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Marine epibioses

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Marine epibioses

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Editorial: Marine epibioses

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Editorial on the Research Topic:
Marine epibioses

The marine domain is often divided into two broad zones: benthic and pelagic. The former consists of habitats and organisms related to the oceanic bottom, whereas the latter refers to the water column and its free-living inhabitants. However, this classification largely overlooks many surface-associated forms that are not necessarily linked to either the bottom or the water column but, rather, utilise any available hard-surfaced substrata. The various properties of water, especially seawater, as a medium favour sessility even in animals. While the availability of hard substrata is high at the ocean floor, the high ratio of the ocean volume to the surface area of its bottom might suggest that much of this environment is, to a great extent, inaccessible to sessile organisms due to a lack of attachment points. However, very often, especially in the open ocean, hard surfaces can be provided by larger organisms, which has encouraged the evolution of a unique lifestyle – epibiosis (Figure 1).

Although the meaning of the term "epibiosis" continues to develop alongside the field of marine biology, most existing definitions describe this phenomenon as the spatial association between a basibiont (substratum organism) and an epibiont (an organism attached to the basibiont's outer surface). Some definitions highlight the lack of trophic dependency of the involved organisms, while others exclude negative interactions such as parasitism. However, recent evidence suggests that the relationships between epi- and basibionts may be more complex and intimate than previously thought. This is especially true for many microepibionts that are only now receiving more research attention. For example, the ability of epibionts to access food will often depend on basibiont behavior and physiology (Wahl et al., 2012). Therefore, the perceived lack of a direct or indirect trophic relationship, or the direction of interactions between epibiont and basibiont, may insufficiently define the true nature of epibiosis. Here, we define epibionts as life forms that live on the external body surfaces of a basibiont, regardless of their trophic relationship

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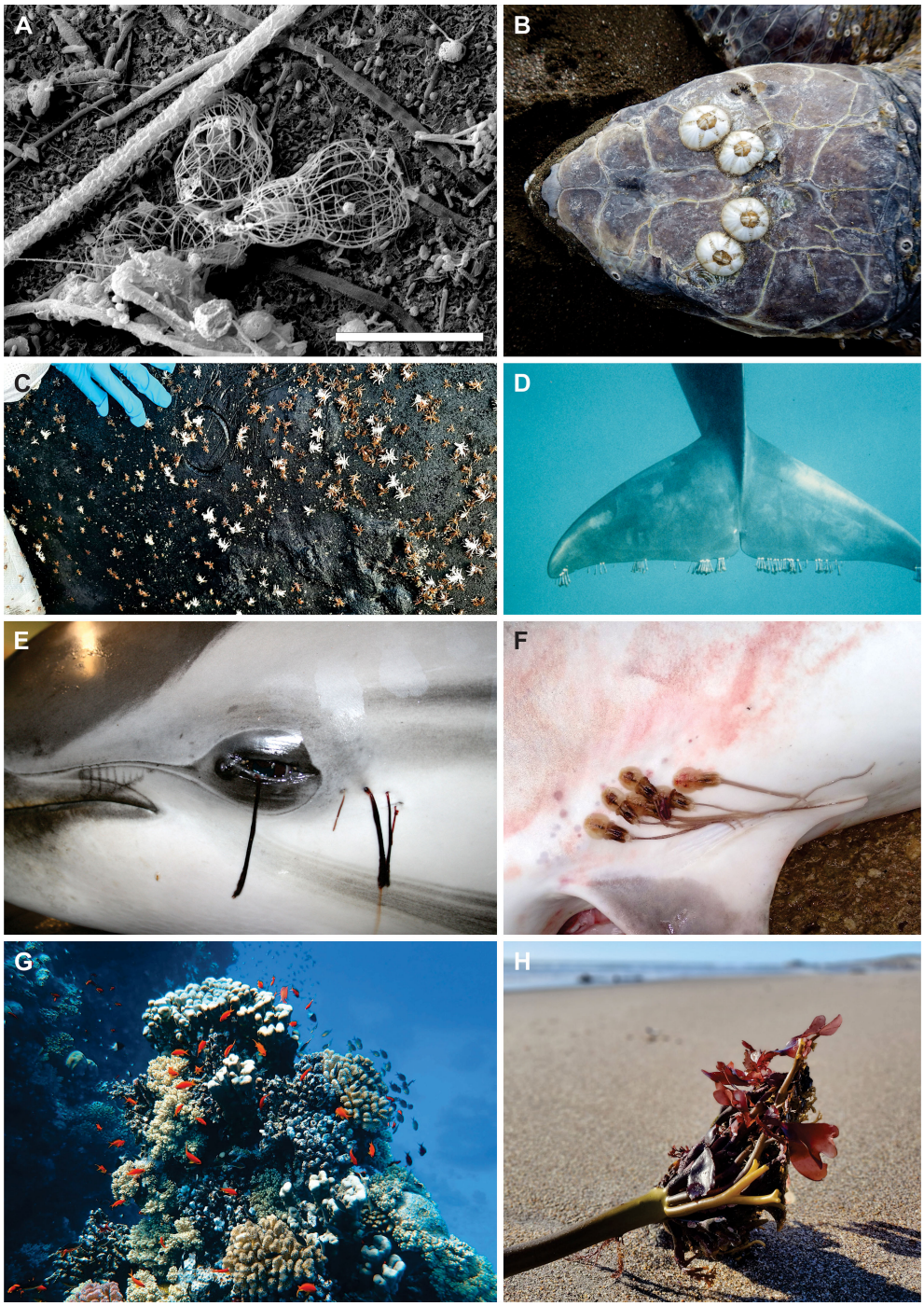

FIGURE 1
Examples of marine epibioses. **(A)** Scanning electron micrograph showing microbial biofilm on the carapace of a loggerhead sea turtle (*Caretta caretta*). Scale bar = 10 µm. **(B)** Barnacles on the head (*Chelonibia testudinaria*) and neck (*Stomatolepas elegans*) of an olive ridley sea turtle (*Lepidochelys olivacea*). **(C)** Whale lice (*Cyamus boopis*) on the surface of a humpback whale (*Megaptera novaeangliae*). **(D)** Barnacles (*Xenobalanus* sp.) on the fluke of an unknown cetacean. **(E)** Copepod parasites (*Pennella* sp.) on the skin of a striped dolphin (*Stenella coeruleoalba*). **(F)** Copepod parasites (*Dinemoura latifolia*) attached to a white shark (*Carcharodon carcharias*). **(G)** Multi-organismal interactions on a coral reef surface. **(H)** Red algae (Rhodophyta) attached to the holdfast of giant kelp (*Ecklonia maxima*).

with that host organism. Such associations may have beneficial, deleterious, or neutral outcomes for both the epi- and the basibiont.

Any physiological cost of evolutionary adaptation to life on the surface of other organisms may be outweighed by the benefits derived from access to a substratum unavailable to less-specialized organisms. Thus, in the oceanic realm, epibioses are omnipresent, with many marine organisms having both a free-living and an epibiotic stage during their life cycles. Nevertheless, despite our

increasingly refined understanding of the relationships between epi- and basibionts, numerous areas of this topic are yet to be identified and explored. For example, questions concerning the consequences of the various types of epibioses on biodiversity, food web dynamics, and biotic responses of the marine systems to environmental changes and disturbance are rarely raised and addressed. Therefore, this Research Topic highlights some of the most recent observations and discoveries in this broad and seemingly simple but poorly understood field.

Sea turtles are one of the most iconic marine basibionts, whose carapaces and skin are almost universally colonized by both macro- and microepibionts. In recent decades, there has been a steadily growing interest in sea turtle epibioses. Numerous studies have shown that the composition of epibiotic communities may give clues about the spatial ecology, behavior, and health of their hosts, thus highlighting the relevance and importance of sea turtle epibiosis research for marine megafauna and habitat conservation (Pinou et al., 2019). Robinson and Pfaller summarize the current knowledge on animal macroepibionts (>1 mm) on sea turtles. They provide an exhaustive list of taxa recorded on sea turtles to identify knowledge gaps and assess biases in the current literature. Even though macroepibionts have long been reported from sea turtles, not all sea turtle species, populations, life stages, geographic regions, and habitats are equally well-researched, and new epibionts will likely be identified with increased and more carefully planned sampling and diagnostic efforts. Loghmannia et al. provide information on epibiont communities from a relatively poorly investigated population of hawksbill turtles nesting in the Persian Gulf. They conclude that epibiont communities, especially at the micro-level, may differ between geographically close nesting beaches due to local environmental conditions. Similar conclusions are drawn by Silver-Gorges et al., who sampled loggerhead turtles nesting in the northern Gulf of Mexico. They used information derived from both stable isotope analysis of sea turtle tissues and the taxonomic composition of the epibiont community to identify animal-associated taxa that could be used as indicators of their hosts' ecology and foraging areas. They observed that smaller meiofaunal taxa were generally more discriminative indicators of sea turtle foraging ecology than larger macroepibionts. This may be related to the fact that some common sea turtle-associated macroepibionts (e.g. barnacles) exhibit host selectivity (Zardus, 2021). Therefore, their presence or absence may be controlled by biological (host species) rather than environmental factors. Boyd et al. show that hawksbill and green turtles with overlapping geographic ranges in the Indian (Madagascar) and Atlantic Oceans (Florida) were each colonized by a single species of *Chelonibia* barnacles. Specifically, *C. testudinaria* colonised only green turtles, whereas *C. carretta* was exclusive to hawksbills. These observations support the previously formulated hypothesis that the larvae of epibiotic barnacles select their host differentially from a shared pool of available species. However, comparably detailed information from many less-researched geographical regions is still required. Kim et al. propose that information derived from the analysis of diversity and abundance of epibionts collected from stranded sea turtle carcasses can serve as a practical alternative when long-term datasets on sea turtle health and physiology are not available. Hayashi, in turn, highlights the potential of Japanese historical monographs (Honzou Gaku) to elucidate life histories and past biogeography of both sea turtles and their macroepibionts.

Sea turtles are not the only marine megafauna members to host diverse and abundant epibiotic communities. Palomba et al. provide novel ecological and molecular data on several species of parasitic copepods associated with pelagic sharks in the Mediterranean region, and Ten et al. present a comprehensive list of epibiotic fauna found on cetaceans worldwide. The latter authors supplement this inventory with comments about the indicator potential of each epibiotic taxon and encourage marine biologists to record and report on epibionts (or their lack) observed during routine research activities. One of the major gaps in our current knowledge about marine epibioses is the need for more information on the ecosystem- and community-level impacts of invasive, non-native basibionts. In their snapshot study from the Damariscotta Estuary (Gulf of Maine, USA), Lazzeri and Auker attempt to evaluate whether non-native basibionts facilitate invasions through epibiosis. Although they conclude that extensive, long-term surveys from diverse regions are necessary to shed light on these complex relationships, it is clear that non-native basibionts do affect the community structure of the local epibionts.

Finally, microepibionts, which also play an essential role in conditioning the living substratum for larger organisms, have only recently started to receive increased research attention. Thus, unsurprisingly, new studies investigating microbial biofilms on marine animals, plants, and algae often reveal unexpected diversity and ecological roles. In one of the first such surveys, Kanjer et al. explore microbiota on the surface of Mediterranean loggerhead turtles. They report a great variety of both bacterial and eukaryotic microbes whose presence and abundance seem to be affected by not only the sea turtle anatomy and substratum tissue type (skin vs carapace), but also environmental factors linked to the sampling locations. Microbial mats may be considered a special case of epibiosis in which entire communities of microorganisms become both basi- and epibionts to other microbes. Although these ecosystems are amongst the oldest on the planet, their taxonomic and metabolic characteristics are often poorly understood. Walter et al. use metagenomic approaches to characterize microbial mats of the hypersaline lagoon system of Araruama (Brazil). Their results reveal a diversity of cooperative niches linked and controlled by microbial interactions that create a habitable environment within an otherwise extreme setting. However, the high metabolic activity of an epibiont may also be a nuisance to its host and sometimes the entire habitat. Zou et al. describe the spatio-temporal distribution of the alga *Prorocentrum concavum* in the tropical coastal lagoon of Xincun Bay (China) and identify the environmental factors linked to its blooms. The new information suggests that seagrass beds, rather than other benthic substrata, constitute important reservoirs of *Prorocentrum* cells

that, under suitable environmental conditions, can seed the harmful algal blooms in the region.

The collection of articles in this Research Topic provides a glimpse into the fascinating research on marine organisms associated with living surfaces, and we hope the next edition of this series will allow readers to stay abreast of this rapidly developing field.

Author contributions

RM drafted the article. RM, SD, NR, and FT reviewed and edited the article. All authors contributed to the article and approved the submitted version.

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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Epibionts Reflect Spatial and Foraging Ecology of Gulf of Mexico Loggerhead Turtles (*Caretta caretta*)

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Sea turtles are exposed to numerous threats during migrations to their foraging grounds and at those locations. Therefore, information on sea turtle foraging and spatial ecology can guide conservation initiatives, yet it is difficult to directly observe migrating or foraging turtles. To gain insights into the foraging and spatial ecology of turtles, studies have increasingly analyzed epibionts of nesting turtles, as epibionts must overlap spatially and ecologically with their hosts to colonize successfully. Epibiont analysis may be integrated with stable isotope information to identify taxa that can serve as indicators of sea turtle foraging and spatial ecology, but few studies have pursued this. To determine if epibionts can serve as indicators of foraging and spatial ecology of loggerhead turtles nesting in the northern Gulf of Mexico we combined turtle stable isotope and taxonomic epibiont analysis. We sampled 22 individual turtles and identified over 120,000 epibiont individuals, belonging to 34 macrofauna taxa (>1 mm) and 22 meiofauna taxa (63 μm–1 mm), including 111 nematode genera. We quantified epidermis $\delta^{13}C$ and $\delta^{15}N$, and used these to assign loggerhead turtles to broad foraging regions. The abundance and presence of macrofauna and nematodes did not differ between inferred foraging regions, but the presence of select meiofauna taxa differentiated between three inferred foraging regions. Further, dissimilarities in macrofauna, meiofauna, and nematode assemblages corresponded to dissimilarities in individual stable isotope values within inferred foraging regions. This suggests that certain epibiont taxa may be indicative of foraging regions used by loggerhead turtles in the Gulf of Mexico, and of individual turtle foraging and habitat use specialization within foraging regions. Continued sampling of epibionts at nesting beaches and foraging grounds in the Gulf of Mexico and globally, coupled with satellite telemetry and/or dietary studies, can expand upon our findings to develop epibionts as efficient indicators of sea turtle foraging and spatial ecology.

Keywords: epibiont, foraging, Gulf of Mexico, isotopes, macrofauna, meiofauna, nematode, loggerhead sea turtle

INTRODUCTION

Sea turtles are highly migratory animals: hatchling turtles may circumnavigate entire ocean basins before maturation (Carr, 1987; Mansfield et al., 2014), and individual mature turtles migrate thousands of kilometers between specific foraging and breeding grounds each year (Plotkin et al., 2002; Broderick et al., 2007; Shillinger et al., 2008). Sea turtles spend much of their lives at foraging grounds (Bolten, 2003; Hawkes et al., 2006), and may be exposed to various threats at these locations (Hart et al., 2018; Fuentes et al., 2020) or while migrating between foraging and breeding regions (Hart et al., 2014).

Knowledge of sea turtle foraging and spatial ecology is critical to identify areas of high/potential use, assess their exposure to threats, and inform management and conservation of sea turtles (Hawkes et al., 2006; Gredzens et al., 2014; Mazor et al., 2016; Rees et al., 2016; Fuentes et al., 2019). However, sea turtle research overwhelmingly relies upon data collected from nesting sea turtles, as they are easier to encounter and sample than foraging, in-water turtles (Hamann et al., 2010; Rees et al., 2016). Satellite telemetry and stable isotope analysis (SIA) are two techniques commonly employed at nesting beaches that provide insight into sea turtle behavior away from nesting beaches (Ceriani et al., 2012; Jeffers and Godley, 2016). Satellite telemetry can be used to track turtles between nesting beaches and foraging grounds and to understand the spatial ecology of sea turtles at foraging grounds (Jeffers and Godley, 2016; Hays and Hawkes, 2018). However, the cost of satellite transmitters is often prohibitive to their use, and many studies only track a small percentage of any nesting assemblage (Rees et al., 2016). Analysis of carbon and nitrogen stable isotopes is less expensive than satellite telemetry and can be used to approximate where sea turtles forage and at what trophic level (DeNiro and Epstein, 1978; Rubenstein and Hobson, 2004; Reich et al., 2007; Vander Zanden et al., 2010). Such inferences depend upon the available baseline stable isotope data in a region, and on isotopic differences between turtles from different foraging grounds, however, baseline data is not always available for a region nor do turtles from different foraging grounds always have different isotopic signatures (Vander Zanden et al., 2015; Ceriani et al., 2017). Therefore, novel, cost-effective and informative tools to explore turtle foraging and spatial ecology would prove useful additions to satellite telemetry and SIA (Rees et al., 2016; Hays and Hawkes, 2018).

Recent attention has turned to sea turtle epibionts as potential natural data loggers of sea turtle migratory and foraging behaviors (Frick and Pfaller, 2013; Pearson et al., 2019; Ten et al., 2019). Epibionts are organisms that colonize other organisms, and are commonly found on the carapaces of all seven sea turtle species (Frick and Pfaller, 2013; Robinson et al., 2016). Epibiotic colonization typically begins after chemical alteration of submerged substrates, followed by the establishment of (1) unicellular bacteria; (2) diatoms and protozoans, and (3) meiofauna and macrofauna (Wahl, 2009; dos Santos et al., 2018). Colonization requires ecological and spatial overlap of epibionts and living substrate (such as sea turtle carapaces), and it is thought that colonization of sea turtle carapaces occurs primarily at foraging grounds, with some colonization occurring at breeding areas (see Figure 15.1 in Frick and Pfaller, 2013; Reeves et al., 2018; Hart et al., 2021).

Few studies have sought to characterize the relationships between epibiont colonization and turtle foraging ecology (Reich et al., 2010; Ten et al., 2019). This is a difficult endeavor, as most epibiont studies report on assemblages sampled from nesting sea turtles, which may share epibiont taxa from recent colonization (Reeves et al., 2018; Hart et al., 2021), and do not pair epibiont sampling with satellite telemetry or stable isotope analysis of turtle tissues to relate epibiosis to foraging or spatial ecology (Frick and Pfaller, 2013; except see Reich et al., 2010; Nolte et al., 2020). Additionally, most studies of sea turtle epibionts focus on large, easily observable species and do not characterize microscopic organisms such as meiofauna that may colonize sea turtle carapaces in large numbers, establishing diverse epibiotic communities (Frick and Pfaller, 2013; notable exceptions include Corrêa et al., 2014; Robinson et al., 2016; dos Santos et al., 2018; Ingels et al., 2020).

Diverse epibiont assemblages, cumulating in over 200 taxa, have been documented on loggerhead sea turtles (*Caretta caretta*; Frick and Pfaller, 2013). A recent study of loggerheads nesting at St. George Island (SGI), Florida, characterized the abundance of 20 meiofauna taxa including 111 nematode genera, and reported discrete groups of epibiont assemblages (Ingels et al., 2020). This implies that some sampled loggerheads underwent similar colonization processes and as such may display similar foraging and spatial ecology. We investigated if and how epibiont assemblages can be informative toward understanding sea turtle foraging and spatial ecology, both alone and when integrated with SIA data. To do so, we analyzed the meiofauna epibiont data from Ingels et al. (2020), along with new data from macrofauna epibiont and stable isotope analyses from the same turtles. We tested to see if and how epibiont assemblages differed between turtles from different inferred foraging regions, and if stable isotope data predicted variation in epibiont assemblages. Our work tests fundamental theories on epibiont colonization and explores the extent to which epibionts can provide information on sea turtle foraging and spatial ecology.

MATERIALS AND METHODS

We encountered nesting loggerhead sea turtles during nightly surveys at SGI (**Figure 1**). St. George Island hosts the largest loggerhead assemblage in the Northern Gulf of Mexico Recovery Unit (NGMRU) for loggerhead sea turtles (FFWCC, 2020). The NGMRU spans beaches from the United States-Mexico border in Texas to Franklin Co., FL, and is a small (Ceriani et al., 2019), genetically discrete subpopulation (Shamblin et al., 2012) of the Northwest Atlantic Ocean Regional Management Unit (RMU) of loggerhead turtles (Wallace et al., 2010), the largest loggerhead RMU globally (Casale and Tucker, 2017; Ceriani et al., 2019). Surveys took place over 2 weeks during the peak of the 2018 nesting season at St. George Island, from June 16th to July 1st (Ingels et al., 2020). We sampled encountered turtles after they had begun covering their egg chambers. We checked all turtles

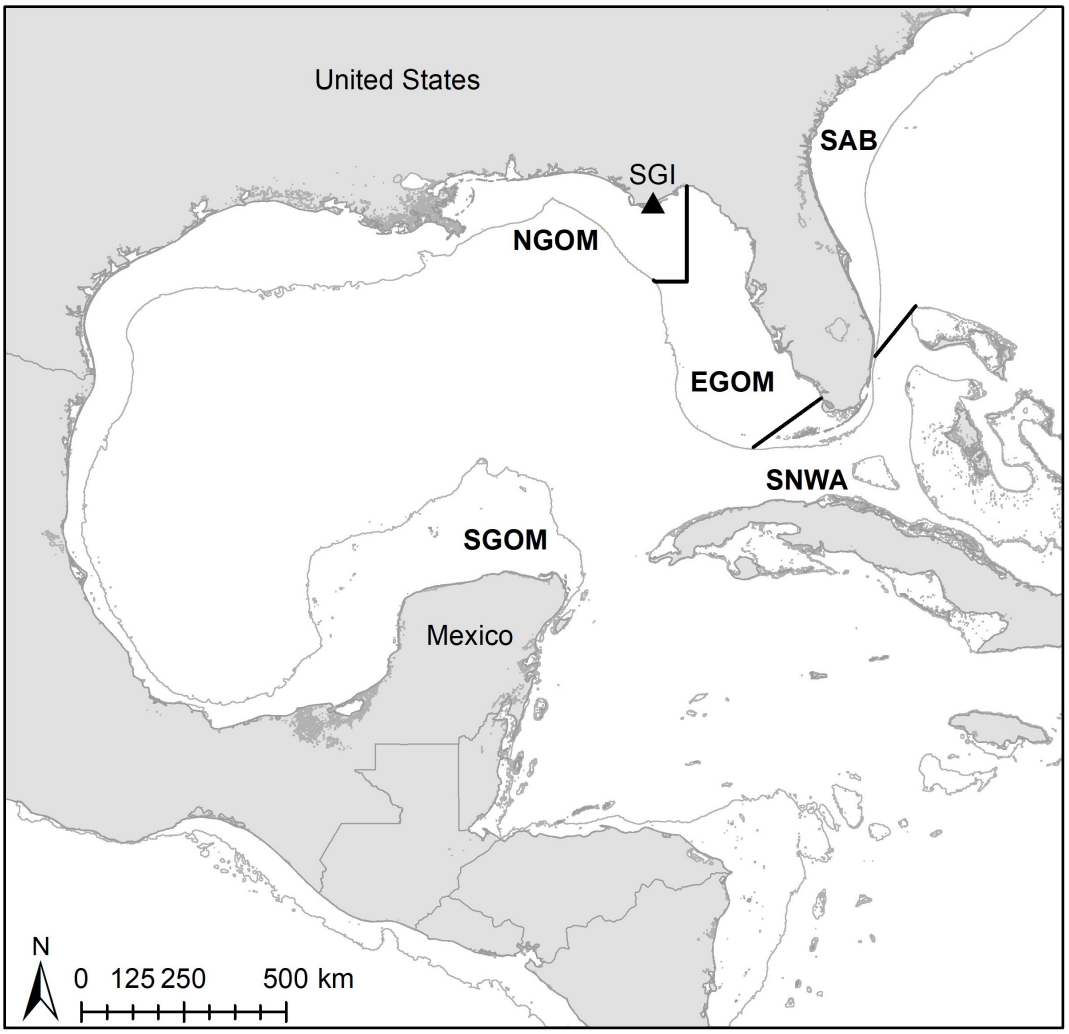

FIGURE 1 | Map of study site (St. George Island; SGI) and broad foraging regions for assignments. NGOM, Northern Gulf of Mexico; EGOM, Eastern Gulf of Mexico; SGOM, Southern Gulf of Mexico; SNWA, Subtropical Northwest Atlantic; SAB, South Atlantic Bight. Thin gray lines indicate 200-m depth contours.

for pre-existing Inconel flipper or PIT tags, and applied these whenever necessary following protocols in the Florida Fish and Wildlife Conservation Commission Marine Turtle Conservation Handbook (FFWCC, 2016). We sampled the entire carapace of each turtle for epibionts following Ingels et al. (2020). Briefly, we scraped off large fauna such as barnacles with a putty knife, and collected small fauna such as nematodes with a sponge by wiping down the entire surface until visibly clean. We stored all epibionts in DESS or a formalin solution until they could be sorted and identified (see below). We collected epidermal tissue samples for SIA from the shoulder of each turtle using 5 mm biopsy punches and stored samples in salt.

We washed and sorted epibiont samples into macrofauna (>1 mm) and meiofauna (63 μm–1 mm) at the Florida State University Coastal and Marine Laboratory. We stored macrofauna and meiofauna separately in a solution of dimethyl sulfoxide, ethylenediaminetetraacetic acid, and saturated sodium chloride (DESS). We identified meiofauna to higher taxa (Higgins and Thiel, 1988; Giere, 2009, following quantitative subsampling procedures from Ingels et al., 2020), and macrofauna to the lowest taxonomic level possible, usually family or a lower level using stereoscopic microscopes and taxa-specific keys to Gulf of Mexico invertebrates (Fauchald, 1977; Culter, 1986). We picked out nematodes *ad hoc* from meiofauna samples (120 individuals per sample), which we then desiccated, and mounted on slides for identification to genera using available nematode keys (Platt and Warwick, 1983; Bezerra et al., 2019).

We prepared turtle tissue samples for SIA at Florida State University Department of Earth, Ocean, and Atmospheric Science. Samples were brushed and then rinsed with deionized water to remove particulate matter and salt, dried in an oven for 2 h at 60°C to remove all moisture, and homogenized using a sterile scalpel (following Lemons et al., 2011; Levin and Currin, 2012; Gillis et al., 2018). We sent homogenized samples to the Paleoclimatology, Paleoceanography, and Biogeochemistry Laboratory at the University of South Florida College of Marine

Science for lipid extraction and SIA. Lipids were extracted from samples using an accelerated solvent extractor (Model 200, Dionex) with petroleum ether (3 cycles of 5 min heating followed by 5 min static purging). Samples were then weighed to 0.5–0.7 mg using a Mettler Toledo micro balance, placed into Costech tin cups, and converted to N_2 and CO_2 using a Carlo-Erba NA2500 Series 2 Elemental Analyzer (Thermoquest Italia). Isotope ratios were measured in a continuous flow mass spectrometer (Delta Plus XP, Thermofinnigan). Sample ratios are expressed as parts per mille (‰) and calculated using the equation:

$$\delta X = \left[\left(\frac{R_{sample}}{R_{standard}}\right) - 1\right] * 1000$$

where X is ^{15}N or ^{13}C, and R is the ratio of ^{15}N:^{14}N or ^{13}C:^{12}C. Standards for ^{15}N and ^{13}C were atmospheric nitrogen and Pee Dee Belemnite, respectively.

SIA values were incorporated into continuous probability surfaces (CPSs) and discriminant functions (DFs) to assign turtles to broad foraging regions within the Gulf of Mexico [as designated by Vander Zanden et al., 2015: Northern Gulf of Mexico (NGOM), Eastern Gulf of Mexico (EGOM), Southern Gulf of Mexico (SGOM), Subtropical Northwest Atlantic (SNWA) and South Atlantic Bight (SAB); **Figure 1**]. We retrieved previously published scute SIA values, foraging locations, and isoscapes from tracked Gulf of Mexico loggerheads from Vander Zanden et al. (2015). We converted SIA values for sampled turtles from epidermis to scute using equations in Vander Zanden et al. (2014), and generated continuous probability rasters for each individual turtle following Ceriani et al. (2017). Individuals were assigned to the foraging region containing the highest probability raster cell. We constructed DFs to assign individuals to foraging regions with the same published SIA data from turtles with known foraging locations in SPSS 27 (IBM; i.e., Ceriani et al., 2014). The first two DFs were constructed based on 39 training individuals and tested with 19 individuals, both groups with known foraging locations. Assignments were made using unequal priors and a leave-one-out cross-validation method (Ceriani et al., 2014; Vander Zanden et al., 2014). Wilks' Lambda was used to assess if the first two DFs adequately explained group membership. Assignment probabilities and odds ratios (Wunder, 2012) were used to assess foraging region assignments for turtles sampled at SGI.

We imported epibiont abundance, SIA, and foraging data into Primer-e V7 (Clarke and Gorley, 2015) with the PERMANOVA+ add on (Anderson et al., 2008) to characterize epibiont assemblages (i.e., diversity indices) and explore relationships between epibiont assemblages, foraging regions, and SIA data. We analyzed macrofauna, meiofauna, and nematodes independently to determine whether and which taxa might relate to the foraging ecology of individual turtles. We square root (sqrt) transformed abundance data to reduce the influence of particularly abundant taxa on multivariate statistics, and presence-absence (p-a) transformed abundance data to determine if the presence or absence of certain taxa related to turtle foraging ecology (Clarke and Gorley, 2015). Square-root transformed data were duplicated and standardized (sqrt-stan) to minimize the influence of variable abundances between samples. Bray-Curtis and Euclidean distance were used as the resemblance measures for epibiont and SIA data, respectively. We used non-metric Multi-Dimensional Scaling (nMDS) plots to visualize data and qualitatively assay for dissimilarities in epibiont assemblages between turtles assigned to different foraging grounds. CLUSTER and SIMPROF (with 5% significance tests) were used to identify similar groups of epibiont assemblages and determine if these groups corresponded to turtles assigned to the same foraging grounds. ANOSIM and PERMANOVA were used to determine if epibiont assemblages differed between turtles assigned to different foraging regions, and SIMPER was used to identify taxa contributing to these differences. RELATE and BEST were used to determine if individual foraging ecology related to epibiont assemblages, and which sample epibiont assemblage dissimilarities correlated significantly (significance level = 5%) to SIA sample dissimilarities. We ran all tests over 10,000 iterations (Clarke and Gorley, 2015).

RESULTS

We sampled 23 individuals for epibionts and 22 individuals for stable isotopes (summary statistics are presented in **Table 1**). The first two foraging region assignment DFs constructed using

TABLE 1 | Epibiont and stable isotope summary statistics and foraging assignment frequencies.

Epibiont summary statistics

Epibionts (*n* = 23)	Total taxa (Range, Mean ± SD)	Abundance Range (Mean ± SD)
Macrofauna	34 (7–19, 12 ± 3)	24–11,569 (3,420 ± 3,172)
Meiofauna (+ Nematodes)	22 (7–16, 12 ± 2)	6,590–146,190 (35,235 ± 29,756)
Meiofauna (− Nematodes)	21 (6–15, 11 ± 2)	4,840–132,530 (26,739 ± 26,220)
Nematodes	111 (8–50, 27 ± 10)	427–20,200 (6,434 ± 4,638)

Stable isotope summary statistics (‰, *n* = 22)

Isotope	Range	Mean ± SD
$\delta^{13}C$	−17.1–10.25	−14.64 ± 1.45
$\delta^{15}N$	6.18–15.74	10.96 ± 2.06

Foraging assignment frequencies

Assignment method	NGoM	EGoM	SGoM	SNWA
CPS	3	8	10	1
DF	6	7	8	1

*Meiofauna statistics are presented with all nematode genera grouped together (+ Nematodes) and without nematodes (− Nematodes). Stable isotope values are presented as parts per mille (‰). Foraging assignment frequencies are presented for both continuous probability surface (CPS) and discriminant function (DF) assignments. NGOM, Northern Gulf of Mexico; EGOM, Eastern Gulf of Mexico; SGOM, Southern Gulf of Mexico; SNWA, Subtropical Northwest Atlantic. Foraging regions are delineated in **Figure 1**.*

the training data were significant ($p >$ Wilks' Lambda < 0.000). The first two DFs had a combined $\chi^2(6) = 98.954$, ($p < 0.000$). Alone, the second DF had a $\chi^2(2) = 9.74$, ($p = 0.008$). The first DF explained 97.4% of the between-group variability, and the second DF explained 2.6% of the between-group variability. 30/39 (76.9%) loggerheads in the training data and 14/19 (73.7%) loggerheads in the testing data were assigned correctly to their known foraging regions. Assignment probabilities ranged from 0.41 to 1 (mean = 0.66 ± 0.18 SD). Odds ratios that described how much more informative the DFs were for assigning individual turtles to foraging regions than a random assignment procedure ranged from 2.04:1 to 6,040,365:1 (mean = 13.6:1 ± 27.4 SD). We have chosen to retain all DF assignments in subsequent analyses (following Kelly et al., 2005; Szymanski et al., 2007; López-Castro et al., 2014). The DF assignments made here had higher probability than if turtles were assigned at random (all turtles had >0.25 probability of being assigned to their most likely foraging region), and we acknowledge the potential for erroneous assignments when not using a probability threshold (i.e., >0.6; see discussion). The CPS and DF foraging ground assignments produced similar results: most turtles were assigned to SGOM (CPS, $n = 10$; DF, $n = 8$), followed by EGOM (CPS, $n = 8$; DF, $n = 7$), NGOM (CPS, $n = 3$; DF, $n = 6$), and finally SNWA (CPS, $n = 1$; DF, $n = 1$; **Table 1**). No turtles were assigned to SAB. Fewer turtles were assigned to NGOM using CPS ($n = 3$) than DF ($n = 6$), and more turtles were assigned to EGOM and SGOM using CPS ($n = 8$ and 10, respectively) than using DF ($n = 7$ and 8, respectively, **Table 1**).

CLUSTER and SIMPROF identified distinct, significant ($p < 0.05$) groups (k) of similar epibiont assemblages within the meiofauna ($k_{sqrt-stan}$, $k_{p-a} = 2$; **Supplementary Figure 1**) and nematodes ($k_{sqrt-stan} = 3$, $k_{p-a} = 2$, $k_{sqrt} = 4$; **Supplementary Figure 2**), but not within macrofauna. SIMPROF groups did not appear to correspond to CPS or DF foraging ground assignments in visual examinations of two dimensional nMDS plots (**Supplementary Figures 1, 2**), but presence-absence transformed meiofauna and square-root transformed nematode assemblages did group visibly according to foraging assignment categories (**Figure 2**). The presence and absence of certain meiofauna contributed to assemblage differences below or near statistical significance (ANOSIM: Rho = 0.158, $p = 0.032$) between turtles assigned to the NGOM and SGOM by DF (ANOSIM: Rho = 0.278, $p = 0.015$; PERMANOVA: pseudo-F = 78.005, $t_{13} = 1.75$, $p = 0.019$), and EGOM and SGOM (ANOSIM: Rho = 0.156, $p = 0.052\%$). SIMPER identified Sarcomastigophorans, Bivalves, Polychaetes, Turbellarians, Limulids, Tanaidaceans, Nauplii, and Acari as contributing to a cumulative 77.18% of the differences between NGOM and SGOM assemblages (**Figure 3A** and **Supplementary Table 1**). Many of the same taxa contributed to differences between EGOM and SGOM assemblages (72.16%) with a few modifications: Nauplii and Tanaidaceans did not contribute to differences, while Hydroids did (**Figure 3A** and **Supplementary Table 2**). The abundance of nematode genera also contributed significantly to differences (ANOSIM: Rho = 0.183, $p = 0.034\%$) between turtles assigned to the EGOM and SGOM by CPS (ANOSIM: Rho = 0.2, $p = 0.013\%$; PERMANOVA: pseudo-F = 37.374, $t_{16} = 1.38$, $p = 0.022$). SIMPER identified 27 nematode genera that contributed to 70.96% dissimilarity between EGOM and SGOM assemblages (**Figure 3B** and **Supplementary Table 3**). Nematode abundance in SGOM assemblages was higher than in EGOM assemblages for nearly all genera that contributed most to dissimilarities between assemblages from these foraging regions (**Figure 3B** and **Supplementary Table 3**).

Dissimilarities in SIA data related to dissimilarities in epibiont assemblages consistently within DF-assigned foraging regions. Dissimilarities in $\delta^{15}N$ correlated moderately with dissimilarities in macrofauna abundances within DF-assigned foraging regions (BEST: Rho = 0.49, $p = 0.01$). Dissimilarities in SIA data between individual turtles correlated significantly with dissimilarities between individual assemblage meiofauna abundance (RELATE: Rho = 0.22, $p = 0.05$) and presence or absence of certain taxa (RELATE: Rho = 0.246, $p = 0.046$) within DF-assigned foraging regions. Dissimilarities in $\delta^{13}C$ and $\delta^{15}N$ correlated also significantly with dissimilarities in nematode genera abundances (BEST: Rho = 0.409, $p = 0.045$) between samples within DF-assigned foraging groups.

DISCUSSION

Epibionts may serve as useful indicators of sea turtle spatial and foraging ecology between and within broad foraging regions. In our study, meiofauna higher taxa and nematode genera proved more discriminative and informative toward broad foraging locations and foraging ecology than macrofauna higher taxa. Previous studies of loggerhead turtle epibiont assemblages elsewhere have suggested that differences in assemblages correspond to a foraging dichotomy between pelagic and neritic habitats (Reich et al., 2010; Nolte et al., 2020). Mature loggerhead turtles in the Atlantic Ocean, particularly in the Gulf of Mexico, restrict foraging to the shallow (<200 m), continental shelf (Hart et al., 2012, 2020; Hardy et al., 2014). Further, individuals exhibit specialized foraging behaviors across their entire foraging range (Vander Zanden et al., 2010, 2016). Variation in epibiont assemblages from adult loggerheads in the Gulf of Mexico therefore likely corresponds to habitat and habitat-use variation between and within foraging regions, rather than pelagic-neritic foraging dichotomies (Reich et al., 2010). Our findings lend support to these paradigms, as we found that some epibiont taxa differed between turtles from different foraging regions, and that epibiont assemblages differed between turtles from within the same foraging region. As colonization depends upon spatial and ecological overlap of epibionts and hosts (Frick and Pfaller, 2013), our analysis of epibionts is informative to the distribution of epibionts among turtle foraging regions and to the behaviors of turtles within those foraging regions.

Many taxa were commonly found on turtle carapaces, regardless of foraging region or SIA data. This may be due to recent colonization, epibiont taxa life-histories, and/or baseline abundance across foraging regions. Turtles that breed and nest in the same region are likely to be colonized in the short-term by the same taxa in similar abundances, particularly if individual turtles are behaving similarly (i.e., reserving resources for generating

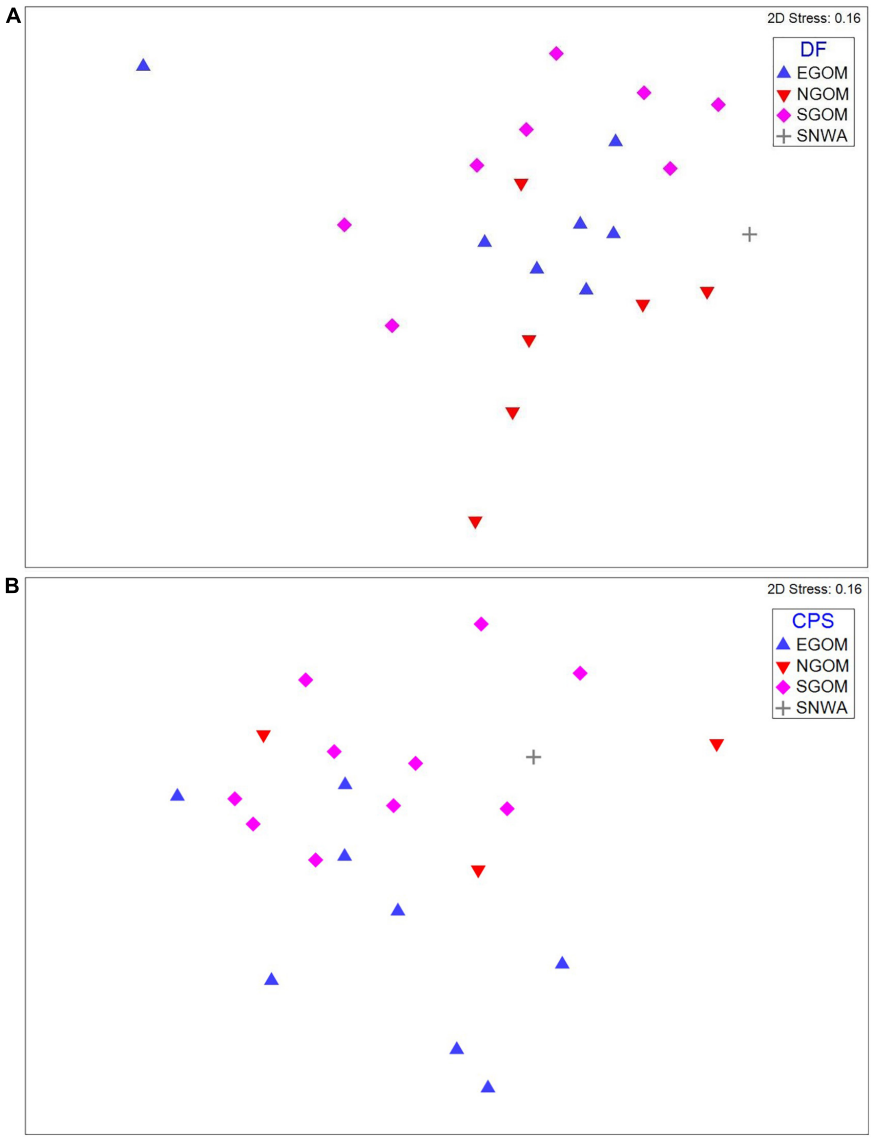

FIGURE 2 | nMDS plots for **(A)** meiofauna presence-absence assemblages and **(B)** nematode square-root abundance assemblages showing evidence for moderate clustering (indicated by points occupying similar space in nMDS plots) by foraging region assignments (symbols). DF, Discriminant Function assignments; CPS, Continuous Probability Surface assignments; EGOM, Eastern Gulf of Mexico; NGOM, Northern Gulf of Mexico; SGOM, Southern Gulf of Mexico; SNWA, Subtropical Northwest Atlantic.

multiple clutches of eggs; Houghton et al., 2002). Organisms such as barnacles settle quite quickly, especially in the northern Gulf of Mexico (Reeves et al., 2018; Hart et al., 2021). It is possible that some ubiquitous epibionts such as turtle barnacles (*Chelonibia testudinaria*) and skeleton shrimp (*Caprella andreae*) settled on sampled turtles as they rested in-between nesting events. Organisms that colonize from a planktonic larval stage may disperse relatively far distances from their original substrate (Thiel, 2003), and therefore be found more commonly on turtles regardless of foraging region, prey-item preferences, or habitat use. This may be one reason why the macrofauna identified here did not differ between turtles from different foraging regions. Twenty-one of the 34 macrofauna taxa identified here have a largely pelagic larval stage before settling, including the highly abundant turtle barnacle and juvenile *Cirripedia* sp. Some taxa without larval dispersal (i.e., skeleton shrimp) were still the most abundant macrofaunal taxa on all turtles. These are abundant benthic species globally (Cabezas et al., 2013), and as such may be abundant turtle epibionts.

The presence and absence of select meiofauna taxa and the differential abundance of nematode genera drove differences between epibiont assemblages of turtles that forage in the NGOM, EGOM, and SGOM. This may have been driven by three factors.

First, colonization frequency may correspond to baseline abundances of these taxa within foraging regions. The presence or absence, or abundance, of taxa in epibiont assemblages can

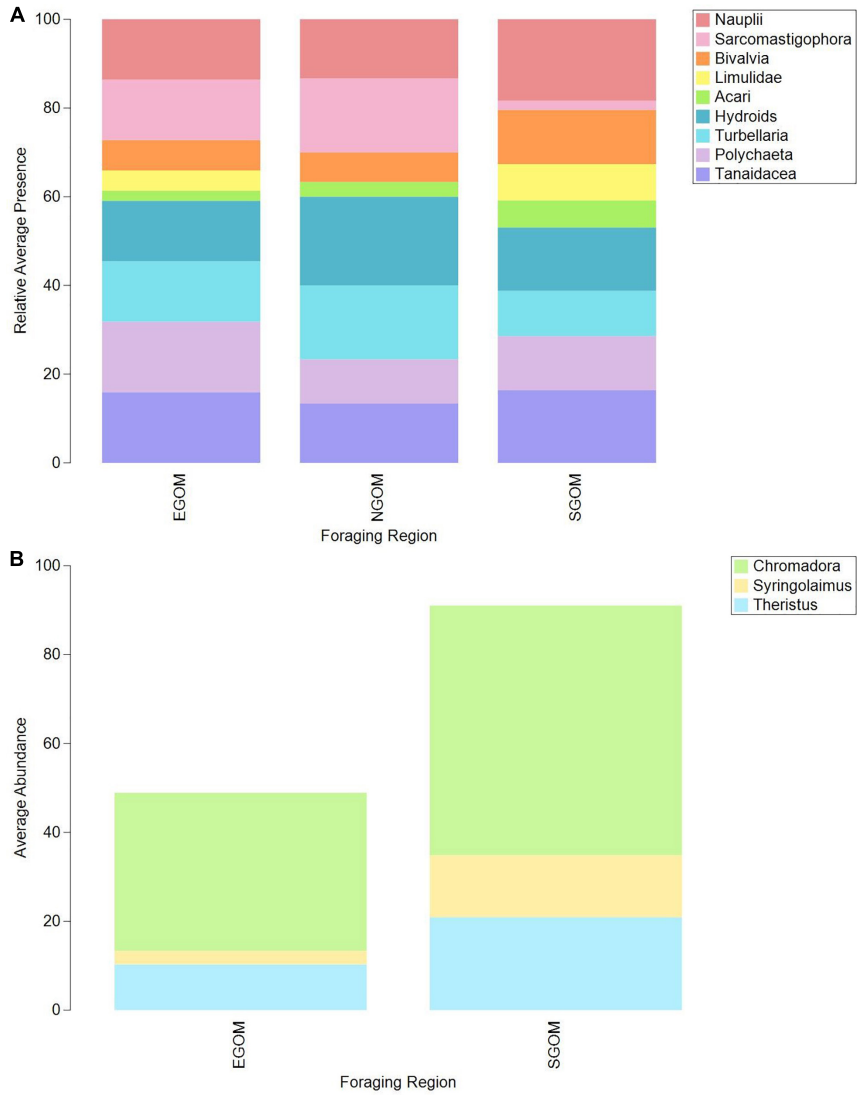

FIGURE 3 | Bar plots of average relative presence of **(A)** meiofauna and **(B)** nematode taxa contributing to dissimilarities between Eastern Gulf of Mexico (EGOM), Southern Gulf of Mexico (SGOM), and Northern Gulf of Mexico (NGOM) DF-assigned turtles, identified via SIMPER. See **Supplementary Tables 1, 2** for specific taxa contributions to dissimilarities between groups (full SIMPER results).

depend upon whether or not those taxa are found commonly within broad foraging regions. If turtles do not frequently encounter certain epibiont species within a foraging region, it is likely that those species will not be present (or will be present in reduced numbers) as epibionts of turtles from that foraging region (Frick and Pfaller, 2013).

Second, the life-histories of specific taxa may vary between foraging regions. For example, larval horseshoe crabs (family *Limulidae*) were not found on turtles that forage in the NGOM, but were present on SGOM and EGOM foragers. In the northwest Atlantic Ocean, horseshoe crabs begin breeding with the onset of warm temperatures (Rudloe, 1980; Cohen and Brockmann, 1983). It is possible that breeding had not begun in the NGOM by the time females migrated to SGI to breed. While it is also possible that turtles and horseshoe crabs do not interact in the NGOM, loggerheads are known to prey on horseshoe crabs (Seney and Musick, 2007; Botton, 2009) and would provide substrate for planktonic larvae in their vicinity (as evidenced here). If potential epibionts are not ready to settle on substrate when they encounter a turtle, they will not colonize.

Third, individual turtle habitat use within foraging regions may influence colonization (Reich et al., 2010; Vander Zanden et al., 2010; Frick and Pfaller, 2013). Turtles forage on specific prey items (or a specific set of prey items) at foraging grounds (Vander Zanden et al., 2010; Nolte et al., 2020). Epibiosis likely occurs in part as a byproduct of foraging; epibionts may colonize from the water column or benthos as turtles search and maneuver for prey items (Frick and Pfaller, 2013). Both potential epibionts and prey items may be found in association with certain habitat types (i.e., mangroves, sea grass beds) in some regions, but

not in others. This could be due to interspecific variation or habitat degradation (and associated declines in diversity; Reed and Hovel, 2006), among other reasons. Colonization cannot occur in certain foraging regions if potential epibionts and prey items are not sympatric where turtles seek out prey items. We cannot determine which of the above factors contributed to differences in meiofauna colonization between NGOM, EGOM, and SGOM foraging turtles with our available data. Nevertheless, our results indicate that epibionts can be informative to turtle spatial ecology, and vice-a-versa.

Differences between epibiont assemblages from turtles assigned to the same foraging region corresponded to differences in SIA values from those turtles (as per RELATE and BEST results). SIA values vary with location and foraging item preference (DeNiro and Epstein, 1978; Rubenstein and Hobson, 2004). Thus, differences in epibiont assemblages within foraging regions may reflect individual specialization in specific prey items or a specific combination of prey items (Vander Zanden et al., 2010; Nolte et al., 2020). Specializing in certain prey may require that turtles utilize specific foraging mechanisms, or frequent certain habitats, that then allow for differential colonization of epibionts. For example, loggerhead turtles that feed on benthic mollusks may dig for their prey, which can suspend benthic organisms that may colonize carapaces (Lazar et al., 2011). Loggerheads that graze on suspended fauna such as gastropods and chondrophores (Hatase et al., 2007) do not perturb sediment, and are colonized by a different suite of organisms. These turtles could exhibit different SIA values and epibiont assemblages, yet be present at the same foraging region. Further, turtles with preferences for specific prey items may have to frequent different habitat types to consume those items (Cardona et al., 2009; Williams et al., 2017). These habitats have their own invertebrate communities, which could contribute to unique colonization of loggerhead carapaces. Epibionts may provide insight into loggerhead turtle foraging preferences and habitat use within foraging regions, and future studies that explicitly relate foraging preferences and habitat use to epibiosis (as discussed below) will allow researchers to garner more information from the epibionts of nesting sea turtles.

Studies such as ours can be most informative by sampling a high proportion of nesting turtles from nesting assemblages, integrating epibiont analysis and SIA with satellite telemetry, and using molecular techniques to identify taxa. Our turtle sample size was representative for the 2018 nesting season at SGI (∼35–40% of nesting turtles; S.C. personal communication, Ceriani et al., 2019), but low relative to the average annual number of nesting turtles at SGI (I.S. unpublished data, Ceriani et al., 2019). Further, this low sample size mitigates the power of inferences that can be made using foraging region assignments. CPS and DF performed slightly differently, and subsequent analyses identified different relationships between epibionts and turtles assigned to foraging regions using the two techniques. It would be unreasonable to expect these methods to perform identically, but increasing sample size could mitigate the bias that slight differences in assignments between CPS and DF have on downstream analyses. Increasing sample size might also allow us to implement assignment probability thresholds for DF assignments (as per Wunder, 2012; Ceriani et al., 2014; Vander Zanden et al., 2014) if future samples have higher probabilities, which would increase certainty in assignments and in the relationships identified here between epibionts, inferred foraging regions, and SIA. Sampling additional turtles for epibionts and SIA would therefore allow us to be more confident in differences between and within inferred foraging regions, and integrating satellite telemetry with our epibiont analysis and SIA would reduce error in foraging ground assignments. However, these efforts would be costly and labor intensive and were beyond the scope of this study.

Molecular techniques such as sequencing of COI barcodes (Hebert et al., 2003) or 16s rRNA (Goetze, 2010) could help further identify epibiont taxa to species, populations, or operational taxonomic units. This could improve taxonomic resolution to demonstrate differences in the presence or abundance of certain common taxa between turtles between and within different foraging regions. This is particularly true for higher taxa identified to phyla or classes and for abundant taxa identified to species (i.e., skeleton shrimp). Certain families, genera, or species within the higher taxa identified here could differ between or within foraging regions, and skeleton shrimp and other common species may exhibit genetic population differences between foraging regions that are not apparent from morphological identification alone. Further, taxa such as diatoms are found ubiquitously on sea turtle carapaces (Robinson et al., 2016), and differ between loggerhead turtles sampled across broad geographic scales (van de Vijver et al., 2020). Diatoms might therefore discriminate between sea turtles at smaller scales, such as in this study. Improving taxonomic resolution and breadth in similar, future studies could render common epibiont taxa more informative toward sea turtle spatial and foraging ecology.

