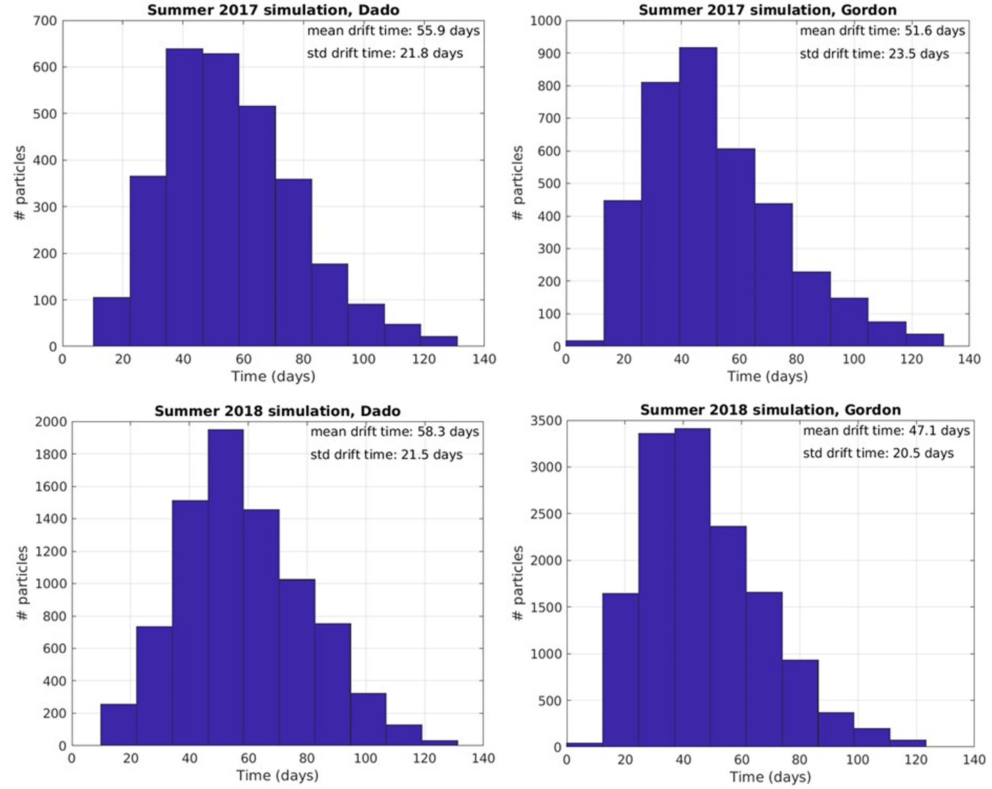

FIGURE 7 | Particle hit distribution on target beaches and mean drift time in days from release locations along the Egyptian coast (Between Alexandria and Bardawil) to Gordon (Tel Aviv) and Dado (Haifa) beaches in 2017 and 2018.

may support this notion. However, for the early summer bathing season during which the large dense mixed-size swarms are recorded, nearshore releases were shown to have a higher hit probability on target beaches than offshore releases (**Figure 5**). Ephyrae released from the Bardawil-Damietta area will typically reach Israeli beaches located 200-300 km down current in 20-40 days, while release from the Alexandria-Baltim area located 300-600 km up current from target beaches will take up to 90 days. This may of course change with variation in current velocities, but an overall mean arrival time of 55-60 days (**Figure 8** and **Table 2**) was found for the study duration in the spring. Similar results are attained when particles are released from Israeli beaches, reaching areas 200 km down current within a month and 400 km (Iskenderun) within three months (**Figure 8** and **Table 2**).

Surprisingly, jellyfish were occasionally spotted in the north (Haifa) before they were reported 100km up current in Tel Aviv (**Figure 2**). When observing the Meduzot Citizen Science database we find that the reason for this may be that at Dado Beach in Haifa, many of the reporters are long-distance swimmers that spotted the jellyfish offshore (twice that of Tel Aviv – **Table 1**) while most Tel Aviv Beach observations were submitted by inshore bathers. This pattern of an offshore positioning of the swarm (>1km from shore) before actually hitting the beaches is also in line with the oceanographic data that predicted a larger number of hits in offshore locations (**Figure 6**).