Sampling and identifying epibionts, especially small meiofauna, from nesting turtles provides promising insights into sea turtle foraging and spatial ecology, but our understanding of carapace colonization should be refined to develop and streamline these techniques. Sampling epibionts from mature turtles in-water at foraging grounds, although difficult, is necessary to establish baseline data for epibionts of turtles within foraging regions (Nolte et al., 2020). Observations of turtle foraging ecology, spatial ecology, and habitat use at foraging grounds (via isotopic mixing models, satellite transmitters and/or animal borne cameras, e.g., Thomson and Heithaus, 2014; Gillis et al., 2018; Hays and Hawkes, 2018) can be paired with epibiont identification to relate epibiosis to specific prey item selection, foraging mechanisms, and habitat use within foraging regions. This would allow studies of epibionts from nesting turtles to draw more specific inferences on the spatial and foraging ecology of individual turtles. Finally, sampling the benthos and water column for invertebrates at known turtle foraging locations at different times throughout the year can establish a baseline for potential colonizers and provide insight into the phenology of potential colonizers within foraging regions. These baseline data may be crucial to identifying epibiont species for future studies of nesting turtles that focus on determining the spatial and foraging ecology of individuals, without necessarily having to rely upon expensive techniques such as satellite telemetry. Nevertheless, our

study has provided new foundations for studies to further explore the relationships between epibionts and sea turtle spatial and foraging ecology in the Gulf of Mexico and elsewhere.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by the Florida State University Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

IS-G, JI, MF, GS, YV, and SC conceived the project and designed methodology. IS-G, JI, GS, YV, LP, ACS, PFN, AS, DP, AR, SS-Z, LA, and AG contributed to epibiont collection, sorting, and identification. IS-G and JI analyzed epibiont data and led the writing of the manuscript. SC and AG generated stable isotope data. IS-G, SC, and MF analyzed stable isotope data. All authors contributed critically to the drafts and gave final approval for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.696412/full#supplementary-material

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Metagenomic Insights Into Ecosystem Function in the Microbial Mats of a Large Hypersaline Coastal Lagoon System

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The hypersaline lagoon system of Araruama (HLSA) is one of the largest in the world and one of the most important sources of evaporative salt in Brazil. The biogeochemical characteristics of this lagoon system led it to be considered a Precambrian relic. The HLSA also harbors extensive microbial mats, but the taxonomic and metabolic attributes of these mats are poorly understood. Our high-throughput metagenomics analyses demonstrated that the HLSA microbial mats are dominated by Proteobacteria, Cyanobacteria, and Bacteroidetes. Among Proteobacteria, Deltaproteobacteria comprises approximately 40% of the total population and it includes sulfate-reducing bacteria such as Desulfobacterales, Desulfuromonadales, and Desulfovibrionales. Differing in composition and function of their reaction centers, other phylogenetic diverse anoxygenic phototrophic bacteria were detected in the HLSA microbial mats metagenomes. The presence of photolithoautotrophs, sulfate reducers, sulfide oxidizers, and aerobic heterotrophs suggests the existence of numerous cooperative niches that are coupled and regulated by microbial interactions. We suggest that the HLSA microbial mats hold microorganisms and the necessary machinery (genomic repertoire to sustain metabolic pathways) to promote favorable conditions (i.e., create an alkaline pH microenvironment) for microbially mediated calcium carbonate precipitation process. Metagenome-assembled genomes (*Ca. Thiohalocapsa araruaensis* HLSAbin6 sp. nov. and *Ca. Araruabacter turfae* HLSAbin9 gen. nov. sp. nov.) obtained support the relevance of Sulfur metabolism and they are enriched with genes involved in the osmoadaptive networks, hinting at possible strategies to withstand osmotic stress. Metabolically versatile bacteria populations, able to use multiple nutrient sources and osmolytes, seem to be a relevant attribute to survive under such stressful conditions.

Keywords: biofilms, microbiome, metagenome, metagenome-assembled genomes, sulfate-reducing bacteria, carbonatogenesis, calcium carbonate, compatible solutes

INTRODUCTION

Microbial mats are one of the oldest known ecosystems on Earth. They support complex consortia of many interdependent species belonging to different functional groups (van Gemerden, 1993; Bolhuis et al., 2014). Fossil records (dated > 3.5 Ga) and modern microbial mats have been investigated extensively from geological, biochemical, and microbiological perspectives (Walter et al., 1980; Vasconcelos et al., 2006; Nutman et al., 2016), raising interesting questions about interactions of microorganisms and its environment. Although modern microbial mats hold taxa that likely arose relatively recently, most metabolic pathways processed by them emerged early in Earth's history and are likely retained at the community level (Bolhuis et al., 2014; Louca et al., 2018). Physicochemical microgradients and taxonomic stratification (Harris et al., 2013) are thus generated to accommodate the coexistence of a wide range of complementary metabolic strategies such as photosynthesis, chemosynthesis, and heterotrophy (Fullmer et al., 2015). Exopolymeric substances (EPSs) excreted by these complex microbial communities protect them against environmental stressors such as desiccation and excessive light. These substances also represent an important source of Organic Carbon under oligotrophic conditions and can serve as nucleation centers for carbonate precipitation processes (Rossi and De Philippis, 2015; Cangemi et al., 2016). These characteristics allow microbial mats to thrive in a variety of harsh environments around the world including hypersaline ecosystems, where intense evaporation and low levels of freshwater input lead to high salt concentrations in the water.

In aquatic ecosystems, the total concentration of inorganic ions such as NaCl (i.e., salinity) is a key environmental factor affecting the distribution of microbial communities (Lozupone and Knight, 2007; Schapira et al., 2009; Dupont et al., 2014). Hypersaline microbial mats are usually composed of extremophile bacteria and archaea that actively regulate cytoplasmic osmotic pressure, thereby maintaining protein integrity under hyperosmotic stress (Oren, 1994; Das et al., 2015). Under high salinity (>10% NaCl), cells tend to lose water to the environment through osmosis, causing dehydration and ultimately cell death. To survive and maintain cell turgor, acclimation processes are needed, including compatible solute accumulation and the expression of channel proteins and osmosensitive enzymes (Das et al., 2015).

The hypersaline lagoon system of Araruama (HLSA) is the largest complex of coastal hypersaline lagoons and salty ponds in Brazil, and one of the largest and commercially most important hypersaline sources of evaporative salt in the world (Kjerfve et al., 1996; Clementino et al., 2008; Laut et al., 2017). HLSA is a rare biogeochemical system, representing an analog for Precambrian environments (Vasconcelos et al., 2006). An excess of evaporation over precipitation maintains the lagoons hypersaline (approximately 52 g/L^{-1} salinity), although some annual unbalance might happen (Moreira-Turcq, 2000). High salinity, together with strong daily fluctuations of temperature, light intensity, UV radiation, and desiccation make this shallow system a harsh environment for any organism. These shifts impose important challenges for the ecosystem function, such as how microbial communities adapt to stay active, while maintaining the characteristic structure of vertically stratified groups of microorganisms. Anthropogenic activities also impose pressure on water quality in these lagoons. Understanding how the HLSA microbial mats are characterized is crucial to provide insights of the system and allow to monitor changes of microbial diversity in these unique ecosystems.

The set of hypersaline lagoons of Araruama has been a subject of study for over 30 years, and the examination of microbial communities inhabiting such environment has been performed through classical cultivation methods and 16S rRNA sequencing (Baeta Neves, 1983; Clementino et al., 2008; Ramos et al., 2017). Clementino et al. (2008) detected a high number of novel prokaryotic phylotypes in the HSLA water column, whereas a better understanding of the cyanobacterial composition in the HLSA microbial mats was given by Ramos et al. (2017). However, little is known about the metabolic diversity of the taxa composing the microbial mats of this lagoon system. In addition, a microbial-induced carbonate precipitation model has been described for the microbial mats of Lagoa Vermelha (L. Vermelha) in Araruama (Vasconcelos et al., 2006, 1995); however, it is not entirely clear what species of bacteria are involved in the carbonate formation in the HLSA mats. The main microbial diversity studies of HLSA lack information about the microorganisms involved in the Sulfur cycling, a key metabolism connected to the calcium carbonate precipitation and dissolution in microbial mats. The aim of the present study was to analyze the taxonomic and metabolic potential of the HLSA microbial mats using shotgun metagenomic sequencing, to avoid the taxonomic primer bias of the 16S rRNA sequencing approach (Jovel et al., 2016). We also sought to investigate the metabolic pathways enabling these microbes to thrive in such a unique environment, by shedding light on the genomic repertoire related to osmoadaptation.

MATERIALS AND METHODS

Study Site and Sample Collection

Microbial mats were sampled in eight salty ponds across the HLSA (16°40′, 19°40′S–39°10′, 37°20′W) (**Figures 1A,B**). This shallow hypersaline lagoon system covers an area of approximately 300 km^2 and is located on the coast 150 km east of Rio de Janeiro where it is subject to a semi-arid climate, an upwelling zone (Kjerfve et al., 1996; Spadafora et al., 2010), and northeast trade winds that promote strong daily fluctuations of temperature, light intensity, and desiccation (Vasconcelos et al., 2006). The low rainfall (annual evaporation rate of 1,390 mm) (Kjerfve et al., 1996) and high evaporation rates in this region result in high salt content in the lagoons (>5.2% total salts) (Kjerfve et al., 1996; Clementino et al., 2008).

The abundant microbial mats are small and organized in stratified (stacked) layers (**Figure 1C**). To obtain a broad representation of the microbial taxonomic composition and metabolic potential, mat samples were collected in two seasons: summer (January 2013) at Brejo do Espinho (Br. Espinho), Monte

FIGURE 1 | (A) Map of the study area. **(B)** Overview of the hypersaline lagoons. **(C)** An excised fraction of the microbial mat ecosystem from the hypersaline lagoon system of Araruama. A stratified structure is observed.

Alto (M. Alto), Mossoró, Queira, Sal Cisne (S. Cisne), and Silva; and winter (June 2013) at BR, Br. Espinho, L. Vermelha, M. Alto, Mossoró, S. Cisne, and Silva (**Figure 1A**). The microbial mats were taken at different stages of maturity or stratification. Samples were collected with a small shovel and sterile metal spatulas, which were sterilized with ethanol and flame between samples. Approximately 75 g samples were collected, transferred to polypropylene tubes in the field and stored in liquid nitrogen. Samples comprised a mixture of the different layers.

DNA Extraction and Sequencing

The samples were separately ground in liquid nitrogen using ceramic mortars and pestles that were washed with SDS detergent, soaked in 10% bleach for 30 min, and autoclaved between samples. Approximately 200 mg of each sample were used for DNA extraction with the DNeasy PowerSoil Kit (Qiagen, Germantown, MD, United States). DNA integrity was evaluated by 1% agarose gel electrophoresis (GelRed^{TM}, Biotium, Inc., Hayward, CA, United States), and DNA purity was assessed with a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, United States). The DNA was quantified with a Qubit® 3.0 Fluorometer (Life Technologies-Invitrogen, Carlsbad, CA, United States). Metagenomic libraries were prepared with the Nextera XT DNA Sample Preparation Kit (Illumina, San Diego, CA, United States). Library size distribution was evaluated with a 2100 Bioanalyzer (Agilent, Santa Clara, CA, United States), and library quantification was carried out with a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, United States) and KAPA Library Quantification Kit (Kapa Biosystems, Wilmington, MA, United States). Paired-end sequencing (2 × 300 bp) was performed on a MiSeq System (Illumina).

Bioinformatics and Statistical Analysis

The fastq files generated by Illumina sequencing were qualitatively evaluated with FASTQC v.0.11.2 (Andrews, 2010). The sequences were preprocessed with PRINSEQ v0.20.4 (Schmieder and Edwards, 2011) to remove low-quality DNA sequences (Phred score < 20), duplicates, and short sequences (<35 bp). The resulting sequences were assembled using MIRA

software (Chevreux et al., 1999) with default parameters. The assembled sequences (contigs) were then annotated *via* Metagenome Rapid Annotation using the Subsystem Technology (MG-RAST) (Meyer et al., 2008) with the following cut-off parameters: *e*-value $\leq 1e^{-5}$, 60% minimum sequence identity, and alignment length $\geq$ 15 bp. Taxonomic annotation was performed using the GenBank database (Benson et al., 2008), the largest (Strasser, 2008; Porter and Hajibabaei, 2018) and reliable (Leray et al., 2019) repository of genetic data for biodiversity; whereas the functional annotation was performed using the SEED Subsystems database (Overbeek et al., 2005), an accurate collection of functionally related protein families.

We compared the HLSA microbial mat metagenomic datasets from the present study ($n = 13$) with microbial mat metagenomic datasets from the following sources: Diamond Fork, Utah, United States (hot spring, $n = 2$) (Gomez-Alvarez et al., 2012), Yellowstone National Park, United States (Mushroom Springs, $n = 4$; Octopus Springs, $n = 2$) (Bhaya et al., 2007; Bolhuis et al., 2014), Guerrero Negro, Mexico ($n = 10$) (Kunin et al., 2008; Harris et al., 2013), Shark Bay, Australia ($n = 6$) (Ruvindy et al., 2016; Wong et al., 2018), Lake Meyghan, Iran ($n = 3$) (Naghoni et al., 2017), Clinton Creek, Canada ($n = 2$) (Unpublished, McCormick, M.)[1], Schiermonnikoog, Netherlands (tidal and intermediate zone mats, $n = 2$) (Bolhuis and Stal, 2011), Abrolhos Bank, Brazil ($n = 19$) (Walter et al., 2016), Neutral Zone, Norway ($n = 1$) (Stokke et al., 2015), Cuatro Cienegas, Mexico (lithifying and non-lithifying microbialites, $n = 2$) (Breitbart et al., 2009; Peimbert et al., 2012), and Highbourne Cay, The Bahamas (stromatolites, $n = 1$) (Khodadad and Foster, 2012). All metagenomes were annotated using the same pipelines and settings, and they are publicly available on the MG-RAST website under the ID provided in **Supplementary Table 1**.

Statistical analyses were performed with R version 3.0.3 (R Core Team, 2011) with the vegan package (Oksanen et al., 2012). One-Way Analysis of Similarities (ANOSIM) were used to test differences between sampling locations at genera, phyla, and SEED Level 1 levels using Bray–Curtis distances and 999 permutations. Non-metric multidimensional scaling (nMDS) analyses were used to display the sampling locations based on Bray–Curtis dissimilarity matrices. The hierarchical clusters were built using Euclidian distances and Ward's clustering method. The relative abundance of microbial taxa and the nMDS results were plotted with the ggplot2 (Wickham, 2009) and reshape (Wickham, 2007) packages.

Metagenome-Assembled Genomes

Cross-assembly of reads from all metagenomes was performed by metaSPAdes v.3.6.2 (Nurk et al., 2017), using the default parameters. Protein sequences were predicted from assembled scaffolds with Prodigal (Hyatt et al., 2010). The predicted protein sequences were searched against the NCBI nr database for functional and taxonomic annotation with DIAMOND (Buchfink et al., 2015) setting an *e*-value cut-off of 10^{-5}. The assembled contigs were binned together using the *super-specific* configuration of MetaBAT (Kang et al., 2015) to obtain partial or complete microbial genomes. Genome quality was assessed by CheckM (Parks et al., 2015). The cross-assembly of reads between metagenomes approach is a central feature in most automated binning algorithms and often implemented in different studies (Sharon et al., 2013; Parks et al., 2017; Stewart et al., 2018). The cross-assembly of reads among the HLSA metagenomes aimed to increase the chances of full-length recovery of genomes from the metagenomes as long as the taxonomic profiles of the individual metagenomes seem to be similar. These data have been deposited in GenBank, https://www.ncbi.nlm.nih.gov, under the BioProject accession number PRJNA675017: BioSample SAMN16710161 and SAMN16710317.

Phylogenomic Analysis of the Reconstructed MAGs

Average amino acid identity (AAI), average nucleotide identity (ANI), and genome-to-genome distance (GGD) were used for genomic taxonomy (species cutoff of 95% AAI/ANI and 70% GGD) (Konstantinidis and Tiedje, 2005). Phylogenomic trees were generated for the two metagenome-assembled genomes (MAGs). Clustal Omega (Sievers and Higgins, 2014) was used to align the 43 phylogenetic markers used by CheckM (Parks et al., 2015) and that were identified in the bins 6 and 9, and in a set of bacterial genomes publicly available in the RefSeq database (O'Leary et al., 2016). These alignments were concatenated and used as input for phylogenomic reconstruction with FastTree 2.0 using default parameters (Price et al., 2010). One thousand bootstrap replications were calculated to evaluate the relative support of the branches.

RESULTS

Overview of the Metagenomic Sequencing Dataset

We sequenced a total of 13 microbial mat samples (corresponding to 12.69 million reads) from eight different HLSA locations in summer ($n = 6$) and winter ($n = 7$) (**Table 1**). After quality control, the number of metagenome sequences pairs per sample ranged from 153,231 to 1,856,780, and the total number of contigs ranged from 36,860 to 1,140,943 (**Table 1**).

Taxonomic Composition of HLSA Microbial Mats

The HLSA microbial mats sustain a taxonomically diverse assemblage of microorganisms (**Figures 2A,B**). A total of 32 phyla and 806 different genera were detected belonging to the prokaryotic fraction in the metagenomes of the HLSA microbial mats. The sequences were predominantly bacterial (95.9% on average) with a relatively minor proportion of Archaea (2.2% on average) (**Supplementary Figure 1**). The most abundant phylum was Proteobacteria (30.9–53.6%), followed by Cyanobacteria (9.7–33.0%), and Bacteroidetes (8.8–22.6%) (**Figure 2A**).

[1]McCormick, M. *Shotgun Metagenome of Clinton Creek Biofilm, Canada*. Clinton, NY: Hamilton College. Available online at: https://www.mg-rast.org/linkin.cgi?project=mgp15973

TABLE 1 | Summary statistics of quality filtering and metagenomic assembly for the hypersaline lagoon system of Araruama.

Metagenome	Number of sequences (pairs)	Number of sequences after quality control (pairs)	Total number of contigs	Largest contig size	Median contigs size	Number of bacterial contigs	Number of archaeal contigs	Number of eukaryotic contigs	Number of viral contigs
Br. Espinho | s	653,686	578,239	192,530	7,782	141	56,964	1,727	1,137	49
Br. Espinho | w	1,176,295	945,015	377,975	2,548	144	122,781	9,068	1,723	94
M. Alto | su	1,396,511	1,204,019	636,979	7,666	154	236,053	11,643	3,307	108
M. Alto | w	1,231,528	1,110,004	380,659	4,922	167	164,706	3,775	1,980	119
Mossoró | s	317,239	300,523	167,792	1,467	120	53,368	980	539	9
Mossoró | w	892,599	797,471	418,036	11,457	187	197,178	5,203	2,301	184
S. Cisne | s	1,961,966	1,856,780	1,140,943	30,227	153	650,386	4,846	7,577	172
S. Cisne | w	155,276	153,231	36,860	914	100	9,205	148	130	5
Silva | s	678,359	644,104	289,430	6,914	148	128,834	1,883	1,482	56
Silva | w	1,054,328	957,287	399,736	13,619	153	134,732	3,801	1,553	83
Queira | s	1,404,748	1,284,214	486,043	34,253	155	260,988	2,879	3,168	127
BR | w	1,281,029	1,231,445	649,467	37,879	162	261,791	7,599	3,005	136
L. Vermelha | w	487,728	424,905	223,730	11,865	126	77,480	1,480	680	28

s, summer; w, winter.

Among Proteobacteria, the most abundant groups included the orders Desulfobacterales (1.3–4.6%), Desulfovibrionales (1.5–4.1%), and Desulfuromonadales (1.0–2.8%) and the genera *Rhodobacter* (0.4–1.8%), *Rhodopseudomonas* (0.3–0.8%), and *Nitrosococcus* (0.4–1.0%) (**Figure 2B**), likely because of the importance of their metabolic roles. Among Cyanobacteria, the difference in the profile abundance was attributed to an increase in reads associated with the orders Chroococcales (4.2–10.4%) and Oscillatoriales (2.1–14.6%). The cyanobacterium genus *Coleofasciculus* (formerly *Microcoleus*) was the most abundant genus in most metagenomes (0.6–11.6%), and the species *Coleofasciculus chthonoplastes* alone represented 23.6% of the total abundance of Cyanobacteria (ranging from 6.4 to 39.9%; $n = 95{,}995$). Reads related to *Cyanothece* sp. PCC 7425 (2.0% in Br. Espinho winter to 5.8% in L. Vermelha winter) were detected in all HLSA metagenomes. Most of the recovered archaeal sequences were assigned to the phylum Euryarchaeota (ranging from 0.7% in S. Cisne summer to 6.4% in Br. Espinho winter), with high relative abundances of the genera Halobacterium (0.1% in S. Cisne summer to 4.1% in Br. Espinho winter) and Methanomicrobia (0.3% in S. Cisne summer to 1.1% in BR winter).

Metabolic Potential of HLSA Microbial Mats

The metagenomic sequences were classified into 28 SEED subsystems (**Supplementary Figure 2**), a wide range of metabolic pathways which allows microbes to detect changes in the environment conditions to survive. Metabolisms related to Carbohydrates, Protein, and Amino Acids and Derivatives subsystems accounted for 35% of all identified sequences. Cyanobacteria were found to be a key component of this system as the main group responsible for Photosynthesis (50.9–82.4%), and the order Chroococcales alone was the main contributor of genes related to Nitrogen Fixation (6.1–27.3%) and Ammonia Assimilation (5.5–36.4%) metabolisms. Proteobacteria was the main contributor of genes related to Respiration (45.3–64.4%) and Fermentation (32.4–50.0%) subsystems. Genes related to Sulfur metabolism (0.5–0.8%) were attributed mainly to Proteobacteria, whereas genes related to Methanogenesis metabolism were attributed mainly to the bacterial phyla Actinobacteria (0.0–61.54%) and Proteobacteria (7.69–100%), and also to the archaeal orders Methanosarcinales (0.0–30.0%) and Methanopyrales (0.0–18.18%).

When examining the taxonomic profile of the HLSA metagenomes, we see that five out of the six most abundant phyla (Proteobacteria, Cyanobacteria, Bacteroidetes, Firmicutes, and Chloroflexi) (**Figure 2A**) contain members that are capable of oxygenic and anoxygenic reaction center-based phototrophy. Metabolic versatility of specific taxa present in the HLSA metagenomes is discussed below. Furthermore, genes related to Osmotic Stress were detected in all HLSA microbial mat metagenomes, following: L-ectoine synthesis (0.01–0.05%), L-proline transport, glycine betaine (0.1–0.2%), hyperosmotic potassium uptake (0.02–0.1%), glutathione-regulated potassium-efflux system (0.05–0.2%), and voltage-gated potassium efflux systems (0.01–0.1%).

Taxonomic and Metabolic Profiles Across Microbial Mats Metagenomic Samples

We compared the HLSA microbial mat metagenomes ($n = 13$) with metagenomes from 11 other microbial communities ($n = 55$). Notwithstanding the similar nature of the structures used for comparison, general bacterial composition differed by sample origin (ANOSIM: $R = 0.706$, $P = 0.001$; **Figure 3**). Cyanobacteria diversity and abundance accounted for much of the variation between samples. The HLSA metagenomes clustered together, without distinction between seasons or samples (**Figures 3**, **4**). The HLSA metagenomes were more closely related to the hypersaline microbial mat metagenomes from Mexico (Guerrero Negro, in Baja

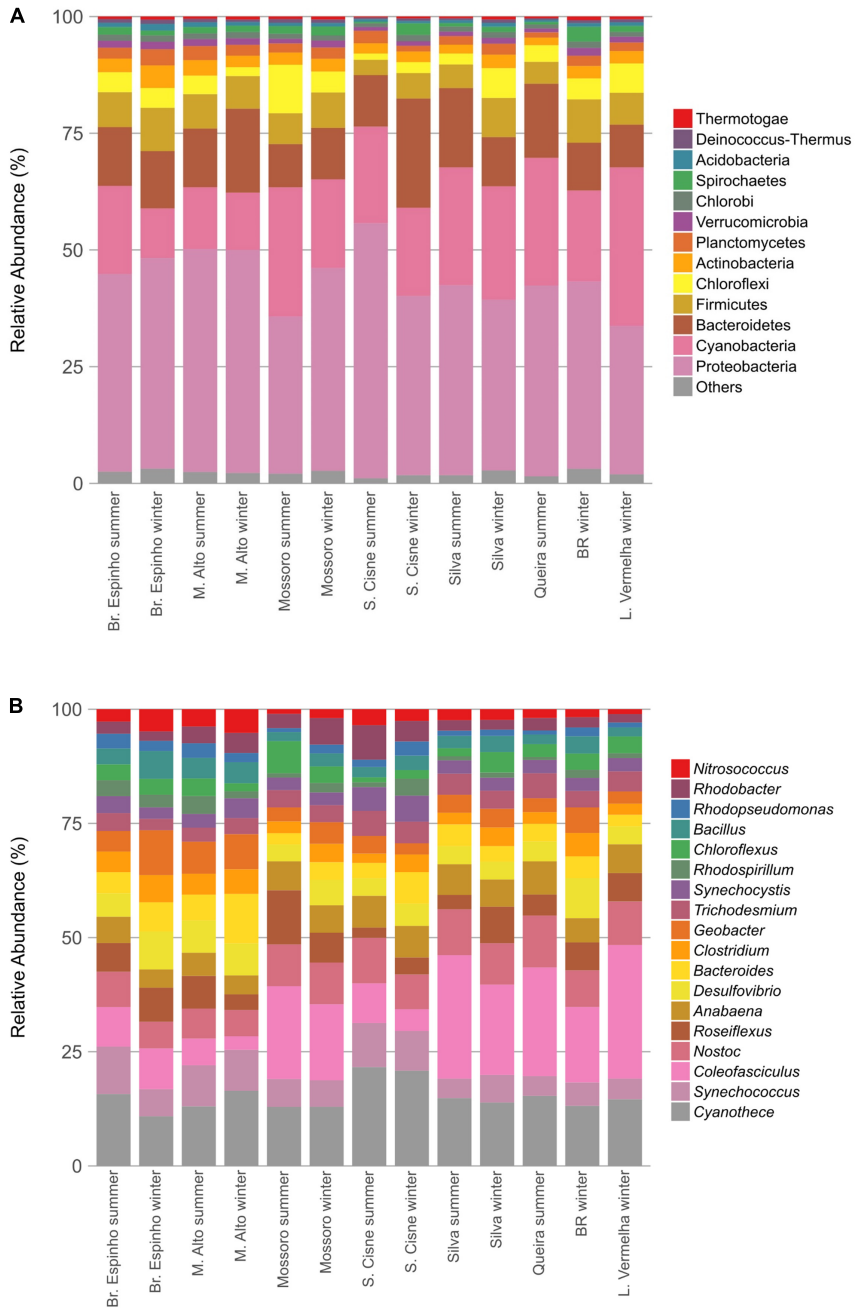

FIGURE 2 | Taxonomic composition of the microbial mats from the hypersaline lagoon system of Araruama. Bar plots depict the relative abundances of bacterial phyla **(A)** and genera **(B)** detected in the metagenomes normalized to 100%. Only the most abundant taxa are shown.

California Sur, and Cuatro Cienegas green mat) (**Figure 4** and **Supplementary Figure 3**).

Comparative Genomics and Functional Complexity of Recovered MAGs

To provide insights into the genomic context of microorganisms interacting within the HLSA microbial mats, we recovered genomes from the metagenomes and explored their taxonomic and functional diversity. Bacterial genomes with completeness >87% and presenting genome sizes of approximately 4 Mbp were obtained from the HLSA metagenomic dataset (**Supplementary Table 2**). Because of the relatively low sequencing depths obtained for the HLSA metagenomes, only the most abundant sequences were binned into individual genomes. Here, we highlighted the annotated taxonomic and functional genes of two reconstructed genomes. Following the standards suggested by Bowers et al. (2017), Bin6 is referred as a high-quality

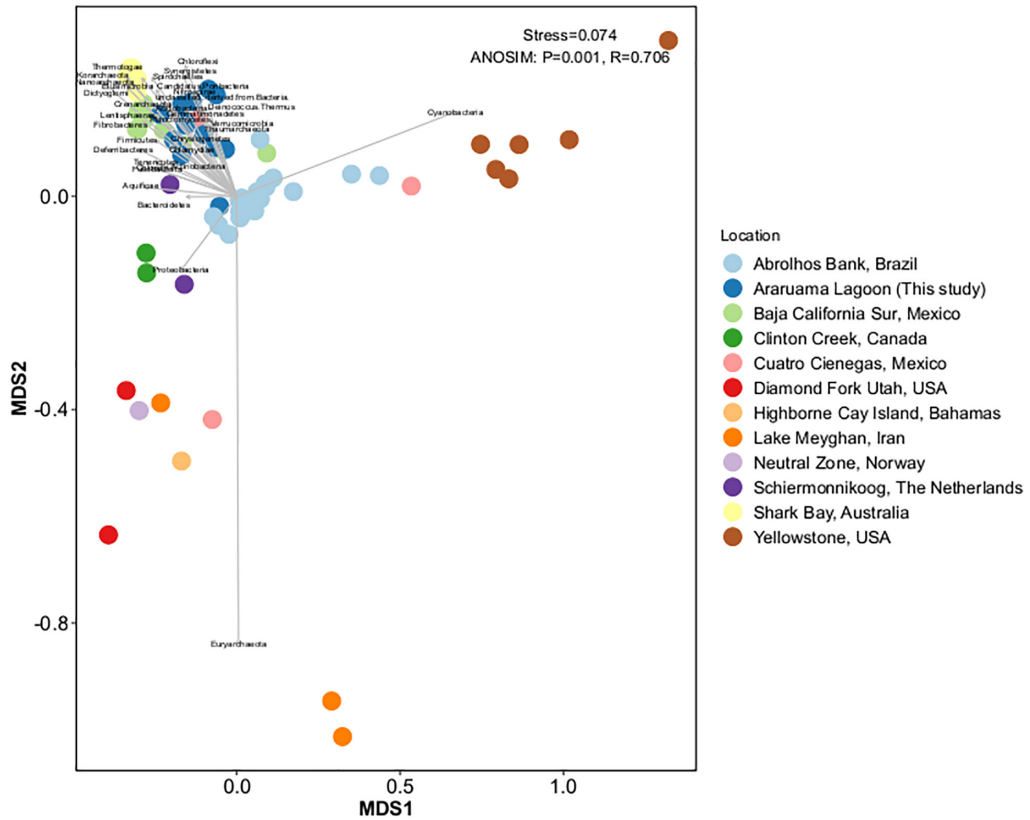

FIGURE 3 | Non-metric multidimensional scaling (nMDS) plot representing the Bray–Curtis similarity of general microbial community structure composition of 68 metagenomes of microbial mats from different habitats. Each dot represents a sample, color represents the sampling location, and the arrows represent the nMDS phyla scores.

draft (>90% complete, <5% contamination), while Bin9 is a medium-quality draft (>50% complete, <10% contamination). A comparison of both reconstructed genomes with their most closely related reference genomes showed that AAI, ANI, and GGD values were much lower than the species cutoff, indicating the novelty of these microorganisms (**Table 2**). Bin6 represents a new species of *Thiohalocapsa* that is closely related to *Thiohalocapsa* sp. ML1 (70.2% AAI), a Gammaproteobacteria belonging to the order Chromatiales (**Table 2** and **Supplementary Figure 4**). Bin9 represents a new genus, and the closest reference genome belongs to a member of Bacteroidetes, *Phaeodactylibacter xiamenensis* (47.6% AAI) (**Table 2** and **Supplementary Figure 5**). To further identify these two reconstructed genomes, phylogenomic analysis were performed. Whereas Bin6 was placed closely with *Thiohalocapsa* sp. ML1 (**Supplementary Figure 4**), the phylogenomic placement of Bin9 shown a relatively distant evolutionary relationship with *P. xiamenensis* (**Supplementary Figure 5**). The novel species were named *Ca. Thiohalocapsa araruaensis* HLSAbin6 sp. nov. (Bin6), and *Ca. Araruabacter turfae* HLSAbin9 gen. nov. sp. nov. (Bin9).

The closest relatives of these two recovered genomes are salt tolerant; therefore, we tested for the presence of genes related to osmoregulation. We identified genes encoding glycine betaine/proline transport systems (ABC transport systems, e.g., *pro*V, *pro*W, and *pro*X), high-affinity choline uptake protein (*bet*T), carnitine/choline transporter (*opu*CB), betaine uptake/biosynthesis systems, genes involved in glucan synthesis, an aquaporin Z, and another outer membrane protein (*omp*A) (**Table 3**).

Key genes related to Carbon, Nitrogen, and Sulfur biogeochemical cycling were compiled and allowed delineation of the functional role of the taxa associated to the bins (**Table 4**). For instance, both bins encode complete and partial sulfate-reduction pathways (**Table 4**), potentially indicating the importance of Sulfur cycling in the HLSA microbial mats. Partial recovery of a determined pathway might be a result of lack of coverage in both not complete MAGs. Annotations for major metabolisms such as Sulfur (*dsr*, *apr* genes, and the *sox*ABHWYZ complex), Nitrogen (*nif* and *nar* genes), and bacteriochlorophyll-based Photoautotrophy (e.g., *bch*, *psb*, *puf*, *rbc*, *ccm*, *apcc*, *coo*) are found in Bin6, which is associated with purple sulfur bacteria.

DISCUSSION

This study aimed at gaining insights into the diversity of microorganisms in the HLSA microbial mats, expanding

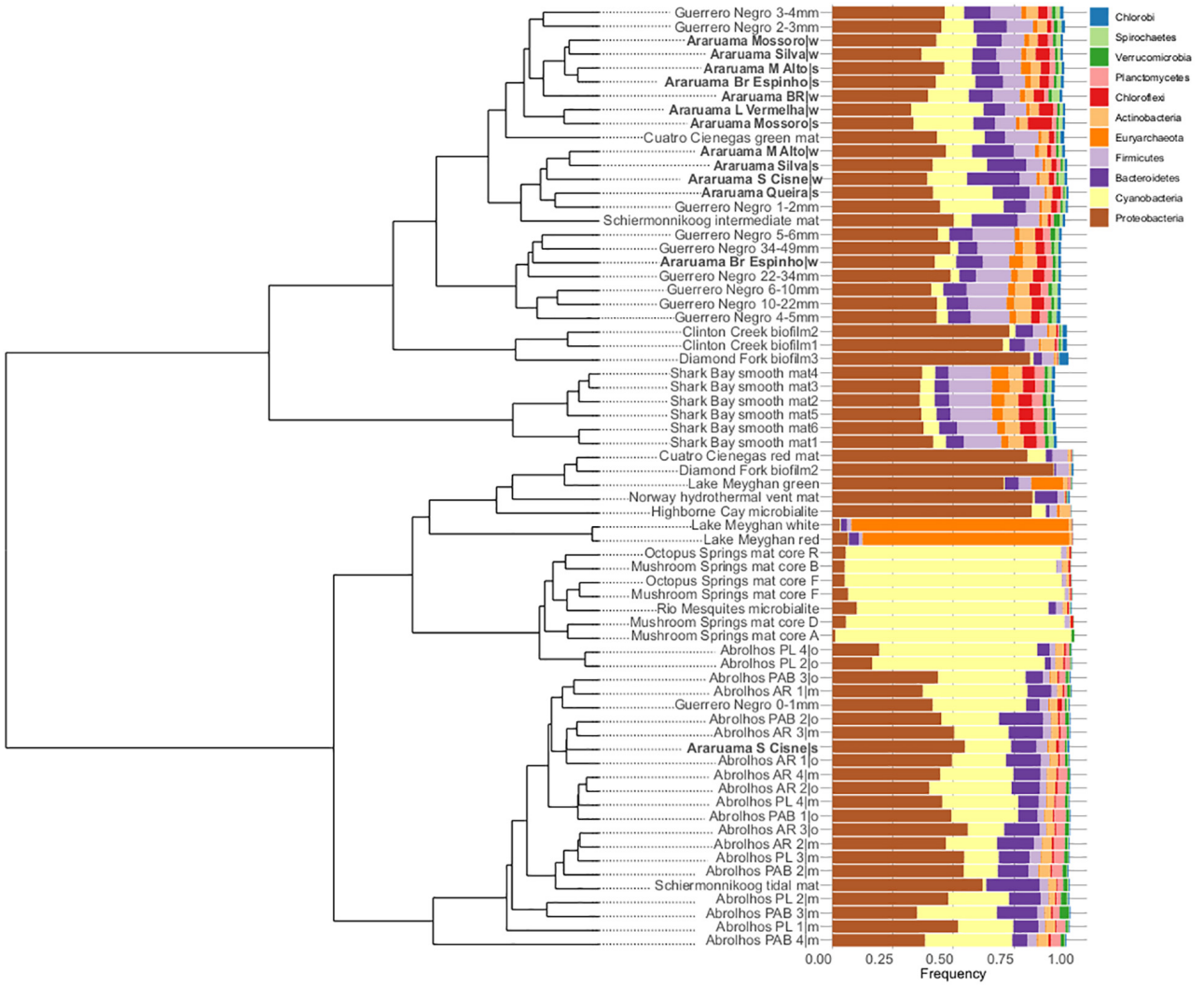

FIGURE 4 | Hierarchical clustering and bar plots of relative abundances of the major microbial phyla found within 68 metagenomes of microbial mats from different habitats. Clustering was based on Euclidian distances and Ward's clustering method.

the knowledge generated by previous studies that employed operational taxonomic unit-based approach to focus on the cyanobacterial populations of these microbial mats (Ramos et al., 2017), and the prokaryotic diversity of the HLSA water column (Clementino et al., 2008). The use of shotgun metagenomics allowed us to delineate a broader characterization of the microbial mats coping with extreme environmental conditions in the coastal Araruama lagoon system. This approach circumvents the use of culture-dependent methodology as part of the polyphasic strategy employed previously (Clementino et al., 2008; Ramos et al., 2017). Although bacterial isolation technique has yielded valuable biodiversity information in the past, currently it provides little information which limits substantial characterization. For the first time in the HLSA ecosystem, shotgun metagenomics was generated, and near-complete genome bins were retrieved from the metagenomic data.

On the southeastern Brazilian continental margin, structures that dominate the HLSA have been extensively studied with focus on the mineralogical and biogeochemical features (Vasconcelos and McKenzie, 1997; van Lith et al., 2002; Delfino et al., 2012; Bahniuk et al., 2015), giving insights into stromatolite genesis. These laminated structures produced by the successive deposition of layers of microbial mat are found in proximity with their modern counterparts. Comprehensive fossil record found worldwide indicates that ancient microbial mat structures are the oldest biological communities known, dating back to 3.7 billion years for structures found in Greenland (Nutman et al., 2016) and 3.5 billion years for Australian stromatolites (Walter et al., 1980; Allwood et al., 2006).

Evaporation, flooding, and salinity fluctuations processes contribute to the dynamic of the naturally occurring shallow HLSA ecosystem. Modern microbial mats descended from stromatolites and are likely to harbor microorganisms adapted

TABLE 2 | Genetic relatedness between the metagenome-assembled genomes and the most closely related reference genomes based on average amino acid identity (AAI), average nucleotide identity (ANI), and genome-to-genome distance (GGD).

	AAI (%)	ANI (%)	GGD (%)
	Bin6		
Thiohalocapsa sp. ML1	70.2	81.0	23.5
Uncultured *Thiohalocapsa* sp. PB-PSB1	55.5	75.6	20.9
Allochromatium vinosum DSM 180^T	51.3	76.6	19.1
Allochromatium warmingii DSM 173^T	47.3	73.8	17.6
Thioflavicoccus mobilis 8321	50.9	76.5	19.9
	Bin9		
Phaeodactylibacter xiamenensis KD52^T	47.6	74.0	19.1
Lewinella nigricans DSM 23189^T	44.1	73.1	18.4
Lewinella agarilytica	39.2	71.3	12.5
Lewinellaceae bacterium SD302^T	39.4	71.8	17.4
Lewinella persica DSM 23188^T	39.0	71.2	16.4

to such stressful conditions. The new sequences generated in the present study substantially increase the representation of all phyla described previously for the HLSA ecosystem (e.g., Proteobacteria and Cyanobacteria) (Clementino et al., 2008; Ramos et al., 2017). The HLSA microbial mats sustain taxonomically diverse assemblage of microorganisms, which exhibit high metabolic diversity.

In contrast to microbial mats in other extreme environments such as hot springs (e.g., Mushroom Springs and Octopus Spring, Yellowstone samples), which are dominated by Cyanobacteria (Bhaya et al., 2007; Bolhuis et al., 2014), the HLSA microbial mats are dominated by Proteobacteria. The prevalence and relative abundances of the three prevailing phyla in the HLSA microbial mats metagenomes were similar to other hypersaline microbial mats from Mexico, such as Guerrero Negro (Kunin et al., 2008; Harris et al., 2013), and Cuatro Cienegas green mat (Breitbart et al., 2009; Peimbert et al., 2012). The dominant proteobacterial groups in the HLSA microbial mats are similar to those in the Mexican microbial mats. Besides, the taxonomic similarity between both locations has been observed previously for the cyanobacterial community (Ramos et al., 2017). Taken together, a taxonomic signature for hypersaline environments may exists. Cyanobacteria were found to be a key component of this system as the main group responsible for photosynthesis and nitrogen fixation. Moreover, the relatively high abundance of the cyanobacterial orders Chroococcales and Oscillatoriales is in agreement with a previous study combining morphology and molecular-based tools to characterize the diversity of Cyanobacteria in the HLSA (Ramos et al., 2017). The abundance of the cyanobacterial genus *Coleofasciculus* in the HLSA microbial mats may be explained by its tolerance to high saline levels and its metabolic flexibility (i.e., ability to perform both photosynthesis and anoxic fermentation) (Burow et al., 2013). OTUs related to this halophilic Cyanobacteria was reported previously in the HLSA ecosystem (Clementino et al., 2008; Ramos et al., 2017), whereas microbial mats dominated by *Coleofasciculus* are found in hypersaline ponds of Guerrero Negro, Mexico (Garcia-Pichel et al., 1996; Marais, 2010; Harris et al., 2013). Acting as the primary producer in the mat, this microorganism maintains high numbers of metabolically active heterotrophs which hold catabolic and transport capabilities, for instance.

When examining the taxonomic profile of the HLSA metagenomes, we see that five out of the six most abundant phyla contain members that are capable of oxygenic and anoxygenic reaction center-based phototrophy. High taxa heterogeneity and metabolic versatility occurs in the HLSA mats, particularly considering the diverse taxa of anoxygenic phototrophic bacteria and oxygenic cyanobacterial communities driving the energetic flow. The utilization of different electron donors is well represented by the photoheterotrophic purple non-sulfur *Rhodobacter* spp., capable of anoxygenic photosynthesis, as well as aerobic and anaerobic respiration (Pérez et al., 2017). Another abundant genus, *Rhodopseudomonas*, is capable to switch among photoautotrophic, photoheterotrophic, chemoautotrophic, and chemoheterotrophic metabolisms (Larimer et al., 2004). *Nitrosococcus*, the most abundant genus of Chromatiales found in the HLSA, is a widespread chemolithoautotrophic ammonia-oxidizing bacterium that possesses monovalent cation transporters that confer salt tolerance (Klotz et al., 2006). Notably, Deltaproteobacteria make up to 37.5% of the Proteobacteria population and include sulfate-reducing bacteria such as Desulfobacterales, Desulfuromonadales, and Desulfovibrionales that obtain energy reducing sulfates to sulfides (Wasmund et al., 2017). Also, very abundant (up to 40.5%) and more diverse is Gammaproteobacteria, which contain anoxygenic phototrophic sulfide-oxidizing members that provide the heterotrophic sulfate reducers with some Organic Carbon, hence closing the Sulfur cycle within the HLSA mats. In correspondence to that, several metabolically versatile microorganisms were identified in the HLSA mats, including a high-quality reconstructed genome related to *Thiohalocapsa* sp. (Bin6), a purple bacterium, which indicates its involvement in the Sulfur metabolism. Bin6 contains annotations for both Sulfur (*dsr*, *apr* genes, and the *sox*ABHWYZ complex) and Nitrogen (*nif* and *nar* genes) metabolisms. These, together with the annotations for bacteriochlorophyll-based Photoautotrophy (e.g., *bch*, *psb*, *puf*, *rbc*, *ccm*, *apcc*, *coo*) suggest a dynamic role of Bin6 in the HLSA microbial mats. This purple sulfur bacteria contain a *puf* operon encoding a type-2 photochemical reaction center (subunits PufL, PufM, and PufH) for aerobic anoxygenic metabolism. Another indication of its anoxygenic metabolism is the presence of BchF, which is exclusively found in those groups of bacteria that can synthetize bacteriochlorophyll *a* (Bryant et al., 2012). Observations of active Sulfur and Nitrogen metabolisms in other purple sulfur bacteria have been shown elsewhere (Bebout et al., 1993; Yurkov et al., 1994).

Another genome recovered from the metagenomes, Bin9, is related to Bacteroidetes. Members of this phylum act as specialists for the degradation of high molecular weight organic matter and complex polysaccharides (Fernandez-Gomez et al., 2013). They have been detected in high abundance and diversity in several hypersaline microbial mats (e.g., Guerrero Negro, Shark

TABLE 3 | Osmoprotectant and osmoregulation profile in the two reconstructed genomes.

Annotation	Gene	Bin6	Bin9
Trehalose synthase (EC 5.4.99.16)	*treS*		
Trehalose-6-phosphate phosphatase (EC 3.1.3.12)	*ostB*		
Trehalose phosphorylase (EC 2.4.1.64)	*treP*		
Malto-oligosyltrehalose trehalohydrolase (EC 3.2.1.141)	*mth*		
Alpha-trehalose-phosphate synthase (EC 2.4.1.15)	*otsA*		
Alpha-amylase (EC 3.2.1.1)	*amyA*		
1,4-alpha-glucan (EC 2.4.1.18)	*glg*		
Glycogen debranching enzyme (EC 3.2.1.-)	*treX*		
Glucoamylase (EC 3.2.1.3)	*ssg*		
Beta-phosphoglucomutase (EC 5.4.2.6)	*pgm*		
YggS, proline synthase	*yggS*		
Gamma-glutamyl phosphate reductase (EC 1.2.1.41)	*gpr*		
Pyrroline-5-carboxylate reductase (EC 1.5.1.2)	*proC*		
Glutamate 5-kinase (EC 2.7.2.11)	*proB*		
RNA-binding C-terminal domain PUA	*pua*		
NADP-specific glutamate dehydrogenase (EC 1.4.1.4)	*gdhA*		
Glycine betaine/L-proline ABC transporter, ATP-binding protein ProV (TC 3.A.1.12.1)	*proV*		
Glycine betaine/L-proline ABC transporter, periplasmic binding protein ProW (TC 3.A.1.12.1)	*proW*		
Glycine betaine/L-proline ABC transporter, protein ProX (TC 3.A.1.12.1)	*proX*		
High-affinity choline uptake protein BetT	*betT*		
Choline-sulfatase (EC 3.1.6.6)	*betC*		
Choline dehydrogenase (EC 1.1.99.1)	*betA*		
Sarcosine *N*-methyltransferase	*bsmA*		
Glycine *N*-methyltransferase (EC 2.1.1.20)	*bsm*		
Dimethylglycine *N*-methyltransferase	*bsmB*		
Aquaporin Z	*aqpZ*		
Outer membrane protein A precursor	*ompA*		
Glucans biosynthesis glucosyltransferase H (EC 2.4.1.-)	*opgH*		
Proline iminopeptidase (EC 3.4.11.5)	*pipX*		
Proline-rich protein/signal peptide	*prb*		
Transporter linked to choline/ethanolamine kinase and OMR	*pnuC*		
Potassium uptake protein TrkA	*trkA*		
Potassium uptake protein TrkH	*trk1*		
Potassium channel protein	*kch*		
Osmosensitive K+ channel histidine kinase KdpD (EC 2.7.3.-)	*kdpD*		
Potassium voltage-gated channel subfamily KQT	*kcn*		
Potassium efflux system KefA protein	*kefA*		
Glutathione-regulated potassium-efflux system protein KefB	*kefB*		
Glutathione-regulated potassium-efflux system protein KefC	*kefC*		
Glutathione-regulated potassium-efflux system ancillary protein KefG	*kefG*		
Glutathione-regulated potassium-efflux system ATP-binding protein	yhe		

Key genes related to halotolerance recovered in genomes obtained from the HLSA metagenomes.
The heatmap displays the presence or absence of key genes related to halotolerance.

Bay), and the occurrence of specialists have been hypothesized. A strain specialized on the scavenging of Cyanobacteria was found in a hypersaline microbial mat (Hania et al., 2017). Complex cyanobacterial exudates become available to the general microbial community thought those bacteria. Therefore, it is likely that Bacteroidetes play a key role in the degradation and cycling of mat compounds.

Altogether, they indicate the importance of the energy flow (e.g., Carbon and Sulfur) (Canfield and Marais, 1993; Baumgartner et al., 2006) in the HLSA microbial mats. The major role of sulfur-bacteria to calcium mineralization has been demonstrated (Visscher et al., 1998; Braissant et al., 2007; Dupraz et al., 2009; Saghai et al., 2015). Previous Oxygen and Sulfur profiles taken at L. Vermelha demonstrated oxygen

TABLE 4 | Functional genetic diversity of biogeochemical cycling (C, N, S) in the two reconstructed genomes.

Cycling	Annotation	Gene	Bin6	Bin9
Assimilatory sulfate reduction	Adenylylsulfate kinase/reductase (EC 2.7.1.25)	*apsK*		
	Adenylylsulfate reductase alpha-subunit	*aprA*		
	Adenylylsulfate reductase beta-subunit	*aprB*		
	Arylsulfatase (EC 3.1.6.1)			
	Dihydrofolate reductase (EC 1.5.1.3)	*lapr*		
Dissimilatory sulfate reduction	Dissimilatory sulfite reductase, gamma subunit	*dsr*		
	DsrE oxidoreductase	*dsrE*		
	Sulfite reduction-associated complex DsrMKJOP multiheme protein DsrJ (=HmeF)	*dsrJ*		
	Sulfite reduction-associated complex DsrMKJOP protein DsrK (=HmeD)	*dsrK*		
	Sulfite reduction-associated complex DsrMKJOP protein DsrM (=HmeC)	*dsrM*		
	Sulfite reduction-associated complex DsrMKJOP iron–sulfur protein DsrO (=HmeA)	*dsrO*		
	Sulfite reduction-associated complex DsrMKJOP protein DsrP (=HmeB)	*dsrP*		
	IscA-like protein, DsrR	*dsrR*		
	DsrS	*dsrS*		
	Sulfate adenylyltransferase, dissimilatory-type (EC 2.7.7.4)	*sat*		
	Sulfite oxidase SoxA protein	*soxA*		
	Sulfite oxidase SoxB protein	*soxB*		
	Sulfite oxidase SoxH protein	*soxH*		
	Sulfite oxidase SoxW protein	*soxW*		
	Sulfite oxidase SoxY protein	*soxY*		
	Sulfite oxidase SoxZ protein	*soxZ*		
Nitrogen	Nitrogenase protein NifA	*nifA*		
	Nitrogenase protein NifB	*nifB*		
	Nitrogenase protein NifE	*nifE*		
	Nitrogenase protein NifN	*nifN*		
	Nitrogenase protein NifO	*nifO*		
	Nitrogenase protein NifQ	*nifQ*		
	Nitrogenase protein NifX	*nifX*		
	Nitrogenase cofactor carrier protein NafY	*nafY*		
Anoxygenic photosynthesis	Light-harvesting LHII, beta subunit	*pufB*		
	Light-harvesting LHI, alpha subunit	*pufA*		
	Photosynthetic reaction center H subunit	*pufH*		
	Photosynthetic reaction center L subunit	*pufL*		
	Photosynthetic reaction center M subunit	*pufM*		
	Photosynthetic reaction center cytochrome *c* subunit	*pufC*		
	Photosystem II proteins	*psb*		
	Putative photosynthetic complex assembly protein			
	2-vinyl bacteriochlorophyllide hydratase	*bchF*		
	Bacteriochlorophyll *c* synthase	*bchC*		
Heterotrophy (fermentation)	Cytochrome *c* oxidase subunit CcoG	*ccoG*		
	Cytochrome *c* oxidase subunit CcoN	*ccoN*		
	Cytochrome *c* oxidase subunit CcoO	*ccoO*		
	Cytochrome *c* oxidase subunit CcoP	*ccoP*		
	2-oxoglutarate oxidoreductase	*kor*		
	2-oxoglutarate dehydrogenase complex (EC 2.3.1.61)	*odh*		
	6-phosphogluconate dehydrogenase, decarboxylating (EC 1.1.1.44)	*pgd*		
	Phosphoglucomutase (EC 5.4.2.2)	*pgm*		
	Phosphoglucosamine mutase (EC 5.4.2.10)	*glmM*		
	Enolase	*ens*		
	Pyruvate kinase	*pyk*		
Carbon fixation	Ribulose bisphosphate carboxylase small chain (EC 4.1.1.39)	*rbc*		
	Ribulose bisphosphate carboxylase large chain (EC 4.1.1.39)	*rbcA*		
	Carbonic anhydrase (EC 4.2.1.1)	*cah*		
	Carboxysome	*ccm*		
	Acetyl-CoA carboxylase	*apcc*		
	Propionyl-CoA carboxylase			
	Carbon monoxide dehydrogenase	*coo*		
	Putative sodium-dependent bicarbonate transporter			

Heatmap displays the presence or absence of a selected subset of genes previously associated with pathways involved in nutrient utilization and energy metabolism.

TABLE 5 | Key genes related to microbial-induced calcium carbonate precipitation.

	BR | w	Br. Espinho | s	Br. Espinho | w	S. Cisne | s	S. Cisne | w	L. Vermelha | w	M. Alto | s	M. Alto | w	Mossoro | s	Mossoro | w	Queira | s	Silva | s	Silva | w	Bin6	Bin9
Carboxysome protein CcmL															
Carboxysome protein CcmM															
Carboxysome protein CcmN															
Carboxysome shell protein CsoS1															
Carboxysome shell protein CsoS2															
Carboxysome shell protein CsoS3															
Putative carboxysome peptide A															
Putative carboxysome peptide B															
Carbonic anhydrase (EC 4.2.1.1)															
Carbonic anhydrase, gamma class (EC 4.2.1.1)															
Carbonic anhydrases/ acetyltransferases															

Heatmap displays the presence or absence of a selected subset of genes previously associated with the mineralization of calcium carbonate in both metagenomes and recovered genomes from the HLSA metagenomes.

peak (oxygen-producing Cyanobacteria) and decrease (oxygen consuming heterotrophs), followed by sulfide consumption (anaerobic sulfide-oxidizing purple bacteria), and sulfide increase (sulfide producing sulfate- and sulfur reducers) (Warthmann et al., 2011). Notably, high sulfate reduction rates coincided with zones of carbonate precipitation in oxygenated zones of another hypersaline microbial mats (Highborne Cay, Bahamas) (Visscher et al., 2000; Dupraz et al., 2004). Different sulfur-reducing bacteria display different tolerances for oxygen exposure and, hence, may present a broad distribution in the microbial mat. Like in the hypersaline mats from Guerrero Negro (Minz et al., 1999), different genera of sulfur-reducing bacteria most likely populate different depths within the HLSA mats, and the oxic zone near the mat surface may present the highest rates of sulfate reduction (Canfield and Marais, 1991). Key genomic repertoire related to calcium carbonate was identified in the HLSA microbial mats metagenomes (**Table 5**). Microbial mats found across the HLSA seem to present thin and discontinuous calcium carbonate deposition, and two lagoons (L. Vermelha and Br. do Espinho) are well-known for containing Ca–Mg carbonate formations alternating with non-lithified organic layers (Vasconcelos et al., 2006; Nascimento et al., 2019). In addition to the cyanobacterial contribution to the precipitation of calcium carbonate, *Cyanothece* sp. PCC 7425 and *Thermosynechococcus elongatus* BP-1 strains are known to accumulate calcium carbonate inclusions in their cytoplasm (Benzerara et al., 2014), and both were abundantly present in the HLSA metagenomes.

Interestingly, the final net production of carbonates depends on the balance of different microbial metabolisms. Metabolisms such as oxygenic and anoxygenic photosynthesis (Dupraz and Visscher, 2005; Bundeleva et al., 2012), sulfate reduction (Visscher et al., 2000; Gallagher et al., 2014), and anaerobic methane oxidation coupled to sulfate reduction (Michaelis et al., 2002) contribute to a state of carbonate saturation, in an alkaline pH, promoted by a matrix of EPS that leads to calcium ions to precipitate as calcium carbonate (Baumgartner et al., 2006; Zhu and Dittrich, 2016). On the other hand, aerobic respiration, sulfide oxidation, and fermentation (Dupraz and Visscher, 2005) tend to promote dissolution by acidification.

Microorganisms adapted to saline and hypersaline environments display different strategies to cope with high osmotic pressure. These microorganisms may use two main strategies to maintain osmotic balance: (1) accumulate (biosynthesize and/or import) organic compatible solutes (osmoprotectants) that do not interfere with enzymatic activity (e.g., L-ectoine, L-proline, sucrose, trehalose, glucosylglycerol, and glycine betaine) and (2) control ion flow across cellular membranes through regulated potassium uptake and efflux pumps (Martinac et al., 1987). Efflux pumps are not sufficient to cope with high osmolarity (Roberts, 2005), because microorganisms may only transiently accumulate potassium ions. Thus most halotolerant organisms use multiple osmolyte strategies to cope with hypersaline environments (Yaakop et al., 2016). The two recovered genomes contain salt tolerant genomic repertoire. Bin6, associated with purple sulfur bacteria, has genes that encode aquaporin Z water channels that may enhance the flux of water across the cellular membrane in response to abrupt changes in osmotic pressure (Calamita, 2000). This species may also achieve osmotolerance by importing proline, glycine, and betaine through the proU operon (*pro*V, *pro*W, and *pro*X). Whereas Bin9, related to the family Saprospiraceae, also possesses osmoregulation genes encoding proteins involved in the biosynthesis of trehalose and proline and in the uptake and biosynthesis of choline and betaine (Chen et al., 2014). In addition, this species has a gene that encodes the OmpA outer membrane protein, which has multiple functions, including osmoprotection (Hong et al., 2006). Osmoprotectant compounds can be used as Carbon and Nitrogen sources and for energy storage (Welsh, 2000), which may help microorganisms, including the novel candidate species identified in this study, to survive under stressful conditions, such as a sudden temperature increase, desiccation, and UV radiation. Indeed, the new candidate species exemplify different strategies for halotolerance as mentioned before.

Although detected in all HLSA metagenomes, the reconstruction of a particular bin associated with a Cyanobacteria representative (the most closely related reference genome was *C. chthonoplastes*) did not pass the binning thresholds (Bowers et al., 2017) due to high level of sequences contamination. Despite that, annotation of the cyanobacterial bin could provide some interesting information (results not shown). This bin contains a genetic repertoire for compatible solute metabolism (e.g., trehalose biosynthesis and glycine betaine uptake and biosynthesis). Interestingly, most mat-forming filamentous Cyanobacteria accumulate trehalose, and the combination of EPS with trehalose protects against desiccation (Potts, 1994). Another compound that may be used by *Coleofasciculus* as an osmoprotectant is carnitine, which can protect against fluctuations in salinity, water content, and temperature (Meadows and Wargo, 2015). Also, we identified sequences related to the permease proteins involved in carnitine transport. Although many bacteria can generate carnitine from direct precursors, these metabolic pathways are not completely understood. *Coleofasciculus* sp. may also use Na^+/H^+ antiporters for ion exclusion (e.g., Na^+) under hypersaline conditions, as described for other Cyanobacteria (Waditee et al., 2002). Na^+ is the main inorganic cation in saline environments and thus, active sodium ion export mechanisms exist in these cells. Clusters of genes encoding the Mrp operon system were also found in all HLSA metagenomes. The Mrp cluster is a monovalent cation/proton antiporter system also involved in Na^+ extrusion (Hagemann, 2011). MAGs allow to disentangle the drivers of functional complexity in other microbial mats (Saghai et al., 2015; Wong et al., 2020), where the genomic repertoire of such candidate microbial taxa was investigated. The genomes recovered from the HLSA metagenomes support the environmental relevance of the microorganisms represented by the assemblies described in this study.

CONCLUSION

Hypersaline lagoon system of Araruama microbial mats have evolved to encompass high taxonomic and metabolic diversity,

illustrated by the autotrophic and heterotrophic guilds found in their metagenomes. The similarity between HLSA, Cuatro Cienegas and Guerrero Negro hint to possible adaptative mechanisms to thrive in hypersaline environments. High metabolic flexibility and the production of osmoprotectant compounds appear to be important for survival in the HLSA microbial mats. Halotolerance, phototrophy, and chemosynthesis pathways by bacterial representatives in both the HLSA microbial mats metagenomes and the recovered genomes are indicative of a diverse metabolic repertoire needed to sustain life in the HLSA. A high proportion of sulfur bacteria is remarkable. Deltaproteobacteria, which includes sulfate-reducing bacteria such as Desulfobacterales, Desulfuromonadales, and Desulfovibrionales, comprise approximately 40% of the Proteobacteria population, the most abundant phylum in the HLSA microbial mat metagenomes. This result supports the relevance of sulfate-reducing bacteria in the hypersaline microbial mats of HLSA, where versatile populations in synergy with other taxa cover most of the metabolic activities within the mat, including the precipitation of calcium carbonate in these unique microbial structures.

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

SD, RC, FT, CT, MN, DB, and AC conceived the study and designed the experiments. LO and DB processed the samples and performed DNA sequencing. JW, LO, DT, and PM analyzed the data. JW, LO, and FT 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.2021.715335/full#supplementary-material

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BRIEF RESEARCH REPORT
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Past Biodiversity: Japanese Historical Monographs Document the Epibiotic Barnacles and Cold-Stunning Event of the Hawksbill Turtle *Eretmochelys imbricata*

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The historical monographs called "*Honzou Gaku*" present the first record of cold-stunning of a hawksbill turtle *Eretmochelys imbricata* (Linnaeus, 1766) in the Echigo region of Japan during the Edo period (1600–1868), and the barnacles attached to the turtle were identified as *Platylepas hexastylos* (Fabricius, 1798). Analysis of this finding adds substantial knowledge to our understanding of the life history of the hawksbill turtles along the coast of Japan. As reported in this study, literature on the historical heritage of other animals or plants can also provide information about their past biodiversity.

Keywords: turtle barnacle, hawksbill turtle, epibionts, *Honzou Gaku*, natural history

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INTRODUCTION

The hawksbill turtle, *Eretmochelys imbricata* (Linnaeus, 1766), is a specialized "sponge-eating" sea turtle that occupies a unique position in coral reef ecosystems. Globally, hawksbill turtles are generally recognized as declining, having been both hunted for their keratinized carapacial scutes called *Bekko* materials for Japanese traditional crafts and recorded as by-catch worldwide (Meylan and Donnelly, 1999; Gillman et al., 2010). Understanding the migration strategies and habitat use of sea turtles is necessary to implement effective conservation strategies (Hamann et al., 2010; Mazor et al., 2016). However, the migration routes and patterns of habitat utilization of hawksbill turtles are rather poorly known compared to other sea turtle species (Godley et al., 2008). Epibiotic organisms such as barnacles are useful to track hosts and understand their life history (Hayashi and Tsuji, 2008; Hayashi, 2009; Fuller et al., 2010), for example, fossil records of epibiotic barnacles presented the past migratory routs of extinct whales (e.g., Bianucci et al., 2006; Collareta et al., 2016; Buckeridge et al., 2019; Taylor et al., 2019).

Before binomial nomenclature was introduced by Linnaeus, observations of these barnacles were reported from western historical scholars. The first reference to the whale barnacle *Coronula diadema* (Linnaeus, 1767) dates back to 1751 (Haelters et al., 2010), and the earliest probable reference to a turtle barnacle *Chelonibia testudinaria* (Linnaeus, 1758) was published by

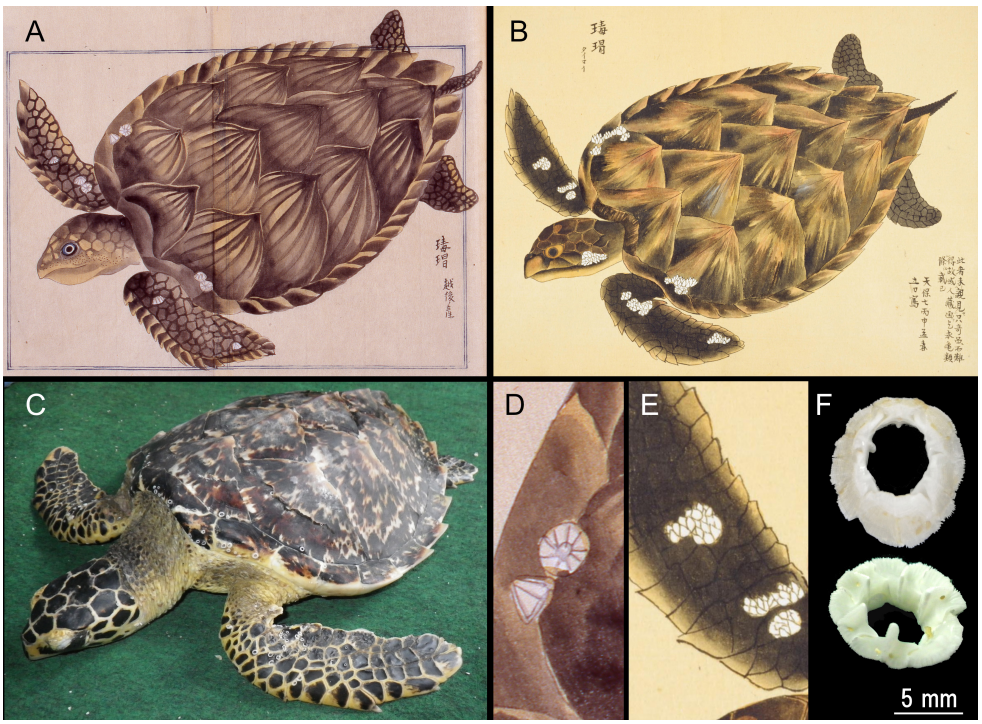

FIGURE 1 | (A,D) Hawksbill turtle with barnacles illustrated by Shunzan Takagi; **(B,E)** Copied illustration by Baien Mouri; **(C,F)** An encrusted living hawksbill turtle and *Platylepas hexastylos*.

Aldrovandi (1606). From the eastern Pacific, Sáenz-Arroyo et al. (2006) reported the 16th–19th century traveler's descriptions of marine wildlife, including sea turtle species.

Animal illustrations by pioneer Japanese naturalists from the Edo period (17th–19th centuries) indicate that the Japanese people of this period were interested in the diversity of life. However, the contributions of their classical natural history records (the so-called "*Honzou Gaku*") to modern biology and ecology are not always recognized. In turn, the *Honzou Gaku* records provide an important resource for understanding past patterns of biodiversity. For example, ancient Japanese naturalists recorded epibiotic barnacles attached to marine vertebrates (Hayashi, 2014) and the trans-Pacific migration of black turtle (Hayashi and Yasuda, 2021) while compiling information on Japanese fauna and flora into monographs. Despite the lack of modern evidence, these historical documents offer insight into past migratory patterns. Here, I present the historical records of a hawksbill turtle with epibiotic barnacles in "*Honzou Gaku*" monographs to elucidate the past life history of these organisms.

MATERIALS AND METHODS

A literature survey was conducted using original illustrations and internet databases. The original illustration in **Figure 1A** is deposited in the Iwase Bunko Library (Takagi, 1852). That in **Figure 1B** is deposited in the National Diet Library of Japan (Mouri, 1839) and it is available to the public online.

RESULTS

To promote domestic production, the early Japanese naturalist Shunzan Takagi (date of birth unknown–1852), was convinced of the need to understand the classical natural history called as "*Honzou Gaku*." With clearly illustrated drawings of Japanese animals and plants, he made a monograph entitled "*Honzou Zusetsu*" (Takagi, 1852). Takagi edited *Honzou Zusetsu* in the 1830s or earlier. However, the 195 volumes of this monograph were not complete at the time of its death in 1852. He included a color drawing of a hawksbill turtle with epibiotic barnacles in his monograph (**Figure 1A**), and described the location of the turtle only as "*captured in Echigo*" [currently the area around Niigata Prefecture (**Figure 2A**), coast of the Sea of Japan].

Baien Mouri (1798–1851), a retainer of the Tokugawa shogunate and early Japanese naturalist, also included a color drawing of a hawksbill turtle with epibiotic organisms in his monograph, *Baien Kaifu* (**Figure 1B**), deposited in the National Diet Library of Japan (Mouri, 1839). Mouri's description of his drawing is as follows:

> *"I did not observe this turtle directly. I asked a person* (presumably Shunzan Takagi) *who had a detailed drawing to copy it, because this is a very rare species and difficult to get. I made a copy on 5*

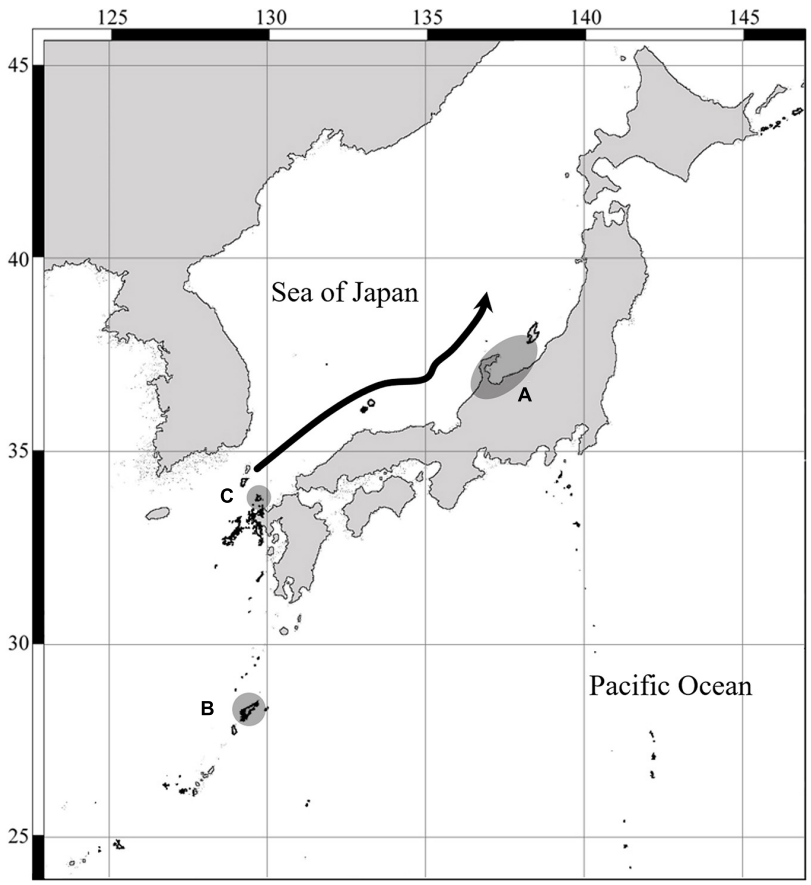

FIGURE 2 | Map of the Japanese Archipelago with indication of the regional currents. **(A)** Echigo region (location of capture for Takagi and Mouri's hawksbill turtle). **(B)** Amami Oshima Island, northernmost nesting record of hawksbill turtle. **(C)** The northernmost coral reefs in Japan. Arrow indicates the Tsushima current.

March 1837. The turtle was captured at Echigo in 1836. A person got this turtle from a fisherman, but this animal was decayed and soon smelled bad. He suspended the animal from a tree, but wind and rain caused it to decay further. I went to his home and saw it, but it was in bad condition. The turtle was a hawksbill turtle and difficult to get, then I asked him to copy his drawing. This turtle was almost the same size as the drawing (ca. 30 cm) *and was encrusted with many organisms".*

As described above, Mouri's figure is a facsimile of *Honzou Zusetsu* (Takagi, 1852) with a detailed description. However, his drawings of epibionts are schematic and difficult to identify (**Figure 1E**). In contrast, Takagi's original drawing, illustrated in **Figures 1A,D**, clearly shows the acorn barnacles. In Japanese waters, the most conspicuous barnacle on the turtle carapace *C. testudinaria* had not been recorded from the hawksbill turtles, on the other hand, *Platylepas hexastylos* (Fabricius, 1798) were commonly found on Japanese hawksbill turtles (**Figures 1C,F**). Distribution of *C. testudinaria* is limited to the hard substrate on turtle body such as carapace or plastron, while that of *P. hexastylos* includes on carapace, plastron, head, flipper, legs, and soft skin of sea turtles. For the above mentioned, the illustrated barnacles are identified as *P. hexastylos*.

DISCUSSION

The northern limit of the hawksbill turtle breeding range was recorded in Amami Oshima Island, Kagoshima (**Figure 2B**, Mizuno, 2013), even though the northernmost coral reef is located on Iki Island (**Figure 2C**, the entrance to the Sea of Japan) and there are no coral reefs in the Sea of Japan (Yamano et al., 2001). The turtle described by Takagi and Mouri might have been carried away from its native habitat by the Tsushima Current (**Figure 2**). Recently, some stranding records of hawksbill turtles were reported from the coast of the Sea of Japan near the Echigo region (Hayashi S., 2012; Ishihara et al., 2017). Local sea surface temperatures are too cold for them and cold-stunning events occur at the upper limits of their native habitat range. Hayashi S. (2012) suggested that the hawksbill turtles were transported by the Tsushima Current and wandered from their native habitat during accidental migration, or vagrancy, caused by sea surface temperature rise due to recent global warming. However, the 19th century records of cold-stunning or accidental migration of the hawksbill turtle indicate that aberrations of sea turtle migration into the Sea of Japan occur frequently and are not only recent events.

Seven species of turtle barnacles including *Platylepas hexastylos* have been recorded from hawksbill turtles (Hayashi, 2013), and *P. hexastylos* has been reported from hawksbill turtle in the Sea of Japan (Hayashi R., 2012). The illustrated barnacles are identified as *P. hexastylos*, and the historical record of epibionts is also consistent with recent records. The *Honzou Gaku* records thus prove precious for understanding sea turtle life history in Japan and can expand our knowledge of the past distribution of species.

Early Japanese naturalists have been recording details of fauna and flora since the Edo period, and there is a large amount of natural history data for Japan. Four basic questions regarding the history of marine animal populations (HMAP) were raised by Holm (2003): How has the extent and diversity of these populations changed over the last 2000 years? Which factors have influenced these changes? What is the anthropogenic and biological significance of these changes? What has been the interplay of changing marine ecosystems and human societies? This paper provides some answer to the questions of HMAP in the case of hawksbill turtles and indicates the importance of natural history to gain insight into past patterns of biodiversity. Evaluating historical natural history materials is a valuable approach to understand the state of the ecosystem in the past and can aid in formulating adequate conservation strategies for endangered species.

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 living hawksbill turtle (reported in **Figure 1**) was fishery bycaught.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Epibiont Assemblages on Nesting Hawksbill Turtles Show Site-Specificity in the Persian Gulf

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Sea turtle epibionts can provide insights into the hosts' habitat use. However, at present, there is a lack of information on sea turtle epibiont communities in many locations worldwide. Here, we describe the epibiont communities of 46 hawksbill turtles (*Eretmochelys imbricata*) in the Persian Gulf. Specifically, we sampled 28 turtles from the Dayyer-Nakhiloo National Park (DNNP) in the northern Gulf and 18 turtles from Shibderaz beach in the Strait of Hormuz. A total of 54 macro, meio, and micro-epibiont taxa were identified, including 46 taxa from Shibderaz and 29 taxa from DNNP. The barnacles *Chelonibia testudinaria* and *Platylepas hexastylos*, as well as harpacticoid copepods and Rotaliid foraminifers, had the highest frequency of occurrence found on almost all turtle individuals. Harpacticoids were the most abundant epizoic taxa (19.55 ± 3.9 ind. per 9 cm^2) followed by forams (*Quinqueloculina* spp.: 6.25 ± 1.5 ind. per 9 cm^2 and Rotaliids: 6.02 ± 1.3 ind. per 9 cm^2). Our results showed significant differences between the study sites in the composition of micro and macro-epibiont communities found on hawksbill turtles. We speculate that the differences in epibiont communities were largely influenced by local environmental conditions.

Keywords: barnacles, epibionts, environmental extremes, Strait of Hormuz, sea turtles

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INTRODUCTION

Epibiosis is a symbiotic relationship where one organism (epibiont) lives on the surface of the other (basibiont) (Wahl and Mark, 1999; Harder, 2008). A wide variety of epibiont communities are found on sea turtles (Wahl, 1989; Pfaller et al., 2008b; Frick and Pfaller, 2013; Majewska et al., 2015) including macro, meio, and micro-epibionts. Macro-epibiont communities encompassing cirripeds, polychaetes, hydrozoans, bryozoans, poriferans, tunicates, periphytic algae, and some motile organisms have been widely studied on different sea turtle species (Caine, 1986; Pfaller et al., 2008b; Fuller et al., 2010; Lazo-Wasem et al., 2011; Robinson N. J. et al., 2017; Robinson et al., 2019), and meiofaunal organisms such as nematodes and copepods have recently been the focus of several studies (Aznar et al., 2010; Corrêa et al., 2013; Domènech et al., 2017; Ingels et al., 2020). Likewise, micro-epibiota on sea turtles, represented mostly by colonizing diatoms, have recently been assessed (Majewska et al., 2015, 2017; Robinson et al., 2016; van de Vijver et al., 2020). Some of these epibionts, such as the barnacle *Chelonibia testudinaria*, have a wide geographical distribution (Rawson et al., 2003; Lazo-Wasem et al., 2011), whereas some others, like some short-lived diatom species, may have a relatively narrow and local distribution (Abarca et al., 2014).

Barnacles are the most prominent epibionts of sea turtles (Casale et al., 2012; Frick and Pfaller, 2013). Turtle barnacles belong to the superfamily Coronuloidea and include three families: Chelonibiidae Pilsbry, 1916, Coronulidae Leach, 1817, and Platylepadidae Newman and Ross, 1976 (Hayashi, 2012, 2013). Members of Chelonibiidae are perhaps the most studied barnacle species recorded on sea turtles. *Chelonibia testudinaria*, the most commonly reported sea turtle barnacle, has been reported on the body surface of all extant sea turtle species (Sloan et al., 2014), sirenians (Zardus et al., 2014), and some crustaceans (Cheang et al., 2013) from distant geographical regions. It is, therefore, considered a host generalist species and should not be assumed as an obligatory turtle barnacle (Cheang et al., 2013; Zardus et al., 2014). In contrast, *Chelonibia caretta,* which is considered a host specialist, is reported only in association with sea turtles, especially loggerheads (*Caretta caretta*) (Torres-Pratts et al., 2009; Farrapeira, 2010).

Several techniques have been successfully used to study habitat use and migration patterns of sea turtles, including satellite telemetry (e.g., Rees et al., 2016; Robinson D. P. et al., 2017; Hays and Hawkes, 2018; Pilcher et al., 2020), aerial surveys (Jean et al., 2010), visual surveys *via* snorkeling (Roos et al., 2005), and stable isotope analysis (e.g., Nolte et al., 2020). However, most of these techniques are costly (Pfaller et al., 2014), and/or logistically difficult to implement. As an alternative, or complementary and relatively low-cost approach, epibiont assemblages living on sea turtles can roughly indicate habitat use and migratory behavior of these highly mobile marine reptiles (e.g., Pfaller et al., 2008b; Lazo-Wasem et al., 2011; Rivera et al., 2018; Robinson et al., 2019; Nolte et al., 2020; Silver-Gorges et al., 2021). For instance, some sea turtle epibionts, e.g., *C. testudinaria* and two lepadid barnacles *Lepas hilli* and *Conchoderma virgatum*, have been proposed to be potentially used as habitat indicators of sea turtles (Casale et al., 2012; Ten et al., 2019). According to previous studies, the barnacles *L. hillii*, *C. virgatum* (Ten et al., 2019), and *Platylepas* spp. (Casale et al., 2012) preferably settle on turtles inhabiting oceanic waters. In contrast, *C. testudinaria*, *Stomatolepas elegans,* and *Stephanolepas muricata* are mainly associated with turtles occupying neritic waters (Casale et al., 2012). Epibiotic barnacles and crabs have also been used as indicators of the distribution and movement of loggerheads (Casale et al., 2004). Thus, epibiont communities could roughly reflect the environment in which the host has recently been living (Casale et al., 2012; Frick and Pfaller, 2013; Nolte et al., 2020; Silver-Gorges et al., 2021). In addition, this method could be very useful in sea turtle conservation planning efforts, as epibionts may affect their health status. Stranded turtles were frequently utilized in studies to examine factors that affect their health and mortality (Sönmez, 2018; Cheng et al., 2019; Wang et al., 2020). Turtle epibionts may cause increased drag (Logan and Morreale, 1994; Wyneken, 1997), which could be energetically expensive for the host turtles, particularly for those undertaking long-distance migrations (Frick and Pfaller, 2013). Additionally, some turtle epibionts such as leeches and barnacles may cause infections in sea turtles (George, 1997; Greenblatt et al., 2004), or enhance their vulnerability to pathogens (George, 1997). The presence of some coronuloid barnacles on eyes and wounds, as well as their penetration into the epidermis of the host's flippers, may have a negative influence on their health (Frick et al., 2011).

The marine environment of the Persian Gulf is characterized by high and wide-ranging temperatures [sea surface temperatures (SST) from 15° to 36°C, Riegl and Purkis, 2012] and high salinities (>39 psu in most areas, Sheppard et al., 2010). This is a challenging environment for many organisms, leading to impoverished biodiversity in this semi-enclosed body of water compared to other coastal habitats of the Indian Ocean (Sheppard et al., 2010). Satellite telemetry has partially revealed habitat use and migratory behavior of the turtles in this region. Hawksbill turtles (*Eretmochelys imbricata*) spend most of their time feeding on foraging grounds in shallow waters near the coasts of Qatar, Saudi Arabia, and UAE while spending only a small portion of their life nesting on Iranian coasts (Pilcher et al., 2014). In summer, when the SST rises to 33°C, hawksbills leave shallow foraging grounds and move northward to deeper waters (30–50 m) of the Persian Gulf (Pilcher et al., 2014; Marshall et al., 2020).

Hawksbill turtles, along with green turtles (*Chelonia mydas*), are the dominant sea turtle species in the Persian Gulf. It is assumed that hawksbill turtles nesting along the Iranian shores of the Gulf may comprise one of the most important nesting populations in the Indian Ocean region (Meylan and Donnelly, 1999). Therefore, obtaining information on epibiont communities of hawksbills in the Gulf, especially those that are likely indicators of nesting ecology, can aid in their management. Additionally, epibionts could be used as bio-indicators of ecological change in the Persian Gulf. Despite this, our knowledge about epibiont communities of the Gulf's hawksbill turtles is restricted to a few studies on turtle barnacles (Loghmani-Devin and Sadeghi, 2010; Razaghian et al., 2019). In this study, we present the first comprehensive dataset on the diversity, assemblage, and abundance of macro, meio, and micro-epibionts of hawksbill turtles nesting at two distant sites along the Iranian coastline of the Persian Gulf, one at the northwest coast, and the other at the Strait of Hormuz. Due to the differences in environmental conditions of the sites, we hypothesized that epibiont assemblages of the two turtle rookeries might show site-specific differences.

MATERIALS AND METHODS

Study Area

Ommolgorm (27° 50′N, 51° 33′E) and Nakhiloo (27° 51′N, 51° 26′E) islands in Dayyer-Nakhiloo National Park (DNNP) and located at the center of Iran's northwestern Persian Gulf coast, and Shibderaz (26° 41′N, 55° 55′E), a 2 km sandy beach on the south coast of Qeshm Island in the Strait of Hormuz (the entrance of the Persian Gulf; **Figure 1**) were used as study sites. Sea surface temperature and salinity data were obtained during 2017 and 2018 for each site (**Table 1**) from the Copernicus Marine Environment Monitoring Service (http://marine.copernicus.eu; product reference: CMEMS-GLO-PUM-001-024).

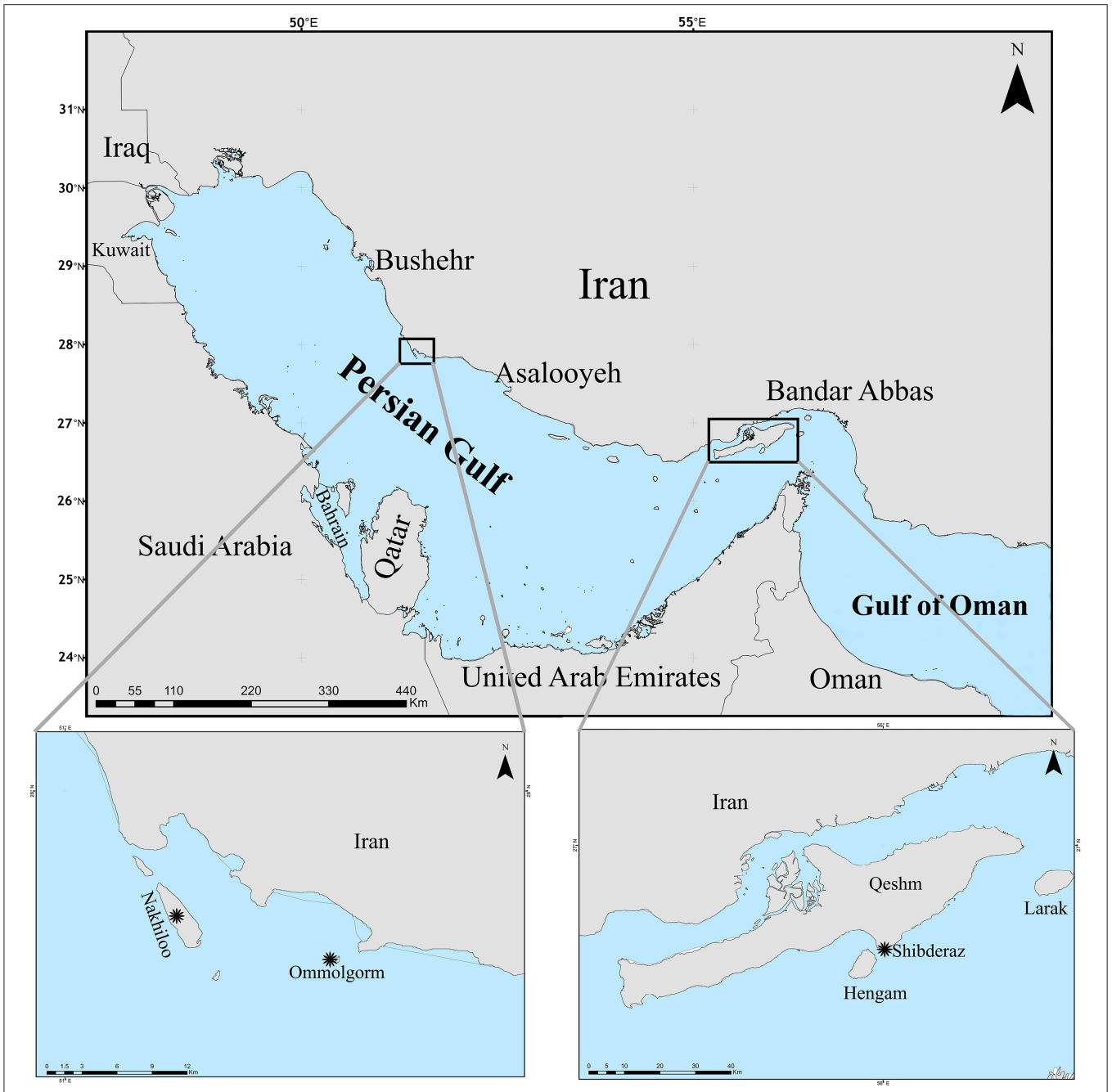

FIGURE 1 | Sampling sites (marked by asterisks) of hawksbill turtle epibionts, Ommolgorm, and Nakhiloo islands in Dayyer-Nakhiloo National Park (DNNP) and Shibderaz on Qeshm Island in the Iranian coasts of the Persian Gulf.

Field Surveys and Sample Collection

The beach areas of both sites were patrolled between March and June in 2017 and 2018. All encountered nesting turtles were examined after the completion of oviposition to avoid interrupting the nesting process. Each turtle was first measured for curved carapace length (CCL) to the nearest 1 mm, and its body was gently washed with clean seawater to remove sand and particles. Following that, a digital camera (Sony DSC-HX9V) was used to photograph the carapace, plastron, head, and soft parts to measure barnacle abundance on each body part using a non-invasive approach. Further, three randomly chosen portions of the carapace surface (9 cm^2) were gently shaved and the keratin materials collected were preserved in 4% formaldehyde solution diluted with filtered seawater. To study diatoms, ∼4 cm^2 of the outer-most layer of the three different scutes were taken and immediately fixed in vials containing 4% formaldehyde solution

TABLE 1 | Sea surface temperature and salinity values at Shibderaz and Dayyer-Nakhiloo National Park (DNNP) during 2017 and 2018.

		Sampling areas	
		Shibderaz	**DNNP**
Geographical coordinates		26° 41′N, 55° 55′E	27° 51′N, 51° 26′E
Temperature (°C)	Average ± SD	28.13 ± 4.12	26.31 ± 5.46
	Min	22.22	17.73
	Max	33.60	35.09
Salinity (PSU)	Average ± SD	37.40 ± 0.48	38.25 ± 0.26
	Min	36.95	37.69
	Max	38.44	38.62

Data were obtained from Copernicus Marine Environment Monitoring Service (http://marine.copernicus.eu; product reference: CMEMS-GLO-PUM-001-024).

diluted by filtered seawater (Majewska et al., 2015). For precise identification of the barnacles, in addition to using photographs, a few barnacle individuals from visually distinct species were physically removed with a safe plastic knife and preserved in vials containing 96% ethanol for laboratory examinations. In total, epibiont samples were collected from 46 nesting hawksbill sea turtles (28 turtles from DNNP and 18 turtles from Shibderaz).

Species Identification and Quantification

Zooepibionts of each collected sample were isolated from algal mats under a stereomicroscope with a magnification of 80x. Specimens were then identified to the lowest possible taxonomic level and their abundance was determined. Scute samples for diatoms identification were subsampled to ca. 1 cm^2, dehydrated through 25, 50, 60, 70, 80, 90, and 100% ethanol series. The samples were then air-dried in a desiccator containing silica gel, placed on microscope slides, sputter-coated with gold, and identified using images taken with a Hitachi SU3500 (Hitachi High-Technologies, Tokyo, Japan) scanning electron microscope (SEM), operating at 15 kV.

We used standard morphological keys following Chan et al. (2009) and Shahdadi et al. (2014) to identify the barnacle species. Sea turtle foraminifera epibionts were identified using the Atlas of Benthic Foraminifera (Holbourn et al., 2013). To identify macroalgae epibionts on hawksbill turtles we utilized the Atlas of the sea algae of the Persian Gulf and Oman Sea coasts (Gharanjik and Rohani Ghadikolaei, 2009) and the Field Guide of Marine Macroalgae of Kuwait (Al-Yamani et al., 2014). Other epibiont taxa were identified using the relevant literature (e.g., Taylor et al., 2007; Guerra-García et al., 2010; Martin et al., 2014).

As the most prominent and visible epibiont taxa, barnacles were analyzed in greater detail. Total and mean barnacle abundance were recorded on each body part (head, carapace, plastron, supra-caudals, and soft parts) using photographs (see above). Image J software (version 1.43 u) was used to measure the basal diameter (Nasrolahi et al., 2013) of each individual barnacles found on turtles.

Statistical Analysis

A Kolmogorov-Smirnov test was used to check for normality, and revealed that the data did not exhibit a normal distribution even after being transformed. The Kruskal-Wallis non-parametric test was performed to compare barnacle abundances, and a Mann-Whitney *U*-test was used to evaluate differences in *C. testudinaria* rostro-carinal diameter (RCD) among different body parts (head, carapace, plastron, supra-caudals, and soft parts). A Mann-Whitney *U*-test was also used to compare *P. hexastylos* RCD between plastron and soft parts of hawksbill turtles encountered in Shibderaz and DNNP.

A PERMANOVA statistical test was used to compare assemblage structure and species composition of sea turtle epibionts between the two study sites. Except for diatoms and other algal taxa, for which only presence-absence data were recorded, the analysis of epibiont structure was based on absolute abundance data. Species composition of the entire epibiont community (including micro, meio, and macro-epibionts) was evaluated based on presence-absence data. A SIMPER (similarity percentage) test was performed to identify the relative contribution of each epibiont taxon to any dissimilarity values between the epibiont assemblages of hawksbill turtles nesting on the two sites. Graphical representation of the similarity was carried out using non-metric multidimensional scaling (nMDS) based on the square-root-transformed abundance data and the Bray–Curtis similarity measure of all identified epibiont taxa for each turtle. Furthermore, a PERMANOVA was used to compare species composition of the macro, meio, and micro-epibionts between the two study sites. Following this, a SIMPER analysis was used to reveal the dissimilarity of epibiont groups between the two sites as well as the contribution of each taxon to the dissimilarity. All the analyses were performed and graphs generated using the statistical software SPSS 26 (George and Mallery, 2019) and Primer 6.0+PERMANOVA (Clarke and Gorley, 2006; Anderson et al., 2008). A significance level of <0.05 was used to reject null hypotheses for all tests.

RESULTS

Examined Turtles

A total of 46 hawksbill turtles were examined from both nesting sites. At Shibderaz, the mean CCL (±SE) was 73.6 ± 0.6 cm (range 69.5–78.0 cm). At DNNP, the mean CCL ± SE was 71.9 ± 0.5 cm (range 67.5–77.0 cm). The overall mean CCL (±SE) for both sites was 72.6 ± 0.4 cm, ranging from 67.5 to 78.0 cm.

Composition and Structure of Epibiont Communities

In total, 54 macro-, meio-, and micro-epibiont taxa including 28 diatoms, five filamentous algae, four barnacles, three foraminifers, and two amphipod species. In addition, single-taxon representatives of bivalves, copepods, cumaceans, gastropods, haptophytes, leeches, hydrozoans, nematodes, ostracods, polychaetes, sponges, and tanaids were identified on hawksbill sea turtles at both nesting sites (**Table 2**). From these, 46 taxa were found on turtles from Shibderaz, whereas only 29 taxa were identified on turtles from DNNP. The difference was

TABLE 2 | Epibiont species list, abundance (ind. per 9 cm^2), and frequency of occurrence on hawksbill (*Eretmochelys imbricata*) turtles ($N = 46$) nesting on Shibderaz (Qeshm Island) and Dayyer-Nakhiloo National Park (DNNP; Bushehr) beaches, Iran.

Main epibiont taxonomic groups	Identified epibionts	Epibiont type	% Frequency of epibiont occurrence on host turtle		Average abundance of epibionts on all hosts	
			Shibderaz $N = 18$	DNNP $N = 28$	Shibderaz $N = 18$	DNNP $N = 28$
Algae: Bacillariophyceae	*Achnanthes* sp.	Micro	*	–	*	–
Algae: Bacillariophyceae	*Achnanthidium* sp.	Micro	–	*	–	*
Algae: Bacillariophyceae	*Actinocyclus* sp.	Micro	*	–	*	–
Algae: Bacillariophyceae	*Amphicocconeis* sp.	Micro	*	–	*	–
Algae: Bacillariophyceae	*Amphora coffeiformis*	Micro	*	–	*	–
Algae: Bacillariophyceae	*Amphora* sp. 1	Micro	*	*	*	*
Algae: Bacillariophyceae	*Amphora* sp. 2	Micro	*	–	*	–
Algae: Bacillariophyceae	*Amphora* sp. 3	Micro	*	–	*	–
Algae: Bacillariophyceae	*Amphora ovalis*	Micro	*	–	*	–
Algae: Bacillariophyceae	*Berkeleya* sp.	Micro	*	–	*	–
Algae: Bacillariophyceae	*Caloneis* sp.	Micro	–	*	–	*
Algae: Bacillariophyceae	*Cocconeis convexa*	Micro	*	–	*	–
Algae: Bacillariophyceae	*Cocconeis distans*	Micro	*	–	*	–
Algae: Bacillariophyceae	*Cocconeis scutellum*	Micro	*	–	*	–
Algae: Bacillariophyceae	*Cocconeis* sp.	Micro	*	*	*	*
Algae: Bacillariophyceae	*Grammatophora* sp.	Micro	*	–	*	–
Algae: Bacillariophyceae	*Licmophora* spp.	Micro	*	–	*	–
Algae: Bacillariophyceae	*Mastogloia horvathiana*	Micro	–	*	–	*
Algae: Bacillariophyceae	*Navicula directa*	Micro	*	–	*	–
Algae: Bacillariophyceae	*Navicula* sp. 1	Micro	*	–	*	–
Algae: Bacillariophyceae	*Navicula* sp. 2	Micro	*	–	*	–
Algae: Bacillariophyceae	*Nitzschia* sp. 1	Micro	*	–	*	–
Algae: Bacillariophyceae	*Nitzschia* sp. 2	Micro	*	–	*	–
Algae: Bacillariophyceae	*Opephora* sp.	Micro	*	–	*	–
Algae: Bacillariophyceae	*Poulinea lepidochelicola*	Micro	*	–	*	–
Algae: Bacillariophyceae	*Psammodictyon* sp.	Micro	*	–	*	–
Algae: Bacillariophyceae	*Tabularia tabulata*	Micro	*	–	*	–
Algae: Bacillariophyceae	*Tabularia* sp. 1	Micro	*	–	*	–
Algae: Chlorophyta	*Chaetomorpha* sp.	Macro	31	–	*	–
Algae: Chlorophyta	*Ulva* sp.	Macro	88	56	*	*
Algae: Rhodophyta	*Ceramium* sp.	Macro	50	59	*	*
Algae: Rhodophyta	*Polysiphonia* sp.	Macro	–	4	–	*
Algae: Rhodophyta	Unknown	Macro	13	63	*	*
Annelida: Hirudinea	*Ozobranchus* sp.	Macro	7	–	0.07 ± 0.1	–
Annelida: Polychaeta	Unknown	Macro	–	35	–	0.36 ± 0.1
Cnidaria: Hydrozoa	Campanulariidae	Macro	47	42	*	*
Crustacea: Amphipoda	*Hyachelia* sp.	Macro	33	35	0.67 ± 0.3	0.46 ± 0.2
Crustacea: Amphipoda	*Caprella* sp.	Macro	–	4	–	0.09 ± 0.1
Crustacea: Cirripedia	*Chelonibia testudinaria*	Macro	100	100	0.21 ± 0.05	0.34 ± 0.4
Crustacea: Cirripedia	*Platylepas hexastylos*	Macro	100	100	4.55 ± 0.44	2.15 ± 0.28
Crustacea: Cirripedia	*Stomatolepas transversa*	Macro	73	85	*	*
Crustacea: Cirripedia	*Stephanolepas muricata*	Macro	73	88	*	*
Crustacea: Copepoda	Harpacticoida	Meio	100	100	22.56 ± 5.1	16.54 ± 2.7
Crustacea: Cumacea		Macro	13	4	0.07 ± 0.0	0.03 ± 0.0
Crustacea: Ostracoda		Meio	60	88	0.82 ± 0.2	4.15 ± 1.13
Crustacea: Tanaidacea	Tanaidacea	Macro	7	27	0.09 ± 0.1	0.51 ± 0.2
Foraminifera: Rotaliida		Meio	100	96	3.22 ± 0.5	8.83 ± 2.1
Foraminifera: Textulariida		Meio	–	12	–	0.08 ± 0.0
Foraminifera: Miliolida	*Quinqueloculina* spp.	Meio	87	92	2.53 ± 0.7	9.97 ± 2.3
Haptophyta: Isochrysidales	*Emiliania huxleyi*	Micro	*	*	*	*
Mollusca: Bivalvia		Macro	–	46	–	0.62 ± 0.2
Mollusca: Gastropoda		Macro	13	58	0.09 ± 0.1	0.81 ± 0.2
Nematoda	Unknown	Macro	7	42	0.02 ± 0.0	0.52 ± 0.2
Porifera		Macro	7	4	*	*

**Taxon represents presence only and individual counts were not undertaken.*

–, Taxon represents absence only.

largely driven by diatoms. Of the 28 total diatom taxa belonging to 17 genera, 25 taxa were identified in samples collected from Shibderaz whereas only five taxa were observed from DNNP (**Table 2**). *Chaetomorpha* sp. and *Ozobranchus* sp. were recorded only from Shibderaz and *Polysiphonia* sp., *Caprella* sp., a bivalve, and a polychaete were only identified in DNNP. Examples of different epibiont taxa are shown in **Figure 2**.

Among macrofauna, *C. testudinaria* and *P. hexastylos* were present on all examined turtles. Among the meiofauna, harpacticoid copepods, and Rotaliid foraminifers were also

FIGURE 2 | Examples of epibiont taxa recorded on the body surface of hawksbill sea turtles in the Iranian coasts of the Persian Gulf: **(a)** *C. testudinaria* on the carapace of hawksbill sea turtle; **(b)** specimens of *Stephanolepas muricata*; **(c)** *Chelonibia testudinaria*; **(d)** *Platylepas hexastylos*; **(e)** Tanaid; **(f)** Rotaliid foraminifer; **(g)** *Chaetomorpha* sp.; **(h)** *Polysiphonia* sp.; **(i)** *Psammodictyon* sp.; **(j)** *Nitzschia* sp.; **(k)** *Tabularia* sp.1; **(l)** *Amphora* sp.1.

observed on almost all sea turtle individuals. Likewise, the filamentous alga *Ulva* sp. showed a high frequency of occurrence on turtles (88 and 56% at Shibderaz and DNNP, respectively, **Table 2**). Harpacticoids (64.5%) followed by *P. hexastylos* (13%) and Rotaliids (9.3%) were the most abundant epizoic taxa on turtles from Shibderaz, whereas harpacticoids (36.4%), *Quinqueloculina* spp. (22%) and Rotaliids (19.5%) were the most dominant taxa on turtles at DNNP (**Figure 3**).

The PERMANOVA analysis identified statistically significant site-based differences in the epibiont species composition and community structure on studied turtles [Pseudo-F = 5.89, *P* (perm) < 0.001; Pseudo-F = 17.51, *P* (perm) < 0.001, respectively, **Supplementary Table S1**]. Similarly, the nMDS plot shows that species composition and community structure were noticeably different between the two sites (**Figure 4**). The SIMPER analysis showed 35.71% dissimilarity between the two sites. Rhodophyta (7.1%), Gastropoda (6.32%), Bivalvia (5.64%), Campanulariidae (5.55%), *Ceramium* sp. (5.54%), *Ulva* sp. (5.11%), and *Hyachelia* sp. (4.93%) contributed to more than 40% of the difference (**Table 3**). When separating the epibionts into macro, meio, and micro-epibiont groups, a significant difference between the two sites in species composition of the micro and macro-epibionts was detected [Pseudo-F = 15.32, *P* (perm) < 0.001, Pseudo-F = 9.02, *P* (perm) = 0.001, respectively]. The SIMPER analysis revealed 97.68 and 39.37% dissimilarity between the two sites, respectively. Diatom species—including *Cocconeis* spp. (23.83%), *Caloneis* sp. (9.43%), *Amphora* sp. 1 (7.14%), and *Amphora ovalis* (6.80%)—contributed around 47% to the differences of the micro-epibionts (**Table 3**). Rhodophyta (10.45%), Gastropoda (9.31%), *Ceramium* sp. (8.28%), Campanulariidae (8.23%), Bivalvia (8.19%), and *Ulva* sp. (7.66%) explained 52% of the macro-epibiont variances (**Table 3**).

Barnacle Composition and Distribution

Four barnacle species, including *P. hexastylos*, *C. testudinaria*, *Stomatolepas transversa*, and *Stephanolepas muricata* were identified on the body surface of examined turtles. About 95% of *P. hexastylos* individuals were found on the flippers and soft parts, while only 5% were recorded on the plastron scutes; no individuals were observed on the carapace. *C. testudinaria* individuals were distributed more broadly, with 51% distributed on the plastron, 37% on the carapace, 10% under the supracaudals, and 2% on the head. Individuals of *S. transversa* were only observed along the plastral sutures and *S. muricata* was only found attached to the leading edges of the front flippers.

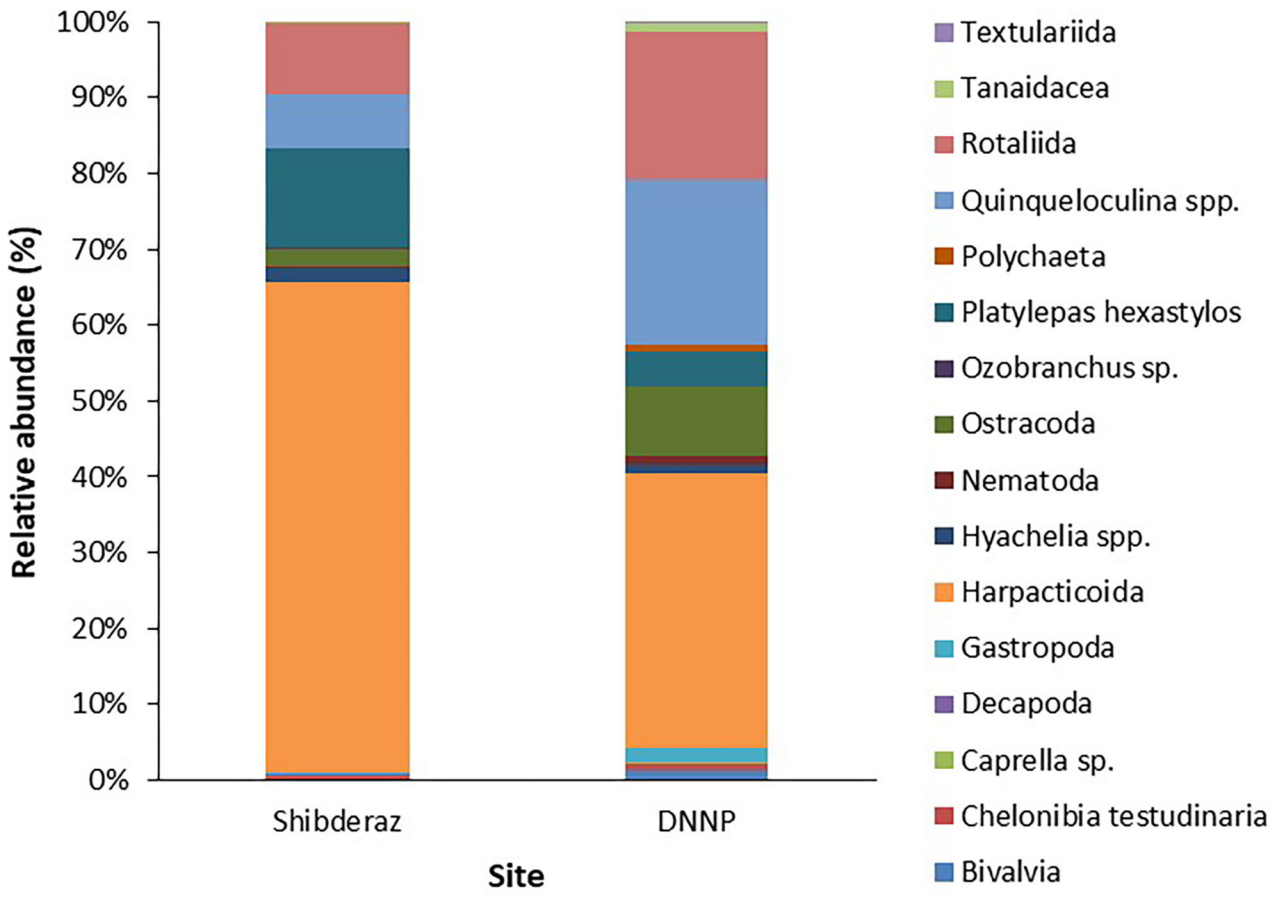

FIGURE 3 | Relative abundance (%) of epibiont taxa on the body surface of the nesting hawksbill turtles in Shibderaz and Dayyer-Nakhiloo National Park (DNNP).

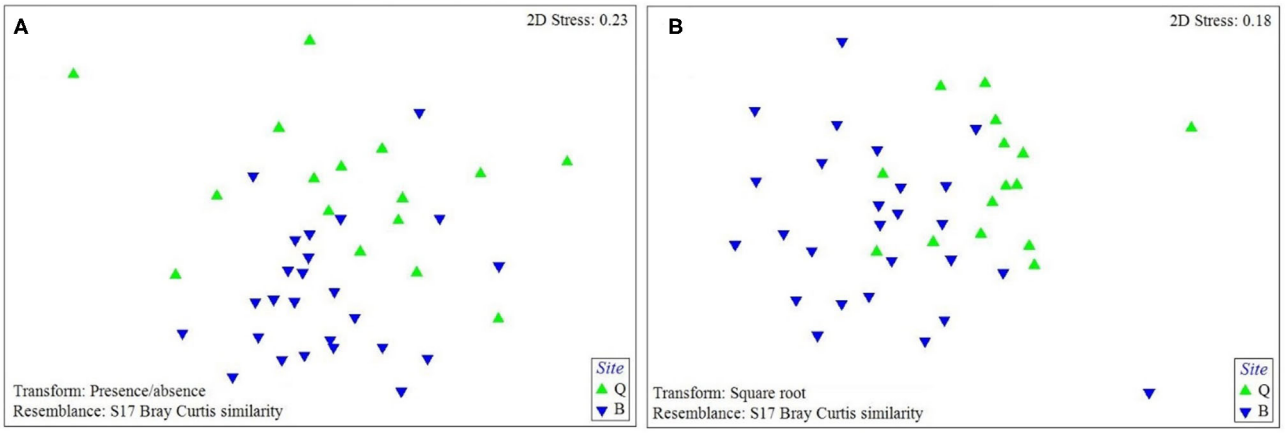

FIGURE 4 | The nMDS plot of **(A)** the species composition and **(B)** assemblage structure of sea turtle epibionts at each study site based on Bray-Curtis similarity matrix performed on presence-absence data for species composition and square-root transformed data for assemblage structure (Q: Shibderaz, Qeshm Island; and B: DNNP, Bushehr province).

In general, 85.3% of all barnacles were attached on flippers and soft parts, 9.7% on the plastron, 3.8% on the carapace, 1% under supracaudals, and 0.2% on the head (**Table 4**).

Barnacle Abundance

As *S. transversa* and *S. muricata* were small and difficult to distinguish from each other, which made it challenging to precisely count them using the images. We visually estimated their total abundance to be <2%. Thus, we only counted *P. hexastylos* and *C. testudinaria*. A total of 68,905 individual barnacles were counted on body parts (including carapace, plastron, head, neck, flippers, and soft parts) of turtles. Of these, there were 61,837 (90%) and 7,068 (10%) individuals of *P. hexastylos* and *C. testudinaria*, respectively. The greatest barnacle load was found on a 72 cm (CCL) turtle that had 3,774 barnacles (3,659 *P. hexastylos* and 115 *C. testudinaria*) and the lowest measured barnacle load was from a 70.5 cm (CCL) turtle that was carrying 212 barnacles (146 *P. hexastylos* and 66 *C. testudinaria*).