Where jellyfish swarm, they are likely to leave polyps on the seabed if the right conditions and suitable habitat exist. The present study only models about a quarter of the Mediterranean distribution area of *R. nomadica* (see Balistreri et al., 2017; Edelist et al., 2020), and while polyp beds located in Egypt may lead to outbreaks on Israeli beaches, Tunisian polyp beds could cause swarms along the Libyan or Egyptian coasts. In 2017, *R. nomadica* swarms were reported from the Israeli coast from June to July while they occurred along the Egyptian coast in mid-June at Port Said (Madkour et al., 2019) and at Baltim and Alexandria from late June to early July (http://www.egyptindependent.com/sea-turtlesto-counter-jellyfish-on-shores/). A swarm covering the entire South-Eastern Mediterranean simultaneous basin thus remains a viable possibility. The availability of observations in the present study was limited to Israeli beaches, and the oceanographic model that tracked them up-current inevitably pointed at potential polyps located in Northern Sinai and the Nile delta region. Similarly, particles released from the Israeli coast showed how Israeli polyp beds may support swarm formation impacting beaches in East Turkey, or even returning (**Figure 8**).

One of the outstanding features of *R. nomadica* in the winter months is their exceptionally large size, up to 90 cm bell diameter

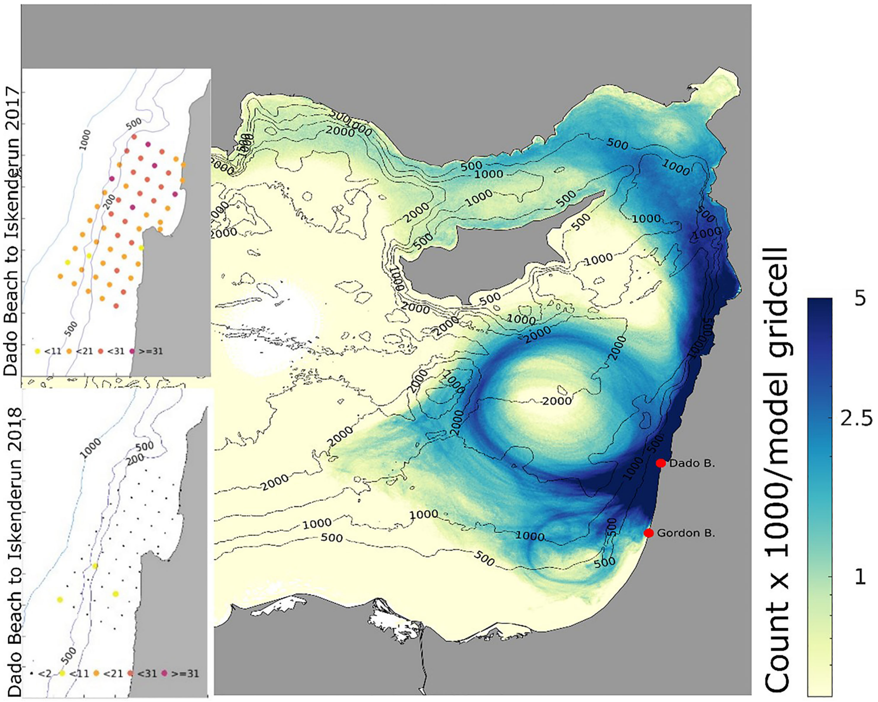

FIGURE 8 | Total number of particle visits within each model gridpoint, released from Dado beach (Haifa) from April 18th to August 31st of 2018. Insets: colour-coded release points off the coast of Haifa according to particle hit distribution on target beaches in 2017 (upper, left) and 2018 (lower, left), averaged across both release depths of 8m and 15m. Inset numbers refer to hits at Iskenderum from the release points, so the red points are connected to the target beach with more than 31 particles (2017) and small black points less than two particles (2018).

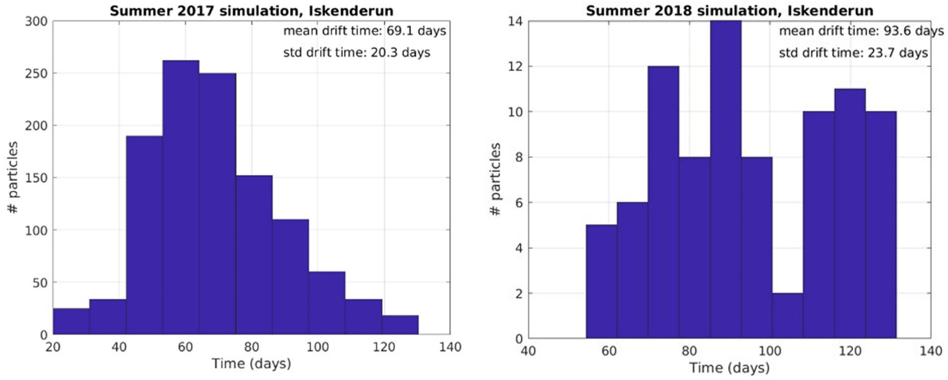

FIGURE 9 | Particle hit distribution on target beaches and mean drift time in days from release locations in Haifa region from April 18th to August 31st to Iskenderun, Turkey.