The overall mean (±SE) barnacle abundance (1497.9 ± 133.7) was significantly different on various body parts of turtles ($p < 0.05$, **Supplementary Table S2**). Mean barnacle abundance was 1278.0 ± 123.4 on the flippers and adjacent soft parts, 144.7 ± 16.4 on the plastron, 56.8 ± 8.2 on the carapace, 15.0 ± 2.5 under the supracaudal scutes, and 3.4 ± 1.1 on the head. The mean abundance for *C. testudinaria* and *P. hexastylos* was 153.7 ± 15.7 and 1344.3 ± 130.1, respectively.

Barnacle Rostro-Carinal Diameter

The results of the Mann-Whitney *U*-test showed that there was no significant difference in the mean RCD of *C. testudinaria* on different body parts of turtles nesting at Shibderaz vs. DNNP ($p > 0.05$). Therefore, the data for both sites were pooled. The RCD of *C. testudinaria* was significantly different among different body parts (i.e., head, carapace, plastron, and supracaudal) (**Figure 5A**, $p < 0.05$, **Supplementary Table S2**). The highest mean barnacle RCD (22.57 ± 9.47) was observed on the head and the lowest (12.27 ± 5.24) on the supracaudal scutes (**Figure 5A**). The mean RCD of *P. hexastylos* was significantly higher in Shibderaz compared to DNNP (**Figure 5B**, $p < 0.05$, **Supplementary Table S2**). The size-frequency distribution of *C. testudinaria* showed a skewness by some large barnacle individuals (**Figure 6**). It showed a peak at 5.01–10 mm followed by two smaller peaks at 10.01–15 and 15.01–20 mm. There were few large barnacles with a size range of 55.01–60 mm (**Figure 6**).

DISCUSSION

The majority of research on turtle epibionts has focused on the epibiont loads on the carapace (see Caine, 1986; Pfaller et al., 2008a,b; Fuller et al., 2010), presuming that the abundance of epibionts is highest on this body part. However, this notion has been contradicted by more recent studies (e.g., Robinson et al., 2019) that have found epibiont abundance on soft skin to be higher than on the carapace and plastron. To provide a holistic qualitative or quantitative data set of the epibiont communities of sea turtles, it is therefore essential to conduct a full-body examination (Robinson et al., 2019). Although most prior research concentrated on macro-epibiota (e.g., Frazier et al., 1985; Fuller et al., 2010; Lazo-Wasem et al., 2011; Casale et al., 2012), meio and micro-epibiota have recently received increasing attention due to their high diversity and abundance in sea turtles, as well as advancements in microscopic techniques that have facilitated greater study of these smaller organisms (e.g., Corrêa et al., 2013; Majewska et al., 2015, 2017; Robinson et al., 2016; Azari et al., 2020; Ingels et al., 2020; Silver-Gorges et al., 2021). Some of these taxa, such as diatoms, are found on all sea turtle species (Majewska et al., 2015; Robinson et al., 2016) and are considered ecological indicators (El-Semary, 2016; Majewska et al., 2017). A comprehensive baseline study on the epibionts of sea turtles should, therefore, encompass both macroscopic and microscopic epibiota to depict a better picture of the turtle epibiont assemblages (Majewska et al., 2015). To the best of our

TABLE 3 | Results of the SIMPER procedure to identify the relative contribution of each epibiont taxa to the dissimilarity between the epibiont assemblages of hawksbills (*Eretmochelys imbricata*) nesting on Shibderaz (Qeshm Island) and Dayyer-Nakhiloo National Park (DNNP; Bushehr) beaches, Iran: (a) all epibionts, (b) micro-epibionts and (c) macro-epibionts.

Systematic group	Epibiont taxon	Shibderaz vs. DNNP		
		Average dissimilarity	Contribution (%)	Cumulative (%)
a				
Algae: Rhodophyta	Unknown	2.53	7.10	7.10
Mollusca: Gastropoda		2.26	6.32	13.42
Mollusca: Bivalvia		2.02	5.64	19.07
Cnidaria: Hydrozoa	Campanulariidae	1.98	5.55	24.62
Algae: Rhodophyta	*Ceramium* sp.	1.98	5.54	30.16
Algae: Chlorophyta	*Ulva* sp.	1.82	5.11	35.27
Crustacea: Amphipoda	*Hyachelia* sp.	1.76	4.93	40.20
Nematoda		1.74	4.88	45.07
Crustacea: Ostracoda		1.51	4.23	49.31
Algae: Chlorophyta	*Chaetomorpha* sp.	1.49	4.18	53.48
Crustacea: Cirripedia	*Stomatolepas transversa*	1.39	3.90	57.38
Annelida: Polychaeta	Polychaeta	1.37	3.84	61.22
Crustacea: Cirripedia	*Stephanolepas muricata*	1.29	3.61	64.83
Algae	Algae sp. 1	1.28	3.58	68.41
Crustacea: Tanaidacea	Tanaidacea	1.16	3.25	71.66
Foraminifera: Miliolida	*Quinqueloculina* spp.	0.75	2.11	73.77
Algae: Bacillariophyceae	*Amphora ovalis*	0.67	1.86	75.63
Crustacea: Cumacea		0.59	1.64	77.27
Algae: Bacillariophyceae	*Amphora* sp. 1	0.52	1.45	78.72
Foraminifera: Textulariida		0.50	1.41	80.13
Algae: Bacillariophyceae	*Cocconeis scutellum*	0.48	1.34	81.47
Porifera		0.42	1.17	82.64
Haptophyta: Isochrysidales	*Emiliania huxleyi*	0.36	1.00	83.64
Algae: Bacillariophyceae	*Cocconeis* spp.	0.30	0.84	84.48
Algae: Bacillariophyceae	*Achnanthes* spp.	0.27	0.77	85.25
Algae: Bacillariophyceae	*Licmophora* spp.	0.27	0.77	86.02
Algae: Bacillariophyceae	*Navicula* sp. 1	0.27	0.77	86.79
Algae: Bacillariophyceae	*Opephora* sp.	0.22	0.63	87.41
Algae: Bacillariophyceae	*Actinocyclus* sp.	0.22	0.61	88.02
Algae: Bacillariophyceae	*Amphicocconeis* sp.	0.22	0.61	88.63
Algae: Bacillariophyceae	*Amphora coffeiformis*	0.22	0.61	89.23
Algae: Bacillariophyceae	*Berkeleya* sp.	0.22	0.61	89.84
Algae: Bacillariophyceae	*Cocconeis distans*	0.22	0.61	90.45
b				
Algae: Bacillariophyceae	*Cocconeis* spp.	23.28	23.83	23.83
Algae: Bacillariophyceae	*Caloneis* sp.	9.21	9.43	33.26
Algae: Bacillariophyceae	*Amphora* sp. 1	6.97	7.14	40.40
Haptophyta: Isochrysidales	*Emiliania huxleyi*	6.68	6.84	47.24
Algae: Bacillariophyceae	*Amphora ovalis*	6.65	6.80	54.04
Algae: Bacillariophyceae	*Achnathidium* sp.	5.66	5.80	59.84
Algae: Bacillariophyceae	*Mastogloia horwatiana*	5.66	5.80	65.63
Algae: Bacillariophyceae	*Cocconeis scutellum*	4.67	4.78	70.42
Algae: Bacillariophyceae	*Achnanthes* spp.	3.44	3.53	73.94
Algae: Bacillariophyceae	*Licmophora* spp.	3.44	3.53	77.47
Algae: Bacillariophyceae	*Navicula* sp. 1	3.44	3.53	80.99
Algae: Bacillariophyceae	*Amphicocconeis* sp.	1.98	2.02	83.02
Algae: Bacillariophyceae	*Grammatophora* sp.	1.98	2.02	85.04

(Continued)

TABLE 3 | Continued

Systematic group	Epibiont taxon	Shibderaz vs. DNNP		
		Average dissimilarity	Contribution (%)	Cumulative (%)
Algae: Bacillariophyceae	*Opephora* sp.	1.98	2.02	87.06
Algae: Bacillariophyceae	*Actinocyclus* sp.	1.23	1.26	88.32
Algae: Bacillariophyceae	*Amphora coffeiformis*	1.23	1.26	89.57
Algae: Bacillariophyceae	*Berkeleya* sp.	1.23	1.26	90.83
c				
Algae: Rhodophyta	Unknown	4.11	10.45	10.45
Mollusca: Gastropoda		3.67	9.31	19.76
Algae: Rhodophyta	*Ceramium* sp.	3.26	8.28	28.05
Cnidaria: Hydrozoa	Campanulariidae	3.24	8.23	36.27
Mollusca: Bivalvia		3.23	8.19	44.47
Algae: Chlorophyta	*Ulva* sp.	3.02	7.66	52.13
Crustacea: Amphipoda	*Hyachelia* sp.	2.82	7.17	59.30
Nematoda		2.79	7.09	66.39
Algae: Chlorophyta	*Chaetomorpha* sp.	2.31	5.87	72.26
Crustacea: Cirripedia	*Stomatolepas transversa*	2.27	5.76	78.02
Annelida: Polychaeta		2.16	5.48	83.50
Crustacea: Cirripedia	*Stephanolepas muricata*	2.09	5.30	88.80
Crustacea: Tanaidacea	Tanaidacea	1.83	4.65	93.45

TABLE 4 | Occurrence of barnacles on different body parts of the hawksbill sea turtles nesting on Shibderaz (Qeshm Island) and Dayyer-Nakhiloo National Park (DNNP) in the Persian Gulf.

Body part	Number of barnacles	Percentage (%)
Flippers and adjucent soft parts	58,790	85.3
Plastron	6,654	9.7
Carapace	2,613	3.8
Under supracaudals	692	1
Head	156	0.2
Total	68,905	100

knowledge, our study is the first study that has simultaneously assessed macro-, meio-, and micro-epibionts on sea turtles. Some of these epibionts may distinguish groups of sea turtles (see Ingels et al., 2020) and reveal their movement pathways. Sea turtle conservation and management might benefit from research into the identification and origin of epibiont species or communities that are likely to be indicators of feeding or nesting sites.

Our results showed a statistically significant difference in the structure and species composition of epibiont assemblages in the two study sites (**Supplementary Table S1**), with higher species diversity in the Shibderaz at the entrance of the Persian Gulf compared to that of DNNP at the mid part of the sea. We suggest that these differences in turtle epibiont assemblages among different habitats in the Persian Gulf might result from differences in environmental conditions at each study site. Extreme and wide-ranging temperature fluctuations and high salinity in the Persian Gulf have led to the selection of tolerant taxa, which may result in impoverished biodiversity in the region (Sheppard et al., 2010). However, the environmental extremes are not similar in all marine habitats of the sea. The Gulf receives incoming currents from the Gulf of Oman *via* the Strait of Hormuz, which flow counterclockwise through the Gulf and exit *via* the bottom of the Strait (Sheppard et al., 2010). Along the Iranian coastline of the Gulf, temperature and salinity increase with incrementing distance from the Strait (Reynolds, 1993). This is also evident from the temperature and salinity data presented in this study for the sites investigated (**Table 1**). Further, as a result of shape, bathymetry, and wind regime, waters close to the Strait of Hormuz are nutrient-rich (German and Elderfield, 1990; Longhurst et al., 1995).

Azari et al. (2020) studied diatoms on foraging green turtles in the Persian Gulf and found that diatom abundance on turtles collected from the Strait of Hormuz was higher than that of on turtles collected from the Gulf habitats found farther from the Strait. However, their findings were based on green turtles that dwell in foraging habitats, while our study examined hawksbill turtles in their nesting habitats, where they reside temporarily. The results of a previous post-nesting satellite tracking study showed that most of the Gulf hawksbills nesting along the Iranian coastline migrate to foraging grounds in the southeastern Persian Gulf and establish home ranges of 40 to 60 km^2 (Pilcher et al., 2014). The same study revealed that the Gulf hawksbill turtles spend only 6% of their time at the nesting grounds, whereas they spend about 68% in foraging grounds, about 20% conducting summer seasonal movements, and 5% migrating between foraging and nesting areas (Pilcher et al., 2014). Therefore, variable epibiont taxonomic composition at each nesting site is thought to be the outcome of various environmental conditions at the nesting grounds during a short period of time (i.e., about 6% of their time as reported by Pilcher

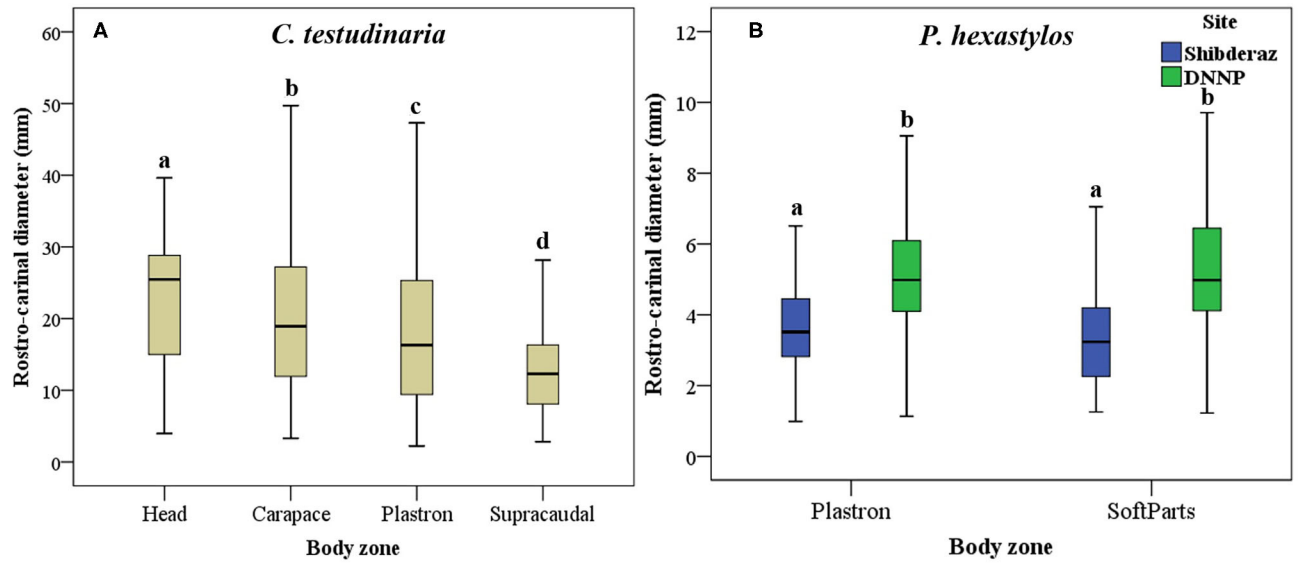

FIGURE 5 | Box plot showing Rostro-Carinal Diameter (RCD) of the turtle barnacles on different body parts of hawksbill turtles (*E. imbricata*) in Shibderaz and Dayyer-Nakhiloo National Park (DNNP): **(A)** *Chelonibia testudinaria* on head, carapace, plastron, and supracaudal and, **(B)** *Platylepas hexastylos* on plastron and soft parts.

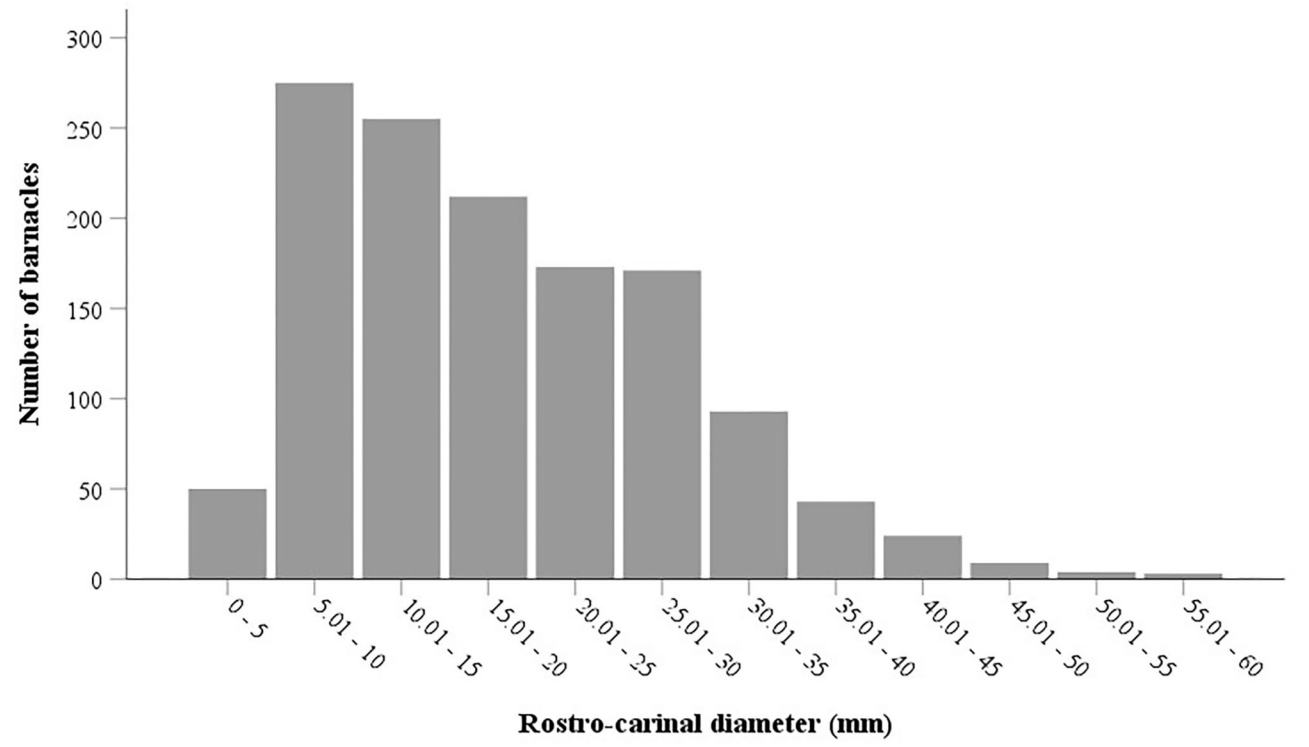

FIGURE 6 | Size frequency distribution of the barnacle *Chelonibia testudinaria* on different body surfaces of the hawksbill sea turtles nesting on Shibderaz and Dayyer-Nakhiloo National Park (DNNP).

et al., 2014). This mostly includes the short-living taxa such as diatoms.

In this research, we found more diverse diatoms on hawksbills and recorded only 11 taxa that were also found by Azari et al. (2020). Although these studies were carried out almost in the same area, the host species was different; hawksbills were studied here whereas green turtles were the focus of Azari et al. (2020). We, therefore, speculate that the difference in diatom species

composition between our study and that of Azari et al. (2020) is partially due to differences in the behavior and local habitat use between these two Gulf turtle species. However, we acknowledge that it could also be a result of annual variations in the abundance and composition of diatom communities.

Some turtle epizoic taxa have a wide geographic distribution. Barnacle species including *C. testudinaria*, *P. hexastylos*, *Stomatolepas* sp., and *S. muricata*, for example, have been found on sea turtles from various locations (see Hayashi and Tsuji, 2008; Fuller et al., 2010; Lazo-Wasem et al., 2011; Casale et al., 2012; Domènech et al., 2015; Robinson et al., 2016, 2019). In addition to barnacles, harpacticoids (especially, *Balaenophilus manatorum*) and the amphipod, *Caprella* sp. have also shown a wide range of distribution on sea turtles (Caine, 1986; Pfaller et al., 2008b; Sezgin et al., 2009; Aznar et al., 2010; Casale et al., 2012; Domènech et al., 2015). The presence of these epibionts is seemingly not affected strongly by local environmental conditions. This wide distribution has also been locally observed in our study shown by the frequency of occurrence of some macro- and meio-epizoic taxa including barnacles (*C. testudinaria* and *P. hexastylos*), harpacticoid copepods, and rotaliid foraminifers. Our results revealed that while macro- and meio-epibiont taxa assemblages are relatively similar at both sites (16 macro- and 4 meio-epibiont taxa at Shibderaz; 18 macro- and 4 meio-epibiont taxa at DNNP, **Table 1**), micro-epibionts (26 taxa at Shibderaz and 6 taxa at DNNP, **Table 1**), represented mostly by diatoms, differ significantly. This was also evident by the high dissimilarity in species composition of micro-epibionts between the two sites (>97%, **Table 3**). We suggest that micro-epibionts may be considered as more sensitive bioindicators.

The most prominent turtle epibionts, barnacles, have shown contrasting spatial patterns on different body parts of studied turtles (Hayashi and Tsuji, 2008; Pfaller et al., 2008b; Fuller et al., 2010; Nájera-Hillman et al., 2012; Razaghian et al., 2019; Robinson et al., 2019). We also found a relative niche partitioning among different barnacle species. *P. hexastylos* individuals were observed mostly on the flippers and soft parts, while *S. transversa* was seen along the plastral sutures and *S. muricata* was mostly embedded in the gaps between scales in the leading edges of the front flippers. *C. testudinaria* showed a wider distribution attaching to both plastron and carapace. These distribution patterns are mainly driven by factors associated with feeding and attachment, including water flow (Pfaller et al., 2008a) and substratum characteristics (Fuller et al., 2010). These factors may also influence the barnacle size as was reflected by the RCD of our measured barnacles. Our results show that the most frequent RCD size range of *C. testudinaria* was 5–10 mm with a unimodal size-frequency distribution probably indicating only a single-age class of barnacles. These results are in line with those of Lim et al. (2020) on the size-frequency distribution of *C. testudinaria* on sea turtles, but are contradictory to Ewers-Saucedo et al. (2015) and Ten et al. (2019) who detected a bimodal size-frequency distribution of *C. testudinaria* in their studies. We speculate that the year-round reproduction of *C. testudinaria* in the Persian Gulf as a warm subtropical sea is the reason for the lack of age classes compared to those from more seasonally affected areas.

In this research, the most abundant barnacle species on sea turtle bodies was *P. hexastylos*. A similar result was also found by Habibi Motlagh et al. (2020) who studied foraging green turtles in the Gulf. Similar to Robinson et al. (2019), we found that barnacle abundance on soft parts, including flippers, neck, and tail was considerably higher than on the carapace and plastron (**Table 4**). In contrast, Razaghian et al. (2019) studied the distribution pattern of epibiont barnacles on nesting hawksbills in DNNP and found that barnacle abundance was much higher on the plastron and carapace than on soft parts. The latter authors did not report *P. hexastylos* in their research but rather introduced only *C. testudinaria* as the epibiont barnacle of the examined turtles. We believe that this might be due to the lack of accurate identification of barnacle species which resulted in the taxonomic assignment of all individuals to *C. testudinaria*. We suggest that, in addition to the hard parts (carapace and plastron), soft parts of sea turtles should also be considered when assessing distribution and abundance of epibionts. Recently, Lim et al. (2020) examined different body parts of hawksbill turtles in Mabul Island (southeastern Sabah, Malaysia). They only examined barnacles larger than 5 mm on the carapace, plastron, and head of the turtles and concluded that *C. testudinaria* mainly settled on the plastron (94.6%) and just a few individuals tended to dwell on the carapace (1.4%) and head (4%). We also found a relatively similar pattern (but with different data values) in the settlement of *C. testudinaria*, with more individuals on the plastron (51%) compared to carapace and head (37 and 2%, respectively). The difference in data values may be a result of differences in the local barnacle larval supply, migratory behavior of turtles, and possibly barnacle removal by local people in some areas.

As a complementary study, these baseline data on turtle epibionts might be highly beneficial for future directions in adopting proper management strategies and making effective conservation decisions for these threatened species. In the face of climate change, the data are highly relevant considering the naturally harsh environment of the Persian Gulf. Furthermore, conducting such qualitative and quantitative assessments as regular monitoring studies can be used to track potential ecological changes in the Gulf. The epibiont assemblages of the two examined nesting turtle rookeries were significantly different, as revealed in this study, and may necessitate separate conservation approaches for the two populations. We encourage assessing epibionts of the other common turtle species in the region, the green turtle, to provide a clearer picture of sea turtle epibionts in the Persian Gulf and to better understand sea turtle habitat use and behavior in the region.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary**

Materials, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because we did the sampling under the Environmental Protection Organization role and received help from their local expert while sampling. As we have done this kind of sampling several times (and have published them), we used totally non-invasive protocols.

AUTHOR CONTRIBUTIONS

JL: conceptualization, methodology, sampling, analyses, and writing the first draft. AN: conceptualization, methodology, analyses, supervision, writing, review, and editing. BK: conceptualization, methodology, and supervision. MR-A: conceptualization, methodology, sampling, supervision, writing, review, and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.690022/full#supplementary-material

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Corrigendum: Epibiont assemblages on nesting hawksbill turtles show site-specificity in the Persian Gulf

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In the published article, there was an error in Figure 2 as published. In the caption, Part **(j)** of the caption should state "*Nitzschia* sp." and not "*Poulinea lepidochelicola.*" The corrected Figure 2 caption appears below.

FIGURE 2. Examples of epibiont taxa recorded on the body surface of hawksbill sea turtles in the Iranian coasts of the Persian Gulf: **(a)** *Chelonibia testudinaria* on the carapace of hawksbill sea turtle; **(b)** specimens of *Stephanolepas muricata*; **(c)** *Chelonibia testudinaria*; **(d)** *Platylepas hexastylos*; **(e)** Tanaid; **(f)** Rotaliid foraminifer; **(g)** *Chaetomorpha* sp.; **(h)** *Polysiphonia* sp.; **(i)** *Psammodictyon* sp.; **(j)** *Nitzschia* sp.; **(k)** *Tabularia* sp.1; **(l)** *Amphora* sp.1

In the published article, there was an error in Table 3 as published. In the text of the table, the systematic group of the epibiont taxon "*Emiliania huxleyi*" was miswritten as Algae: Bacillariophyceae, whereas the correct name is Haptophyta: Isochrysidales. The corrected Table 3 and its caption appear below.

In the published article, we neglected to explain whether all the various micro, meio, and macro epibionts were quantified or not. A correction has been made to **Materials and methods**, *Statistical analysis*, 2. This sentence previously stated:

"The analysis of epibiont structure was based on abundance data whereas species composition was evaluated based on presence-absence data."

The corrected sentence appears below:

"Except for diatoms and other algal taxa, for which only presence-absence data were recorded, the analysis of epibiont structure was based on absolute abundance data.

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TABLE 3 Results of the SIMPER procedure to identify the relative contribution of each epibiont taxa to the dissimilarity between the epibiont assemblages of hawksbills (*Eretmochelys imbricata*) nesting on Shibderaz (Qeshm Island) and Dayyer-Nakhiloo National Park (DNNP; Bushehr) beaches, Iran: (a) all epibionts, (b) micro-epibionts, and (c) macro-epibionts.

Systematic group	Epibiont taxon	Shibderaz vs. DNNP		
		Average dissimilarity	Contribution (%)	Cumulative (%)
a				
Algae: Rhodophyta	Unknown	2.53	7.10	7.10
Mollusca: Gastropoda		2.26	6.32	13.42
Mollusca: Bivalvia		2.02	5.64	19.07
Cnidaria: Hydrozoa	Campanulariidae	1.98	5.55	24.62
Algae: Rhodophyta	*Ceramium* sp.	1.98	5.54	30.16
Algae: Chlorophyta	*Ulva* sp.	1.82	5.11	35.27
Crustacea: Amphipoda	*Hyachelia* sp.	1.76	4.93	40.20
Nematoda		1.74	4.88	45.07
Crustacea: Ostracoda		1.51	4.23	49.31
Algae: Chlorophyta	*Chaetomorpha* sp.	1.49	4.18	53.48
Crustacea: Cirripedia	*Stomatolepas transversa*	1.39	3.90	57.38
Annelida: Polychaeta	Polychaeta	1.37	3.84	61.22
Crustacea: Cirripedia	*Stephanolepas muricata*	1.29	3.61	64.83
Algae	Algae sp. 1	1.28	3.58	68.41
Crustacea: Tanaidacea	Tanaidacea	1.16	3.25	71.66
Foraminifera: Miliolida	*Quinqueloculina* spp.	0.75	2.11	73.77
Algae: Bacillariophyceae	*Amphora ovalis*	0.67	1.86	75.63
Crustacea: Cumacea		0.59	1.64	77.27
Algae: Bacillariophyceae	*Amphora* sp. 1	0.52	1.45	78.72
Foraminifera: Textulariida		0.50	1.41	80.13
Algae: Bacillariophyceae	*Cocconeis scutellum*	0.48	1.34	81.47
Porifera		0.42	1.17	82.64
Haptophyta: Isochrysidales	*Emiliania huxleyi*	0.36	1.00	83.64
Algae: Bacillariophyceae	*Cocconeis* spp.	0.30	0.84	84.48
Algae: Bacillariophyceae	*Achnanthes* spp.	0.27	0.77	85.25
Algae: Bacillariophyceae	*Licmophora* spp.	0.27	0.77	86.02
Algae: Bacillariophyceae	*Navicula* sp. 1	0.27	0.77	86.79
Algae: Bacillariophyceae	*Opephora* sp.	0.22	0.63	87.41
Algae: Bacillariophyceae	*Actinocyclus* sp.	0.22	0.61	88.02
Algae: Bacillariophyceae	*Amphicocconeis* sp.	0.22	0.61	88.63
Algae: Bacillariophyceae	*Amphora coffeiformis*	0.22	0.61	89.23
Algae: Bacillariophyceae	*Berkeleya* sp.	0.22	0.61	89.84
Algae: Bacillariophyceae	*Cocconeis distans*	0.22	0.61	90.45
b				
Algae: Bacillariophyceae	*Cocconeis* spp.	23.28	23.83	23.83
Algae: Bacillariophyceae	*Caloneis* sp.	9.21	9.43	33.26
Algae: Bacillariophyceae	*Amphora* sp. 1	6.97	7.14	40.40

(Continued)

TABLE 3 (Continued)

Systematic group	Epibiont taxon	Shibderaz vs. DNNP		
		Average dissimilarity	Contribution (%)	Cumulative (%)
Haptophyta: Isochrysidales	*Emiliania huxleyi*	6.68	6.84	47.24
Algae: Bacillariophyceae	*Amphora ovalis*	6.65	6.80	54.04
Algae: Bacillariophyceae	*Achnathidium* sp.	5.66	5.80	59.84
Algae: Bacillariophyceae	*Mastogloia horwatiana*	5.66	5.80	65.63
Algae: Bacillariophyceae	*Cocconeis scutellum*	4.67	4.78	70.42
Algae: Bacillariophyceae	*Achnanthes* spp.	3.44	3.53	73.94
Algae: Bacillariophyceae	*Licmophora* spp.	3.44	3.53	77.47
Algae: Bacillariophyceae	*Navicula* sp. 1	3.44	3.53	80.99
Algae: Bacillariophyceae	*Amphicocconeis* sp.	1.98	2.02	83.02
Algae: Bacillariophyceae	*Grammatophora* sp.	1.98	2.02	85.04
Algae: Bacillariophyceae	*Opephora* sp.	1.98	2.02	87.06
Algae: Bacillariophyceae	*Actinocyclus* sp.	1.23	1.26	88.32
Algae: Bacillariophyceae	*Amphora coffeiformis*	1.23	1.26	89.57
Algae: Bacillariophyceae	*Berkeleya* sp.	1.23	1.26	90.83
c				
Algae: Rhodophyta	Unknown	4.11	10.45	10.45
Mollusca: Gastropoda		3.67	9.31	19.76
Algae: Rhodophyta	*Ceramium* sp.	3.26	8.28	28.05
Cnidaria: Hydrozoa	Campanulariidae	3.24	8.23	36.27
Mollusca: Bivalvia		3.23	8.19	44.47
Algae: Chlorophyta	*Ulva* sp.	3.02	7.66	52.13
Crustacea: Amphipoda	*Hyachelia* sp.	2.82	7.17	59.30
Nematoda		2.79	7.09	66.39
Algae: Chlorophyta	*Chaetomorpha* sp.	2.31	5.87	72.26
Crustacea: Cirripedia	*Stomatolepas transversa*	2.27	5.76	78.02
Annelida: Polychaeta		2.16	5.48	83.50
Crustacea: Cirripedia	*Stephanolepas muricata*	2.09	5.30	88.80
Crustacea: Tanaidacea	Tanaidacea	1.83	4.65	93.45

Species composition of the entire epibiont community (including micro, meio, and macro-epibionts) was evaluated based on presence-absence data."

In the published article, we stated *Emiliania huxleyi* was a diatom species. A correction has been made to **Results**, 4. This previously stated:

"The SIMPER analysis revealed 97.68 and 39.37% dissimilarity between the two sites, respectively. Diatom species—including *Cocconeis* spp. (23.83%), *Caloneis* sp. (9.43%), *Amphora* sp. 1 (7.14%), *Emiliania huxleyi* (6.84%), and *Amphora ovalis* (6.80%)—contributed around 54% to the differences of the micro-epibionts (Table 3)."

The corrected sentence appears below:

"The SIMPER analysis revealed 97.68 and 39.37% dissimilarity between the two sites, respectively. Diatom species—including *Cocconeis* spp. (23.83%), *Caloneis* sp. (9.43%), *Amphora* sp. 1 (7.14%), and *Amphora ovalis* (6.80%)—contributed around 47% to the differences of the micro-epibionts (Table 3)."

In the published article, there was an error. Diatoms were the microepibionts focused on in this study and thus should not be described as dominating within this group. A correction has been made to **Discussion**, 5. This sentence previously stated:

"Our results revealed that while macro- and meio-epibiont taxa assemblages are relatively similar at both sites [...], micro-epibionts (26 taxa at Shibderaz and 6 taxa at DNNP, Table 1), dominated by diatoms, differ significantly"

The corrected sentence appears below:

"Our results revealed that while macro- and meio-epibiont taxa assemblages are relatively similar at both sites [...], micro-epibionts (26 taxa at Shibderaz and 6 taxa at DNNP, Table 1), represented mostly by diatoms, differ significantly"

The authors apologize for these errors and state that they do not change the scientific conclusions of the article in any way. The original article has been updated.

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Evidence for Host Selectivity and Specialization by Epizoic *Chelonibia* Barnacles Between Hawksbill and Green Sea Turtles

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Epibionts are organisms that utilize the exterior of other organisms as a living substratum. Many affiliate opportunistically with hosts of different species, but others specialize on particular hosts as obligate associates. We investigated a case of apparent host specificity between two barnacles that are epizoites of sea turtles and illuminate some ecological considerations that may shape their host relationships. The barnacles *Chelonibia testudinaria* and *Chelonibia caretta*, though roughly similar in appearance, are separable by distinctions in morphology, genotype, and lifestyle. However, though each is known to colonize both green (*Chelonia mydas*) and hawksbill (*Eretmochelys imbricata*) sea turtles, *C. testudinaria* is >5 times more common on greens, while *C. caretta* is >300 times more common on hawksbills. Two competing explanations for this asymmetry in barnacle incidence are either that the species' larvae are spatially segregated in mutually exclusive host-encounter zones or their distributions overlap and the larvae behaviorally select their hosts from a common pool. We indirectly tested the latter by documenting the occurrence of adults of both barnacle species in two locations (SE Florida and Nose Be, Madagascar) where both turtle species co-mingle. For green and hawksbill turtles in both locations (Florida: n = 32 and n = 275, respectively; Madagascar: n = 32 and n = 125, respectively), we found that *C. testudinaria* occurred on green turtles only (percent occurrence – FL: 38.1%; MD: 6.3%), whereas the barnacle *C. caretta* was exclusively found on hawksbill turtles (FL: 82.2%; MD: 27.5%). These results support the hypothesis that the larvae of these barnacles differentially select host species from a shared supply. Physio-biochemical differences in host shell material, conspecific chemical cues, external microbial biofilms, and other surface signals may be salient factors in larval selectivity. Alternatively, barnacle presence may vary by host micro-environment. Dissimilarities in scute structure and shell growth between hawksbill and green turtles may promote critical differences in attachment modes observed between these barnacles. In understanding the co-evolution of barnacles and hosts it is key to consider the ecologies of both hosts and epibionts in interpreting associations of chance, choice, and dependence. Further studies are necessary to investigate the population status and settlement spectrum of barnacles inhabiting sea turtles.

Keywords: turtle barnacle, epibiont, assortative epibiosis, substratum specificity, basibiont preference, carapace, Madagascar, Florida (United States)

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INTRODUCTION

Barnacles in the family Chelonibiidae (superfamily Coronuloidea) are common epibionts of sea turtles (Zardus, 2021). Their highly mobile hosts provide them with a substratum that protects them from predators (Foster, 1987) while also aiding their dispersal (Rawson et al., 2003) and supporting their reliance on passive feeding (Lane et al., 2021). Chelonibiid barnacles occupy multiple turtle and non-turtle host species (Zardus et al., 2014) but there has been minimal characterization of differences in their ecological niches and settlement preferences, limiting our understanding of the association between barnacles and hosts. While predominantly associating with marine turtles, chelonibiids have also been observed on other aquatic reptiles including the American alligator, diamondback terrapins, and saltwater crocodilians (Monroe and Garrett, 1979; Seigel, 1983; Nifong and Frick, 2011), as well as manatees (Zardus et al., 2014) and various crabs and other arthropods (Ortiz et al., 2004; Cheang et al., 2013; Ewers-Saucedo et al., 2017).

Barnacles in the genus *Chelonibia* have been evolving as commensals of marine turtles since the late Miocene (Ross, 1963) and several extinct forms illuminate their evolutionary history with diverse hosts (Collareta and Newman, 2020; Collareta et al., 2021). It has recently been recognized that there are but two extant species in the genus, *Chelonibia testudinaria* and *Chelonibia caretta* (Cheang et al., 2013; Zardus et al., 2014), both occurring globally on marine turtles. Though sometimes confused for each other, with informed examination they can usually be readily distinguished. Along with several morphological differences between the two (Monroe, 1981) are distinctions in their attachment modes that leave diagnostic marks on their hosts.

Typical of other acorn (balanomorph) barnacles, *C. testudinaria* (and presumably *C. caretta*) develops through multiple swimming larval stages in the plankton before being able to find a host and becoming competent to attach and metamorphose (Zardus and Hadfield, 2004). The terminal larval stage, the cyprid, searches for a suitable substratum and attaches by gluing down a pair of organs, the antennules, specialized for surface adhesion though, surprisingly, not obviously specialized for adhering to particular surface types (Dreyer et al., 2020). Subsequent to attaching, metamorphosis follows within hours which involves forming a calcareous shell cemented to the substratum. Most barnacles are immovably fixed in place at this point but *C. testudinaria's* capability for slow movements across the substratum allows it to modify its feeding position throughout life (Chan et al., 2021). Paradoxically, despite its mobility, this species is otherwise extremely passive, exhibiting no active feeding behavior as an adult, probably as a consequence of having evolved to live on mobile hosts (Lane et al., 2021). But how the planktonic larvae of both of these barnacles optimize a rendezvous with sea turtles and identify their itinerant hosts remains enigmatic. Available evidence suggests the two likely meet up along coastlines where larvae can become entrained in harbors, embayments, and lagoons (Sloan et al., 2014; Lim et al., 2021) where juvenile and adult sea turtles forage, as opposed to open-ocean locations.

Habitat characteristics for epibionts of marine turtles potentially vary due to differences in host species' behavior and carapace growth and composition. With the exception of leatherback turtles, sea turtle shells are covered with a varying number of enlarged, keratinous epithelial scales known as "scutes," which are known to provide suitable substratum for attachment of a variety of epibionts (Frazier et al., 1991; Scharer, 2001; Frick et al., 2004). However, differences in both scute development and carapace grooming behavior among sea turtle species may influence the type, placement, and persistence of epibiotic growth found on each. In contrast to green turtle scutes that maintain smooth seams along their edges as they expand, the anterior edge of each hawksbill scute subducts the one in front of it, producing the characteristic "imbrication" of the scutes unique to hawksbill turtles (Palaniappan, 2007). Though very little is known of ecdysis in sea turtles, in contrast to green turtles, hawksbills appear not to shed outer layers of their scutes, which consequently thicken over time, making them famous for their particular and unfortunate suitability in the international tortoiseshell trade (Mrosovsky, 2000; Pederson, 2021).

Hawksbill (*Eretmochelys imbricata*) and green sea turtles (*Chelonia mydas*) are globally distributed marine turtles currently listed by the IUCN as "Critically Endangered" and "Endangered," respectively, throughout their ranges (Seminoff, 2004; Mortimer and Donnelly, 2008). Though hawksbills typically prefer coral reef/hard bottom habitats, while green turtles prefer seagrass pastures, the often-close proximity of these habitat types to one another can result in overlapping ranges between the two turtle species (Bjorndal and Bolten, 2010, Wood pers. obs.). Co-occurring populations of green turtles and hawksbills have been documented in the same coastal reef habitats, e.g., south Florida, Turks and Caicos, and Northwestern Indian Ocean (Bourjea et al., 2006; Makowski et al., 2006; Taquet et al., 2006; Wood et al., 2013; Bechhofer and Henderson, 2018). As juveniles and subadults, both hawksbill and green turtles frequently remain in relatively small home ranges for extended periods (10 years+) prior to embarking on reproductive migrations (Berube et al., 2012; Hazel et al., 2013; Wood et al., 2017). The swimming behaviors of adult hawksbills and green turtles are similar, using their foreflippers to propel themselves through the water column and hind flippers for directional movement (Wyneken, 1996). Green turtles are known to actively groom their carapaces with their flippers and/or by rubbing on underwater surfaces, which could strongly influence patterns of epibiotic recruitment (Heithaus et al., 2002, Wood pers. obs.). Symbiosis through mutualistic behaviors exhibited by reef fishes foraging on marine turtle epibionts is another factor that may preclude barnacles from successful settlement (Sazima et al., 2010). Hawksbill individuals have been observed displaying postures that signal fishes to clean their exterior (Grossman et al., 2006) and cleaner fishes have been recorded cleaning the carapace and skin of green turtles as well (Losey et al., 1994; Sazima et al., 2010). Booth and Peters (1972) reported a barnacle removal behavior in moon wrasse, *Thalassoma lunare*, in which individuals targeted skin barnacles for consumption. Further stomach analysis of moon wrasses confirmed the presence of barnacle material as a dietary item. The active removal of epibiota by green

turtle self-cleaning behaviors and symbiotic fishes presents major limitations to the settlement success of various epibiota. As *Chelonibia* barnacles are obligate associates of sea turtles, it is imperative to examine the abundance, distribution, and settlement preferences of these co-evolved symbionts to properly assess their conservation status, particularity in relation to the conservation status of the host sea turtle species. Identifying key host-commensal species relationships is a first step in properly determining turtle barnacle population abundances and distributions.

The objective of the present study was to provide insight into the host preferences of *Chelonibia* barnacles when access to multiple host species was available in the wild. The overlap of habitat use between hawksbills and green turtle in southeast Florida and Nosy Be, Madagascar provided an opportunity for assessing biases in the presence of *C. testudinaria* and *C. caretta* among these two host turtle species. We also related the attachment modes of *C. caretta* and *C. testudinaria* (cementation and down-cutting, respectively) to what is known of scute growth and host behavior in these two turtle species to explore the possibility that one or both of these barnacles is specialized for a particular host.

MATERIALS AND METHODS

Between 2007 and 2020, juvenile and subadult green and hawksbill turtles were captured from co-occurring populations in the nearshore waters of SE Florida United States (Palm Beach through Monroe Counties) (**Figure 1**), and the islands of Nosy Sakatia, Nosy Tanikely, and Nosy Komba, which are part of the Nosy Be Island complex located in the northwest region of Madagascar (**Figure 1**). In Florida, the hawksbills were encountered in 2–26 m of water along the Southeast Florida Continental Reef Tract, a relatively high-latitude reef system with varied community structure that includes reef-building *Acropora* corals in the southern portion (FL Keys), gradually transitioning to algae/sponge/octocoral-dominated habitats near its northern terminus in Palm Beach County (Jaap and Hallock, 1990; Banks et al., 2008). This highly variable, non-uniform seascape is in close proximity to the Florida Current, a branch of the Gulf Stream that

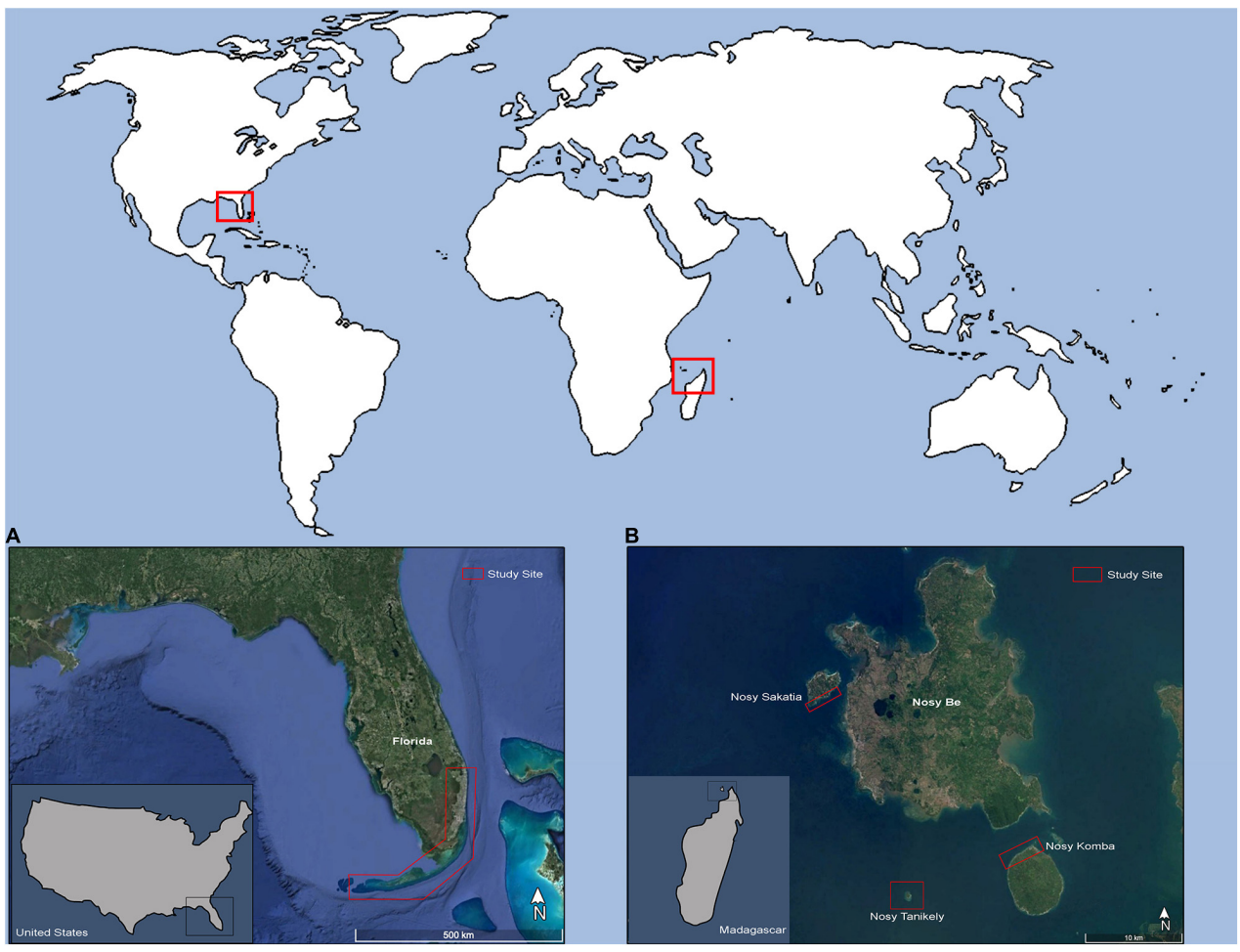

FIGURE 1 | Map of study sites in southeast Florida **(A)** and Nosy Be, Madagascar **(B)**.

carries warm tropical water northward along the SE Florida Coast from the Gulf of Mexico and Caribbean. The green turtles were encountered in 1–3 m of water in Palm Beach County Florida at Lake Worth Lagoon (West Palm Beach, FL) and Jupiter Inlet (Jupiter, FL), two well-flushed seagrass-dominated tidal lagoons with open-ocean access via nearby major navigable inlets.

In Madagascar, green turtles were observed at a shallow, seagrass shoal with surrounding fringing reefs on the coast of Nosy Sakatia with a tidal range depth of approximately 0–4 m (McKenna and Allen, 2003). Juvenile and adult male and female green turtles forage and rest at this site (Sagar, 2001). Adult and sub-adult hawksbill turtles were encountered between 2 and 8 m of water in shallow coastal coral reef systems off the coasts of Nosy Tanikely and Nosy Komba. Fringing reefs in these areas are dominated by Acropora corals; however, live coral coverage has decreased around 20% since 1998 with significant changes every 4 of 5 years due to isolated coral bleaching events (Webster and McMahon, 2002; McKenna and Allen, 2003; Obura, 2012; Obura et al., 2017). In the northwest of Madagascar predominant currents move in a northward direction toward Mozambique in a counterclockwise direction (McKenna and Allen, 2003). While captured at different locals within the Nosy Be Island complex, sea turtle species were found to co-occur within each island, particularly at Nosy Sakatia and Nosy Komba, most likely due to the nearshore seagrass beds that are more extensive at these two islands (Knauer pers. obs.).

Depending on water depth, turtles were either dip-netted from a boat or hand-captured with the use of snorkel or SCUBA gear, with hand-capture via snorkeling being the only method of capture for Madagascar turtles. Turtles were brought up onto the boat and the incidence of two barnacle species (*C. testudinaria* and *C. caretta*) on the carapaces of green and hawksbill turtles were quantified and recorded from photographs taken directly above each subject (**Figure 2**). Photos were analyzed to enumerate barnacle abundance. Data was analyzed in Excel and RStudio. The abundance of *C. caretta* on hawksbill carapaces and *C. testudinaria* on green turtle carapaces between the two study sites were compared using a Welch's two-sample *t*-test ($\alpha = 0.05$).

RESULTS

Discriminating *Chelonibia testudinaria* from *C. caretta* was possible from photographs because in the former, wall sutures widen upward and the parieties become splayed at their tips with radii extended in between; whereas, in the latter species the seams between the parieties remain pressed close together with no radii visible but with alae visibly underlapping the parities at their apex (**Figure 3**). *Chelonibia testudinaria* attaches via adhesive cementation of its basal membrane which spreads underneath an even, supporting platform made of numerous

FIGURE 2 | Representative photographs of hawksbill sea turtle (left) and green sea turtle (right) from the present study with presence of barnacle species (*C. caretta* and *C. testudinaria*, respectively).

FIGURE 3 | The scute-attaching turtle barnacles: *Chelonibia testudinaria* collected from a green sea turtle in Japan (specimen courtesy of Hiroyuki Suganuma), apical view **(A)** and basal view **(B)**; and *C. caretta* collected from a hawksbill sea turtle in Barbados (specimen courtesy of Marina Fastigi), apical view **(C)** and basal view **(D)**. Scale bars = 2 cm.

septal wall termini (**Figure 3B**). Its adhesion is impermanent which, uniquely among barnacles, facilitates slow movement by this species across its substratum (Moriarity et al., 2008; Chan et al., 2021). *Chelonibia caretta* on the other hand, though also attaching by cementation and possessing a similar construction with membranous base, has a less expansive basal platform that has sharp marginal edges (**Figure 3D**) that down-cut into the carapace for permanent, entrenched attachment. Attachment by *Chelonibia* barnacles can leave marks on host scutes that also distinguish the species. *Chelonibia testudinaria*, which cements superficially to the surface and is facultatively mobile, sometimes leaves behind harmless "skid" marks or traces of trailing adhesive on the surface (**Figure 4**) while *C. caretta*, which has a more invasive form of attachment, can leave behind physical indentations or incisions, even cutting entirely through the scutes at times (**Figure 5**).

In both study sites of mixed turtle species, the barnacle *C. caretta* occurred only on hawksbills. Its incidence was highest in Florida where it was hosted by 82.2% of hawksbills (n = 275) compared to 27.5% in Madagascar (n = 120) (**Figure 6**). The other species of barnacle, *C. testudinaria*, in both localities occurred exclusively on green turtles where its percent occurrence on green turtles was 38.1% in Florida (n = 21) compared to 6.3% in Madagascar (n = 32) (**Figure 6**). The abundance of *C. caretta* on hawksbills in Madagascar ranged from 0 to 15 barnacles per turtle with an average of 0.98 ± 0.21 (SE) (**Figure 7A**). In Florida, barnacle abundance ranged from 0 to 65 barnacles per hawksbill with an average of 12.01 ± 0.81 (SE). The results of the Welch's two sample t-test between the abundance of *C. caretta* on hawksbill carapaces in the two study sites was significantly different (t_{310} = −13.23, p = <0.0001). The abundance of *C. testudinaria* on green turtles in Madagascar ranged from 0 to 1 individuals per turtle with an average of 0.08 ± 0.05 (SE) barnacles (**Figure 7B**). In Florida, the range was from 0 to 6 with an average of 0.86 ± 0.33 (SE) barnacles. The difference in mean abundance of *C. testudinaria* on green turtle carapaces in the two study sites was not significant (t_{35} = 0.29, p = 0.77).

DISCUSSION

In the mixed stocks of green and hawksbill sea turtles in this study, the epizoic barnacles *C. caretta* and *C. testudinaria* exhibited strongly contrasting biases in host occupancy. This follows a general pattern described by Zardus (2021) globally in which both species of barnacles have been reported on both species of turtles, but *C. caretta* is almost always more abundant on hawksbills and infrequent on greens or other sea turtles, while *C. testudinaria* is common on most other sea turtles but less so on hawksbills.

Though exact drivers remain unknown, differences in host utilization by these barnacle species may be due to preferences at larval settlement, to various post-settlement selection pressures, or some combination of the two. If larvae of these barnacles preferentially select their substratum, this raises the question of what in the surface features or surface environments of

FIGURE 4 | Traces of adhesive cement left by the barnacle *Chelonibia testudinaria* on the thin carapacial scutes of a green sea turtle, demonstrating the non-destructive attachment and movements of this mobile barnacle (photo made possible by the South Carolina Aquarium, United States).

green and hawksbill turtles differs and what cues do *Chelonibia* barnacles detect at attachment? Settlement signals for larval barnacles, though intensively studied, are not exhaustively defined. Seemingly tuned less to the material composition of a substratum (Pomerat and Weiss, 1946; Lohse, 1993), barnacles are generally more responsive to physical properties such as texture, hydrophobicity, and surface flow (Crisp, 1955; Wethey, 1986; Mullineaux and Butman, 1991; Di Fino et al., 2014), and especially to chemical cues, either from other attached barnacles (Gabbott and Larman, 1987; Matsumura et al., 1998; Ferrier et al., 2016) or from microbial biofilms (Neal and Yule, 1994; Lau et al., 2005; Dreanno et al., 2006; Bacchetti de Gregoris et al., 2012; Siddik and Satheesh, 2019). It is highly conceivable that *Chelonibia* barnacles are able to detect and discriminate between hosts chemically. However, host detection by chemoreception in barnacles has rarely been demonstrated and is not known for *Chelonibia*. In the few studies demonstrating this phenomenon, Pasternak et al. (2004a,b) have confirmed that the cyprids of barnacles commensal with corals and parasitic with crabs can track host chemical plumes in flow and Nogata and Matsumura (2006) have shown that whale-barnacle cyprids successfully metamorphose in petri dishes supplied with bits of whale skin over dishes of plain seawater.

Alternatively for *Chelonibia*, host selectivity at the larval stage, though certainly operating at least at the level of choosing a turtle, may be subordinate to survivorship at the adult stage. Turtle behavior, where and how they forage, and whether they self-groom or not, may have the greater influence on barnacle distribution patterns. Green turtles are known for actively swiping their carapaces with their flippers and rubbing against reefs and rock ledges to remove epibiota (Parrish, 1958; Limpus, 1980; Heithaus et al., 2002), while hawksbills typically do not engage in such behavior. The lower aspect, domed shell of *C. testudinaria*, and its temporary, peripatetic attachment may better suit it to host-grooming activities whereas the higher aspect, immobile *C. caretta* may survive better on a non-grooming host. Additionally, post-settlement pressures on barnacle survival may include diet. As suspension feeders, these barnacles may acquire some or much of their nutrition from their hosts' foraging spillover, either obtaining food items from turtles directly or indirectly from material resuspended by host feeding activities. Thus, the diet and/or foraging habitat of each host turtle may differentially influence the sustenance of their barnacle epibionts. Despite these factors, it does seem improbable that post-settlement selection would result in absolute removal of only particular barnacles from both hosts.

FIGURE 5 | Empty shells of the barnacle *Chelonibia caretta* entrenched in the thick carapacial scutes of a deceased hawksbill sea turtle, demonstrating the destructive downward cutting action of the barnacle shell margin (photo courtesy of Nicolas Winkler).

Rather, the remarkable, mutually exclusive pattern of barnacle occurrence we observed suggests that larval selectivity is the primary cause of this pattern. Because we did not observe any small barnacles (i.e., recently settled individuals), perhaps due to limitations of photographic analysis, we did not compare patterns relative to barnacle size; which, if such individuals had been present might have provided further insight. Timing of larval development and recruitment for *Chelonibia* likely varies with latitude but is imprecisely known. In Charleston, South Carolina, United States, latitude 32.8° N, recruitment has been recorded for *C. testudinaria* in early spring (Sloan et al., 2014). In tropical locales reproduction may occur year-round but at Mabul Island, Malaysia, 4.3° N, barnacle size classes for *C. testudinaria* were larger in May than November, suggesting recruitment periodicity (Lim et al., 2021). Barnacles can also settle on the plastron of turtles (Hayashi and Tsuji, 2008), and in several cases have been found to do so more abundantly there than on the carapace (Ling and Palaniappan, 2011; Razaghian et al., 2019; Loghmannia et al., 2021). But, limited to photographing just the carapace in this study, we were unable to assess occurrence on the entirety of each host which could conceivably alter observed patterns.

If these barnacles are indeed adapted for particular hosts as we suspect, at least in the case of *C. caretta* with hawksbills, regardless of selection occurring either at the larval or adult stage, what advantage does host specificity provide them? The simplest answer is that each is optimized for retaining their attachment on their respective hosts. The thick, enduring scutes of hawksbill turtles and the thinner, deciduous scutes of green and other sea turtles may have been the primary selective agent in shaping the attachment modes of these barnacles. In general, barnacles secrete a very strong adhesive cement (Liang et al., 2019) which makes them suited to turtle shell and keeps them well-secured to their substratum. Thick scutes in hawksbills may have influenced entrenched attachment (and possibly greater longevity) in *C. caretta*, while intermittent shedding of relatively thin scute layers by green sea turtles may have promoted temporary adhesion and mobility in *C. testudinaria*. The dynamics and periodicity of scute shedding in sea turtles generally is an understudied aspect of their biology that requires further understanding. Apart from attachment, niche specialization in these barnacles may also be advantageous by reducing interspecific competition for space and food while also improving access for mating. Typically hermaphroditic,

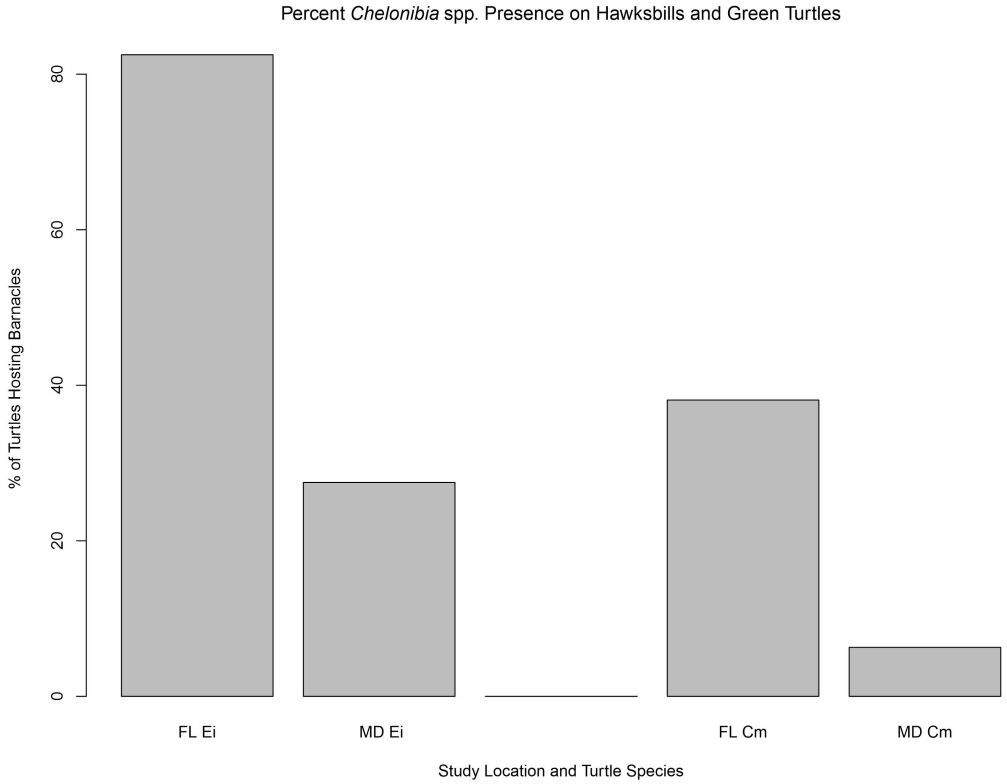

FIGURE 6 | Percent occurrence of *Chelonibia* barnacles per host species per geographic location, for hawksbill (Ei) and green turtles (Cm) in Florida (FL) and Madagascar (MD). At both locations, hawksbill turtles hosted the barnacle *C. caretta* only and green turtles only *C. testudinaria*.

acorn barnacles are not self-fertile and must cross-copulate with neighboring individuals for reproduction (Anderson, 1994). Eliminating other species as a physical barrier could increase chances for mating. Surprisingly, the species with mobility, *C. testudinaria*, has the most versatile mating system comprised of tiny complemental males, sometimes many in number, that attach to and ride around with larger hermaphrodites (Zardus and Hadfield, 2004). *Chelonibia caretta* on the other hand is not known for complemental males, but we have observed it is more commonly found in aggregations of multiple hermaphrodites with shell plates fused together.

For future investigation, it would be valuable to know how feeding and growth vary between these species of barnacles. *Chelonibia caretta* does not become as large as *C. testudinaria* and perhaps entrenched attachment is a factor that limits its size. Not growing as wide as *C. testudinaria* either, it does, however, grow as tall or taller. Along these lines, it would be interesting to know how it expands its base while also growing entrenched. Life expectancies may also vary between these species. *Chelonibia testudinaria* lives approximately 2 years (Doell et al., 2017) but ages for *C. caretta* are not known, yet by being a hawksbill specialist, *C. caretta* may have a significantly longer lifespan and perhaps slower growth rate than its congener. In addition to growth, identifying the reproductive period of each species in areas where they co-occur would help in knowing if their larval stages develop simultaneously. Even better would be to identify their larval distribution in the plankton, perhaps by molecular genetic methods (Chen et al., 2013).

Investigating epibiont occurrence within co-occurring populations of multiple turtle species is a valuable but uncommon approach to understanding selectivity of epibionts (Robinson et al., 2017). Examining larger spatial scopes and different assemblages of hosts would help provide a more complete perspective of barnacle epibiosis of marine turtles. Expanding the area of study beyond exclusively the carapace would provide a more holistic understanding of barnacle settlement on sea turtle individuals. Indeed, differences in settlement abundance on the carapace, plastron and facial scales has been documented for barnacles on some turtles (Hayashi and Tsuji, 2008; Ling and Palaniappan, 2011; Razaghian et al., 2019; Chan et al., 2021; Loghmannia et al., 2021), though the meaning of these patterns remains elusive. Loggerhead sea turtles are another species known to host a diverse array of marine epibionts, including *C. testudinaria* and *C. caretta* (Caine, 1986; Zardus, 2021). Cross comparisons of barnacle assemblages in sites where green, loggerhead, and hawksbills are all present would be a valuable contribution. But settlement choice experiments in the laboratory would address the question of larval selectivity more directly and potentially provide the most definitive answers.

Because larval distribution of these epizoic barnacles is presumably limited to the ranges and source populations of

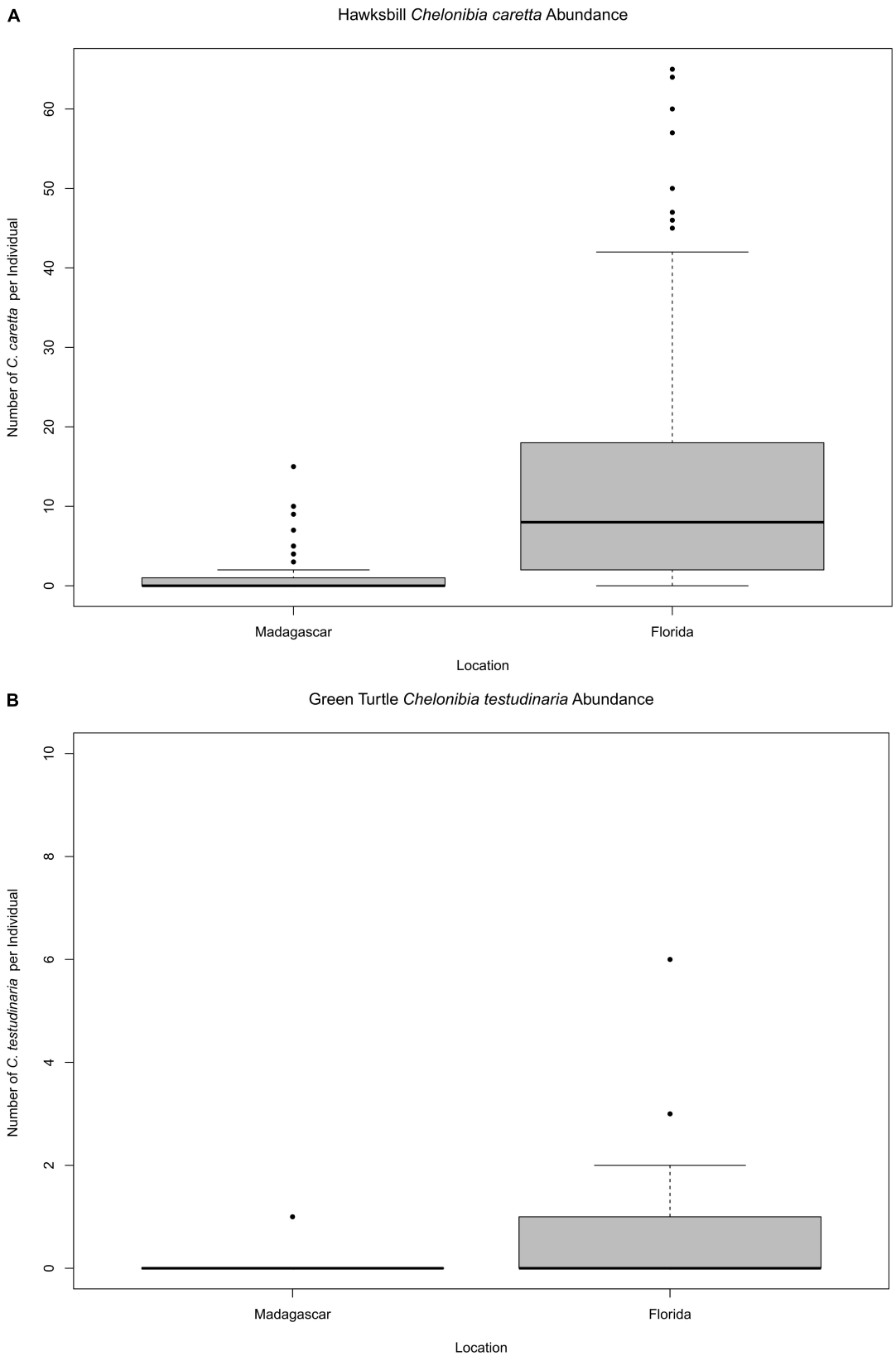

FIGURE 7 | Quantity of respective barnacles present on **(A)** hawksbill and **(B)** green turtles in Madagascar and Florida.

their sea turtle hosts, declines of hawksbill and green sea turtles may be of consequence to them, particularly *C. caretta* whose hawksbill host populations have diminished by over 80 percent over the last several hundred years (Mortimer and Donnelly, 2008). *Chelonibia testudinaria,* which associates with all sea turtle species (Zardus, 2021) and even some non-turtle hosts (Zardus et al., 2014), has greater substratum choice and widespread occurrence and may be at less peril. Intra-oceanic host migrations undoubtedly assist in genetically diversifying their associated epibiota across widely dispersed populations, and further understanding the degree of population connectivity of the epibionts of sea turtles is crucial to evaluating their conservation status.

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, Florida International University Office of Research Integrity.

AUTHOR CONTRIBUTIONS

LB, JZ, and LW contributed to the original concept and design of the study. LB, CK, and LW collected data from the field. LB conducted the data analysis and led the manuscript. All authors wrote and edited the manuscript.

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Corrigendum: Evidence for Host Selectivity and Specialization by Epizoic *Chelonibia* Barnacles Between Hawksbill and Green Sea Turtles

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Occurrence and Molecular Characterization of Some Parasitic Copepods (Siphonostomatoida: Pandaridae) on Pelagic Sharks in the Mediterranean Sea

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Copepods of the family Pandaridae are typically ectoparasites of elasmobranch fishes. They display a cosmopolitan distribution and limited host specificity. Published literature on their occurrence on pelagic sharks in the Mediterranean is scarce, often from the past century, or scattered through fish parasite surveys. Moreover, of the 64 valid pandarid species known at present, molecular data from GenBank exists for only 10 species and there are no data from the Mediterranean. In this study, we begin addressing this knowledge gap by exploring the molecular features of some pandarid copepods (i.e., *Dinemoura latifolia*, *Echthrogaleus coleoptratus, Pandarus satyrus,* and *Phyllothyreus cornutus*) and their phylogenetic relationships using new material from pelagic sharks (i.e., *Prionace glauca*, *Isurus oxyrinchus*, and *Carcharodon carcharias*) in the Mediterranean. Genetic distances analysis showed intraspecific variation in the mitochondrial DNA cytochrome oxidase *c* subunit 1 (mtDNA *cox*1) sequences and interspecific variations of 0.001–0.081 and 0.196–0.288, respectively, for the small subunit ribosomal DNA (SSU rDNA) and the *cox*1 gene locus. Phylogenetic analyses of pandarid copepods based on sequences available in GenBank plus the sequences generated by our study revealed two major clades: the first, with strong nodal support, included species of *Pandarus*, *Phyllothyreus*, *Pannosus,* and *Pseudopandarus*; the second, with weaker nodal support, included species of *Achtheinus*, *Perissopus*, *Echtrogaleus, Nesippus*, and *Dinemoura.* As most pandarid species are missing from the present analyses, we discuss the limitations of our phylogenetic results. Nevertheless, this study represents a first step toward to yielding new information about the phylogeny of parasitic copepods on pelagic sharks in the Mediterranean.

Keywords: parasitic copepods, Pandaridae, *Carcharodon carcharias*, *Isurus oxyrinchus*, *Prionace glauca*, molecular identification, phylogenetic analysis

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INTRODUCTION

The Order Siphonostomatoida Thorell, 1859 includes 39 families of copepods and encompasses about 75% of all parasitic copepods on fishes (Gunn and Pitt, 2012). Members of the family Pandaridae Edwards, 1840, are typically parasites of external surfaces of elasmobranchs (Kabata, 1979; Izawa, 2010; Bernot and Boxshall, 2017). Pandaridae consists of 23 valid genera with at least

64 recognized species (Walter and Boxshall, 2021). Pandarid life cycles have been not elucidated, however, a life cycle similar to that of Caligidae Burmeister, 1835 has been proposed based on the close phylogenetic relationships between these taxa (Huys et al., 2007; Dippenaar, 2009). The supposed life cycle of Pandaridae includes two free-living nauplius stages, one infective copepodid stage, four parasitic chalimus stages, two parasitic preadult stages, and the parasitic adult stage (Wilson, 1907).

Pandarid copepods are characterized by attachment organs named adhesion pads (Kabata, 1988). Their adhesive surface is formed by a thick cushion of skin with a ridged outer layer (Wilson, 1907). The site of attachment on the host varies depending on tissue tropism and the fundamental niche of the parasite species; they can colonize fins, gills, the cloacal aperture, the mouth, or nasal passages (Benz, 1981, 1986; Rokicki and Bychawska, 1991). Pandarid species are cosmopolitan in their distribution, occurring in warm and temperate waters with most of the species capable of parasitizing more host species (Alvarez and Winfield, 2001).

Published literature on the occurrence of pandarid copepods on pelagic sharks in the Mediterranean is scarce, often from the past century, or scattered through fish parasite surveys (Brian, 1906; Öktener and Trilles, 2009; Öktener et al., 2020). According to the most recent studies, members of Siphonostomatoida remain largely unexplored in terms of their molecular characterization and phylogenetic relationships (Dippenaar, 2009; Bernot et al., 2021). In particular, of the 64 valid pandarid species listed at present, molecular data from GenBank exists for only 10 species and there are no data from the Mediterranean. The present study aimed to report the occurrence (and characterize using a molecular approach) of pandarid copepods obtained opportunistically on shark species off the coast of Sicily (Italy) and to provide newly generated molecular and phylogenetic data to improve knowledge of the poorly known Pandaridae parasites infecting sharks.

MATERIALS AND METHODS

Sampling and Parasitological Analysis

The material here studied comprised undetermined copepod parasites collected by two co-authors (GI and BZ) under the framework of a project of the Museo Civico di Storia Naturale (MSNC) in Comiso on non-native and rare marine species of the Mediterranean Sea (see Katsanevakis et al., 2020; Deidun et al., 2021). The MSNC is a scientific institution registered at the CITES Secretariat, D.M. 23.03.1994 (Cod. IT030), authorized to take, keep, use and display dead endangered fauna.

The present material encompassed copepod parasites collected from 2003 to 2021 from the coast of Sicily on six pelagic sharks [i.e., three blue sharks, *Prionace glauca* (Linnaeus, 1758), two shortfin mako sharks, *Isurus oxyrinchus* Rafinesque, 1810 and one great white shark, *Carcharodon carcharias* (Linnaeus, 1758)]. The blue sharks were from strandings; the shortfin mako sharks and the great white shark were caught as bycatch (**Table 1**).

The taxonomic identification of sharks followed Compagno (1984). The fishes were weighed, measured (total length) to the nearest 0.1 cm and sexed by visual observation of external characteristics. Copepods from the skin were carefully removed using forceps while gills were removed from carcasses and examined for copepods in Petri dishes under a stereomicroscope. Copepod parasites were counted, washed in physiological saline, and preserved in 70% ethanol (Santoro et al., 2014, 2020). For identification, copepods were sent to the Stazione Zoologica Anton Dohrn in Naples where they were studied using a stereomicroscope and an optical microscope both equipped with the ZEN 3.1 imaging system (Zeiss). Morphological identification of copepods followed the identification keys of Lewis (1966) and Cressey (1967, 1968). After examination, the sharks were prepared and incorporated into the museum collections of the MSNC under inventory numbers as listed in **Table 1**, except the blue shark #2 which was a live individual rescued, rehabilitated, and released back into the wild after the external examination.

Molecular and Phylogenetic Analyses

Following the morphological identification, genomic DNA was extracted from the antennae of six specimens of *D. latifolia*, collected from a shortfin mako shark (n = 3) and a great white shark (n = 3), and two specimens of *Echthrogaleus coleoptratus* (Guérin-Méneville, 1837), three specimens of *Pandarus satyrus* Leach, 1816 and two specimens of *Phyllothyreus cornutus* (Milne Edwards, 1840), collected from the blue shark. Genomic DNA extraction was performed using a Quick-gDNA Miniprep Kit (ZYMO RESEARCH), following the manufacturer-recommended protocols, with modification of the incubation period with proteinase K to 3 h.

The small subunit ribosomal DNA (SSU rDNA) (∼1,795 bp) was amplified using the primers 18Sf (5′-TACCTGGTTGATCCTGCCAG-3′) and 18Sr (5′-TAATGA TCCTTCCGCAGGTTCAC-3′) (Huys et al., 2007). The partial sequence of the mitochondrial cytochrome *c* oxidase subunit 1 (mtDNA *cox*1) (∼600 bp) was amplified using the primers LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer et al., 1994). Both polymerase chain reactions (PCRs) were performed in a 25 μL volume containing 0.6 μL of each primer 10 μM, 2 μL of MgCl2 25 mM (Promega), 5 μL of 5 × buffer (Promega), 0.6 μL of dNTPs 10 mM (Promega), 0.2 μL of Go-Taq Polymerase (5 U/μL) (Promega) and 2 μL of total DNA. PCR temperature conditions for the SSU rDNA were the following: 94°C for 5 min (initial denaturation), followed by 35 cycles at 94°C for 30 s (denaturation), 57°C for 30 s (annealing), 72°C for 30 s (extension) and followed by post-amplification at 72°C for 5 min. PCR cycling parameters for the mtDNA *cox*1 amplifications were: 95°C for 5 min (initial denaturation), followed by 40 cycles at 95°C for 1 min (denaturation), 45°C for 1 min (annealing), 72°C for 1 min (extension) and followed by post-amplification at 72°C for 7 min. PCR amplicons were purified using the AMPure XP kit (Beckman coulter) following the standard manufacturer-recommended protocol and Sanger sequenced from both strands, with the same primers, through an Automated Capillary Electrophoresis Sequencer 3730 DNA Analyzer (Applied Biosystems), using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies).

TABLE 1 | Available data of shark individuals examined for pandarid copepods from the coast of Sicily.

ID (MSNC*inventory number)	Stranding date	Stranding locality	Total length (cm)	Sex	Weight (Kg)	Parasites (n females/n males)	Site on the host
Great white shark *Carcharodon carcharias* (MSNC 4636)	August 20, 2003	Cava d'Aliga (Ragusa)	122	f	10.8	*Dinemoura latifolia* (6 f/1 m)	Skin around the pelvic fins
Shortfin mako shark *Isurus oxyrinchus* 1 (MSNC 4848)	May 23, 2020	Ognina di Catania (Catania)	318	f	350	*Dinemoura latifolia* (14 f/2 m)	Skin around the pelvic fins
Isurus oxyrinchus 2 (MSNC 4638)	May 2, 2017	Marzamemi (Siracusa)	104	f	10	*Dinemoura latifolia* (1 f)	Skin around the pelvic fins
Blue shark *Prionace glauca* 1 (MSNC 4768)	April 4, 2010	Port of Milazzo (Messina)	310	m	130	*Phyllothyreus cornutus* (5 f/1 m)	Gills
Prionace glauca 2 (released back into the wild)	August 27, 2020	Pozzallo (Ragusa)	312	f	120	*Echthrogaleus coleoptratus* (2 m/2 f); *Pandarus satyrus* (10 f/1 m)	Skin
Prionace glauca 3 (MSNC 4850)	April 4, 2021	Marina di Ragusa (Ragusa)	250	m	63.1	*Pandarus satyrus* (9 f/2 m)	Skin

**MSNC, Museo Civico di Storia Naturale of Comiso.*

Contiguous sequences were assembled and edited using MEGAX v. 11 (Kumar et al., 2018). Sequence identity was checked using the Nucleotide Basic Local Alignment Search Tool (BLASTn) (Morgulis et al., 2008). The SSU and *cox*1 data sets were, respectively, aligned with all sequences of Pandaridae available in GenBank (see **Table 2**), using ClustalX v. 2.1 (Larkin et al., 2007). Sequences of both genes (SSU + *cox*1) were concatenated using SequenceMatrix (Vaidya et al., 2011), while the best partition schemes and best-fit models of substitution were identified using Partition Finder (Lanfear et al., 2012) with the Akaike information criterion (AIC; Akaike, 1973). The analyses were performed using the GTR + invgamma substitution model.

Sequences obtained in the present study were deposited in GenBank under the accession numbers as listed in **Table 2**. Genetic distances were computed using the Kimura 2-Parameters (K2P) model (Kimura, 1980) with 1,000 bootstrap re-samplings, by MEGA Software, version 7.0.

The phylogenetic trees were constructed using the maximum likelihood (ML) method by IQ-TREE (Nguyen et al., 2015) with 1,000 ultrafast bootstrap replicates (BS). Clades were considered to have high nodal support if the ML bootstrap resampling $\geq$ 70%. Due to the phylogenetic position of Pandaridae (see Dippenaar, 2009), the phylogenetic trees were rooted using *Alebion* Krøyer, 1863, as outgroup. The SSU and *cox*1 sequences from GenBank included in the phylogenetic trees are listed in **Table 2**. To corroborate the taxonomic assessment obtained according to the phylogenetic species concept, the species delimitation method on *cox*1 gene locus was also performed using the assemble species by automatic partitioning (ASAP) (Puillandre et al., 2020).

RESULTS

General Data

Available data from shark individuals examined for pandarid copepods, and species, number, and sex of pandarid copepods found are listed in **Table 1**. A total of four species of pandarid copepods were morphologically identified. A single species (i.e., *D. latifolia*) (**Figures 1A,B**) was found on the skin surfaces of the shortfin mako shark and great white shark, and three species (i.e., *E. coleoptratus*, *P. satyrus and Ph. cornutus*) (**Figures 1C–H**) were collected on the skin and gills of the blue shark (**Table 1**). Voucher specimens have been deposited in the collection of the Stazione Zoologica Anton Dohrn in Naples with the following accession numbers: SZN-CRUOO2A-2B (*D. latifolia*), SZN-CRU003A-3B (*P. satyrus*), SZN-CRUOO4A-4B (*Ph. cornutus*) and SZN-CRU005A-5B (*E. coleoptratus*).

Molecular and Phylogenetic Analyses

High quality sequences for both SSU and *cox*1 gene loci were successfully obtained for *D. latifolia*, *E. coleoptratus,* and *P. satyrus*. High quality sequences for *Ph. cornutus* were obtained only for SSU rDNA. The six SSU gene locus sequences obtained from *D. latifolia* collected from both the shortfin mako shark and the great white shark were identical to each other, and all sequences showed 100% similarity with the sequence (DQ538501) of *D. latifolia* available in GenBank. The present six *cox*1 sequences of *D. latifolia* showed 83–84% similarity with that (KF483702) of *Caligus robustus* Bassett-Smith, 1898, available in GenBank. Unfortunately, no sequence of *D. latifolia* for the *cox*1 gene locus was retrieved from GenBank for comparison.

The sequence of the SSU rDNA obtained from *Ph. cornutus* showed 100% similarity with the sequence (FJ447449) of *Ph. cornutus* previously deposited in GenBank. Sequences of *E. coleoptratus* and *P. satyrus* were here generated for the first time. The SSU and *cox*1 sequences obtained for *E. coleoptratus* showed 98.38 and 86.71% similarity with the sequences of *Achtheinus oblongus* Wilson, 1908 (FJ447452) and *Caligus mutabilis* Wilson, 1905 (KF483685) available in GenBank, respectively. The present SSU and *cox*1 sequences of *P. satyrus* showed 99.88% and 87% similarity with the sequences of *Pandarus* sp. 2 (FJ447454-FJ447387) available in GenBank, respectively.

Pairwise distances among specimens and species for the obtained SSU and *cox*1 sequences are given in **Table 3**. While

TABLE 2 | Species, host, locality, and accession numbers of *cox*1 and SSU sequences of pandarid copepods included in the phylogenetic analysis shown in **Figure 2**.

Species	Host	Locality	*cox*1	SSU	References
Achtheinus oblongus	*Carcharodon carcharias*	South Africa	FJ447385	FJ447452	Dippenaar, 2009
Dinemoura latifolia	-	-	-	DQ538501	Huys et al., 2007
Dinemoura latifolia	*Isurus oxyrhinchus*	Mediterranean Sea	MZ934715 OL415941-42	MZ935642 OL333874-5	This study
Dinemoura latifolia	*Carcharodon carcharias*	Mediterranean Sea	OL415938-40	MZ935643 OL333872-3	This study
Echtrogaleus coleoptratus	*Prionace glauca*	Mediterranean Sea	OL348230-1	MZ935645 OL333879	This study
Nesippus crypturus	*Sphyrna mokarran*	South Africa	FJ447379	FJ447444	Dippenaar, 2009
Nesippus orientalis	*Carcharodon carcharias*	South Africa	FJ447383	FJ447448	Dippenaar, 2009
Nesippus vespa	*Rhina ancylostoma*	South Africa	FJ447378	FJ447443	Dippenaar, 2009
Pandarus satyrus	*Prionace glauca*	Mediterranean Sea	OL457303-5	OL333876-8	This study
Pandarus smithi	-	-	-	DQ538502	Huys et al., 2007
Pandarus sp. 1	*Carcharias taurus*	South Africa	FJ447390	FJ447457	Dippenaar, 2009
Pandarus sp. 2	*Sphyrna lewini*	South Africa	FJ447387	FJ447454	Dippenaar, 2009
Pandarus sp. 3	*Carcharodon carcharias*	South Africa	FJ447388	FJ447455	Dippenaar, 2009
Pandarus sp. 4	*Isurus oxyrhinchus*	South Africa	FJ447391	FJ447458	Dippenaar, 2009
Pannosus japonicus	*Sphyrna lewini*	South Africa	FJ447384	FJ447450	Dippenaar, 2009
Phyllothyreus cornutus	*Isurus oxyrhinchus*	South Africa	-	FJ447449	Dippenaar, 2009
Phyllothyreus cornutus	*Prionace glauca*	Mediterranean Sea	-	OL333880 MZ935644	This study
Perissopus dentatus	*Carcharhinus obscurus*	South Africa	FJ447386	FJ447453	Dippenaar, 2009
Pseudopandarus longus	*Carcharhinus obscurus*	South Africa	-	FJ447451	Dippenaar, 2009
Alebion sp. (outgroup)	*Carcharhinus obscurus*	South Africa	FJ447377	FJ447442	Dippenaar, 2009

no intraspecific variations were found between SSU sequences, intraspecific variations were found in the *cox*1 sequences of *D. latifolia* (K2P = 0.008 ± 0.003) and *P. satyrus* (0.0032 ± 0.002). SSU sequence divergence among species (i.e., interspecific variation) was found to range from a minimum of 0.001 ± 0.000 between *P. satyrus* and *Pandarus* sp. 4 to a maximum of 0.081 ± 0.007 between *Ph. cornutus* and *Nesippus vespa* Cressey, 1964 (**Table 3**). *Cox*1 sequence divergence among species was found to range from a minimum of 0.196 ± 0.023 between *E. coleoptratus* and *Nesippus crypturus* Heller, 1865 to a maximum of 0.288 ± 0.029 between *P. satyrus* and *Perissopus dentatus* Steenstrup and Lütken, 1861 (**Table 3**).

Phylogenetic analyses were conducted using both separately (**Supplementary Figures 1, 2**) and combined *cox*1 and SSU gene loci (**Figure 2**). The resulting tree for SSU (**Supplementary Figure 1**) showed Pandaridae as a monophyletic group, with high support (BS = 100), and the existence of two main clades. The first clade, with strong nodal support (BS = 100) involved two lineages, that included the genera *Phyllothyreus* Norman, 1903, *Pannosus* Cressey, 1967, *Pseudopandarus* Kirtisinghe, 1950 and the paraphyletic genus *Pandarus* Leach, 1816. At species level, the new generated sequences of *P. satyrus* clustered with that of *Pandarus* sp. 2 previously deposited in GenBank in a well-supported lineage (BS = 98). The obtained sequences of *Ph. cornutus* clustered with high nodal support (BS = 98) with the sequences of *Ph. cornutus* and *Pannosus japonicus* (Shiino, 1960) previously deposited in GenBank.

The second major clade, with weaker nodal support (BS = 71), involved three lineages, that included the species of *Achtheinus* Wilson, 1908, *Perissopus* Steenstrup and Lütken, 1861, *Echtrogaleus* Steenstrup and Lütken, 1861, *Nesippus* Heller, 1865, and *Dinemoura* Latreille, 1829. At species level, the SSU tree topology placed the new sequences of *E. coleoptratus* within a well-supported lineage (BS = 99) with the sequences of *Achtheinus oblongus* and *Pe. dentatus*, previously deposited in GenBank. The present new generated and the previously deposited sequences of *D. latifolia* clustered in a separate lineage with high nodal support (BS = 99).

In the resulting tree obtained only for *cox*1, Pandaridae was also a monophyletic group (BS = 100). Two major clades were generated, the first well-supported (BS = 100) formed by all sequences of *Pandarus*, *Pa. japonicus*, *A. oblongus*, the new generated sequences of *E. coleoptratus, N. vespa* and *Pe. dentatus,* and the second (BS = 34) formed by the obtained sequences of *D. latifolia* and the sequences of *N. orientalis* and *N. crypturus* previously deposited in GenBank, highlighting the monophyly of *D. latifolia*.

The species delimitation analyses of the *cox*1 gene locus highlighted a total of 10 taxonomic entities, revealing that the sequences of *Pandarus* spp. belonged to two distinct taxonomic entities (as shown in **Supplementary Figure 2**). The sequences of *P. satyrus* obtained in the present study belonged to the same taxonomic entity that included the sequences of *Pandarus* sp. 2, *Pandarus* sp. 3, and *Pandarus* sp. 4 from GenBank (**Supplementary Figure 2**).

The tree inferred by concatenating the SSU and *cox*1 gene loci (**Figure 2**) showed the same topology of the SSU tree (**Supplementary Figure 1**).

DISCUSSION

This study provides the first molecular data on the occurrence of four species of pandarid copepods from the Mediterranean. To our knowledge prior to of the present study only *Ph. cornutus*, *D. latifolia*, *E. coleoptratus,* and *Pandarus bicolor* have been

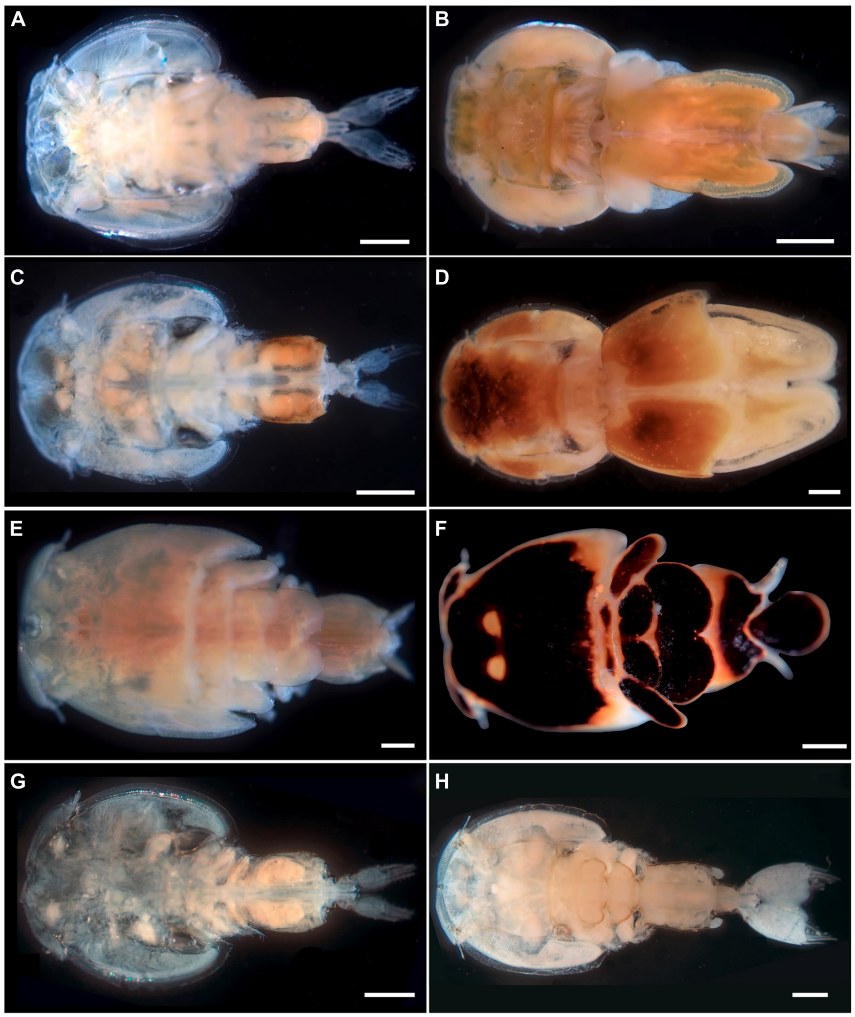

FIGURE 1 | Specimens of pandarid copepods sequenced in the present study. *Dinemoura latifolia* dorsal view of male **(A)** (bar scale: 1,000 μm) and female (GenBank: OL415938 and MZ935643) **(B)** (bar scale: 2,000 μm) from the great white shark; *Echtrogaleus coleoptratus* dorsal view of male **(C)** (bar scale: 1,000 μm) and female (GenBank: OL348230 and MZ935645) **(D)** (bar scale: 1,000 μm) from the blue shark; *Pandarus satyrus* dorsal view of male **(E)** (bar scale: 500 μm) and female (GenBank: OL457303 and OL333876) **(F)** (bar scale: 1,000 μm) from the blue shark; *Phyllothyreus cornutus* dorsal view of male **(G)** (bar scale: 1,000 μm) and female (GenBank: MZ935644) **(H)** (bar scale: 2,000 μm) from the blue shark.

recorded on shark species in the Mediterranean Sea (Richiardi, 1880; Brian, 1906; Öktener and Trilles, 2009; Öktener et al., 2020).

In general, pandarid copepods are widely distributed mirroring the movements and distribution of their hosts. In particular, *Dinemoura* parasitizes the skin of large pelagic sharks. After Cressey (1967), *Dinemoura* comprises four valid species including *D. discrepans* Cressey, 1967, *D. ferox* (Krøyer, 1838), *D. latifolia* and *D. producta* (Müller, 1785). The only reports of *D. latifolia* in the Mediterranean were on blue, shortfin mako, and thresher sharks *Alopias vulpinus* (Brian, 1906). However, along with its geographical distribution range *D. latifolia* has been found at least, on other three shark species (i.e., the great white shark, the porbeagle *Lamna nasus*, and the school shark *Galeorhinus galeus*) from North and South Atlantic, East, and West Pacific, Indian Ocean, and West Indies (see Williams, 1978).

Phyllothyreus cornutus, the only species in its monotypic genus, infects the gills of several pelagic sharks (i.e., the blue shark, the shortfin mako shark, the porbeagle, the smooth hammerhead *Sphyrna zygaena*, the sandbar shark *Carcharhinus plumbeus*, and the tiger shark *Galeocerdo cuvier*) from the North and South Atlantic and North Pacific (Hewitt, 1967; Schaeffner and Smit, 2019). In the Mediterranean Sea, it has been reported exclusively on the blue shark (Richiardi, 1880).

The genus *Echthrogaleus* comprises eight species including *E. asiaticus* Ho, Liu and Lin, 2012, *E. coleoptratus*, *E. denticulatus* Smith, 1873, *E. disciarai* Benz and Deets, 1987, *E. mitsukurinae* Izawa, 2012, *E. pellucidus* Shiino, 1963, *E. spinulus* Morales-Serna, Crow, Montes and González, 2019 and *E. torpedinis* Wilson, 1907. *Echthrogaleus coleoptratus* has been reported from the North and South Atlantic, the Pacific and Indian Oceans, and the Mediterranean Sea (Hewitt, 1967). It parasitizes the skin of

TABLE 3 | K2P genetic distances ± standard error among specimens and species of pandarid copepods.

	D. latifolia	***E. coleoptratus***	***P. satyrus***	***Ph. cornutus***
A. oblongus	0.037 ± 0.004	0.015 ± 0.003	0.052 ± 0.005	0.054 ± 0.001
	0.239 ± 0.026	0.209 ± 0.023	0.300 ± 0.030	-
D. latifolia	0.000 ± 0.000	-	0.050 ± 0.005	
	0.008 ± 0.003	-	0.267 ± 0.028	
E. coleoptratus	0.032 ± 0.004	0.000 ± 0.000		0.049 ± 0.005
	0.222 ± 0.025	0.000 ± 0.000		-
N. crypturus	0.049 ± 0.005	0.056 ± 0.006	0.061 ± 0.006	0.062 ± 0.006
	0.216 ± 0.026	0.196 ± 0.023	0.257 ± 0.029	-
N. orientalis	0.045 ± 0.005	0.048 ± 0.005	0.057 ± 0.006	0.059 ± 0.006
	0.221 ± 0.027	0.260 ± 0.029	0.282 ± 0.031	-
N. vespa	0.058 ± 0.006	0.068 ± 0.006	0.078 ± 0.006	0.081 ± 0.007
	0.219 ± 0.025	0.201 ± 0.023	0.280 ± 0.029	-
P. satyrus	0.050 ± 0.005	0.047 ± 0.005	0.000 ± 0.000	
	0.267 ± 0.028	0.302 ± 0.030	0.003 ± 0.002	
P. smithi	0.048 ± 0.005	0.045 ± 0.005	0.006 ± 0.001	0.006 ± 0.001
	-	-	-	-
Pandarus sp. 1	0.048 ± 0.005	0.045 ± 0.005	0.004 ± 0.001	0.005 ± 0.001
	0.252 ± 0.029	0.242 ± 0.027	0.256 ± 0.030	-
Pandarus sp. 2	0.049 ± 0.005	0.047 ± 0.005	0.000 ± 0.000	0.006 ± 0.001
	0.239 ± 0.026	0.218 ± 0.026	0.189 ± 0.023	-
Pandarus sp. 3	0.048 ± 0.005	0.045 ± 0.005	0.006 ± 0.001	0.007 ± 0.002
	0.237 ± 0.026	0.258 ± 0.027	0.222 ± 0.024	-
Pandarus sp. 4	0.045 ± 0.005	0.043 ± 0.005	0.001 ± 0.000	0.004 ± 0.001
	0.214 ± 0.025	0.221 ± 0.026	0.152 ± 0.021	-
Pa. japonicus	0.050 ± 0.005	0.047 ± 0.005	0.007 ± 0.002	0.001 ± 0.001
	0.256 ± 0.029	0.283 ± 0.031	0.205 ± 0.027	-
Ph. cornutus	0.052 ± 0.005	0.049 ± 0.005	0.007 ± 0.001	0.000 ± 0.000
	-	-	-	-
Pe. dentatus	0.040 ± 0.005	0.021 ± 0.003	0.053 ± 0.005	0.055 ± 0.006
	0.261 ± 0.029	0.238 ± 0.027	0.288 ± 0.029	-
Ps. longus	0.048 ± 0.005	0.045 ± 0.005	0.007 ± 0.002	0.004 ± 0.001
	-	-	-	-

The SSU K2P-values are in the upper row, while in the bottom row are reported the cox1 K2P-values (0.000 indicates identity between specimens; - indicates missing data).

about 13 species of sharks; however, it is commonly found on the great white shark, the porbeagle and the blue shark (Hewitt, 1967, 1979; Cressey and Lachner, 1970; Rokicki and Bychawska, 1991; Henderson et al., 2002; Benz et al., 2003; Luque and Tavares, 2007). In the Mediterranean, it has been reported on the blue shark, the gulper shark *Centrophorus granulosus* (Bloch and Schneider, 1801) and the great white shark (Brian, 1906).

The genus *Pandarus* comprises 14 nominal species including *P. ambiguous* (Scott, 1907), *P. bicolor*, *P. brevicaudis* Dana, 1852, *P. carcharhini* Ho, 1963, *P. cranchii* Leach, 1819, *P. floridanus* Cressey, 1967, *P. katoi* Cressey, 1967, *P. niger* Kirtisinghe, 1950, *P. rhincodonicus* Norman, Newbound and Knott, 2000, *P. rouxii* Risso, 1826, *P. satyrus* Dana, 1849, *P. sinuatus* Say, 1818, *P. smithii* and *P. zygaenae* Brady, 1883. *Pandarus satyrus* has a wide geographical distribution including Atlantic, Pacific, and Indian Ocean; however, it has never been reported from the Mediterranean. According to Cressey (1967); Benz (1986), and Rojas et al. (2001), *P. satyrus* has been predominantly found on the blue shark. It is closely related to *P. cranchii* with which it was synonymized by Shiino (1954) but considered as valid species by Cressey (1967). According to Cressey (1967) the two species are easily separated on the basis of the caudal rami. The rami of *P. cranchii* extend at least to the tip of the abdominal plate (often beyond) whereas the rami of *P. satyrus* extends only about half the length of the abdominal plate. The only other species of *Pandarus* reported from the Mediterranean is *P. bicolor* found on the blue shark, the dusky smooth-hound, the common smooth-hound, the angular rough shark *Oxynotus centrina* Linnaeus, 1758 and the picked dogfish *Squalus acanthias* Linnaeus, 1758 (Richiardi, 1880; Brian, 1906; Öktener and Trilles, 2009; Öktener et al., 2020). *Pandarus bicolor* can be distinguished from *P. satyrus* as the cephalon only occupies 1/3 of the total body length and its caudal rami are small and scarcely visibly dorsally (Cressey, 1967).

Based on specific morphological characters, pandarid copepods have been arranged into two major groups: (i) species with all three thoracic segments provided with dorsal or

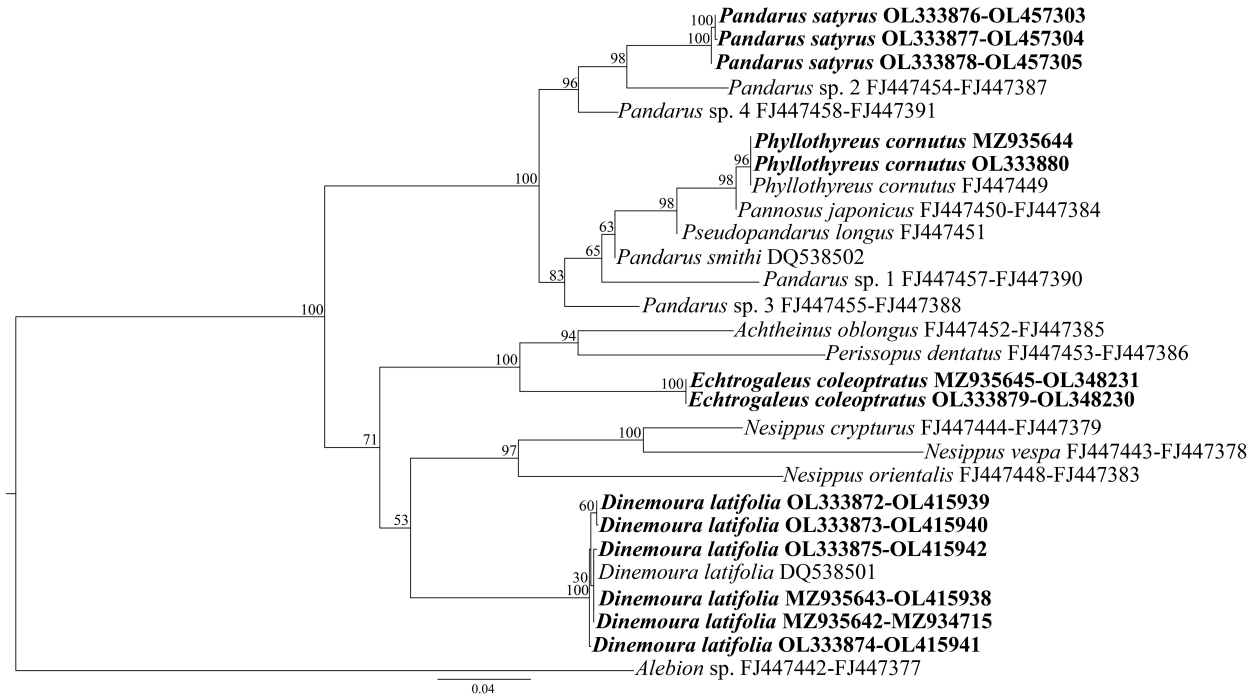

FIGURE 2 | Phylogenetic concatenated tree from maximum likelihood based on SSU and *cox*1 sequences of pandarid species obtained in the present study, with respect to the pandarid sequences at the same gene loci available in GenBank. *Alebion* sp. was used as outgroup. The sequences obtained in this study are in bold.

dorsolateral plates (*Pandarus*-group), and (ii) species with the second free thoracic segment without plates (*Dinemoura*-group) (Kabata, 1979). *Pandarus* and *Phyllothyreus* have been included in the first group with *Achtheinus*, *Perissopus*, *Gangliopus* Gerstaecker, 1854, *Pannosus* and *Pseudopandarus*; *Dinemoura* and *Echtrogaleus* have been included in the second group with *Demoleus* Heller, 1865, *Dinemoleus* Cressey and Boyle, 1978, *Nesippus*, *Paranesippus* Shiino, 1955 and *Pagina* Cressey, 1963 (see Kabata, 1979).

In contrast, based on the results of more recent phylogenetic analyses, pandarid copepods have been placed into two major clades: the first clade included the species of *Nesippus*, and the second clade included the species of *Phyllothyreus*, *Pannosus*, *Pandarus*, *Pseudopandarus,* and *Achtheinus* (Dippenaar, 2009). However, Dippenaar (2009) focused mainly on relationships among families of the Siphonostomatoida, while no phylogenetic relationships were deepened among the genera.

Maximum-likelihood analysis inferred by concatenated SSU + *cox*1 data set placed the sequences of pandarid copepods available in GenBank plus the new generated sequences into two major clades, however, some differences were observed when the present results were compared to those of Dippenaar (2009). For example, the present specimens of *P. satyrus* and *Ph. cornutus* were included in a first clade with *Pandarus* spp., *Pa. japonicus*, *Ps. longus*, and *P. smithi* with strong nodal support. Within this clade, *Ph. cornutus*, *Pa. japonicus*, *Ps. longus*, *P. smithi*, *Pandarus* sp. 1, and *Pandarus* sp. 3 were placed in a subclade not supported by the posterior probabilities and bootstrap analysis. Finally, *D. latifolia* and *E. coleoptratus* were included in a second clade with a weaker nodal support with *Nesippus orientalis* Heller 1865, *N. vespa*, *N. crypturus*, *A. oblongus*, and *Pe. dentatus*.

The phylogenetic pattern for the species here collected was congruent with the morphological characters of the two species groups above mentioned, except for *A. oblongus* and *Pe. dentatus* which were placed into the second major clade with the genera *Echtrogaleus*, *Nesippus* and *Dinemoura*. In contrast, the phylogenetic clustering among the members of the two clades seems to be not related to the host preference. For instance, both clades included parasites capable of infecting shark species belonging to six orders and 11 families, with the second clade that also included parasites capable of infecting five additional families of sharks. Nevertheless, we cannot exclude a coevolutionary hypothesis between copepods and their hosts species. Indeed, little is known regarding the nature of host-copepod association in elasmobranchs (Bernot et al., 2021). Huys et al. (2007) suggested a host switching event in the siphonostomatoid copepods highlighting monostrilloids' alterations in the host utilization, body plan, and life cycle strategy. The scarcity of data regarding the host association and life cycle strategy of pandarid copepods does not help to resolve phylogenetic relationships among species. The present phylogenetic analysis included only a small subset (12) of the 64 valid species of Pandaridae. Therefore, it is possible that the present phylogenetic results may not reflect the true relationships, as a large majority of species is missing from the present analysis. Indeed, as already discussed above, our phylogenetic results were not congruent with those obtained by Dippenar (2009). More thorough sequencing of Pandaridae

species will be needed to better resolve the phylogenetic relationships among the members of this family.

CONCLUSION

In conclusion, we provide additional DNA sequences for *D. latifolia* and *Ph. cornutus*. Furthermore, new molecular data for *E. coleoptratus* and *P. satyrus* are reported, based on, both the nuclear (SSU) and mitochondrial (*cox*1) gene loci. Whilst the single use of the SSU gene locus permitted the molecular identification of the copepod species, the mtDNA *cox*1 could represent a suitable marker to infer population structure of pandarid copepods, and consequently of their hosts (Criscione et al., 2006; Baldwin et al., 2011). In this sense, intraspecific variation of *cox*1 was actually detected for some of the species in the present study. Nonetheless, the scarce reference sequence information, hampered any further understanding on the population structure of these copepod parasites. This study represents the first attempt to yield new molecular and phylogenetic data of pandarid copepods on pelagic sharks in the Mediterranean Sea that could contribute to a better characterization of these poorly known parasites. Future molecular and genetic studies should also provide a more detailed assessment of the host-parasite interactions, ecological data, and life cycle strategy. *Pandarus satyrus* represents a new record for the Mediterranean.

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: NCBI accession numbers: MZ935642-45, OL333872-80, MZ934715, OL415938-42, OL348230-31, and OL457303-05.

ETHICS STATEMENT

The material here studied has been collected from two co-authors (GI and BZ) of the present paper under the framework of a project of the Museo Civico di Storia Naturale (MSNC) in Comiso on alien and rare marine species of the Mediterranean Sea. The MSNC is a scientific institution registered at the CITES Secretariat, D.M. 23.03.1994 (Cod. IT030), authorized to take, keep, use and display dead endangered species of wild fauna.