TABLE 2 | Mean and standard deviation of drift times to and from Haifa from simulations.

Period	Alexandria-Port Said to Haifa Mean/standard dev.	Haifa to Iskenderun Mean/standard dev.
Summer 2017	55.9 d/21.8 d	69.1 d/20.3 d
Summer 2018	58.3 d/21.5 d	93.6 d/23.7 d

TABLE 3 | Sample sizes and standard diversity indices for partial mtCOI sequences of *Rhopilema nomadica* sampled at three locations (North, Centre and South) and two seasons (summer 2020 and winter 2021).

Population	N	h	H	π (%)
North	9	8	0,97 ± 0,06	0,63 ± 0,18
Centre	19	11	0,90 ± 0,06	0,33 ± 0,23
South	14	9	0,91 ± 0,06	0,47 ± 0,48
Summer 2020	18	12	0,95 ± 0,03	0,62 ± 0,12
Winter 2021	21	11	0,88 ± 0,06	0,43 ± 0,43

Summer 2020 and winter 2021 samples were collected from Centre and Centre North area, as no specimens from South were recovered (N, number of specimens; h, number of haplotypes; H, haplotype diversity and π nucleotide diversity).

TABLE 4 | Pairwise FST values among samples of Rhopilema nomadica in three locations the coast of Israel (p>0.05).

	North	Centre
Centre	-0.034	–
South	-0.011	-0.012

as observed in Israeli coastal waters (Edelist et al., 2020). This size disparity may be a result of currents that carry the summer populations into the gyres and return them to the Levantine shores in winter. The population genetic data found in this study supports this possibility since the specimens sampled did not show any strong temporal or spatial genetic differentiation of the populations. Another possibility is that such large specimens (>60cm) could have been released as ephyrae in Western Egypt, Libya or even Tunisia (where autumn *R. nomadica* swarms have been reported in recent years by Balistreri et al., 2017). Peculiarly, *R. nomadica* swarms are seldom reported in Cyprus (www.ciesm.org/gis/JW/build/JellyBlooms.php) despite currents that may carry them both north and south of the Island (**Figures 4**, **8**). A wider population genetic study with specimens collected from these regions is imperative for the study of both origins and spread of *R. nomadica* in the region.

Kawahara et al. (2006) and Moon et al. (2010) showed that *Nemopilema nomurai* swarms can travel thousands of km in the Tsushima current along Japanese coasts from July to November. *R. nomadica* may similarly traverse the ~2000km distance from Tunisia to Israel, or the ~3000km long Egypt-Israel-Lebanon-Syria-Turkey-Rhodes-Egypt circuit (**Figure 6**). Said et al. (2013) showed that drogue drifters released at 15 m depth near the Rhodes gyre may reach the Egyptian longshore current and drift eastwards at mean velocities of 20-25 cm/s (17.2-21.6 km/day). These values change geographically and throughout the year and the longshore current may at times be an order of magnitude slower. Mean monthly current velocities over the Israeli shelf as measured by Rosentraub and Brenner (2007) were only 0.86 km/

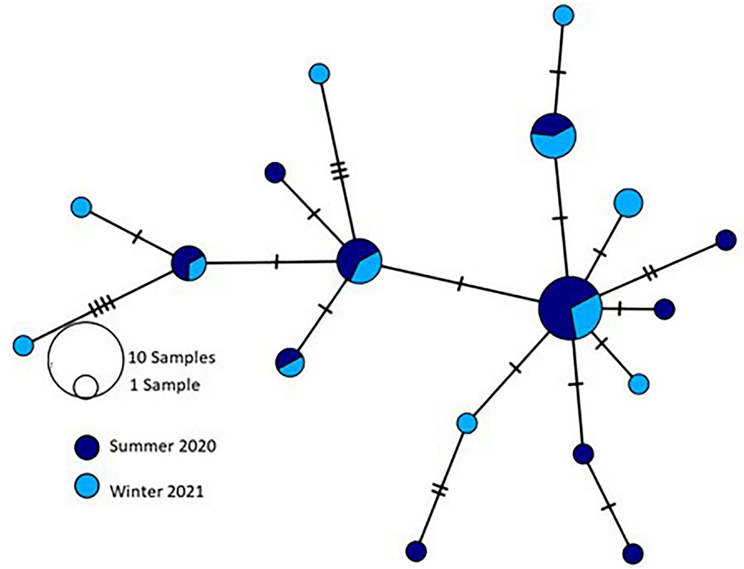

FIGURE 10 | Median-joining network showing the relationships between 18 haplotypes detected by sequencing *R. nomadica* collected from the coast of Israel during summer 2020 and winter 2021 for the partial mitochondrial DNA cytochrome oxidase I (mtCOI) region. Circle sizes are approximately proportional to haplotype frequency: the smallest circle represents a single individual; the largest circle represents 10 individuals. Each connection represents a single mutation, and small open black dots represent missing intermediate haplotypes.