AUTHOR CONTRIBUTIONS

MP and MS design of experiment, performed morphological and molecular analyses, and wrote the manuscript. GI and BZ collected the host data and parasitic copepods. All authors 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.2021.778034/full#supplementary-material

Supplementary Figure 1 | Phylogenetic tree from maximum likelihood based on SSU sequences of pandarid species obtained in the present study, with respect to the pandarid sequences at the same gene locus available in GenBank. *Alebion* sp. was used as outgroup. The sequences obtained in this study are in bold.

Supplementary Figure 2 | Phylogenetic tree from maximum likelihood based on *cox*1 sequences of pandarid species obtained in the present study, with respect to the pandarid sequences at the same gene locus available in GenBank. *Alebion* sp. was used as outgroup. The sequences obtained in this study are in bold. Braces indicate the specimens that according to the species delimitation analyses are part of the same taxonomic entity. Numbers in brackets indicate the 10 taxonomic entities revealed by the species delimitation analyses.

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Barnacle Epibiosis on Sea Turtles in Korea: A West Pacific Region With Low Occurrence and Intensity of *Chelonibia testudinaria* (Cirripedia: Chelonibiidae)

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Loggerhead and green turtles inhabit all oceans except the polar regions. External surfaces of sea turtles are often colonized by epibiotic chelonibiid barnacles. Barnacle taxonomy studies in Korea began in 1985, but until present, no turtle barnacles were recorded. This suggests that either the diversity and frequency of occurrence of turtle barnacles in Korean waters are low or the turtle barnacles have been understudied. This study complies with data collected over 6 years of sea turtle stranding events in Korea (2015–2020). We examined the diversity, frequency, and intensity of turtle barnacle occurrence. Of the 55 recorded strandings, loggerhead turtles were the most common (58%), followed by green turtles (33%). Only one species of barnacle, *Chelonibia testudinaria*, was found on both loggerhead and green turtles. The frequency of barnacle occurrence on loggerhead turtles was 28%, with an intensity of 2.4 ± 2.7 barnacles per turtle. Notably, 11% of green turtles had barnacles, with an average of one individual per turtle. The frequency and intensity of barnacle occurrence on green turtles analyzed in this study were five times lower than that on green turtle populations in Okinawan, Bornean, and Australian waters in the Indo-Pacific. Based on these new data and the available literature, we speculated that the barnacle larval pools in cold, high-latitude Korean waters are smaller than those occurring in other locations in the Indo-Pacific. The frequency and intensity of occurrence of barnacles on loggerhead turtles in Korea fall within the range recorded in other Indo-Pacific locations. The longer migratory routes of loggerhead turtles allow them to pass through different larval pools in the Indo-Pacific water, exposing them to higher barnacle abundances.

Keywords: stranding, turtle barnacles, green sea turtles, loggerhead sea turtles, Indo-Pacific

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INTRODUCTION

The loggerhead turtle (*Caretta caretta*) and green turtle (*Chelonia mydas*) are latitudinally and longitudinally widespread marine reptiles (FitzSimmons and Limpus, 2014). Carcasses of loggerhead and green turtles often carry epibiotic assemblages, including algae, meiofauna, decapods, and barnacles (Pfaller et al., 2008; Silver-Gorges et al., 2021). Chelonibiid barnacles

are common epibionts on both loggerhead and green turtles (Zardus, 2021). Currently, sixteen confirmed species of turtle-associated barnacles are known (Zardus, 2021). The examples include the commonly occurring *Chelonibia testudinaria* attaching on the turtle carapaces (Zardus et al., 2014). *Chelolepas cheloniae*, in turn, bores into turtle carapaces, and *Platylepas* spp. attach to the skin (Hayashi, 2012; Zardus, 2021).

The diversity of epibiotic barnacle species on turtles in the Indo-Pacific has been studied in specific regions, including the Pacific coast of Japan (10 species, Hayashi, 2012), Malaysia (one species, Lim et al., 2021), Taiwan (two species, Chan et al., 2009), and China (nine species, Liu and Ren, 2007). These studies indicate a considerable diversity of species. Korean waters cover both the subtropical region around Jejudo Island and temperate waters around the Korean Peninsula, which is at the northern limit of the range of green turtles in the Western Pacific. Sea turtles have most often been recorded in Jejudo Island (Kim et al., 2017) and the east coast of Korea, in the East Sea (Sea of Japan).

Barnacle fauna of Korea have been comprehensively described by Kim (2011) and Kim H. K. et al. (2020), but there are no reports of turtle-associated barnacles, suggesting that either the diversity and occurrence rate of turtle barnacles in Korea is low or the local turtle barnacles have been understudied. It is also possible that commensal barnacles on green turtles inhabiting the northern edge of their range may show lower frequency and intensity of occurrence when compared to tropical turtle populations. This study reports the diversity and occurrence of turtle-associated barnacles based on the stranding records (2015–2020) of loggerhead and green turtles in Korean waters. We tested the hypothesis that the frequency and intensity of barnacle occurrence on sea turtles are lower in Korean populations than other previously investigated sea turtle populations in the Indo-Pacific.

MATERIALS AND METHODS

Korean Marine Ecoregions

There are three distinct ecoregions within the marine environment around the Korean Peninsula: the Yellow Sea ecoregion on the west coast, the East China Sea ecoregion along the southern coast and outlying islands, including Jejudo Island, and the East Sea ecoregion on the east coast, facing the East Sea. The Yellow Sea ecoregion is affected by the Korean Coastal Current which has opposite flow directions in summer and winter (**Figure 1**). The East China Sea ecoregion is affected by the Cheju and Tsushima Warm Currents (**Figure 1**). The East Sea ecoregion is influenced by the North Korean Cold Currents and the Tsushima Warm Current (Kim H. K. et al., 2020). The ecoregions differ mainly in winter water temperature (**Figure 1**). Waters in the Yellow Sea ecoregion have the lowest winter temperatures (∼2°C), followed by those in the East Sea ecoregion (∼10°C). The South Sea ecoregion is the warmest, with winter water temperature ranging from 12°C to 15°C (**Figures 1B,C**; Kim H. K. et al., 2020).

Stranded Turtles and Epibiotic Barnacles

Stranded sea turtle carcasses were collected from the Korean coastal regions from 2015 to 2020 by the National Marine Biodiversity Institute of Korea (MABIK). For each stranding, we recorded ecoregion, specific location, and turtle species. The curved carapace length of turtles was measured using tape rulers to the nearest millimeter. Barnacles were collected from the sea turtle head, plastron, and carapace using a stainless steel hand scraper. They were subsequently identified and counted.

Comparison of the Frequency and Intensity of Occurrence of *C. testudinaria* on Sea Turtles in the Indo-Pacific

The frequency of occurrence of barnacles on loggerhead and green turtles in this study was calculated as the ratio between the stranded turtles with barnacles and the total number of stranded turtles (2015–2020). The intensity of barnacle occurrence on a given sea turtle species was expressed as the average number of barnacles per turtle.

The frequency and intensity of *C. testudinaria* in other Indo-Pacific locations were extracted from the previously published studies conducted in Okinawa (Hayashi and Tsuji, 2008), Australia (Limpus et al., 1994; Doell et al., 2017), Aldabra Atoll, Seychelles (Frazier, 1971), Mabul Island in N. Borneo (Lim et al., 2021), Kyushu, Japan (Matsuura and Nakamura, 1993), and Natal, South Africa (Hughes, 1974; **Supplementary Table 1**). Although Hughes (1974) identified the barnacles observed on loggerheads as *Chelonibia* sp., we included his observations in the current dataset due to the general scarcity of distribution data for *C. testudinaria* on loggerhead turtles in the Indo-Pacific region (also refer to the literature cited by Zardus, 2021). To study the variation in sea surface temperature among the studied regions of *C. testudinaria* in the Indo-Pacific, a time-averaged overlay sea surface temperature map of the Indo-Pacific region was created using the NASA Giovanni Database version 4.36[1].

RESULTS

Frequency and Intensity of Barnacle Occurrence

From 2015 to 2020, we recorded 55 sea turtle stranding events. The following sea turtles were observed (**Figures 1**, **2**): 32 loggerheads, 18 green turtles, two leatherbacks, two olive ridleys, and one hawksbill (**Figure 2**). Loggerhead and green turtle strandings were recorded in all three ecoregions: East Sea (23 and 5, respectively), East China Sea (8 and 12, respectively), and Yellow Sea (1 and 1, respectively) (**Figure 1**). Most of the stranded sea turtle carcasses were relatively fresh with no signs of decomposition. Most of the strandings occurred from May to October (**Supplementary Table 2**).

[1]https://giovanni.gsfc.nasa.gov/giovanni/

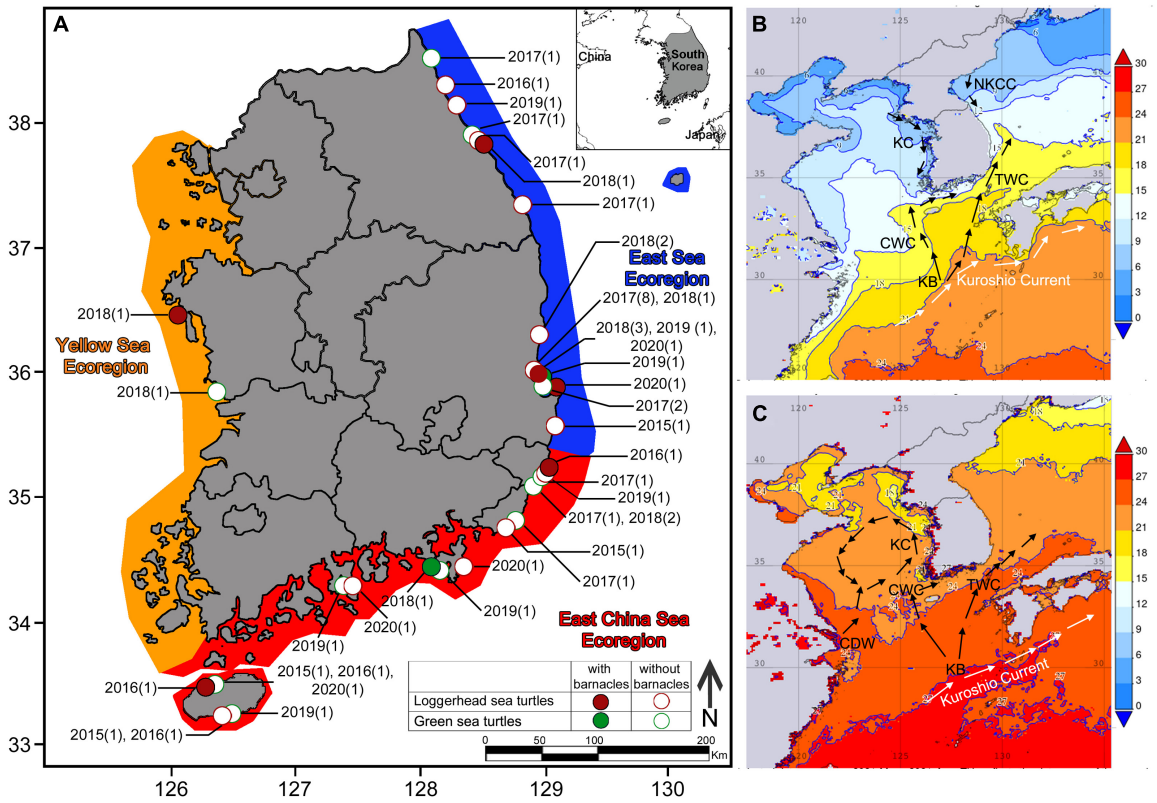

FIGURE 1 | (A) Records of stranded loggerhead and green sea turtles in Korea (Orange: Yellow Sea ecoregion, Red: East China Sea ecoregion, Blue: East Sea ecoregion). Locations of a loggerhead sea turtle with barnacles are represented by red circles. Locations of a loggerhead sea turtle without barnacles are represented by open red circles. Locations of a green sea turtle with barnacles are represented by green circles. Locations of a green sea turtle without barnacles are represented by open green circles. In each location, the year and number of strandings are stated in brackets; **(B)** Averaged winter surface seawater temperature (November 2020–February 2021) and oceanographic currents in Korean and adjacent waters; **(C)** Averaged summer surface seawater temperature (May–August 2021) and oceanographic currents in Korean and adjacent waters. Sea surface temperature map created from NASA Giovanni database version 4.36. KC, Korean Coastal Current; KB, Branch of Kuroshio Current; TWC, Tsushima Warm Current; CWC, Cheju Warm Current; CDW, Changjiang Diluted Water; NKCC, North Korean Coastal Current.

Loggerhead and green turtles hosted barnacles, while leatherback, olive ridley, and hawksbill turtles had no barnacles. *C. testudinaria* on stranded loggerhead turtles was mainly found in the East China Sea and East Sea ecoregions, with one record in the Yellow Sea ecoregion (**Figure 1**). On loggerhead turtles, the frequency of occurrence was 28%, and the intensity of occurrence was 2.4 ± 2.7 barnacles per turtle. Among the recorded barnacles, 82% of *C. testudinaria* were found on the carapace and 18% on the plastron (**Figure 2**). The curved carapace length of loggerhead turtles with barnacles ranged from 588 to 861 mm (subadults to adults).

Stranded green turtles with barnacles were only found in the East China Sea and East Sea ecoregion (**Figure 1**). In green turtles, the frequency of occurrence was 11%, and the intensity of occurrence was 1.0 ± 0.2 barnacles per turtle (**Figure 2**). The carapace lengths of green turtles with barnacles on their carapaces ranged from 460 to 740 mm (one juvenile and one subadult) (**Figure 3**). Stranded adult green turtles carried no barnacles (**Figure 3**).

Literature-Based Comparison of Frequency and Intensity of Occurrence of *C. testudinaria* on Sea Turtles in the Indo-Pacific

Based on the available data, we were able to calculate the following parameters. The frequency of occurrence of *C. testudinaria* on loggerheads from South Africa ranged from 18% (juvenile specimens from Natal) to 73% (**Supplementary Table 1** and **Figure 4**). In green turtles, the frequency of occurrence exceeded 50% in all analyzed populations (**Supplementary Table 1** and **Figure 4**).

The intensity of occurrence of *C. testudinaria* on loggerheads from Kyushu and Australia was 6.4 and 1.9 barnacles per turtle, respectively (**Supplementary Table 1**). In green turtles, the intensity of occurrence was 2.6 barnacles per turtle in Australia, 8.4 in Okinawa, and 19.3 in North Borneo. In the latter location, up to 43 barnacles per turtle were recorded in adult female populations (**Supplementary Table 1** and **Figure 4**).

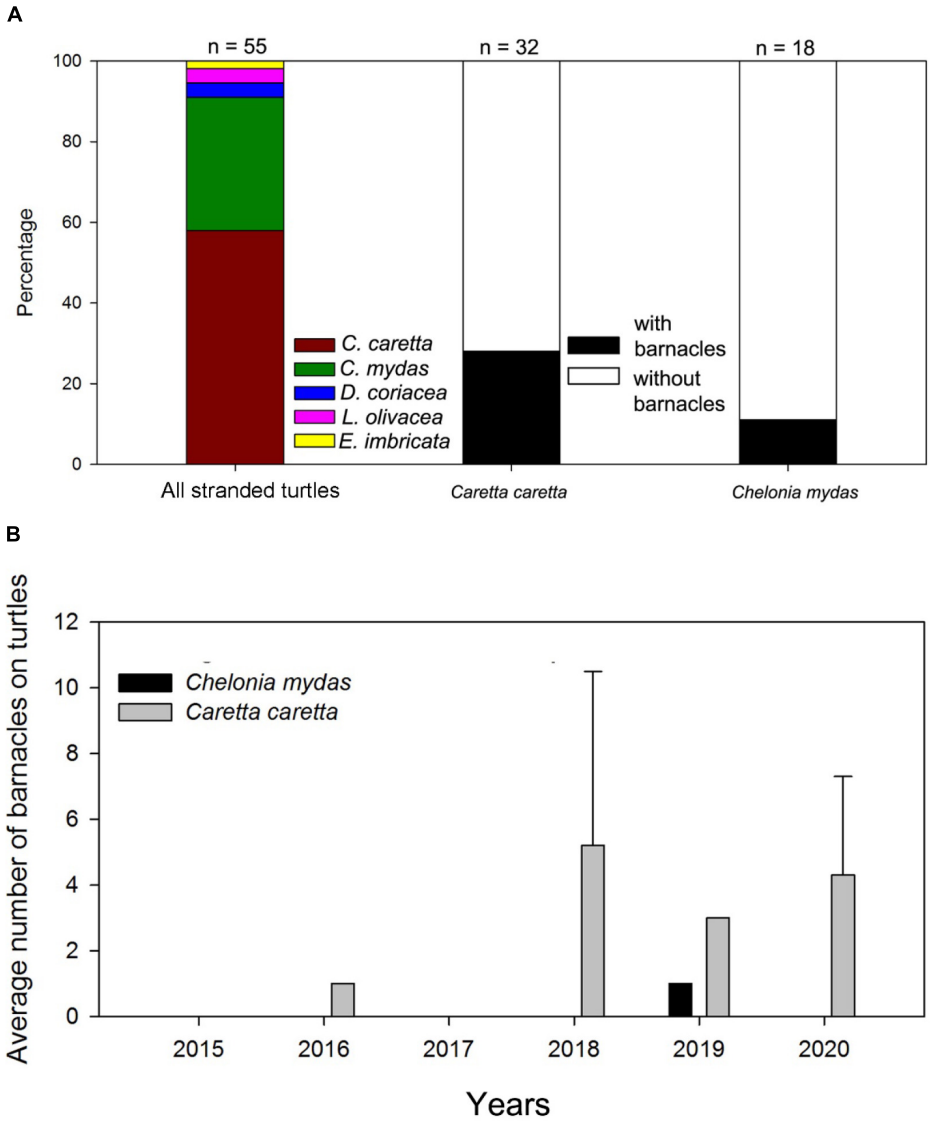

FIGURE 2 | Stranded sea turtles collected from 2015 to 2020 in Korea. **(A)** Percentage of stranded turtles collected from 2015 to 2020 and percentage of loggerhead and green sea turtles with barnacles; **(B)** Density (average number of barnacles per turtle) on stranded loggerhead and green sea turtles from 2015 to 2020.

DISCUSSION

Only one barnacle species, i.e., *C. testudinaria*, was found for the first time as a turtle-associated barnacle in Korean waters. Prior to this study, this species had not been recorded in Korea, although the taxonomic studies of Korean barnacles began in 1985 (Kim, 2011). Among the 13 sea turtle barnacles reported from the Indo-Pacific region (Zardus, 2021), *Platylepas hexastylos* was recorded from a "stuffed turtle specimen" kept in a university museum without any information on the collection site (Kim, 2011) and thus may not be of Korean origin. It has been suggested that the diversity of epibiotic turtle-associated barnacles in Korea was likely lower than that in adjacent regions, including the Pacific coast of Japan (Hayashi, 2012). Thus, *C. testudinaria* is not expected to be common in Korean sea turtle populations. Some of the sea turtle populations from the adjacent Pacific waters may be separated from Korea, resulting in differences in the diversity of turtle-associated barnacles among regions (Jang et al., 2018). The relatively low temperature of Korean waters, as compared to the adjacent regions, may limit the diversity of turtle barnacles in Korea.

The frequency and intensity of occurrence of *C. testudinaria* on Korean loggerhead turtles (28%, 2.4 barnacles per turtle) fell within the ranges estimated for other Indo-Pacific populations (18–73%, 1.9–6.4 barnacles per turtle). Both the frequency and intensity of occurrence of *C. testudinaria* on green turtles (11%, one barnacle per turtle), in turn, were several times lower than the values calculated for other Indo-Pacific populations.

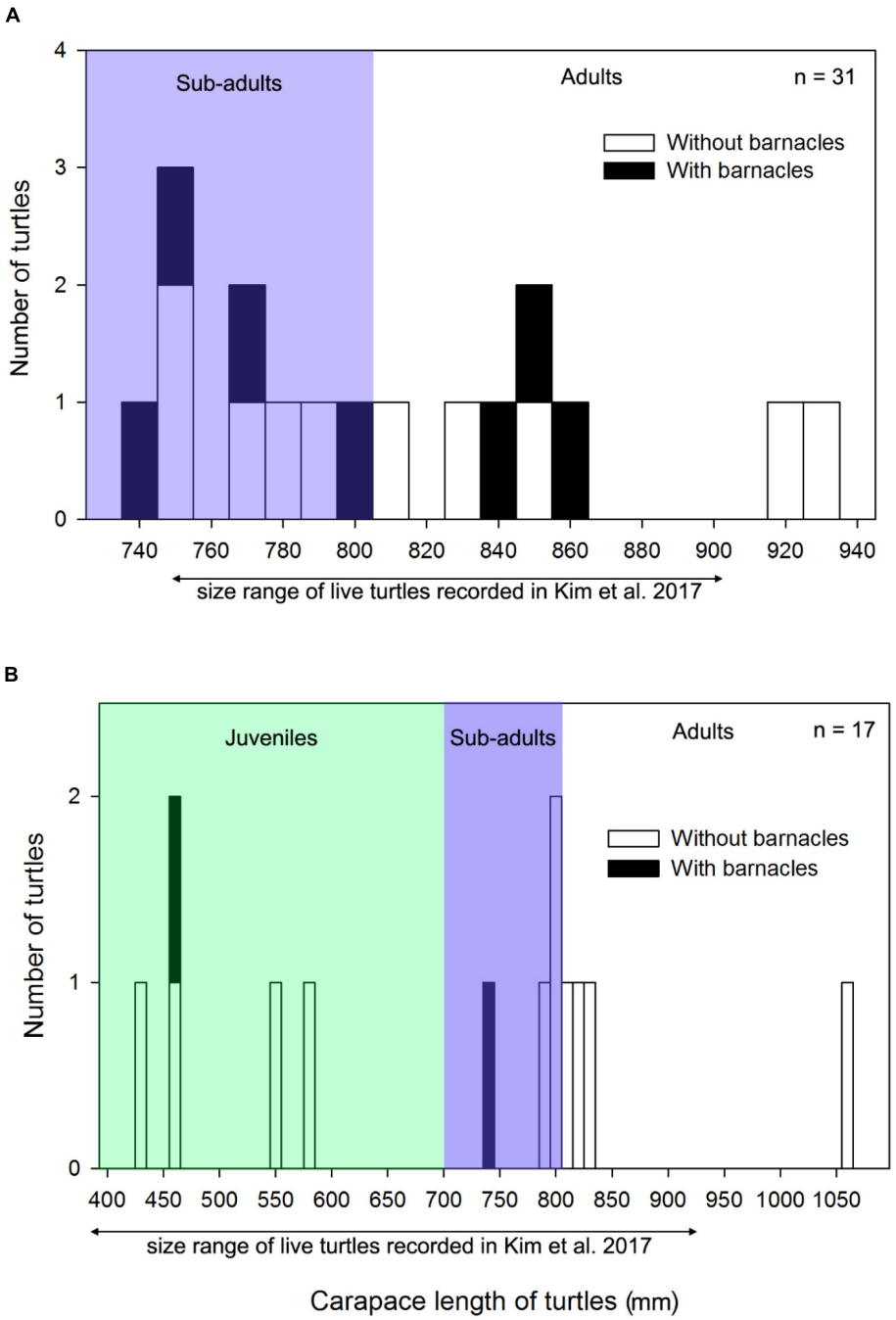

FIGURE 3 | Frequency histograms of carapace length (mm) of **(A)** Loggerhead sea turtles and **(B)** Green sea turtles, with (black bars) and without (white bars) barnacles in Korea. The size range of live loggerhead and green sea turtles recorded in Korea in the study by Kim et al. (2017) is provided for comparison with the stranded turtles in this study. The definition of juveniles, subadults, and adults is based on the size range defined in the study by Hughes (1974).

Another study investigating Korean green turtles (Moon et al., 2009) presented a photograph of a wild turtle carrying a single barnacle on its carapace. Although the specimen was identified as a *Balanus* species, the photograph clearly indicates that the taxon in question was *C. testudinaria* (refer to Figure 8 in Moon et al., 2009). Observations made by us over the years (the personal observations of Y. N. Choi, C. Yi, I.-H. Kim, and Y. N. Choi), as well as photographs provided by other authors (Moon et al., 2009; Kim et al., 2017, 2019; Kim I. H. et al., 2020), suggest that green turtles in Korea (but also hawksbills and olive ridleys) rarely carry any barnacles.

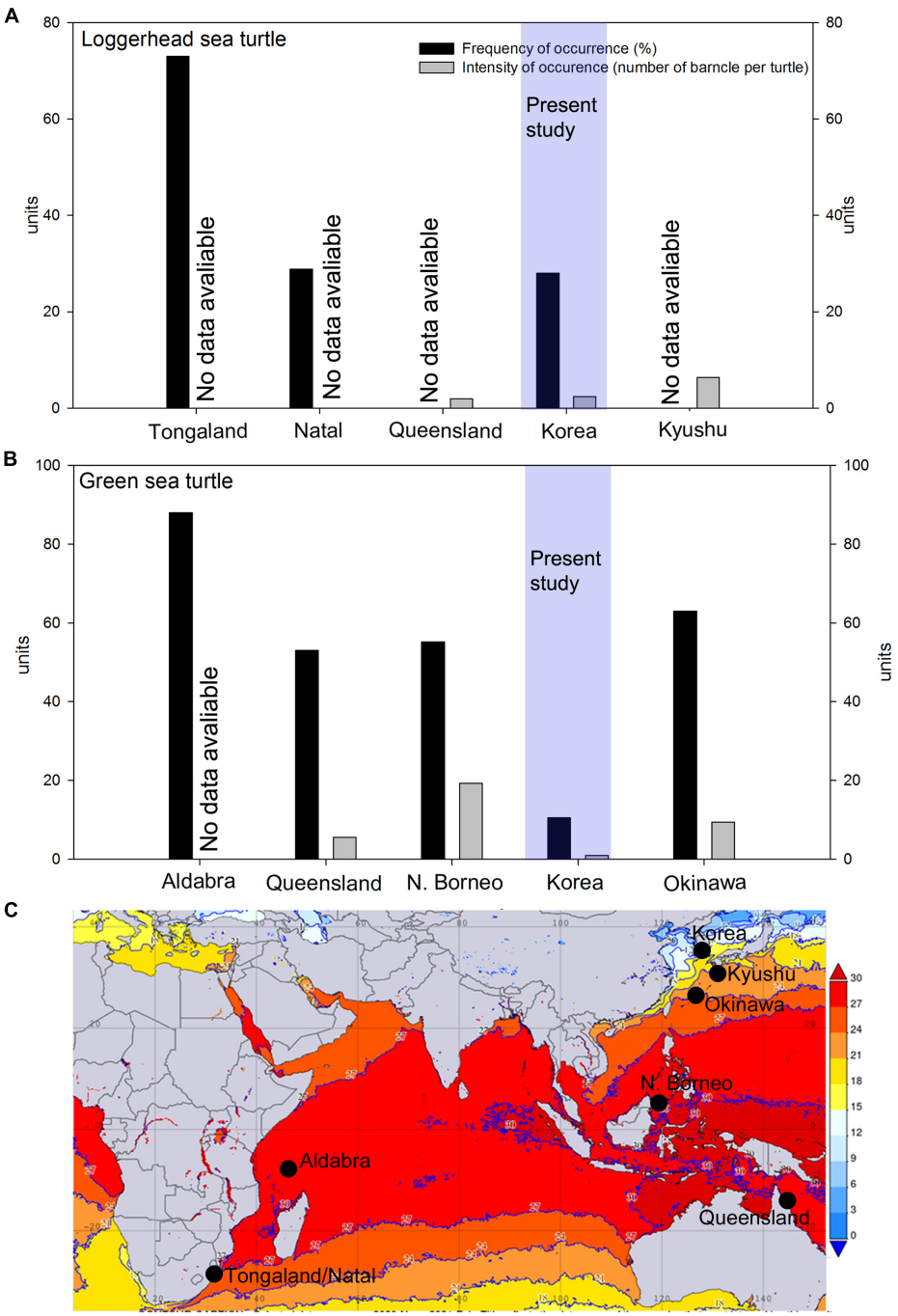

FIGURE 4 | Comparison of the frequency of occurrence (%, black bar) and intensity of occurrence (number of barnacles per turtle, gray bars) in the Indo-Pacific of **(A)** Loggerhead sea turtles and **(B)** Green sea turtles in Korea. Data sources from geographical population: Okinawa (Hayashi and Tsuji, 2008), Queensland in Australia (Limpus et al., 1994; Doell et al., 2017), Aldabra Atoll (Frazier, 1971), Mabul Island in N. Borneo (Lim et al., 2021), and Natal and Tongland (Hughes, 1974). The frequency of occurrence of barnacles on loggerhead sea turtles on Natal and the frequency of occurrence and intensity of occurrence of barnacles in green sea turtles in N. Borneo were averaged from the data on juveniles, subadults, and adults. For details, refer to **Supplementary Table 1**; **(C)** Averaged winter sea surface temperature map of the Indo-Pacific (November 2020–February 2021), generated from NASA Giovanni database version 4.36. Black dots show the locations in panels **(A,B)**.

Satellite tracking studies of green turtles in Korea and Japan indicate that these animals can stay in waters surrounding Jejudo Island for up to 1 year and migrate between Kyushu, the Korean Peninsula, and Jejudo Island in the East Sea region (Jang et al., 2018). As previously suggested (Kim H. K. et al., 2020), lower water temperatures in this region may limit the larval pools of

C. testudinaria and other barnacles. For example, in Malaysia, leatherback, olive ridley, and hawksbill turtles are often colonized by *C. testudinaria* (Lim et al., 2021). In Korean waters, these sea turtle species have not yet been observed to host *C. testudinaria*.

We speculated that the local loggerhead turtles do not follow this trend due to their longer migratory routes (Bowen et al., 1995). Satellite tracking of juvenile loggerheads revealed that they are restricted to the pelagic feeding grounds located in the Central North Pacific, where they stay for up to 1–2 years (Briscoe et al., 2016). During non-reproductive periods, numerous loggerheads are also observed in the East China Sea enclosed by Korea, eastern China, Taiwan, and Kyushu (Kobayashi et al., 2011). Therefore, loggerhead turtles in the West Pacific would have had to pass through the more diverse and possibly larger larval pools than green turtles during their migrations. Considering the life span of *C. testudinaria* (up to 2 years; Doell et al., 2017), barnacles present on Korean loggerheads might have settled on these animals in different geographical regions.

It has to be highlighted that this study analyzed barnacles that present solely on the carcasses of stranded sea turtles, and it is possible that such data would not accurately reflect the actual trends in living sea turtle populations. However, based on the low degree of decomposition of the carcasses used in this study, we are confident that all animals resided in Korean waters. Somewhat in support of this statement, the distribution patterns of the Korean sea turtles shown by Kim et al. (2017) largely agree with the stranding locations (this study). Similarly, the size range of sea turtles recorded in Korean waters (Kim et al., 2017) overlaps with that of the analyzed carcasses.

We acknowledged the relatively small sample size of 55 individual sea turtles, as well as the general scarcity of similar studies in the Indo-Pacific region, and emphasized that our results should be interpreted with caution. It is likely that the local sea turtle barnacle diversity remains underestimated, and more studies (preferably including living turtles) are necessary to further investigate the correctness of some of our assumptions. Given the possible significant effect of water temperature on sea turtle barnacle abundance and ranges, in the future, more barnacle species may be observed in Korea due to global warming. The analyses of preserved sea turtle carcasses and carapaces may help detect such temperature-related changes over different time scales.

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

HKK and BKKC conceived and designed the study. CY and I-HK collected the field data. BKKC, HKK, I-HK, and CY wrote the manuscript and prepared the figures. All authors read 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/fevo.2022.785692/full#supplementary-material

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Sea Turtle Epibiosis: Global Patterns and Knowledge Gaps

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Competition for space drives many marine propagules to colonize the external surfaces of other marine organisms, a phenomenon known as epibiosis. Epibiosis appears to be a universal phenomenon among sea turtles and an extensive body of scientific literature exists describing sea turtle-epibiont interactions. When viewed in isolation, however, these epibiont "species lists" provide limited insights into the factors driving patterns in taxonomic diversity on a global scale. We conducted an exhaustive literature review to collate information on sea turtle-epibiont interactions into a global database. As studies involving meio- and micro-epibionts, as well as plants, are limited, we exclusively focused on animal, macro-epibionts (>1 mm). We identified 304 studies that included a combined total of 1,717 sea turtle-epibiont interactions involving 374 unique epibiont taxa from 23 Higher Taxon categories (full Phylum or select phyla differentiated by Subphylum/Class/Subclass). We found that loggerhead turtles hosted the highest taxonomic richness (262 epibiont taxa) and diversity, including representative taxa from 21 Higher Taxon categories, followed by hawksbill, green, olive ridley, leatherback, Kemp's ridley, and flatback turtles. In addition, the taxonomic richness for all turtle species except leatherbacks was projected to increase with additional studies. We found that taxonomic richness not only varies between species but also between well-studied populations of loggerhead turtles. Lastly, we assessed biases in the current literature and identified knowledge gaps for certain species (e.g., Kemp's ridleys and flatbacks), life stages (e.g., juveniles), habitats (e.g., oceanic habitats), and geographic regions (e.g., central Pacific, east Atlantic, and east Indian oceans). Our hope is that this database will serve as a foundational platform for future studies investigating global patterns of the diversity, ecological function, and evolutionary origins of sea turtle epibiosis.

Keywords: commensalism, epibiont, parasite, species richness, symbiosis

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INTRODUCTION

Competition for space drives marine propagules to colonize almost any exposed, undefended surface in the marine environment (Harder, 2009; Wahl, 2009). While colonization frequently occurs on inanimate structures (e.g., submerged bedrock and dock pilings), the external surfaces of other marine organisms can also provide suitable substrata for settlement—resulting in

a phenomenon known as epibiosis. Epibiosis involves a single host species and one or more colonizers called *epibionts*. Most epibionts are opportunistic organisms found "free living" in the surrounding environment, while others are obligate commensals of specific hosts. In complex and captivating cases of epibiosis, epibionts colonize the bodies of migratory marine megafauna, such as sea turtles and cetaceans, and are transported as "hitchhikers" across marine habitats and even entire ocean basins (Ingels et al., 2020).

Sea turtles are renowned for hosting diverse communities of epibionts, including representative taxa from almost every Phylum within Animalia (e.g., Frick et al., 1998; Lazo-Wasem et al., 2011; Corrêa et al., 2014). While robust, encrusting forms, such as barnacles, tend to be more common and exhibit greater diversity (Zardus, 2021), other taxonomic groups that are less resistant to abrasion and dramatic shifts in environmental conditions tend to be rarer and less diverse (e.g., Lazo-Wasem et al., 2007; Perrault et al., 2015). Because epibiosis necessitates ecological overlap between host turtles and "free living" populations of epibionts and/or their propagules, the assemblages of epibionts found on sea turtles also tend to reflect the regions and habitats where host turtles spend time (e.g., Reich et al., 2010; Pfaller et al., 2014; Ten et al., 2019). Consequently, the presence of certain epibiont species or assemblages that occupy specific regions (e.g., tropical, temperate, or polar) and/or habitats (e.g., oceanic/pelagic or neritic/benthic) can serve as indicators of the migratory movements and habitat preferences of sea turtles (Casale et al., 2004; Frick and Pfaller, 2013). Similarly, the diversity of sea turtle epibionts and the equally diverse ways they interact with their hosts, means that the presence or absence of particular epibiont taxa can also serve as indicators of the hosts' foraging preferences (Pfaller et al., 2014), social or reproductive behavior (Domènech et al., 2017; Robinson et al., 2017a), body condition and/or health status (Lazo-Wasem et al., 2007; Nolte et al., 2020), and more.

Following a rich history of anecdotal reports dating back to Darwin (1851, 1854) and Pilsbry (1916), the epibiont communities of sea turtles have received considerable attention (Frick and Pfaller, 2013). Most of this work has focused on animal, macro-epibionts (>1 mm) as they are relatively easy to identify and sample; however, it is increasingly being realized that sea turtles also frequently host meio- and micro-epibionts as well as various plant species (Robinson et al., 2016; Ingels et al., 2020). There is now an extensive body of scientific literature describing the epibiotic diversity of sea turtle populations worldwide. However, most studies only report the species of epibionts found on a single sea turtle species at a single locality. Viewed in isolation, these "species lists" provide limited inferences for understanding the factors driving patterns in epibiont richness and diversity on a global scale (Lazo-Wasem et al., 2011; Pinou et al., 2019; Zardus, 2021). Moreover, because measures of species richness are inherently connected to sampling effort, comparisons of epibiotic diversity among turtle species and regions may be biased by variations in sample size (i.e., the number of studies or turtles surveyed) (Robinson et al., 2017b). Collating these "species lists" along with their associated metadata (e.g., host species and life stage, geographic region, habitat, etc.), while also accounting for differences in sampling effort, could therefore provide a foundation to analyze broad-scale patterns in sea turtle epibiosis. Such an effort would also help identify understudied species or regions, thereby guiding productive directions for future research.

To enact this important step in the field of sea turtle epibiosis, we conducted an exhaustive review of published scientific articles and gray literature (i.e., government reports, theses and dissertations, and conference presentations) to collate information on sea turtle-epibiont species pairs as well as their associated metadata. Because studies involving meio- or micro-epibionts, as well as plants, are limited, we exclusively focused on animal, macro-epibionts (>1 mm) (Hereafter, we use the term "epibionts" to exclusively refer to animal macro-epibionts unless stated otherwise). As an initial investigation of the information amassed in this global database, we first quantified and compared the taxonomic diversity of epibionts for each sea turtle species to answer two questions. (1) Which sea turtle species hosts the greatest epibiont diversity? (2) Does the current literature for each turtle species fully encompass the taxonomic richness of their epibiont communities? Next, we investigated similar questions among three well-studied populations of loggerhead turtles (*Caretta caretta*). Last, we characterized the current epibiont literature for each sea turtle species in terms of turtle life stage, habitat type, and geographic distribution to assess biases and identify knowledge gaps for future research.

METHODS

Database Development

Between March 2018 and December 2020, we conducted a two-tiered literature search to compile all records of sea turtle-epibiont interactions. A structured search was conducted in Web of Science, Google Scholar, and Sea Turtle Online Bibliography (Archie Carr Center for Sea Turtle Research, University of Florida) using the following Boolean search terms: epibiont, epibiosis, epifauna, epibiota, and both common and scientific names of the seven marine turtle species: loggerhead turtle (*C. caretta*), green turtle (*Chelonia mydas*), leatherback turtle (*Dermochelys coriacea*), hawksbill turtle (*Eretmochelys imbricata*), olive ridley turtle (*Lepidochelys olivacea*), Kemp's ridley turtle (*Lepidochelys kempii*), and flatback turtle (*Natator depressus*). Secondly, an unstructured literature search was conducted by reviewing the reference lists of relevant publications and reports from the structured search. We included any peer-reviewed scientific article, thesis/dissertation, conference presentation, and official report that contained information on sea turtle epibiosis. When the same data were presented in separate publications by the same author/s, we only included the data from the original source publication. We did not include references published after December 2020 (i.e., those published between the completion of our literature search and the publication of this article).

We constrained our two-tiered literature search to only include records of sea turtle-epibiont interactions from (1) turtles surveyed in the wild, (2) animal epibionts (i.e., Kingdom

Animalia), and (3) macro-epibionts (>1 mm). We excluded records from turtles reared in captivity (e.g., Crespo-Picazo et al., 2017) to focus on naturally occurring instances of epibiosis. We excluded epibiotic interactions involving plants (namely algae) as very few studies have included plants and, those that have, tend to show relatively low taxonomic diversity. Lastly, we focused our review exclusively on macro-epibionts (>1 mm) even though sea turtles are known to host meio- or micro-epibionts (<1 mm) (e.g., Robinson et al., 2016; Ingels et al., 2020). This was because the collection and identification of meio- or micro-epibionts generally requires specialist equipment (e.g., light and/or scanning electron microscopes), while this is not the case for macro-epibionts.

From each applicable reference, we extracted data on all reported sea turtle-epibiont interactions according to the data parameters and descriptions listed in **Table 1**. If a single study included more than one host-epibiont pair (e.g., multiple epibiont taxa from one host species or multiple host species or life stages for one epibiont taxon), each specific host-epibiont pair was listed on a separate row within the database. At minimum, we recorded the host turtle species, the epibiont taxon (the lowest taxonomic level reported), the study site, and the type of survey conducted. When data were available, we also recorded the number of turtles surveyed, the life stage of the sampled turtles, the habitat where turtles were encountered, the percent frequency of occurrence per host for each epibiont taxa, the total number of recorded individuals of each epibiont taxon, the turtle body part/s on which each taxon was found, and the deposition location for collected specimens.

To extract further information on each epibiont taxon, we recorded the Higher Taxon (either full Phylum or select phyla differentiated by Subphylum/Class/Subclass) and the taxonomic rank (e.g., species, genus, family, etc.) of the taxonomic name reported in the study. Five phyla were not differentiated further: Bryozoa, Nemertea, Platyhelminthes, Porifera, and Sipuncula. However, the diversity of taxa within six phyla warranted further differentiation by either Subphylum, Class, or Subclass: Annelida (Hirudinea, Polychaeta, and Oligochaeta), Arthropoda (Arachnida, Insecta, Malacostraca, Pycnogonida, Ostracoda, and Thecostraca), Chordata (Tunicata and Vertebrata), Cnidaria (Anthozoa and Hydrozoa), Echinodermata (Asterozoa and Echinozoa), and Mollusca (Bivalvia, Gastropoda, and Polyplacophora). To ensure that we used the most up-to-date taxonomic nomenclature and rank for each epibiont taxon, we referred to the World Register of Marine Species (WoRMS Editorial Board, 2021).

To define the geographic location of each study site, we recorded the latitude and longitude (either provided in the study or plotted using Google Earth) and the ocean region that best described the study site (see **Table 1** for a list of potential ocean regions). Lastly, to further characterize the sea turtle population from which epibionts were sampled for each sea turtle-epibiont interaction, we recorded the regional management unit (RMU) following designations by Wallace et al. (2010) as these are considered geographic cohorts of turtles on independent evolutionary trajectories. When a study site was within overlapping RMUs, we used the best available data (e.g.,

TABLE 1 | Data parameters and descriptions extracted for each sea turtle-epibiont interaction identified during an exhaustive review of published articles and gray literature (i.e., government reports, theses and dissertations, and conference presentations), including possible data options for each category.

Parameter	Description	Data options
Host species	Species of host sea turtle.	*Caretta caretta* (CC), *Chelonia mydas* (CM), *Dermochelys coriacea* (DC), *Eretmochelys imbricata* (EI), *Lepidochelys kempii* (LK), *Lepidochelys olivacea* (LO), *Natator depressus* (ND).
Epibiont taxon	Taxonomic name of recorded epibiont (lowest possible taxonomic level), reflecting the most up-to-date taxonomic nomenclature from the World Register of Marine Species (WoRMS Editorial Board, 2021).	See database for full list.
Taxonomic rank	Taxonomic rank of the taxonomic name for the recorded epibiont.	Species, Genus, Subfamily, Family, Superfamily, Infraorder, Suborder, Order, Infraclass, Subclass, Class, Subphylum, Phylum.
Higher taxon	Phylum of the epibiont taxon including further differentiation by Subphylum, Class, or Subclass for select phyla (when applicable).	Annelida (Hirudinea, Polychaeta, Oligochaeta), Arthropoda (Arachnida, Insecta, Malacostraca, Pycnogonida, Ostracoda, Thecostraca), Bryozoa, Chordata (Tunicata and Vertebrata), Cnidaria (Anthozoa and Hydrozoa), Echinodermata (Asterozoa and Echinozoa), Mollusca (Bivalvia, Gastropoda, and Polyplacophora), Platyhelminthes, Porifera, Nemertea, Sipuncula.
Site	Country and/or name of sampling site.	See database for full list.
Ocean region*	Geographic region of study.	Northwest Atlantic (including Gulf of Mexico), Caribbean, North Central Atlantic, Northeast Atlantic, Mediterranean Sea, Southwest Atlantic, South Central Atlantic, Southeast Atlantic, North Indian (including Red Sea and Persian Gulf), Southwest Indian, South Central Indian, Southeast Indian, Northwest Pacific, North Central Pacific (Hawaii), Northeast Pacific, Southwest Pacific (including Gulf of Carpentaria and Melanesia), South Central Pacific (Polynesia excluding Hawaii), Southeast Pacific, Indonesian Archipelago, Unknown.
Regional Management Unit (RMU)	Regional Management Unit, as defined in Wallace et al. (2010), of the sampled turtles (see Host Species - Data Options for species acronyms)	CC-A-NE, CC-A-NW, CC-A-SW, CC-MED, CC-I-NE, CC-I-NW, CC-I-SW, CC-I-SE, CC-P-S, CC-P-N, CM-A-E, CM-A-NW, CM-A-SC, CM-A-SW, CM-A-SCR, CM-I-NE, CM-I-NW, CM-I-SE, CM-I-SW, CM-MED,

(Continued)

TABLE 1 | (Continued)

Parameter	Description	Data options
		CM-P-E, CM-P-NC, CM-P-NW, CM-P-SC, CM-P-SW, CM-P-WC, CM-P-WPSEA, CM-P-W*, DC-A-NW, DC-A-SE, DC-A-SW, DC-I-NE, DC-I-SW, DC-P-E, DC-P-W, EI-A-E, EI-A-SW, EI-A-WCAR, EI-I-NE, EI-I-NW, EI-I-SE, EI-I-SW, EI-P-E, EI-P-NC, EI-P-SC, EI-P-SW, EI-P-WC, EI-P-WPSEA, LO-A-E, LO-A-W, LO-I-NE, LO-I-NE(a), LO-I-W, LO-P-E, LO-P-E(a), LO-P-W, LK-A-NW, ND-I-SE, ND-P-SW, UNK.
Latitude/ Longitude	Latitude and longitude of sampling site (either provided in the study or plotted using Google Earth).	Latitude and longitude coordinates.
Stage	Life stage of turtle sampled (as described in each study or based on reported body size at maturity).	Juvenile, Adult, Unknown.
Habitat	Habitat type of sampling site.	Nesting beach, Neritic, Oceanic, Stranding/Dead, Unknown.
N (T)*	Number of turtles sampled.	An integer.
Freq (%)*	Percent frequency of occurrence per host for the epibiont taxon.	A percentage.
N (E)*	Number of individuals of the epibiont taxon recorded in the study.	An integer.
Body part*	Body part(s) from which the epibiont taxon was collected.	Carapace, Head/Neck, Front flippers, Rear flippers, Plastron, Inguinal Area/Tail, Unknown.
Deposition*	Location where epibiont specimens were curated after the study.	Not Collected, Museum, Personal Collection, Unknown.
Survey type	Method of epibiont sampling: Did the study sample all possible taxa (All Taxa) or only focused on a subset of taxa (Taxon Specific). Also, were the turtles sampled exhaustively for epibionts (Exhaustive) or were only a subset of epibionts sampled (Non-Exhaustive)	Exhaustive/All Taxa, Exhaustive/Taxon Specific, Non-Exhaustive/All Taxa, Non-Exhaustive/Taxon Specific.
Primary reference	Reference for data source.	See database for full list.
Secondary reference(s)	References that also presented these data but were not the primary data source.	See database for full list.

Asterisks indicate data categories that were included in the global database (Pfaller and Robinson, 2022) but were not analyzed in this study.

life stage and habitat) to select the most likely RMU for the surveyed turtles.

Comparing Epibiont Diversity

We collated the total number of distinct epibiont taxa documented for each turtle species and for three loggerhead RMUs (Northwest Atlantic, Mediterranean, and North Pacific). We selected these specific RMUs because each had been the focus of >15 studies. To avoid overestimating the number of taxa when studies identified epibionts to different taxonomic ranks (e.g., species versus genus level), we counted all potentially equivalent taxa as one taxon. For example, if three different studies on olive ridley turtles identified *Lepas anatifera* (species rank), *Lepas* sp. (genus rank), and Lepadidae (family rank), we combined these three taxa into one taxon and only counted them once. We therefore defined taxonomic richness as the total number of unique epibiont taxa that could not be further hierarchically combined. Because most studies reported epibiont taxa at the species level, the mean percentage of taxa that we hierarchically combined in this way within each host species and within the three loggerhead RMUs was relatively low (10.2 and 9.0%, respectively).

For each turtle species, as well as the three loggerhead RMUs, we plotted the number of taxa and proportion of taxa documented within each Higher Taxon category (either full Phylum or select phyla differentiated by Subphylum/Class/Subclass as detailed in **Table 1**). To minimize the number of categories displayed in these figures, we combined Higher Taxon categories within a given Phylum (e.g., Echinodermata – Asterozoa and Echinodermata – Echinozoa) when no individual turtle species was documented hosting eight or more taxa within a Higher Taxon category. All remaining Higher Taxon categories with fewer than seven total host-epibiont pairs across all turtle species were also combined into a single category called "Other taxa." One exception to these rules was the Higher Taxon category Annelida – Hirudinea, which includes two globally distributed species of marine turtle leech that retained in the diversity plots because of their important role in sea turtle health and disease transmission (Greenblatt et al., 2004; Köhnk et al., 2021).

Extrapolating Taxonomic Richness

To evaluate whether the current scientific literature fully encompasses the taxonomic richness of each turtle species, as well as the three selected loggerhead RMUs, we used rarefaction curves following the Bernoulli product model to estimate the rate at which epibiont taxonomic richness increased with increasing sample sizes (Colwell et al., 2012). This allowed us to account for differences in samples sizes when comparing the taxonomic richness between different turtle species and RMUs. Because rarefaction curves can reasonably extrapolate species richness up to double or triple the reference sample size (Colwell et al., 2012), we estimated taxonomic richness after 150 studies for each turtle species and after 80 studies for each loggerhead RMU. We excluded Kemp's ridley and flatback turtles from the rarefaction analyses because there were <15 studies available for these two species and this was not sufficient to provide accurate extrapolations of taxonomic richness.

We used individual studies as the baseline sampling unit and built sample-based rarefaction curves instead of individual-based rarefaction curves. While it would have been preferential to use individual turtles as the sampling unit for the rarefaction analyses, most studies only presented the combined epibiont

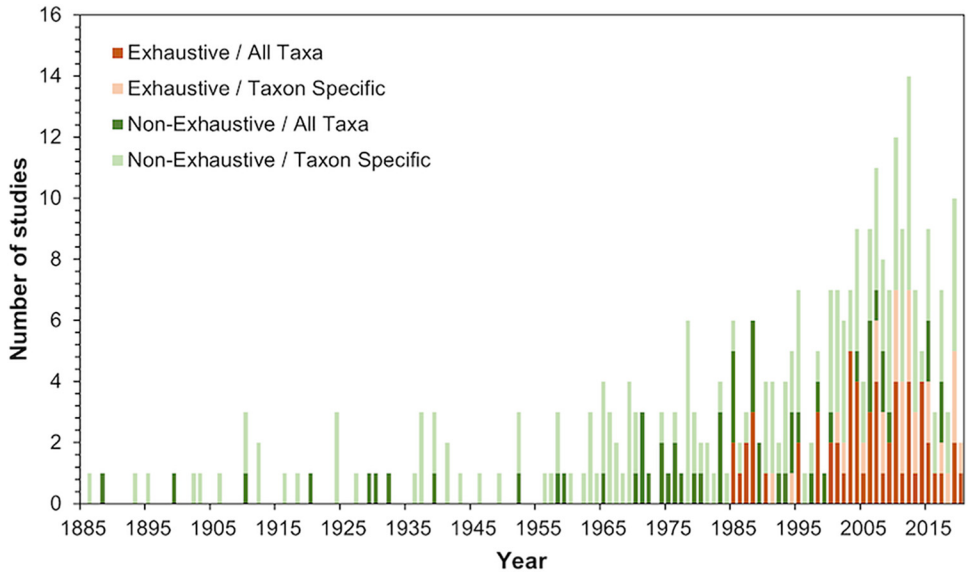

FIGURE 1 | Number of All Taxa, Taxon Specific, Exhaustive, and Non-Exhaustive studies by year that included data on sea turtle-epibiont interactions identified during our two-tiered search of both published scientific articles and gray literature (e.g., government reports, theses and dissertations, and conference presentations). All Taxa studies attempt to document all possible epibiont taxa, while Taxon Specific studies focus on one or more specific taxonomic groups. Exhaustive studies sampled all observed epibionts, while Non-Exhaustive only sampled a subset of the observed epibionts.

communities of all turtles sampled and not the unique epibiont communities on each turtle. We acknowledge this violates two key assumptions of rarefaction curves: samples are collected at random from the population and samples are collected with equal effort. Indeed, global epibiont studies were not conducted at random between different RMUs (see section "Results: Identifying Knowledge Gaps") and the same number of turtles were not sampled in each study. For these reasons, the sample-based rarefaction curves in this study likely underestimate total taxonomic richness. Sample-based rarefaction and extrapolation curves as well as their 95% confidence intervals were calculated using the program EstimateS V.9.1.

Identifying Knowledge Gaps

To identify knowledge gaps in the literature, we quantified sampling effort (i.e., the number of studies) for each turtle species in terms of turtle life stage and habitat type. For turtle life stage, we collated the number of studies documenting epibionts on juveniles (i.e., non-sexually mature), adults (i.e., sexually mature), and turtles of unknown size or reproductive status (i.e., not indicated in the study). For habitat type, we collated the number of studies documenting epibionts on turtles that were intercepted while nesting on beaches, captured in neritic habitats (<200 m depth), captured in oceanic habitats (>200 m depth), found dead/debilitated in the marine environment or washed ashore, and those in which the habitat type was unknown. Studies in which epibionts were surveyed on both juvenile and adult turtles or where turtles were surveyed in more than one habitat type were counted in each applicable category.

Additionally, we assessed knowledge gaps in terms of geographic distribution by plotting every sea turtle epibiont study onto global maps that delineated RMUs for each turtle species following Wallace et al. (2010). We distinguished between studies that reported only the presence of a subset of epibiont taxa (Taxon Specific surveys) from those that focused on all potential taxa (All Taxa surveys) because these differences may bias geographic patterns in taxonomic richness among sea turtle RMUs. For example, studies investigating the phylogenetics of a specific sea turtle barnacle will only report the collection localities of that barnacle species and not on the other epibiont taxa that may have also occurred on the host turtles sampled in those regions (e.g., Pinou et al., 2013). In contrast, All Taxa studies attempt to document and report on all epibiont taxa detected on surveyed turtles (e.g., Robinson et al., 2017b). Maps were created in ArcGIS v10.6.

RESULTS

Database Summary

From our literature review, we identified 304 studies that contained data on a combined total of 1,717 sea turtle-epibiont interactions. Across the seven sea turtle species, we recorded 374 unique epibiont taxa representing 11 Phyla, separated into 23 Higher Taxon categories. The earliest record included was published in 1886. Since then, there was a steady increase in the annual number of publications reporting on sea turtle epibiosis until approximately 2010, after which there was a slight decline (**Figure 1**). This increase included both All Taxa and Taxon Specific studies, although Exhaustive studies only began to increase after 1985.

To encourage further exploration of the information amassed in this database, we have made it freely available in the Dryad Digital Repository[1] (Pfaller and Robinson, 2022). Data compiled for several categories listed in **Table 1** were not analyzed in this study but were included in the database because they provide important biological and/or methodological information specific to each sea turtle-epibiont interaction (e.g., frequency of occurrence, body part, etc.) that may be used in future studies. We expect future authors to update this database with information from new publications (after 2020), as well as any sea turtle-epibiont interactions that were missed during our literature review.

Comparing Epibiont Diversity

Of the 374 epibiont taxa representing 23 Higher Taxon categories (either full Phylum or select phyla differentiated by Subphylum/Class/Subclass) that were documented on sea turtles globally, loggerheads hosted 262 taxa from 21 categories (**Figure 2** and **Supplementary Table 1**). Loggerheads from the Northwest Atlantic RMU hosted 162 taxa from 18 Higher Taxon categories, while loggerheads from the Mediterranean and North Pacific RMUs hosted 85 taxa from 12 categories and 27 taxa from six categories, respectively (**Supplementary Figure 1**). Hawksbills hosted a similar level of diversity as loggerheads (20 Higher Taxon categories), but the total taxonomic richness was lower (87 taxa). The richness and diversity of epibionts hosted by the other five sea turtle species were considerably lower than that of loggerheads and somewhat lower than that of hawksbills: greens (56 taxa from 12 Higher Taxon categories), olive ridleys (51 taxa from 14 categories), leatherbacks (15 taxa from 4 categories), Kemp's ridley (7 taxa from 3 categories), and flatbacks (7 taxa from 3 categories) (**Figure 2** and **Supplementary Table 1**).

Among the 13 Higher Taxon categories not combined into "Other taxa," loggerheads and hawksbills were the only species that hosted epibiont taxa from all categories (**Figure 2** and **Supplementary Table 1**). Moreover, loggerheads and hawksbills hosted roughly similar proportions of taxa from each category, with the predominant categories being Annelida – Polychaeta (i.e., polychaete worms), Arthropoda – Malacostraca (e.g., crabs, shrimps, and amphipods), and Arthropoda – Thecostraca (e.g., acorn and goose-necked barnacles). Northwest Atlantic loggerheads hosted epibiont taxa from 12 Higher Taxon categories not combined into "Other taxa," while Mediterranean and North Pacific loggerheads hosted epibiont taxa from nine and five categories, respectively (**Supplementary Figure 1** and **Supplementary Table 1**). Green and olive ridley turtles hosted epibiont taxa from all but one category (Echinodermata – Asterozoa, Echinozoa were not recorded for either host species), while leatherbacks, Kemp's ridleys, and flatbacks hosted epibiont taxa from four, three, and three categories, respectively. Arthropoda – Thecostraca was the only Higher Taxon category reported from all seven sea turtle species and its predominance (% of taxa) tended to increase as the total epibiont richness of a host species decreased.

[1]http://doi.org/10.5061/dryad.x3ffbg7m8

Extrapolating Taxonomic Richness

Rarefaction curves indicated that loggerhead turtles both host the highest total taxonomic richness of all seven sea turtle species and have had the greatest sampling effort in terms of number of studies (**Figure 3**). Extrapolating beyond the current 135 studies documenting the epibiont diversity of loggerheads, the rarefaction curve approaches 300 epibiont taxa after 150 studies. This suggests that the current scientific literature has not yet fully described the taxonomic richness for loggerhead epibionts on a global scale.

The rarefactions curves for loggerheads from the Northwest Atlantic, Mediterranean, and North Pacific RMUs also suggested that further studies will reveal additional epibiont diversity (**Figure 4**). Extrapolating up to 80 studies per RMU indicated that the taxonomic richness of Northwest Atlantic loggerheads could reach an estimated 202 epibiont taxa, while Mediterranean and North Pacific loggerheads could reach an estimated 137 and 55 epibiont taxa, respectively. While extrapolations suggest that current studies have not fully described the taxonomic richness within each loggerhead RMU, the lack of overlap between the 95% confidence intervals suggests that geographic variation in taxonomic richness among loggerhead RMUs represents a true biological pattern.

Hawksbill turtles hosted the second highest taxonomic richness after loggerheads. However, because this diversity was recorded from comparably fewer studies (N = 58), the extrapolated rarefaction curve for hawksbills reached 148 epibiont taxa after 150 studies (**Figure 3**). The 95% confidence intervals for hawksbills overlapped with that of olive ridleys. Like hawksbills, olive ridleys hosted relatively high diversity from comparably fewer studies (N = 32), causing the extrapolated rarefaction curve to estimate approximately double the diversity (101 taxa) after 150 studies. Additional studies investigating the epibiont diversity of hawksbills and olive ridleys globally are needed to unequivocally determine whether the epibiont communities of hawksbill turtles are more taxonomically rich than olive ridley turtles.

Green turtles, despite having the second most studies (N = 111), were recorded hosting significantly fewer epibiont taxa than hawksbills and approximately the same number of taxa as olive ridleys. Consequently, the extrapolated rarefaction curve for green turtles estimated only 64 epibiont taxa after 150 studies, which was only eight taxa higher than is currently reported (**Figure 3**). Similarly, epibiont research on leatherbacks has appeared to reach a plateau: 15 taxa have been recorded from 35 studies to date, but only 16 total taxa are expected to be recorded after 150 studies are conducted.

Identifying Knowledge Gaps – Life Stage and Habitat

Turtle life stage (adult and/or juvenile) was only reported in 52% of studies (**Figure 5A**). Within each turtle species, the percentage of studies reporting turtle life stage ranged from 49% in greens and 84% in olive ridleys. Among studies that reported turtle life stage, there were strong biases toward adult turtles for flatbacks (100%), leatherbacks (86%), and olive ridleys (84%),

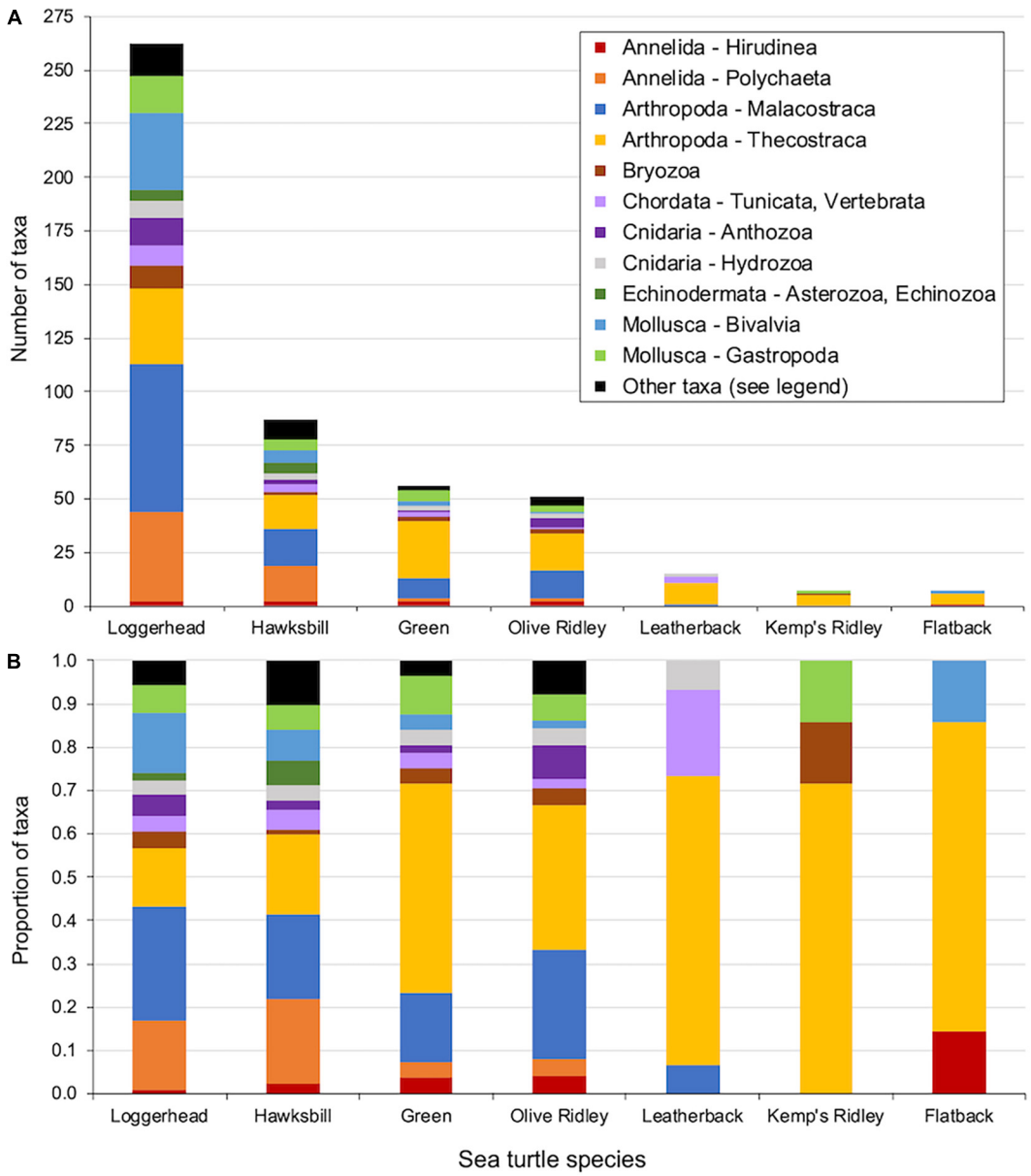

FIGURE 2 | Taxonomic composition of the epibiont communities reported from all seven sea turtle species. **(A)** Number of taxa per Higher Taxon category (either full Phylum or select phyla differentiated by Subphylum/Class/Subclass) and **(B)** proportion of taxa per Higher Taxon category. The category "Other taxa" combines taxa from 10 Higher Taxon categories in which fewer than six total taxa were documented across all turtle species (Loggerhead: Annelida – Oligochaeta, Arthropoda – Ostracoda, Arthropoda – Pycnogonida, Mollusca – Polyplacophora, Nemertea, Platyhelminthes, Porifera, and Sipuncula; Hawksbill: Annelida – Oligochaeta, Arthropoda – Insecta, Arthropoda – Ostracoda, Mollusca – Polyplacophora, Platyhelminthes, Porifera, and Sipuncula; Green: Arthropoda – Arachnida and Platyhelminthes; Olive Ridley: Arthropoda – Pycnogonida, Mollusca – Polyplacophora, Platyhelminthes, and Porifera). See **Supplementary Table 1** for numerical summary of the number of epibiont taxa reported within each Higher Taxon for each sea turtle species.

while significant biases toward adult turtles were not as apparent for hawksbills (59%), loggerheads (54%), and green turtles (48%). Kemp's ridleys were the only species with a strong bias toward juveniles (25% adult). Nevertheless, the two species with the strongest biases (Kemp's ridleys and flatbacks) also had relatively few studies (seven and two total studies, respectively).

Habitat type (nesting beach, neritic, oceanic, and/or stranding/dead) was reported for only 63% of studies (**Figure 5B**). Within each turtle species, the percentage of studies that reported habitat type ranged from 50% in flatbacks and 92% in olive ridleys. Among studies that reported habitat type, there were biases toward nesting beaches for flatbacks (100%), leatherbacks (54%), and olive ridleys (53%), reflecting their biases toward studies on adult turtles. The habitat types among studies on leatherbacks and olive ridleys were similar in proportion: nesting beaches (54 and 53%, respectively), neritic

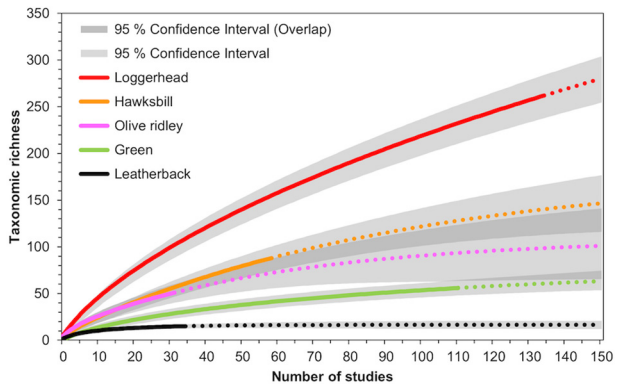

FIGURE 3 | Rarefaction curves to estimate taxonomic richness of epibionts for loggerhead, hawkbill, olive ridley, green, and leatherback sea turtles. Solid lines indicate data curves calculated from previous studies, while dashed lines represent extrapolated data.

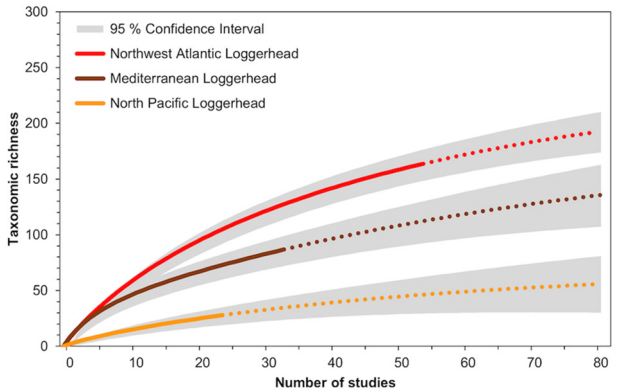

FIGURE 4 | Rarefaction curves to estimate the taxonomic richness of epibionts for three loggerhead Regional Management Units: Northwest Atlantic, Mediterranean, and North Pacific. Solid lines indicate data curves calculated from previous studies, while dashed lines represent extrapolated data.

(22 and 23%, respectively), oceanic (11 and 12%, respectively), and stranding/dead (14 and 12%, respectively). Studies on loggerheads were more balanced between habitat types: nesting beach (33%), neritic (30%), oceanic (17%), and stranding/dead (20%). Studies on hawksbills were biased toward nesting beaches (42%) and neritic habitats (44%) and away from oceanic habitats (only 2%). Studies on greens and Kemp's ridleys were biased toward neritic habitats (59 and 50%, respectively) and away from nesting beaches (19 and 0%, respectively) and oceanic habitats (6 and 0%, respectively), but studies on Kemp's ridleys also suffered from a small sample size (only three neritic, three stranding/dead, and three unknown habitats for Kemp's ridleys). Studies on flatbacks were also biased by low sample sizes (two studies on nesting beaches and two in unknown habitats). Among all studies from all species, including those that included turtles sampled from more than one habitat type (i.e., those counted in more than one category), 38% were in neritic waters, 34% were on nesting beaches, 11% were in oceanic waters, and 17% were from stranded or dead turtles.

Identifying Knowledge Gaps – Geography and Regional Management Units

For loggerheads, epibiont studies were geographically concentrated in the Northwest Atlantic (33%) and Mediterranean (22%) RMUs, where a high percentage of studies were All Taxa surveys (42 and 33%, respectively) (**Figure 6A**). Conversely, loggerhead studies in the western portion of the North Pacific RMU were relatively numerous (15%) but were predominantly Taxon Specific surveys (87%). For green turtles, epibiont studies were globally distributed but most studies were Taxon Specific (79%) (**Figure 6B**). Both Taxon Specific and All Taxa surveys were geographically concentrated in the East Pacific RMU and the area of overlap between the Southwest Atlantic and South Central Atlantic RMUs in southern Brazil and Uruguay. Taxon Specific surveys were also concentrated in the Northwest Pacific, Southwest Pacific, and Northwest Indian RMUs, while All Taxa surveys were also concentrated in the North Central Pacific RMU (i.e., Hawaii).

For hawksbills, Taxon Specific surveys were more common (69%) and geographically more homogeneous (**Figure 7A**), with the West Pacific/Southeast Asia RMU and East Pacific RMU having the most Taxon Specific surveys (75 and 100%, respectively) and only one All Taxa survey each. All Taxa surveys for hawksbills were geographically concentrated in the Western Caribbean/United States (100%) and Southwest Pacific RMUs (71%). For leatherbacks, both Taxon Specific and All Taxa surveys were geographically concentrated in the Northwest Atlantic RMU (**Figure 7B**), which included epibiont studies conducted in both eastern North America and the Caribbean as well as western Europe in the Northeast Atlantic. Two All Taxa surveys have been conducted on leatherbacks in the northern portion of the East Pacific RMU. The West Pacific RMU was the subject of several Taxon Specific surveys. However, these surveys covered a very wide geographic area, ranging from Japan to Malaysia to New Zealand.

For Kemp's and olive ridleys, by far the greatest concentration of both Taxon Specific and All Taxa surveys were in the East Pacific olive ridley RMU (**Figure 8A**), representing 81% of the All Taxa surveys and 64% of the Taxon Specific surveys for ridleys. Kemp's ridley studies in the Gulf of Mexico and Northwest Atlantic were few with only seven studies but were balanced between Taxon Specific and All Taxa surveys. For flatbacks, only four studies have been conducted (**Figure 8B**): one All Taxa survey in the Southeast Indian RMU, and one All Taxa and two Taxon Specific surveys in the Southwest Pacific RMU.

DISCUSSION

The first studies documenting epibionts of sea turtles were conducted over a century ago and since then data available on sea turtle epibiosis has grown extensively. To utilize this growing body of knowledge, we developed a global database of sea

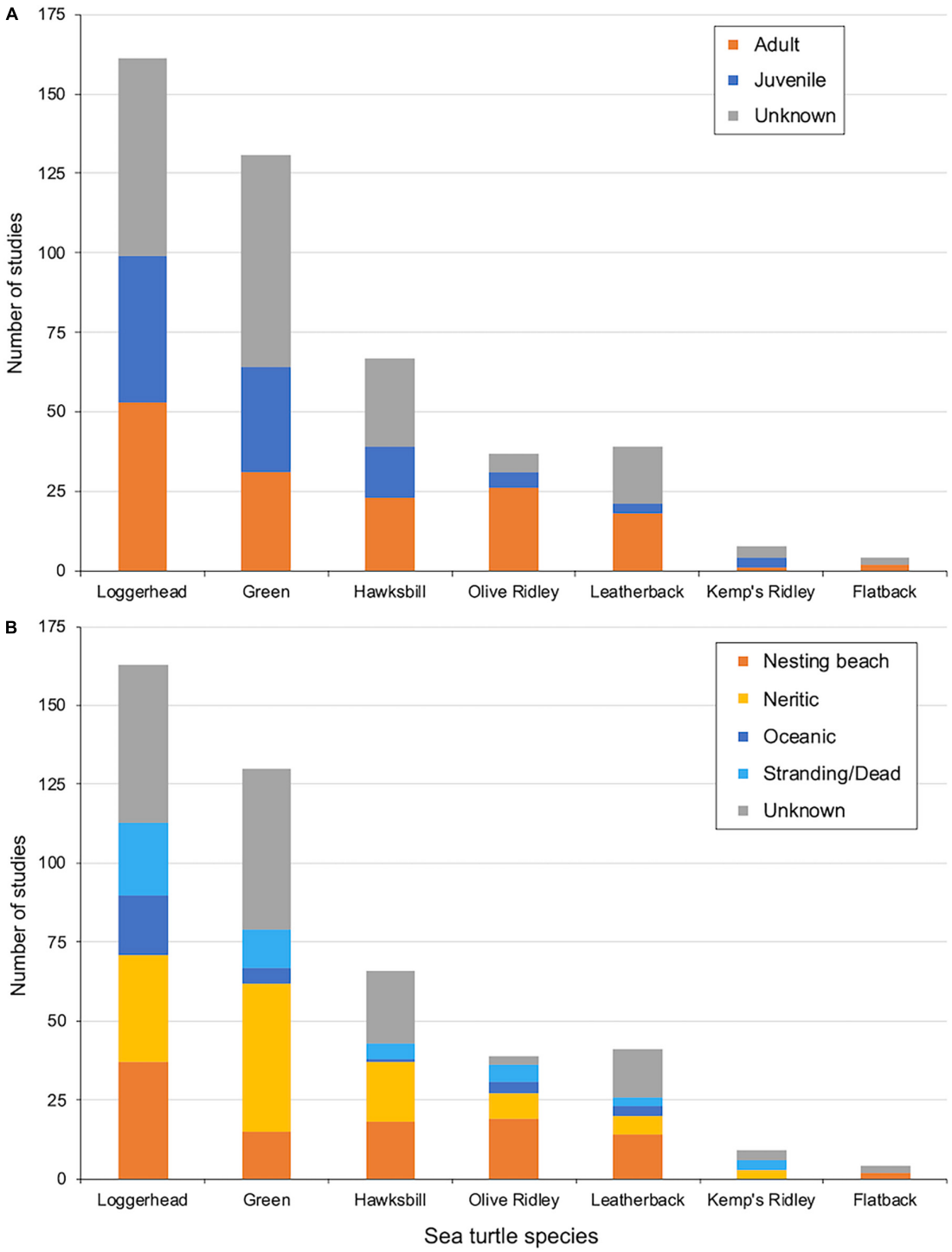

FIGURE 5 | (A) Number of epibiont studies separated by turtle species and life stage (either adult, juvenile or unknown). **(B)** Number of epibiont studies separated by turtle species and habitat type (nesting beach, neritic, oceanic, stranding/dead animals, or unknown).

turtle-epibiont interactions. We compiled data from 304 studies, spanning both published scientific articles and gray literature, and explored global patterns in epibiont diversity. In doing so, we synthesized over 100 years of sea turtle epibiont research. We demonstrate that additional epibiont diversity remains to be documented, even within the most well-studied sea turtle species and populations, and we identify biases in sampling effort that may reveal additional diversity in future studies.

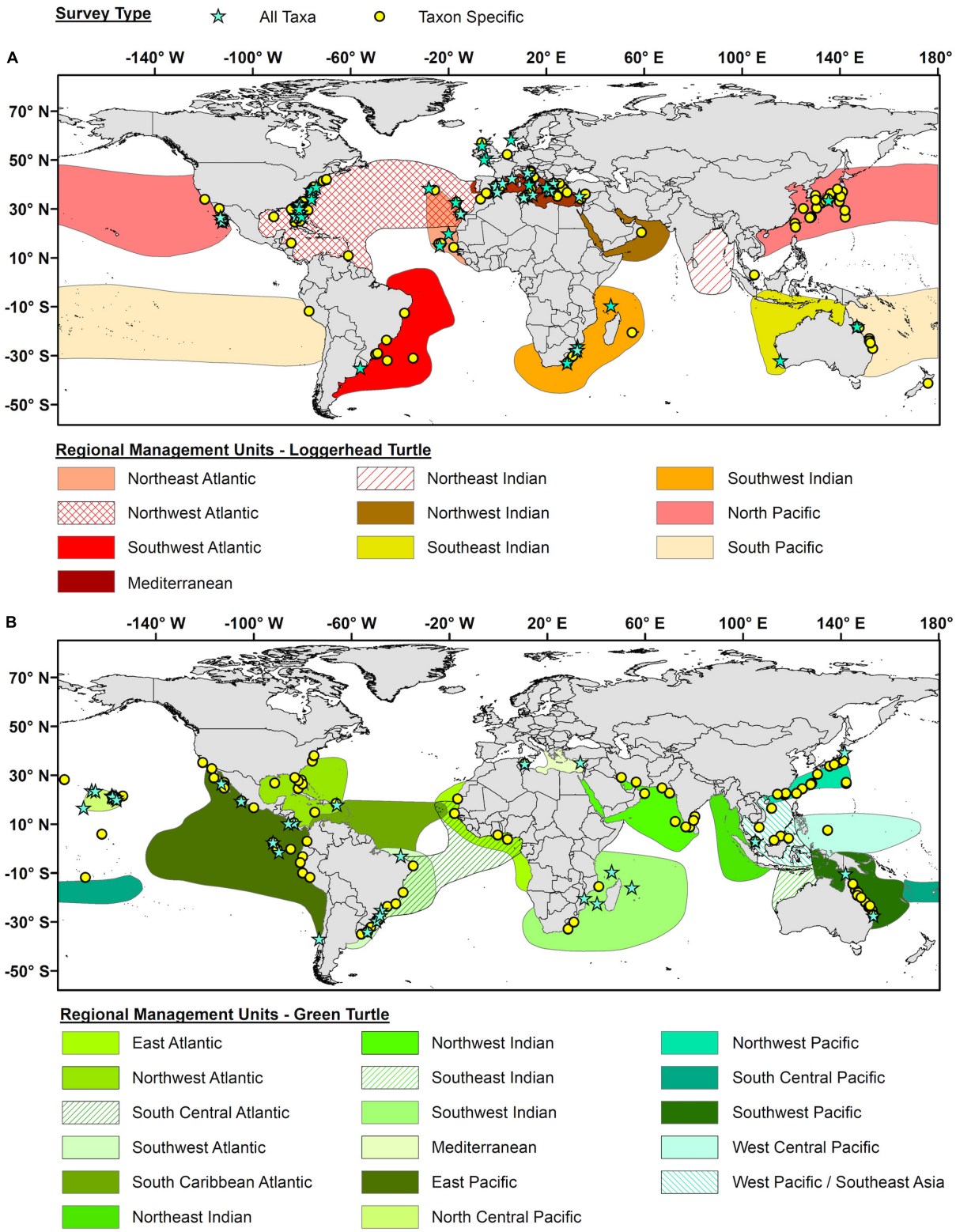

FIGURE 6 | Geographic distribution of all epibiont studies for **(A)** loggerhead turtles (*Caretta caretta*) and **(B)** green turtles (*Chelonia mydas*). Blue stars represent studies that report on all potential taxa (All Taxa), while yellow circles represent studies that only focus on specific taxa (Taxon Specific). The geographic outlines for each Regional Management Unit as represented by differentially colored polygons (based on Wallace et al., 2010).

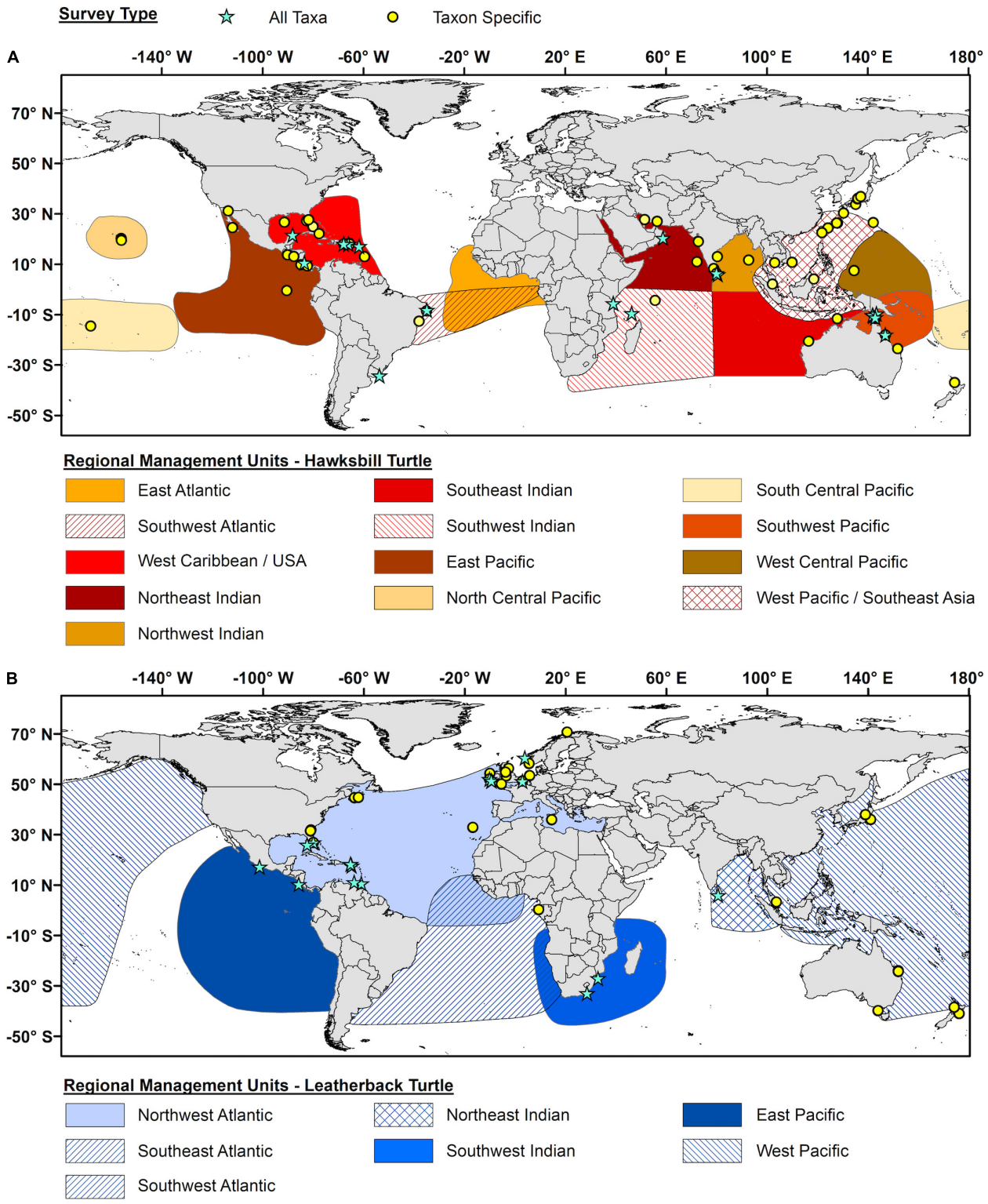

FIGURE 7 | Geographic distribution of all epibiont studies for **(A)** hawksbill turtles (*Eretmochelys imbricata*) and **(B)** leatherback turtles (*Dermochelys coriacea*). Blue stars represent studies that report on all potential taxa (All Taxa), while yellow circles represent studies that only focus on specific taxa (Taxa Specific). The geographic outlines for each Regional Management Unit as represented by differentially colored polygons (based on Wallace et al., 2010).

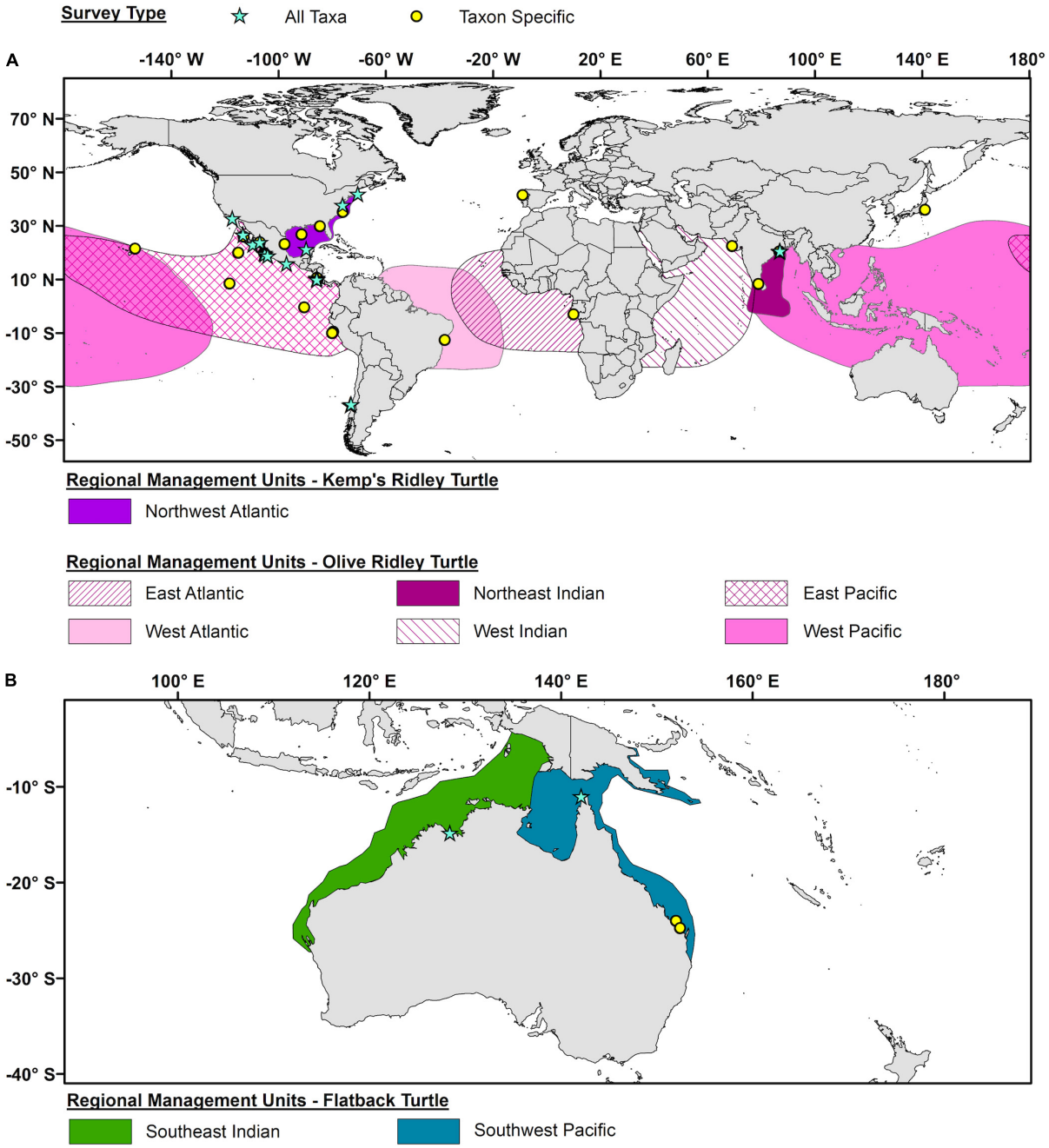

FIGURE 8 | Geographic distribution of all epibiont studies for **(A)** Kemp's and olive ridley turtles (*Lepidochelys kempii* and *L. olivacea*) and **(B)** flatback turtles (*Natator depressus*). Blue stars represent studies that report on all potential taxa (All Taxa), while yellow circles represent studies that only focus on specific taxa (Taxa Specific. The geographic outlines for each Regional Management Unit as represented by differentially colored polygons (based on Wallace et al., 2010).

Which Sea Turtle Species Hosts the Greatest Epibiont Diversity?

Based on the current literature and confirmed *via* the rarefaction analyses, loggerhead turtles host the most diverse epibiont communities both in terms of total taxonomic richness and number of higher taxa represented (262 taxa from 21 Higher Taxon categories). This level of epibiont diversity is only rivaled by hawksbill turtles (20 Higher Taxon categories). Nevertheless, the total taxonomic richness reported for hawksbills (87 taxa) is still far lower than loggerheads. As for the other five sea turtle species, the richness and diversity of their epibionts communities were considerably lower than that of loggerheads and somewhat lower than that of hawksbills, especially for leatherbacks, Kemp's ridleys, and flatbacks. Differences in the taxonomic richness and

diversity among the epibiont communities of different sea turtles may be attributed to several, non-mutually exclusive factors.

First, because epibiosis necessitates spatial overlap between the ranges of host turtles and their epibionts (Frick and Pfaller, 2013), sea turtle species with wider geographic ranges would theoretically encounter a greater diversity of potential epibionts and thus host greater richness and diversity. Consistent with this hypothesis, Kemp's ridleys and flatbacks have the smallest geographic ranges of the seven sea turtle species and host the lowest epibiont richness and diversity. However, among the remaining sea turtle species, this hypothesis is not consistent with the observed patterns. Leatherbacks have the widest geographic range, spanning all tropical to sub-polar oceans of the world (James et al., 2006), yet host comparably low epibiont richness and diversity. In contrast, loggerheads, greens, hawksbills, and olive ridleys have comparable circumglobal ranges, yet exhibit considerable differences in epibiont richness and diversity. Moreover, for example, North Pacific loggerheads have a vastly wider geographic range than Mediterranean loggerheads yet host lower epibiont richness and diversity. Clearly, geographic range size is not the only factor driving differences in epibiont richness and diversity among sea turtle species or populations.

Second, sea turtles occupying a wider array of habitats during their life cycle may overlap with a greater diversity of potential epibionts and thus host greater richness and diversity. In support of this hypothesis, loggerheads, which host the greatest epibiont diversity, not only exhibit an extended oceanic developmental phase (Bolten, 2003; Avens et al., 2013) but are also well known for a behavioral polymorphism in which some individuals transition to neritic foraging areas while other remain oceanic (Hatase et al., 2010; Rees et al., 2010; Vander Zanden et al., 2010). In contrast, greens and hawksbills exhibit a more truncated oceanic developmental phase and primarily inhabit neritic habitats (Vander Zanden et al., 2013; Martinez-Estevez et al., 2021), while leatherbacks and olive ridleys occupy primarily oceanic habitats (Fossette et al., 2010; Pikesley et al., 2013). Moreover, not all habitats will contain equally diverse communities of potential epibionts. For example, the coastal hard-bottom and coral reef habitats occupied by loggerheads and hawksbills not only support high invertebrate biodiversity but also exhibit intense competition for space, both of which may contribute to greater epibiont diversity in these habitats. In contrast, the oceanic habitats occupied by leatherbacks and olive ridleys and the coastal seagrass meadows occupied by green turtles support lower biodiversity and less intense competition for space. As such, our results support the idea that the number and type of habitats occupied by sea turtles, rather than the size of their geographic range, is likely a more important factor driving differences in epibiont richness and diversity.

Third, sea turtles presenting more suitable conditions for epibiont settlement would be more likely to host greater epibiont richness and diversity. Especially for sessile epibionts, the surface properties (e.g., chemical signals, rugosity, and wettability) of the skin and carapace of different sea turtle species may provide more (or less) favorable conditions for larval attachment and subsequent growth. Leatherbacks, in particular, have uniquely smooth skin covering their carapace and this may provide a less favorable substrate for epibionts than the rigid keratin covering the carapace of "hard-shelled" sea turtles (Wyld and Brush, 1983). This could be one of the primary reasons why leatherbacks host lower epibiont richness and diversity than other sea turtles. Among "hard-shelled" sea turtles, the thick, overlapping scutes of hawksbills and characteristically "rough" scutes of loggerheads may facilitate epibiont attachment and persistence to a greater degree than the "smooth" scutes of green and ridley turtles and the thin, waxy scutes of flatbacks. Moreover, the presence of certain sessile epibionts may also provide settlement cues for other epibionts, including many motile forms (e.g., Arthropoda – Malacostraca). Sea turtles that provide favorable conditions for these "pioneer" species may develop more diverse epibiont communities that begin to resemble the faunal assemblages found in the surrounding environment (e.g., nesting loggerheads in the Northwest Atlantic RMU; Frick et al., 1998). Many of these hypotheses have yet to tested empirically but are likely important biological factors driving differences in epibiont richness and diversity.

Overall, it is unlikely that one single factor that explains why some sea turtles host greater epibiont richness and diversity than others. Instead, a complex suite of factors including geographic range, habitat use and behavior, surface properties, and likely others we have not discussed here, collectively drive the observed patterns. To evaluate the relative importance of various factors, we recommend more detailed comparisons between the epibiont communities of turtles from (1) different RMUs of the same species and (2) different species with overlapping RMUs. Current data may be sufficient to compare some RMUs (e.g., Northwest Atlantic, Mediterranean, and North Pacific loggerhead RMUs). However, for most other RMUs, the acquisition of more data is needed to make such comparisons.

Does the Current Literature Fully Encompass the Taxonomic Richness of Sea Turtle Epibionts?

Despite over 100 years of research describing the epibiont diversity of sea turtles, the current literature has not yet fully encompassed the taxonomic richness of most sea turtle species (**Figure 3**) or even three well-studied loggerhead RMUs (**Figure 4**). Rarefaction analyses indicate that the rate at which taxonomic richness has increased with additional sampling effort (number of studies) has not plateaued for any species or RMU, except leatherbacks.

For the "hard-shelled" or Cheloniid sea turtles, additional studies are expected to continue to reveal more undiscovered taxonomic richness in all species and regions. That said, some species are projected to be greater sources of additional richness than others. Green turtles, which have already been included in over 100 studies, host relatively modest epibiont diversity, and appear to be approaching a plateau in taxonomic richness around 70 taxa globally. Conversely, the taxonomic richness of hawksbill and olive ridley turtles, which have received comparably less attention than green turtles, are projected to almost double after 150 studies, adding an estimated 61 and 50 new epibiont taxa, respectively. Even within the most well-studied sea turtle RMUs, like Northwest Atlantic and Mediterranean loggerheads, dozens of new epibiont taxa are expected to be found with

additional sampling effort. These patterns suggest that addressing the knowledge gaps identified in this study (see below), as well as conducting further sampling in well-studied RMUs, will both be productive and interesting areas of future research.

Epibiont research on leatherbacks has appeared to reach a plateau in taxonomic richness: only 16 taxa are projected after 150 studies, only one higher than currently described. While there are still sampling gaps for leatherbacks in terms of life stage, habitat type, and geography (see below), their oceanic lifestyle and inhospitable surface properties (i.e., leathery skin) have likely constrained the diversity of their epibiont communities to a limited number and type of taxa. Indeed, the epibionts of leatherbacks tend to either be oceanic/pelagic specialists or taxa found only on sea turtles, including two species of barnacle (*Platylepas coriacea* and *Stomatolepas dermochelys*) that are essentially exclusive to leatherbacks (Zardus, 2021; this study). Future studies on leatherbacks are therefore unlikely to reveal additional undocumented diversity.

What Knowledge Gaps in Life Stage and Habitat Type Were Identified?

As with many aspects of sea turtle biology, the relative ease of accessing adult female turtles on nesting beaches has created biases in epibiont research as well. Although nesting females represent a relatively small proportion of the total individuals in any sea turtle population (Heppell et al., 2003), the majority of epibiont studies that report life stage and/or habitat for leatherbacks, olive ridleys, and flatbacks come from adult turtles (>80% of studies) and nesting beaches (>50%). While approximately half of epibiont studies that report life stage for loggerheads, greens, and hawksbills come from adult turtles, there is a better balance of studies between nesting beaches and neritic habitats for loggerheads and hawksbills and an emphasis toward neritic habitats for green turtles. Only Kemp's ridleys have yet to be surveyed for epibionts on nesting beaches. While nesting beaches provide an excellent opportunity to initiate epibiont research in many understudied RMUs, the full richness and diversity of epibionts in those RMUs will not be discovered until in-water studies (both neritic and oceanic) are also conducted.

Relative to their abundance, juvenile turtles have received considerably less attention than adult turtles, especially for leatherbacks and olive ridleys. Similarly, the number of non-breeding turtles inhabiting neritic and oceanic foraging areas is always far greater than the number of breeding females (Heppell et al., 2003), yet the percentage of epibiont studies conducted at in-water sites only exceeds that of nesting beaches for green turtles. The difficulty of sampling turtles in oceanic habitats is evident from the relatively low percentages of epibiont studies conducted in these habitats. Nevertheless, the turtle species that spend more time in oceanic habitats (leatherbacks, olive ridleys, and, to a lesser extent, loggerheads) tend to have proportionately more epibiont studies in those habitats (11–17%) than the turtle species that have a truncated oceanic stage (hawksbill and green turtles; 2 and 6%, respectively). Epibiont studies on Kemp's ridleys and flatbacks were too few to assess biases in life stage and habitat.

Two issues regarding life stage and habitat type emerged from our assessment of the sea turtle epibiont literature. First, a significant percentage of studies that report sea turtle epibionts did not indicate the life stage (48%) or habitat type (37%) of the sampled turtles (**Figure 5**). We strongly encourage researchers interested in reporting epibionts from sea turtles to also collect and provide these important pieces of metadata. Efforts to gain insights from the epibionts of specific turtles and understand global patterns in sea turtle epibiosis require these important data. Second, excluding Kemp's ridleys and flatbacks, between 12 and 20% of the studies that report habitat type came from stranded/dead turtles. Because stranded and/or dead turtles may acquire epibiont taxa after their debilitation or death, their epibiont communities may not be characteristic of the surrounding population of healthy turtles. Instead, the processes involved in the development of their epibiont communities may be quite different. For this reason, inferences gleaned from epibiont taxa found on stranded/dead should be made with caution.

What Knowledge Gaps Among Regional Management Units Were Identified?

Because sea turtle RMUs are defined by their shared geography, critical habitats, and evolutionary trajectory (Wallace et al., 2010), individual RMUs should also be considered the basic unit of sampling for epibiont research. Indeed, we demonstrated that epibiont communities vary dramatically not only between sea turtle species but also between conspecific RMUs. Developing a holistic checklist of sea turtle epibionts on a global scale would therefore require sampling turtles from all RMUs.

When initially assessing the sea turtle epibiont literature for knowledge gaps, it was evident that the two turtle species with the smallest geographic ranges and fewest RMUs were also the most understudied. Kemp's ridleys and flatbacks have been the subjects of seven and four epibiont studies, including just three and two All Taxa studies, respectively. Compared to other turtle species, far more sampling effort would be needed to fully describe the epibiont communities of these hosts. However, when comparing among RMUs, the one global RMU of Kemp's ridleys (Northwest Atlantic) and the two global RMUs for flatbacks (Southeast Indian and Southwest Pacific) have in fact received more attention than many RMUs for the other "well-studied" sea turtle species. Most notably, the following RMUs have never been the focus of a single epibiont study: Northeast Indian loggerheads, Northeast Indian greens, and East Atlantic hawksbills. Moreover, unlike the RMUs for Kemp's ridley and flatback turtles, many RMUs for the other turtle species have also never been the focus of an All Taxa study (e.g., Northwest Indian loggerheads, West Pacific and Southwest Atlantic leatherbacks, and many RMUs for hawksbills, greens, and olive ridleys). Because individual RMUs should be considered the basic unit for epibiont sampling, future epibiont research should focus not only on understudied host species but also on understudied RMUs within well-studied species.

Based on the geographic distribution of epibiont studies as well as their study types (All Taxa versus Taxon Specific), we have

identified the most prominent knowledge gaps among RMUs for each turtle species to help guide future research:

- Loggerheads: eastern portion of the South Pacific RMU (Peru and Chile), Northeast Indian RMU (India, Bangladesh, and Myanmar), and Southeast RMU (NW Australia and southwestern Indonesia), as well as the globally important Northwest Indian RMU, which includes Oman and countries surrounding the Red Sea, Persian Gulf, and gulfs of Oman and Aden.
- Greens: South Caribbean Atlantic RMU (northern South America and Lesser Antilles), Northeast Indian RMU (Bangladesh, Myanmar, and eastern Indonesia), Southeast Indian RMU (northern Australia and south-central Indonesia), West Central Pacific RMU (Philippines and west Micronesia), and South Central Pacific RMU (eastern Melanesia and central Polynesia).
- Hawksbills: East Atlantic RMU (Mauritania to Angola and into the central South Atlantic), Southeast Indian RMU (Western Australia), West Central Pacific RMU (Micronesia), and South Central Pacific RMU (central Polynesia).
- Leatherbacks: central and eastern portions of the West Pacific RMU, the southern portion of the East Pacific RMU (western South America), Southeast and Southwest Atlantic RMUs (eastern South America to western Africa), and Northeast Indian RMU (India, Bangladesh, Myanmar, and eastern Indonesia).
- Olive ridleys: excluding the East Pacific RMU, all five remaining RMUs (West Atlantic, East Atlantic, West Indian, Northeast Indian, and West Pacific).
- Kemp's ridleys: northern and eastern Gulf of Mexico and southeast Atlantic coast of United States (North Carolina thru Florida).
- Flatbacks: western and northern portions of the Southeast Indian RMU (northern Western Australia and southwestern Papua) and the western and southern portion of the Southwest Pacific RMU (Gulf of Carpentaria and central and southern Queensland, as well as southeastern Papua New Guinea).

Future Directions

We built this global database from over 100 years of sea turtle epibiont research and made the first effort to use it by analyzing broad-scale patterns and identifying knowledge gaps. However, the capacity for the information amassed in this database to answer additional questions in this field is extensive. Our hope is that this database will serve as a foundational platform on which a novel array of hypothesis-driven questions can be tested with respect the taxonomic diversity, ecological complexity, and evolutionary origins of sea turtle epibiosis.

Research questions that we consider important for promoting scientific progress in the field of sea turtle epibiosis include but are not limited to:

(1) How does geographic range and habitat use influence the epibiont richness and diversity of different sea turtle species and RMUs?
(2) How do turtle behaviors (e.g., migrating, diving, mating, nesting, etc.) and habitats influence epibiont diversity, as well as the frequency and intensity of different epibiont taxa?
(3) How do surface properties and settlement cues affect the epibiont communities of different turtles?
(4) How does the diversity of meio- and micro-epibionts compare and/or contrast with macro-epibiont communities?
(5) What characteristics make certain "free living" taxa common sea turtle epibionts?
(6) How do the geographic ranges of "free living" populations of common sea turtle epibionts correspond with that of their hosts?
(7) Are obligate epibiont taxa more abundant than facultative taxa on certain turtle species?
(8) Does the frequency and intensity of "pioneer" epibiont taxa facilitate higher species richness?
(9) Which epibiont taxa/communities are best suited to serve as ecological indicators of the behaviors and habitat preferences of their turtle hosts?
(10) How do local oceanographic and climatic factors influence epibiont diversity?
(11) Can epibiont communities be used as indicators of the health status of sea turtles and/or sea turtle populations?
(12) Are certain obligate epibiont taxa of comparable conservation concern as their endangered sea turtle hosts?

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: NR and JP (2022) Data from: Sea turtle epibiosis: global patterns and knowledge gaps. Dryad Digital Repository (http://doi.org/10.5061/dryad.x3ffbg7m8).

AUTHOR CONTRIBUTIONS

Both authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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The Role of Invasion Status and Taxon of Basibionts in Marine Community Structure

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Studies on non-native epibionts typically focus on the organismal-level impacts of epibiosis on basibionts, rather than community-level impacts of this relationship. The purpose of our study was to evaluate if non-native basibionts in general facilitate invasions through epibiosis in Maine compared to native basibiont species. We collected 64 basibiont assemblages including replicate samples of 10 different basibiont taxa on the central Maine coast in October 2019. Each basibiont and associated epibionts were identified to genus, classified as native or non-native to the region where they were collected, and weighed. We found that while there was no association between invasion status of the epibiont and the basibiont, native basibionts had a significantly higher Shannon Diversity Index than non-native basibionts. Although diversity of epibionts was greater on native basibionts, the percentage of invaders varied across basibiont taxa. Specific basibiont taxon characteristics may be more important than status because different taxa have different surface topographies, resulting in varying settlement among epibiont species. Our study indicates that there is differential settlement of epibiont taxa across basibiont taxa, which may help predict, based on surface characteristics, which species support more epibiont taxa. This study, as a snapshot of floating dock fouling communities within a 10 km radius, may indicate that non-native basibionts play a role in changing community structure. Expanding the scope of this initial study to include a wider taxonomic and geographic range should help determine if epibiosis is truly a facilitative process in invasions.

Keywords: invasive species, community ecology, epibiosis, biodiversity, basibiont, facilitation

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INTRODUCTION

Novel ecosystems are the product of new combinations of native and non-native species, resulting in potential changes in ecosystem functions. These are the result of anthropogenic activity – intentional or inadvertent – and result from degradation or invasion of native ecosystems (Hobbs et al., 2006). Non-native species are a threat to marine biodiversity, and invasions have been found in over 84% of marine ecoregions (Molnar et al., 2008). Regions with low diversity may be at a higher risk for invasion, as well as regions with high numbers of transport vectors or disturbances (Cohen and Carlton, 1998).

A potential facilitative mechanism for invasions that has not been directly examined in the literature is epibiosis. Epibiosis occurs when basibionts, organisms that provide a habitable surface, are colonized by epibionts, the organisms that settle on basibiont surfaces (Wahl, 1989). In the Gulf of Maine, a non-native epibiont *Membranipora membranacea* indirectly facilitates the invasion by *Codium fragile* as the epibiont causes decreased growth and increased mortality in native kelp, a competitor for space, on which the bryozoan settles (Levin et al., 2002). Floerl et al. (2004) described the entrainment of propagules of other species by a non-native bryozoan on the hull of a boat covered in antifouling paint. The bryozoans provided an additional habitable space not otherwise available on the boat surface. Prenter et al. (2004) suggest that such facilitative interspecific interactions increase the success and impact of non-native species. However, there are few studies that look at the community-wide impacts of epibiosis, particularly whether epibiosis is a mechanism for facilitating invasion.

The purpose of this study is to analyze the impact of non-native species in marine ecosystems by focusing on invasion status, taxonomy, and frequency of associated epibionts on basibiont specimens at one location on the central coast of Maine. We aim to determine if facilitation *via* epibiosis is impacting community structure in a marine ecosystem in the Gulf of Maine. We ask if the invasion status of the basibiont impacts the frequency of settled non-native epibionts and if there are any differences in epibiont diversity on native and non-native basibionts. Our predictions are that, if non-native basibionts directly facilitate further invasion, we expect to see an increased frequency of non-native epibiont settlement on non-native basibionts. Because patterns in marine communities suggest there is a relationship between invasion and diversity (Stachowicz et al., 2002), we also predict that there may be a difference in species diversity on non-native basibionts versus native basibionts.

MATERIALS AND METHODS

Study Sites

The samples (n = 64) used in this study were collected from floating docks at three sites in the Damariscotta estuary of Maine on October 11, 2019: a scallop farm at Peter's Island (43°54′32.68″ N, 69°34′05.05″ W), South Bristol Fishermen's Co-op (43°51′50.07″ N, 69°33′16.67″ W), and the Darling Marine Center (43°56′3.16″ N, 69°34′46.41″ W; **Figure 1**).

Collection and Processing of Samples

Each sample consisted of a single basibiont and all associated epibionts. The basibionts were haphazardly selected and removed from the floating docks by hand or by gently scraping the dock surface with a net (mesh size = 4.8 mm). Epibionts were considered any sessile species attached to the basibiont at time of collection. Each sample was placed in a numbered 50 mL polystyrene vial and preserved initially in 99% isopropyl alcohol with menthol crystals for relaxation. In the laboratory, the samples were placed in 70% ethanol for longer preservation. For each sample, all basibiont and epibiont specimens were identified to genus using dichotomous keys (e.g., Weiss, 1995) and classified by invasion status (native, including cryptogenic taxa if present, or non-native). The blotted wet weight of each basibiont and associated epibionts were individually determined, in addition to the number of epibionts in each sample. We used blotted wet weight as it is a reliable method for estimating size of most common invertebrates (Ricciardi and Bourget, 1998). To ensure reliability of our measurements, we repeated blotting and weighing until the weights of each sample were consistent. We calculated the Shannon Diversity Index (SDI) of the epibiont assemblages on each basibiont, using weight to measure abundance of the organisms as it applies to both colonial and solitary organisms.