day (July) to 10.36 km/day (February). If jellyfish do travel with the currents at these velocities, it should take a specimen only 18 days in February and 30 days in July to cover a distance of 200 km.

Initially, low winter temperatures were thought to be the main barrier to westward spread of *R. nomadica* beyond Greece in the northern Mediterranean, and an absence of *R. nomadica* from the coastal waters of Egypt was thought to result from lack of sufficient rocky substrates necessary for polyp settlement (Lotan et al., 1992). Since then, however, these supposed barriers proved no match for *R. nomadica*, which is now known to swarm beaches as far west as Sardinia and Tunisia (Balistreri et al., 2017) with numerous recent reports of swarms off Egyptian coasts (Abu El-Regal and Temraz, 2016; Madkour et al., 2019). Swarms may thus be much larger than the 100 km estimated by Galil and Zenetos (2002) as Alexandria and Baltim are located >500 km up current from Israel. Moreover, a synchronous bloom across the entire SE Mediterranean basin also seems reasonable, with numerous polyp beds contributing to these pan-basin blooms. Seascape genetics can potentially align hydrodynamic modelling with population genetic data and help identify population differentiation in swarms over local vs. broader spatial scales. Tools that are more advanced and better suited for populations genetic studies than mtCOI exist today and can be applied. For *R. nomadica*, future studies should also extend to pan-basin and cross-basin swarm dynamics, including reports from more target beaches over longer periods and sampling of specimens from countries across the Mediterranean for a full population genetics study.

A recent genetic study of *R. nomadica* populations indicates that the Israeli medusae are all part of a single Levantine population (Giallongo et al., 2021), with phylogenetic trees showing no geographical or temporal partitioning, but haplotype numbers indicating a small north-south gradient. Our findings agree with these results, and no significant spatial and temporal gradients were found in the medusae analysed. Some scyphozoan species, such as *Pelagia noctiluca* (Stopar et al., 2010), *Nemopilema nomurai* (Dong et al., 2016) and *Periphylla periphylla* (Majaneva pers. comm), have shown similar lack of genetic structure at comparable geographical scales over several hundred kilometres. However, other species have shown a clear geographically structured pattern; for example, *Cyanea capillata* and *Aurelia aurita* (Majaneva et al. In Prep)., particularly when observations cover different bodies of water (e.g. the Baltic and North Sea). The single unstructured Israeli population, and high number of haplotypes and singletons in *R. nomadica* were proposed by Giallongo et al. (2021) to indicate recurring independent introductions, or an open corridor augmenting gene flow. While this may be the case inside the Mediterranean, *R. nomadica* is unique in the sense that large swarms have never been recorded anywhere outside the Mediterranean, and the nearest 'origin' specimens ever recorded were >2,000 km away from the mouth of the supposed invasion vector, the Suez-Canal. The unimodal distribution found here for mtCOI haplotypes resembles that of a population that has experienced a recent demographic expansion (Roman and Darling, 2007; Giallongo et al., 2021). Therefore, while it is still unknown how (and whether) *R. nomadica* first entered the Mediterranean, ballast water or hull fouling scenarios should be favoured over direct drift\swimming through the canal. Recurring wide scale swarm observations and the oceanographic modelling of the currents driving them from various locations suggest a decentralized, inshore origin for *R. nomadica* swarms in the Mediterranean, rather than a single polyp bed.

The many unresolved questions regarding *R. nomadica*'s successful ongoing colonization of the Mediterranean merit a wide spatial future study, that includes examination of specimens from all Mediterranean areas alongside Indo-Pacific 'origin' populations, if such can be found. As shown here, a combination of citizen-science with oceanographic and molecular approaches can be instrumental in such applications.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: European Nucleotide Archive OW055719-OW055735.

AUTHOR CONTRIBUTIONS

DE and DA drafted the manuscript and oversaw acquisition of jellyfish observation data, ØK and IE carried out oceanographic modelling and spatial analysis, NA, SM and HD carried out genetic sampling and analysis. All authors contributed to manuscript writing and editing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.869619/full#supplementary-material

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