Statistical Methods

In our statistical analyses, we pooled the data across all three sites, because these sites were in close proximity (all within 10 km of one another) and had similar fouling communities. We used a chi-square test to determine if there was an association between epibiont and basibiont status using the proportion of non-native organisms in each sample. A t-test was conducted to determine if the percentage of epibionts that were non-native differed among epibionts by their basibiont host status. An additional t-test was used to compare the mean number of epibiont taxa per basibiont genus on native and non-native basibionts to determine if different genera of basibionts supported varying number of taxa.

Four analysis of variance (ANOVA) tests were used to compare across basibiont taxa. First, we compared the epibiont-basibiont weight ratio among basibiont phyla. The second and third ANOVA tests were used to compare the percentage of epibionts that were non-native among basibiont phyla and basibiont genera, respectively. Finally, we compared the SDI of epibionts (by weight) across basibiont phyla. Significant values ($P < 0.05$) were further examined with a Tukey's test.

Finally, we used an analysis of covariance to test the combined effects of basibiont weight and status on each of the following: the percentage of epibionts that were non-native on each basibiont, and the total epibiont weight on each basibiont host. All statistical analyses were conducted in R (R Project for Statistical Computing, SCR_001905).

RESULTS

A total of 64 basibiont assemblages were collected across all sites; 26 basibionts were non-native species, and 38 basibionts were native. The basibionts and epibionts, as well as their associations, are shown in **Table 1**.

Does Invasion Status Matter?

We tested the hypothesis that there is an overall general difference in epibiont characteristics between native and non-native basibionts. Non-native epibionts were equally distributed among native and non-native basibionts (chi-squared = 1.934, df = 1, p = 0.1643). On native basibionts, there were 63 occurrences of non-native epibionts and 37 occurrences of native

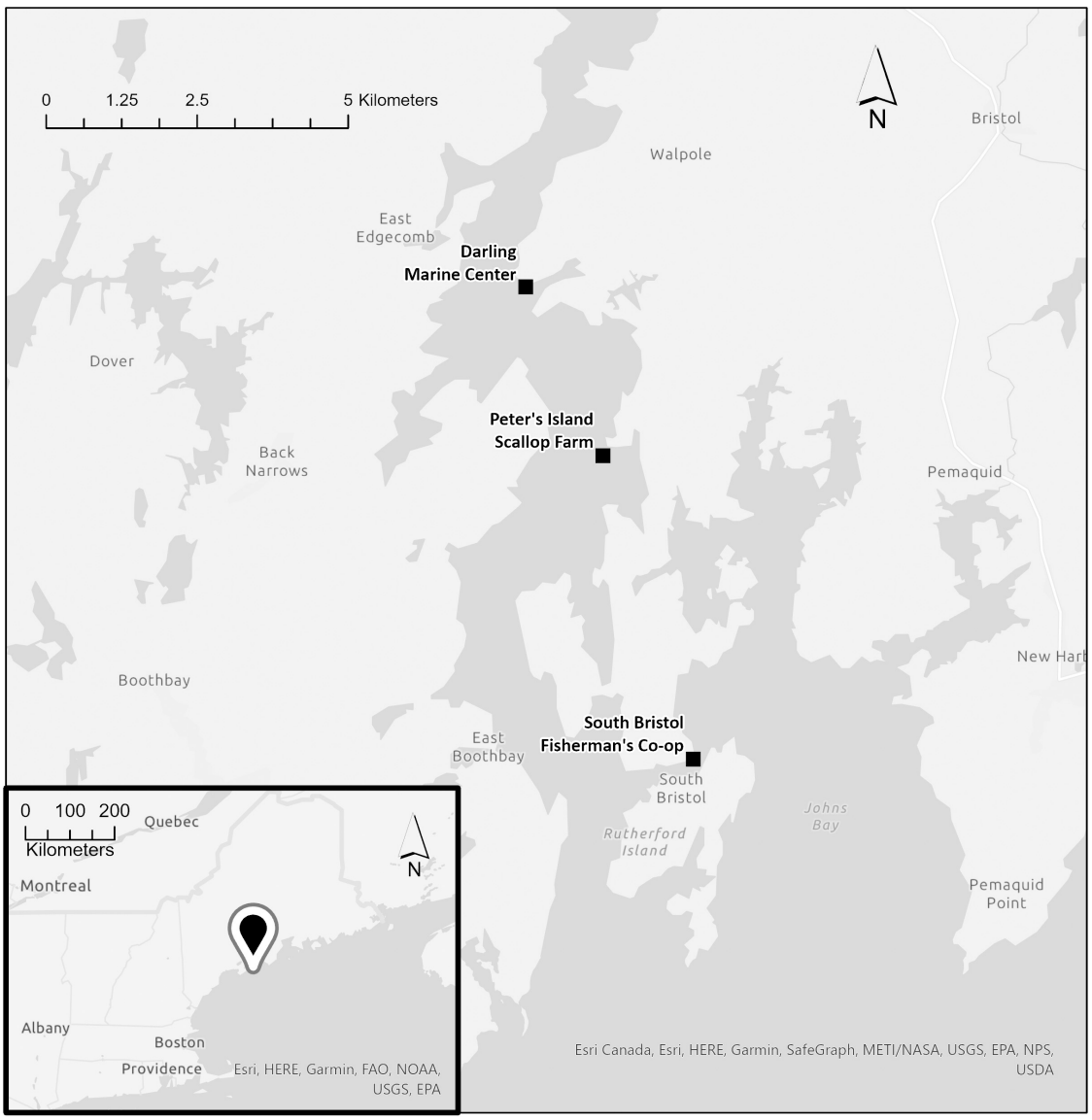

FIGURE 1 | Map of study sites in the Damariscotta Estuary. Inset map shows the general location of the sites in the Gulf of Maine, indicated by the pointer symbol.

epibionts. Overall, there were 27 occurrences of each non-native and native epibionts on non-native basibionts (**Figure 2**). Of non-native epibionts, 30% settled on non-native basibionts, and 70% settled on native basibionts.

Quantity, by weight, of non-native epibionts on non-native basibionts (68.988 ± 8.48%) and on native basibionts (69.303 ± 6.09%) were not significantly different in our study (t = 0.03, df = 48.6, P = 0.976; **Figure 3A**). Native basibiont genera yielded significantly greater epibiont diversity (SDI: 0.296 ± 0.064) than on non-native basibiont genera (SDI: 0.540 ± 0.054; t = –2.9081, df = 54.658, $P < 0.01$; **Figure 3B**).

There was no significant difference between mean number of epibiont genera on each native basibiont genus (8.67 ± 2.42) than on non-native basibiont groups (5.00 ± 1.58; t = –1.269, df = 7.81, P = 0.241; **Figure 3C**). There was also no significant difference in the epibiont-basibiont weight ratio when comparing non-native (0.929 ± 0.169) to native (1.833 ± 0.483) basibionts (t = –1.77, df = 45.655, P = 0.084; **Figure 3D**).

Does the Basibiont Taxon Matter?

Cumulatively, molluscan basibionts supported the most epibiont phyla (n = 7). Following that were Chordata and Rhodophyta (n = 5 each), Ochrophyta (n = 4), and Bryozoa (n = 2). Only molluscan basibionts supported all of the observed epibiont phyla; and mollusks were the only substrate for arthropod and cnidarian epibionts. However, note that there were varying number of samples for each basibiont phylum (**Table 1**). Chordate epibionts dominated the percent occurrence on all basibiont phyla (n = 47.54 ± 8.85%), followed by molluscan epibionts (n = 28.40 ± 3.41%).

TABLE 1 | The occurrences of epibionts and basibionts collected in this study.

		Phylum Bryozoa	Phylum Chordata				Phylum Mollusca	Phylum Ochrophyta			Phylum Rhodophyta	
		Bugula (n = 2)	*Ciona* (n = 11)	*Didemnum* (n = 2)	*Molgula* (n = 1)	*Styela* (n = 12)	*Mytilus* (n = 14)	*Ascophyllum* (n = 2)	*Desmarestia* (n = 2)	*Laminaria* (n = 14)	*Chondrus* (n = 4)	Total Occurrences as Epibiont
Phylum Arthropoda	*Balanus*	0	0	0	0	0	4	0	0	0	0	4
	Chthamalus	0	0	0	0	0	1	0	0	0	0	1
Phylum Bryozoa	*Bugula*	0	0	0	0	1	2	1	0	10	0	15
	Electra(*)	0	0	0	0	0	1	0	0	0	0	1
	Membranipora	0	0	0	0	0	0	0	0	8	2	10
	Tricellaria	0	0	0	0	0	0	0	0	1	0	1
Phylum Chlorophyta	*Ulva(*)*	0	0	0	0	3	1	0	0	0	1	5
Phylum Chordata	*Botrylloides*	1	1	0	0	0	1	1	1	6	2	13
	Ciona	0	1	1	0	0	0	0	0	2	0	4
	Didemnum	1	9	0	1	6	8	0	2	2	3	32
	Molgula	2	4	0	0	3	1	1	2	1	1	15
Phylum Cnidaria	*Obelia(*)*	0	0	0	0	0	1	0	0	0	0	1
Phylum Mollusca	*Hiatella*	0	1	0	0	0	0	0	0	0	0	1
	Macoma	0	0	0	0	0	1	0	0	0	0	1
	Mytilus	0	5	2	0	4	9	1	0	5	3	29
	Tellina	0	2	1	0	0	3	0	0	0	0	6
Phylum Ochrophyta	*Ascophyllum*	1	0	0	0	0	0	0	0	0	0	1
	Desmarestia	0	0	1	0	7	1	0	0	1	1	11
	Dictyosiphon	0	0	0	0	0	0	0	0	1	0	1
	Laminaria	0	0	0	0	1	0	0	0	0	0	1
Number epibionts		**5**	**23**	**5**	**1**	**25**	**34**	**4**	**5**	**37**	**13**	
Total taxon richness		**4**	**7**	**4**	**1**	**7**	**13**	**4**	**3**	**10**	**5**	

Each column represents a different basibiont genus, and each row represents a different epibiont genus. Gray columns and rows indicate that the genus is non-native. Boxes with bold borders show occurrences of non-native epibionts colonizing non-native epibionts. Genera marked with (*) indicate cryptogenic taxa.

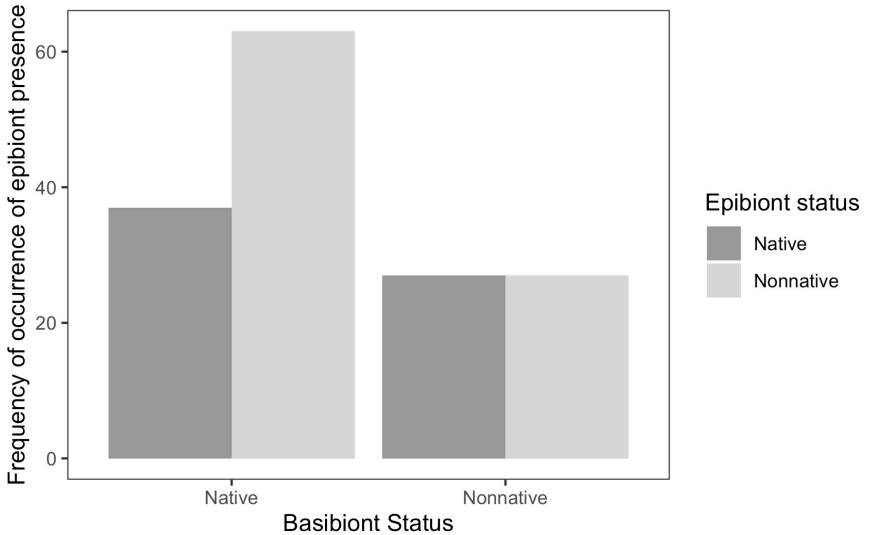

FIGURE 2 | Frequency of occurrence by basibiont status. Non-native epibionts have a higher abundance on native basibionts.

There was a significant difference in epibiont-basibiont weight ratio among basibiont phyla (F = 3.72; df = 4, 59; $P < 0.01$). Only Ochrophyta supported more than its own weight in epibionts (3.151 ± 0.905). The epibiont-basibiont weight ratio for Ochrophyta is significantly higher than that of Chordata (Tukey's, $P < 0.05$) and Mollusca (Tukey's, $P < 0.05$).

There was a significant difference in the percentage of epibionts that were non-native among basibiont phyla (F = 0.3.15; df = 4, 59; $P < 0.05$). Ochrophyta supported significantly more non-native epibionts (mostly bryozoans) than Phylum Mollusca (Tukey's, $P < 0.05$). The only non-native basibiont phylum was Chordata. Interestingly, there is a significant difference in percent epibionts that are non-native among *genera* (F = 3.551; df = 9, 54; $P < 0.05$), in which *Ciona* harbored a higher percentage of non-native epibionts (98.093 ± 0.717%) than *Styela* (44.905 ± 13.677%), *Mytilus* (45.910 ± 11.416%), and *Laminaria* (89.845 ± 5.470%; Tukey's, $P < 0.05$).

Shannon Diversity Index varied with basibiont phyla. There was a significant difference in SDI among phyla (F = 3.78; df = 4, 59; $P < 0.01$). Phylum Rhodophyta (mean SDI: 0.846 ± 0.116, n = 4) had a significantly higher epibiont SDI than Phylum Chordata (mean SDI: 0.296 ± 0.064, n = 26; Tukey's, $P < 0.05$).

Does Size and Status Matter?

Due to the variation in size among the collected basibionts, we were interested in determining if size (approximated by blotted wet weight) and basibiont status were useful in predicting either

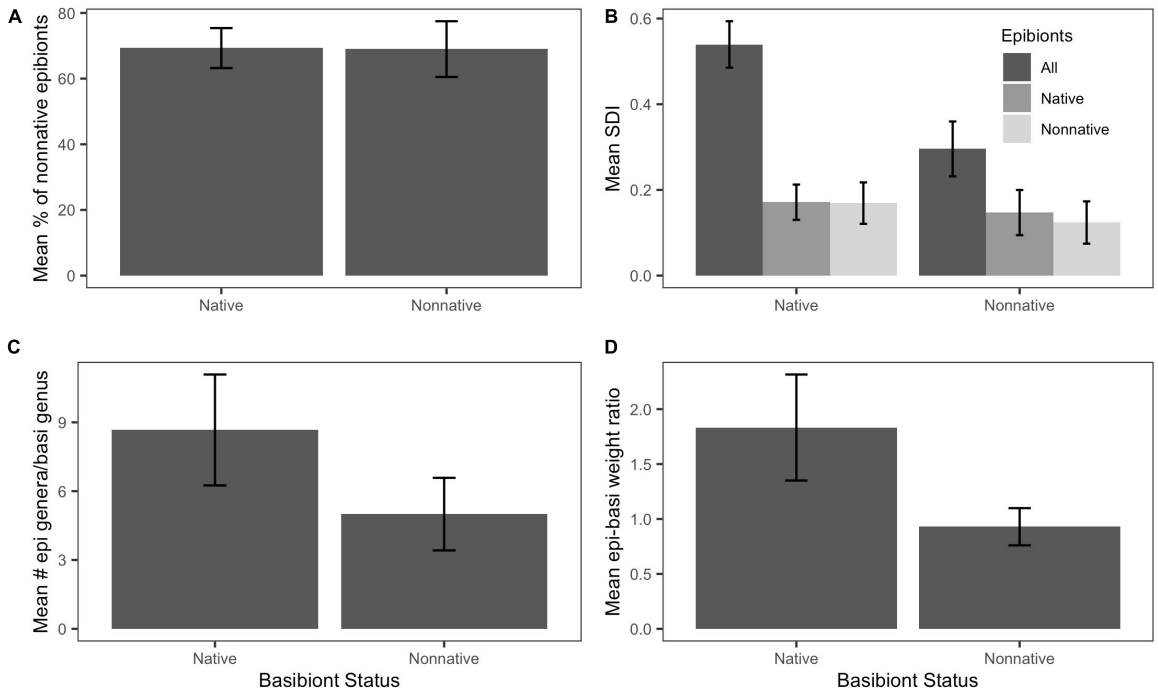

FIGURE 3 | Distribution and diversity of epibionts by basibiont status. Error bars indicate standard error. **(A)** Mean percentage of non-native epibionts by weight. There was no significant difference in percentage of non-native epibionts between native and non-native basibionts (t = 1.37, df = 49.6, P = 0.17). **(B)** Mean Shannon Diversity Index (SDI). There was a significantly higher SDI on native basibionts than non-native basibionts (t = –2.91, df = 54.66, P < 0.01). **(C)** Mean number of epibiont genera per basibiont genus. There was no significant difference between native and non-native basibionts (t = –1.27, df = 7.81, P = 0.241). **(D)** Mean epibiont-basibiont weight ratio. There was no significant difference between basibiont groups (t = –1.77, df = 45.66, p = 0.08).

percent of non-native epibionts present or total epibiont weight colonizing a basibiont. We tested the hypothesis that the effect of basibiont weight on the percentage of epibionts that are non-native depended on basibiont invasion status. We found no evidence for an interaction between basibiont weight and status on the percent of non-native epibionts (F = 0.0285; df = 1, 60; P = 0.867; **Figure 4**). We also tested the hypothesis that the effect of basibiont weight on total epibiont weight depended on the invasion status of the basibiont. We again found no interaction between basibiont weight and status on total epibiont weight (F = 0.401; df = 1, 60; P = 0.529; **Figure 5**). In both cases, the effects of basibiont and status were additive. All larger basibionts (weighing above 20 grams) were native.

DISCUSSION

In our study, there was no relationship between epibiont and basibiont status. However, while we found no preference for basibiont by status, there was a significantly more diverse assemblage of epibionts on native basibionts. This suggests that non-native basibiont species do not support as many epibiont species as native basibiont taxa.

Basibiont Status

The data we gathered may suggest that non-native basibiont organisms inhabiting an area may result in lower biodiversity of associated epibionts. There is evidence in the literature that non-native species may lead to community diversity decreases overall (Blackburn et al., 2004; Gaertner et al., 2009); though the extent of such declines was dependent on the invading species and the ecosystem in which the study was conducted (Gaertner et al., 2009). Arnold et al. (2016) found that fewer epibiotic species settled on the non-native alga *Undaria pinnatifida*, compared to native members of the same phylum (*Laminaria ochroleuca, Saccharina latissima,* and *Saccorhiza polyschides*). However, contrast these findings to Munari (2008), who found that in the Mediterranean, a non-native mussel basibiont *Musculista senhousia* ultimately led to higher biodiversity in areas that it invaded. These mussels supported over double the number of non-native epibionts, when compared to native basibionts. In these studies, as well as in the current study, the focus was primarily on the diversity of epibionts on each basibiont; however, an additional factor to consider in community structure is how foundational basibiont species may ameliorate stressful habits for mobile species (e.g., the non-native *Mytilus galloprovincialis* in South Africa, per Robinson et al., 2007).

Basibiont Taxon

This contradicting evidence from Munari (2008) may simply mean that basibiont taxon characteristics matter more than basibiont invasion status in determining which epibionts will

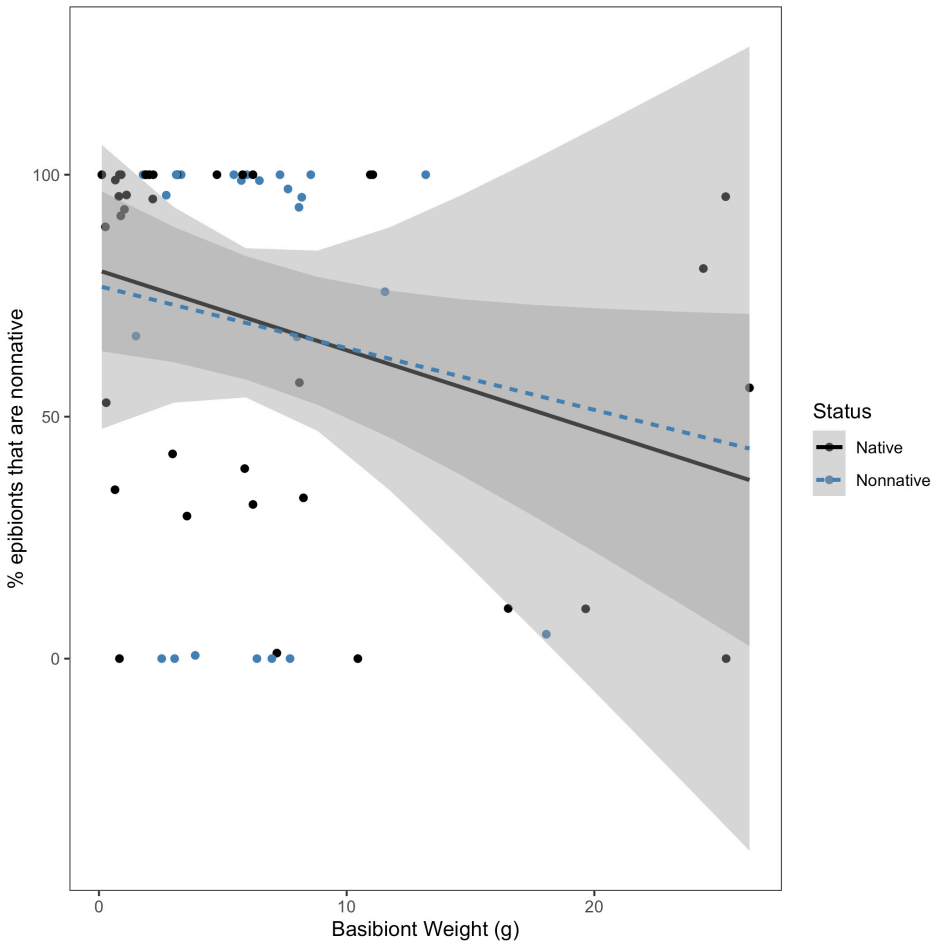

FIGURE 4 | The effect of basibiont weight (g) on percent of epibionts that are non-native, by basibiont status. There was no interaction between status of the basibiont and basibiont weight in determining the percentage of non-native epibionts on each basibiont ($F = 0.0285$; df = 1, 60; $p = 0.867$).

settle. In our study, mussels were the most common native basibiont taxa; therefore, mussel surface topography may be an important factor that determines settlement potential. We also found no overall relationship between basibiont status coupled with either basibiont weight (a proxy for size) on either the total weight of the epibionts on each basibiont sample or the percent of basibionts that were non-native. Instead, surface microtopography and basibiont defenses may regulate the species that settle as epibionts (Wahl, 2009). Therefore, the specific species of basibiont present in the community may determine the quality, and perhaps quantity, of settling species.

Variation of multiple surface characteristics and morphology have been shown to impact which epibionts settle on a basibiont. Basibiont microtopographies and ability to chemically control epibiosis may also determine whether epibionts will settle on a basibiont (Marszalek et al., 1979; Stachowitch, 1980; Davis et al., 1989; Wahl et al., 1998; Lee and Qian, 2003; Dobretsov et al., 2006). Defenses against epibionts vary by species (Wahl, 2009). For example, mussels possess an antifouling periostracum (Bers et al., 2006), while algal species produce secondary metabolites that prevent colonization by epibionts (Nylund et al., 2005; Dobretsov et al., 2006). Ascidians also use mechanical and chemical defenses to minimize surface fouling (Wahl and Banaigs, 1991). Basibiont morphology also matters. Colonial animals are better competitors due to their indeterminate growth and asexual reproduction, while solitary animals survive due to their size and aggregation (e.g., mussel beds; Jackson, 1977). Drakard and Lanfranco (2016) found that macroalgal basibiont age (estimated by size) and surface area (estimated by coarseness) best predicted total abundance of epibionts, with the former measurement positively predicting species richness.

In our study, mussels supported the most epibiont phyla. Mussels, as ecosystem engineers, are a fundamental part of marine communities because they alter the substrate and facilitate interactions with many other species resulting in complex habitats (Jones et al., 1994; Gutiérrez et al., 2003; Robinson et al., 2007; Gutiérrez et al., 2019). Species richness increases in the presence of aggregating mussels (Seed, 1996; Chintiroglou et al., 2004; Borthagaray and Carranza, 2007). Mussels provide both substrate and food resources for interstitial and other associated species (Thiel and Ullrich, 2002). Çinar et al. (2008) found that *Mytilus galloprovincialis* assemblages

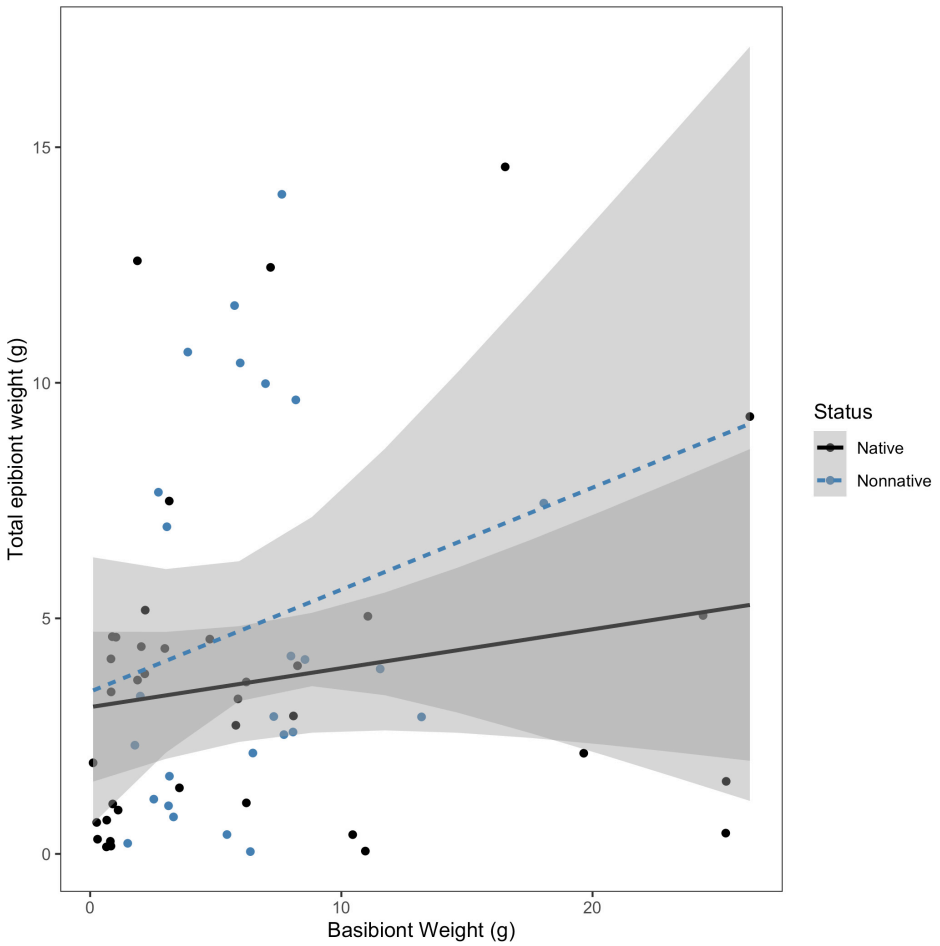

FIGURE 5 | The effect of basibiont weight (g) on total epibiont weight (g), by basibiont status. There was no interaction between status of the basibiont and basibiont weight in determining the total epibiont weight on each basibiont (F = 0.4013; df = 1, 60; p = 0.529).

supported non-native species, about 31% of the total individuals found in their study.

What Is the Role of Epibiosis in This Community?

Repeated invasion events, in which a non-native species facilitates additional invasions, may create a positive feedback loop (Simberloff and Von Holle, 1999). This "invasional meltdown" results in a snowball effect in which facilitation of one invader by another further exacerbates non-native species' impacts on native species (Von Holle, 2011). Our study certainly did not demonstrate invasional meltdown as described in Simberloff (2006a). In fact, few studies directly demonstrate meltdown (Simberloff, 2006a). Gurevitch (2006) posits that invasional meltdown may be relatively uncommon and difficult to demonstrate. Very few papers show examples of marine meltdown (e.g., Grosholz, 2005; Geraldi et al., 2020); the majority of claimed meltdown events are based on terrestrial or freshwater data (e.g., Ricciardi, 2001; Jackson, 2015). Furthermore, Green et al. (2011) observe that the invasional meltdown hypothesis is controversial as few studies show positive feedback loops between invaders, in which amplified facilitative effects exist, and no studies at the time of their paper demonstrate facilitation of entry or spread of secondary invaders. Simberloff (2006a) agrees that specific evidence to show meltdown in the literature is rare.

Simberloff (2006a,b) and Gurevitch (2006) distinguish between facilitation and positive feedback. Facilitation occurs when one species aids another but does not necessarily receive a benefit in return. Positive feedbacks describe mutual facilitation, a population-level interaction where species facilitate one another. For example, an invader facilitates a species through predator deterrence while that species in turn ameliorates a harsh environment allowing the invading species to further establish. Gurevitch (2006) further claims that meltdown is an imprecise term that may refer to positive interactions, facilitative interactions, or actual meltdown, which includes feedback between invaders, amplifying their impacts, acting as an "autocatalytic" community-level process that accelerates replacement of native communities (Simberloff, 2006b; Green et al., 2011). Both Simberloff (2006b) and Gurevitch (2006) agree that studying positive feedbacks are likely to help in

understanding invasion. Where, then, does epibiosis fall on the spectrum of facilitation, positive feedback, and meltdown? Epibiosis is not necessarily a mutualism since there are more benefits for epibiont species than basibiont species (Wahl, 1989). For example, basibionts provide additional substrate, and favorable hydrodynamic positions for filter feeding epibionts. In return, some epibionts provide potential protection against predation for basibionts, while many epibionts have been shown as harmful to basibionts (due to surface damage, competition, added weight leading to reallocation of resources away from growth, etc.; Wahl, 1989). If this is the case, then epibiosis may result in facilitation of invaders, but it is unclear if it is truly a mechanism for meltdown.

However, epibiosis may provide a vehicle for *facilitation*. Grosholz (2005) describes a model in which invaders arriving early in community establishment produce a change in the system, facilitating the establishment and spread of later invaders. In the case of epibiosis, a non-native basibiont, particularly if it is a strong competitor for space, may provide additional space for other settling species. While our study showed no preferential settlement on non-native or native basibionts, the availability of novel three-dimensional surfaces is expected to support additional species settlement. Improving habitat complexity is likely to increase potential for additional species, thereby increasing species richness (Crooks, 2002). Wonham et al. (2005) describe the facilitation of other species, including invaders, by the presence of the non-native Asian hornsnail (*Batrillaria attrimentaria*) on a mudflat. Generally, a fluctuating resource supply should increase invasions (Davis et al., 2000).

Several factors may determine invasibility of a community. Sher and Hyatt (1999) propose that both invader and environmental traits are incorporated into models predicting invasibility. Stachowicz et al. (1999) studied invasibility of marine ecosystems by looking at recruitment in sessile, suspension-feeding invertebrate communities and found that less diverse communities harbored more non-native invaders. Stachowicz et al. (2002) determined that this relationship was present at multiple scales. They found that the factors that control space availability also contributed to invasion success. Levine (2000) describes species loss at small scales reducing invasion resistance (that is, making them more prone to invasion), however, at community-level scales, diverse communities may be more likely to be invaded due to additional factors such as propagule supply (Kolar and Lodge, 2001).

Future Steps and Comments on the Study

This study is intended as an initial observational snapshot of communities in geographically close sites in Maine; it is not sufficient nor intended to be representative of patterns or the full range of taxa along coastal communities in other regions. It is not appropriate to assume that the impacts of non-native basibionts would have a similar impact in all marine ecosystems, as other factors may be at play in determining community structure (i.e., propagule pressure and other biotic components, as well as abiotic components; Lodge, 1993). The impact of an invader may very well depend on the factors leading to formation of the community in which it has invaded (Parker et al., 1999). With an observational focus, our study used basibionts that varied in size, and likely age, which may have affected the epibionts that were present. Longer-lived and larger species may be colonized by species at a different time period than younger or smaller species, which may impact epibiont communities on each basibiont.

There are multiple opportunities to expand on our study to better understand the role of epibiosis and facilitation in marine communities. This study may further be broadened to examining the impacts of within-species size variation on epibiont composition and diversity. In addition, a phenological time-series study to determine population impact of native species both before and after invasion would be ideal (Simberloff, 2006a). We do not know how the presence of basibionts changes overall community diversity because we did not measure the diversity before and after invasion. Furthermore, by expanding this study geographically, we can look at larger scale impacts on biodiversity in areas dominated by native and non-native basibiont foundational taxa. Future research should also investigate how epibiont-basibiont relationships differ by location to determine whether trends observed in this study are universal or vary with location. According to Jackson (2015), specific interactions between associated non-native species should be studied further as it is "a critical area of ecology." As epibiosis is a relationship between two different pairs of species, it is one of the relationships that requires additional attention in understanding the structure of marine communities, and this interaction provides an excellent model to understand facilitation in nearshore communities.

CONCLUSION

Our study has indicated that while there is no direct association between epibionts and basibionts by status, native basibionts support a more diverse group of organisms. Hobbs et al. (2006) asks if novel systems are on the increase and whether such ecosystems will predominate at the end of the present century. How does this change our understanding of "wild" or "natural" ecosystems? Is invasional meltdown and other specialized concepts necessary for understanding the changes ecosystems are facing, or are they simply a typical example of ecosystem dynamics? Studies on epibiosis, particularly between non-native epibionts and their host, have focused primarily on the impacts on the basibiont (e.g., Saier and Chapman, 2004; Auker, 2010; Dijkstra and Nolan, 2011), rather than facilitative, community-wide effects. This study sheds light on the connection between epibiosis, invasion, and facilitative effects, with the hope that more studies will investigate epibiosis as a facilitator of invasions.

DATA AVAILABILITY STATEMENT

The original contributions presented in this study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

LA conceived of the research and collected the samples. KL processed and analyzed the samples. LA and KL wrote and edited the manuscript. Both authors contributed to the article and approved the submitted version.

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Spatial-Temporal Distribution of Prorocentrum concavum Population in Relation to Environmental Factors in Xincun Bay, a Tropical Coastal Lagoon in China

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A harmful benthic Prorocentrum concavum bloom was recorded in August 2018 in Xincun Bay, China, which is the location of a national seagrass nature reserve. Annual ecological surveys have been conducted to study the population dynamics of *P. concavum* in the benthic community and water column. Seasonal variations in benthic *P. concavum* abundance were found and the abundances on seagrass and macroalgae in the wet season were 2.5 and 2.82 times higher, respectively, than those in the dry season, although the differences were not statistically significant. The abundance of *P. concavum* in the water column differed significantly between seasons. The maximum abundances of benthic and planktonic *P. concavum* were (1.7 ± 0.59) × 106 cells (100 cm2)–1 on Thalassia hemperichii in July and 2.0 × 104 ± 4.7 × 103 cells L–1 in June, respectively. High spatial heterogeneity in P. concavum abundance was observed among five sampling sites. Abundances were significantly higher in seagrass beds than those in macroalgae beds, mangroves, and coral reefs. The abundance of *P. concavum* at site A (in a seagrass bed and close to a cage-culture area) was 5.6 times higher than that at site D (seagrass bed and distant from the cage-culture area). Planktonic *P. concavum* showed a similar spatial distribution and presented a maximum density at site A. Moreover, the abundance of benthic *P. concavum* also showed heterogeneity on host substrates, and the abundance on *T. hemperichii* was significantly higher than that on sediment. Based on a Spearman's test, temperature, dissolved organic phosphorus, and dissolved organic nitrogen were the three important factors driving the spatiotemporal distribution of benthic *P. concavum* in Xincun Bay. Planktonic *P. concavum* were derived from cells on the substrates and were influenced by concentrations of dissolved oxygen. In conclusion, seagrass beds may be a reservoir of harmful benthic algal blooms in Xincun Bay and the dense cage-culture area provides sufficient organic nutrients for the growth and reproduction of benthic dinoflagellates.

Keywords: benthic dinoflagellate, *Prorocentrum concavum*, Xincun Bay, lagoon, dissolved organic nutrient, seagrass bed

1 INTRODUCTION

Benthic *Prorocentrum* is a harmful benthic dinoflagellate group, which is widely distributed from tropical to temperate zones (Hoppenrath et al., 2013; Luo et al., 2017; Chomérat et al., 2019; Zou et al., 2021). To date, nine species of epibenthic *Prorocentrum* have been identified that produce okadaic acid (OA), and/or its analogs (Dickey et al., 1990; Morton et al., 1998; Ten-Hage et al., 2002; An et al., 2010; Rodríguez et al., 2018; Nishimura et al., 2020). This toxin causes diarrhea, vomiting, abdominal pain, and nausea in humans that ingest shellfish contaminated by OA (Toyofuku, 2006), which called as diarrhetic shellfish poisoning (DSP). In extreme cases, gastric cancer can result from chronic exposure to OA (Aune and Yndestad, 1993). Also, harmful benthic *Prorocentrum* blooms have been increasingly reported in recent decade (Koike, 2013; Turkoglu, 2016; Cicily et al., 2020), which represents a concerning issue for marine benthic ecosystems and public health (Zou et al., 2020).

Considering the threat of toxic benthic *Prorocentrum* to marine benthos and humans, ecological studies related to blooms, including the spatial-temporal dynamics of their distribution and relationships with environmental parameters, should be clarified. Significant spatial and temporal distributions of benthic *Prorocentrum* have been observed, and these distributions are often related to environmental factors including temperature, nutrients, and depth (Glibert et al., 2012; Hachani et al., 2018; Gharbia et al., 2019). Distinct seasonal distribution characteristics are also observed in benthic *Prorocentrum* (mainly *P. concavum* and *P. lima*), with a high abundance in the wet season (Tindall and Morton, 1998). The results of a two-year study in the Mediterranean Sea showed a high abundance of *P. lima* in the summer (July to October) when high temperatures were observed (27–30°C) (Aissaoui et al., 2014). An 18-month survey at two tropical islands in the Caribbean Sea showed significant spatial heterogeneity between the islands and among different sites within islands (Boisnoir et al., 2019). In the Gulf of Tunis, Mediterranean Sea, the abundance of benthic *Prorocentrum*, which was related to nutrients, decreased with depth and the maximal abundance was observed at a depth of 0.5–1.5 m (Hachani et al., 2018). However, an investigation explored the vertical distribution of benthic dinoflagellates in the Caribbean Sea in the dry season and then showed the highest density of benthic *Prorocentrum* at 1.5 m depth, which differed significantly from the abundance at 3 m depth (Boisnoir et al., 2018). A previous study showed that the highest abundances of *Prorocentrum* occurred at 7–8 m depth and no significant differences were observed between different depths at 20 m in the wet season (Boisnoir et al., 2018). Overall, the distribution of benthic *Prorocentrum* is a complicated ecological process and is correlated with different environmental parameters.

As mentioned above, the development of benthic *Prorocentrum* is a complicated phenomenon related to environmental parameters, including both physical and chemical factors. Previous ecological studies discovered that hydrological parameters can affect the growth and toxin production of benthic *Prorocentrum* (Aissaoui et al., 2014; Accoroni et al., 2018; Aquino-Cruz et al., 2018). For example, the proliferation of *P. lima* was positively related to temperature, salinity, and dissolved oxygen in a two-year survey in the coastal waters of the Gulf of Tunis (Aissaoui et al., 2014). Similarly, a field survey of the epiphytic abundance of *P. lima* found that density increased on most macrophytes from April to August (summer) in a lagoon in the UK (Foden et al., 2005). Climate change may expand the geographic distribution of benthic dinoflagellates (Tester et al., 2010). Surveys of the dynamics of benthic *Prorocentrum* in relation to temperature, to some extent reflect the influences of global warming on the benthic ecosystem. Chemical factors (nutrients) also play an essential role in the proliferation of benthic *Prorocentrum* (Glibert et al., 2012; Aissaoui et al., 2014). Numerous studies about the physiology of benthic *Prorocentrum* in the laboratory have found that this dinoflagellate shows preferences for certain forms and ratios of nutrients (Glibert et al., 2012). *P. lima* from Mahone Bay, Canada, preferentially consumed ammonium over nitrate and nitrite (Pan et al., 1999). Similarly, *in situ* studies revealed that benthic *Prorocentrum* had nutrient preferences (Aissaoui et al., 2014), and the occurrence of *P. lima* blooms was strongly correlated with nutrients in the Gulf of Tunis (Hachani et al., 2018). However, except for *P. lima*, little is known about the interactions of noxious benthic *Prorocentrum* with variations in environmental factors.

Benthic dinoflagellates can live and/or attach to the surface of seagrass, macroalgae, and sediment based on their flagellates or mucus (García-Portela et al., 2016), and they can also swim in the water column (Gharbia et al., 2019; Zou et al., 2020). Many previous studies have revealed substrate preferences of benthic *Prorocentrum* (Boisnoir et al., 2019; Gharbia et al., 2019). However, a number of reports found that the three-dimensional architecture of hosts can affect the attachment of benthic dinoflagellates, and those substrates with flexible and complex structures are most suitable (Accoroni and Totti, 2016). Therefore, differences in abundance should not be compared based on a universal unit (i.e., cells g^{-1} fresh or dry weight macrophytes); the sampling of benthic dinoflagellates needs to be standardized and eliminate the influence of host architecture (Berdalet et al., 2017). Tester et al. (2014) firstly applied a scientific method of artificial screening to investigate the abundance of benthic dinoflagellates. However, this approach is not suitable for some studies, such as substrate preferences. Cells per square 100 centimeters [cells (100 $cm^2)^{-1}$] is a more reliable unit and fully considers the substrate structure (Tester et al., 2014; Berdalet et al., 2017).

Prorocentrum concavum, a tychoplanktonic dinoflagellate, was first described on a coral reef in French Polynesia by Fukuyo (1981). Among nine toxic epibenthic *Prorocentrum*, results on the production of OA by *P. concavum* are often contradictory. Some studies report that this dinoflagellate can produce OA (Dickey et al., 1990; Hu et al., 1992; Juranovic et al., 1997), while other recent studies demonstrated no detectable OA using liquid chromatography-tandem mass spectrometry (Luo et al., 2017; Verma et al., 2019). Despite the debate regarding whether *P. concavum* produces OA or not, this species is certainly a toxic dinoflagellate and can be lethal to marine invertebrates (Zou et al., 2020). As mentioned by Morton et al. (2002), a red tide of *P. arabianum* [synonymized with *P. concavum* by Mohammad-Noor et al. (2007)] was collected from plankton samples but there

were no data on the abundance in either the plankton or benthos. In August 2018, a toxic and tychoplanktonic *P. concavum* bloom was detected in a seagrass bed in Xincun Bay, Hainan Island, South China Sea, with high abundances observed on seagrasses and macroalgae, and in the water column (Zou et al., 2020). This bloom presented a suitable model to analyze the population dynamics of toxic benthic *Prorocentrum*. The present study aims to determine the spatiotemporal distribution of *P. concavum* and its relationship with environmental factors in a coastal tropical lagoon, to improve our knowledge of the ecology of harmful benthic *Prorocentrum* blooms.

2 MATERIALS AND METHODS

2.1 Study Area

The study area, Xincun Bay (18°24′–18°27′N, 109°58′–110°58′E), is a tropical coastal lagoon situated in the southeast of Hainan Island, China (**Figure 1**). The lagoon has a high annual temperature and abundant rainfall. The bay (~22.6 km^2) has a narrow canal connection to the open sea (Yang et al., 2017), hence, the water flow is driven by daily tidal movements. The largest fish aquaculture area (~0.05 km^2) on Hainan Island is located in the bay, with annual production of 1105 tons. A mixed seagrass meadow (2 km^2) is situated in the shallow waters of southeast Xincun Bay, based on observation in 2002 (Huang et al., 2006). *Enhalus acoroides*, *Thalassia hemprichii*, and *Cymodocea rotundata* are the main species in the meadow, and the former two dominate (**Figure 2**). Sampling was carried out at stations A, B, C, and D in Xincun lagoon and an outside station (E; **Figure 1**). Stations A and D were situated in the southwest of the lagoon where seagrasses grow; station A was close to an aquaculture area and station D was located some distance away (**Figure 1**). Stations B and C were located in the northeast and northwest of the lagoon, respectively. Macroalgae, but no seagrasses, were present at station B during the study period, and the other site (C) was in a mangrove area with no seagrasses or macroalgae. Station E (reference site) was on a coral reef located outside the Xincun lagoon. This station was relatively devoid of human activities.

2.2 Sample Collection

Sampling was conducted bimonthly from December 2018 to December 2019 in the Xincun lagoon and once a month in the summer of 2019 as a result of the *P. concavum* bloom present at station A in August 2018 (Zou et al., 2020). The wet season ranged from May to October and the other months were the dry season in the tropical Xincun Bay. At stations A and D, each type of seagrass, macroalga (**Table 1**), and sediment was collected in triplicate. Macroalgal and sediment samples (triplicate) were collected at station B, but only sediment was sampled (in triplicate) at stations C and E. In addition, samples from the water column were also collected in triplicate at each station. At each station, well-developed floating leaves on which benthic dinoflagellates attach were slightly cut and then placed in sealed bags containing a small amount of the surrounding seawater. The cells of benthic dinoflagellate on the sediment were sampled based on the method of Xie et al. (2022). Briefly, the surface sediments (~300 cm^{-2}) were collected in the bags with 1 L surrounding seawaters. A 1.5 L water column (triplicate samples) was sampled using a 1 L white plastic bottle. The samples were collected between 0.2 and 1.5 m depth within the lagoon and between 2 and 3 m depth in the open sea (**Table 1**).

2.3 Environmental Parameters

Surface water temperature (T), pH, salinity (Sal), and dissolved oxygen (DO) were measured using a YSI meter (YSI-professional plus, YSI Inc., USA) at each station during the sampling period. Each 0.1 L water column sample collected from each station was filtered with GF/F filters (Whatman, USA). The concentrations of nitrate (NO_3), nitrite (NO_2), ammonium (NH_4), silicate (SiO_3), phosphate (PO_4), total dissolved nitrogen (TDN), and total dissolved phosphorus (TDP) were measured using a flow injection analyzer (LACHAT QC8500, USA) (Murphy and Riley, 1962; Solrzano, 1969; Strickland and Parsons, 1972; Jeffries

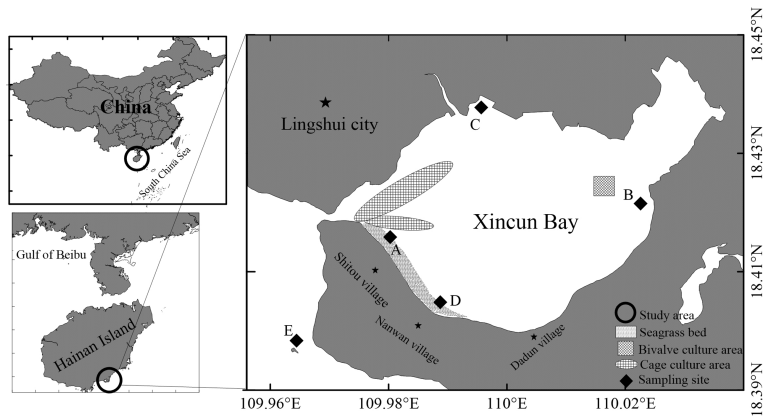

FIGURE 1 | Map of sampling stations of Xincun Bay.

TABLE 1 | Sampling locations characteristics, seagrasses and macrophytes sampled in Xincun Bay.

Sites	Latitude	Longitude	Habitat type	Bottom	Depth (m)	Seagrasses	Macroalgae
A	109°58′38.2″E	18°24′34.9″N	Seagrass bed	Sand	0.5-1.5	Enac, Thhe	Ulla
B	110°1′1.5″E	18°25′28.5″N	Seaweed bed	Sand	0.5-1.5	None	Ulla
C	109°58′48.2″E	18°25′25.1″N	Mangrove	Sand	0.5-1.5	None	None
D	109°59′16.8″E	18°24′7.2″N	Seagrass bed	Sand	0.2-1.5	Enac, Thhe, Cyro	Ulla
E	109°57′48.9″E	18°23′13.2″N	Coral	Sand	2-3	None	None

Enhalus acoroides (Enac); Thalassia hemperichii (Thhe); Cymodocea rotundata (Cyro); Ulva lactuca (Ulla).

et al., 1979). In addition, dissolved organic nitrogen (DON) and dissolved organic phosphorus (DOP) were calculated by subtracting the concentrations of dissolved inorganic nitrogen and PO_4 from TDN and TDP, respectively.

2.4 Sample Processing

The seagrass, macroalgal, and sediment samples were shaken vigorously and washed three times using filtered water to ensure microalgal cells were separated from the substrate. Benthic dinoflagellates were concentrated using 10 μm nylon filters and then saved in 15 mL centrifuge tubes containing acidic Lugol's solution (final concentration, 1.5%). The biological substrates (seagrasses and macroalgae) were removed and then weighed. Similarly, the 1 L water column samples were concentrated and reserved in 15 mL centrifuge tubes containing a suitable concentration of acidic Lugol's solution.

In addition to weighing, the biological substrates were photographed with a DSLR camera and the surface area was measured using Image Pro Plus V. 7.0. In the present study, at least ten weights and surface areas of every substrate were recorded to establish standard curves for the surface area and weight.

2.5 Benthic Dinoflagellate Counts

Samples were quickly transferred to the laboratory and counted using a light microscope (Olympus CX31, Tokyo, Japan) at a magnification of 100×. The morphology of the bloom-forming species, *P. concavum*, was obvious and easy to identify. Other epibenthic dinoflagellate species were extremely similar and were recorded as genera (*Prorocentrum*, *Coolia*, *Amphidinium*, *Gambierdiscus*, and *Ostreopsis*). The abundances of benthic dinoflagellates were expressed as cells $(100\ cm^2)^{-1}$ and cells L^{-1} on substrate and in the water column, respectively. In addition, we defined more than 1×10^5 cells $(100\ cm^2)^{-1}$ as benthic *P. concavum* bloom based on the results described by Zou et al., 2020.

2.6 Statistical Analysis

All abundances of *P. concavum* were displayed as means ± standard error. Analysis of seasonal variations in *P. concavum* in Xincun Bay was restricted to data collected from the seagrass meadow (stations A and D) to avoid errors resulting from the null data collected from the other sites. One-way ANOVA was used to assess the distributions of environmental factors and assess *P. concavum* abundances among five stations and between two seasons. Non-parametric tests were carried out when the variances were not homogenous. Spearman correlation tests were used to assess the relationships between planktonic and benthic *P. concavum* and the effects of environmental factors on the development of the *P. concavum* population. Aall analyses were performed using SPSS Statistics 25 (IBM Corp., USA).

3 RESULTS

3.1 Environmental Factors

Figure 3 showed the spatiotemporal distributions of the environmental factors. Temperature fluctuated from 21.3°C in

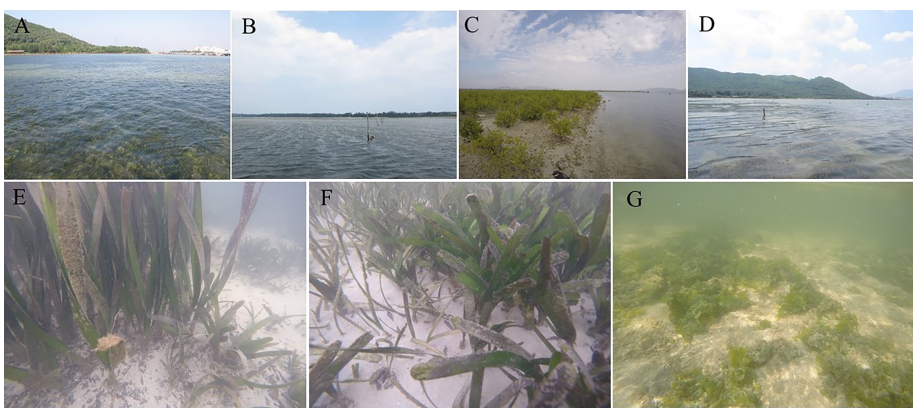

FIGURE 2 | Sampling stations in Xincun Bay. **(A–D)** represented sampled stations **(A)** (seagrass bed), **(B)** (macroalgae meadow), **(C)** (mangrove) and **(D)** (seagrass bed), respectively. **E-G** represented *Enhalus acoroides, Thalassia hemperichii and Cymodocea rotundata, Ulva lactuca*, respectively

December 2019 at station B to 32.8°C in July at station B (**Figure 3A**). The average temperatures of the dry and wet seasons were 26.36°C and 29.32°C, respectively. Salinity ranged from 29.08 to 32.59, with an average value of 31.61 (**Figure 3B**). pH and concentrations of DO ranged from 7.8 to 8.85 and 3.48 mg L^{-1} (50.2% of saturation level) to 12.62 mg L^{-1} (182% of saturation level), respectively (**Figures 3C, D**). No significant differences were discovered among the five sampling stations for these physical factors ($p = 0.814$, $p = 0.478$, $p = 0.969$, and $p = 0.283$ for temperature, salinity, pH, and DO, respectively). However, the temperature (Mann-Whitney U test, $p = 0.002$) and salinity (Mann-Whitney U test, $p < 0.01$) fluctuated significantly between dry and wet seasons. There were no seasonal differences in pH (Mann-Whitney U test, $p = 0.909$) or DO concentration (Mann-Whitney U test, $p = 0.192$).

In terms of the nutrient levels in Xincun Bay, concentrations of nitrate ranged from 0.06 to 14.16 μmol L^{-1}, with an average of 2.66 ± 3.07 μmol L^{-1} (**Figure 3E**). The concentrations of nitrite and ammonium ranged from 0.05 at station B to 2.53 μmol L^{-1} at station C, and from 0.89 at station C to 12.21 μmol L^{-1} at station A, respectively (**Figures 3F, G**). The mean concentrations of silicate and phosphate were 8.24 ± 5.32 and 0.54 ± 0.43 μmol L^{-1} (**Figures 3H, I**). DON and DOP showed a wide range of concentrations, from 5.37 to 111.82 μmol L^{-1} (means of 28.85 ± 15.21 and 44.26 ± 24.45 μmol L^{-1} in the dry and wet seasons, respectively) and from 0.06 to 6.09 μmol L^{-1} (means of 1.17 ± 1.73 and 1.47 ± 1.29 μmol L^{-1} in the dry and wet seasons, respectively), respectively. Each nutrient showed a similar range of values among the five sampling stations, except silicate concentrations, which were significantly higher at stations B and C than those at stations A and E (Kruskal-Wallis test, $p < 0.01$). Concerning the seasonal variations, significant differences were only observed for concentrations of nitrite, phosphate, and DON (Mann-Whitney U test, $p < 0.01$).

3.2 Population Dynamics of *P. concavum*

3.2.1 Linear Curve Between Surface Area and Weight of Macrophytes

The surfaces of four biotic substrates (three seagrasses, *E. acoroides*, *T. hemperichii*, *C. rotundata*; one macroalga, *Ulva*

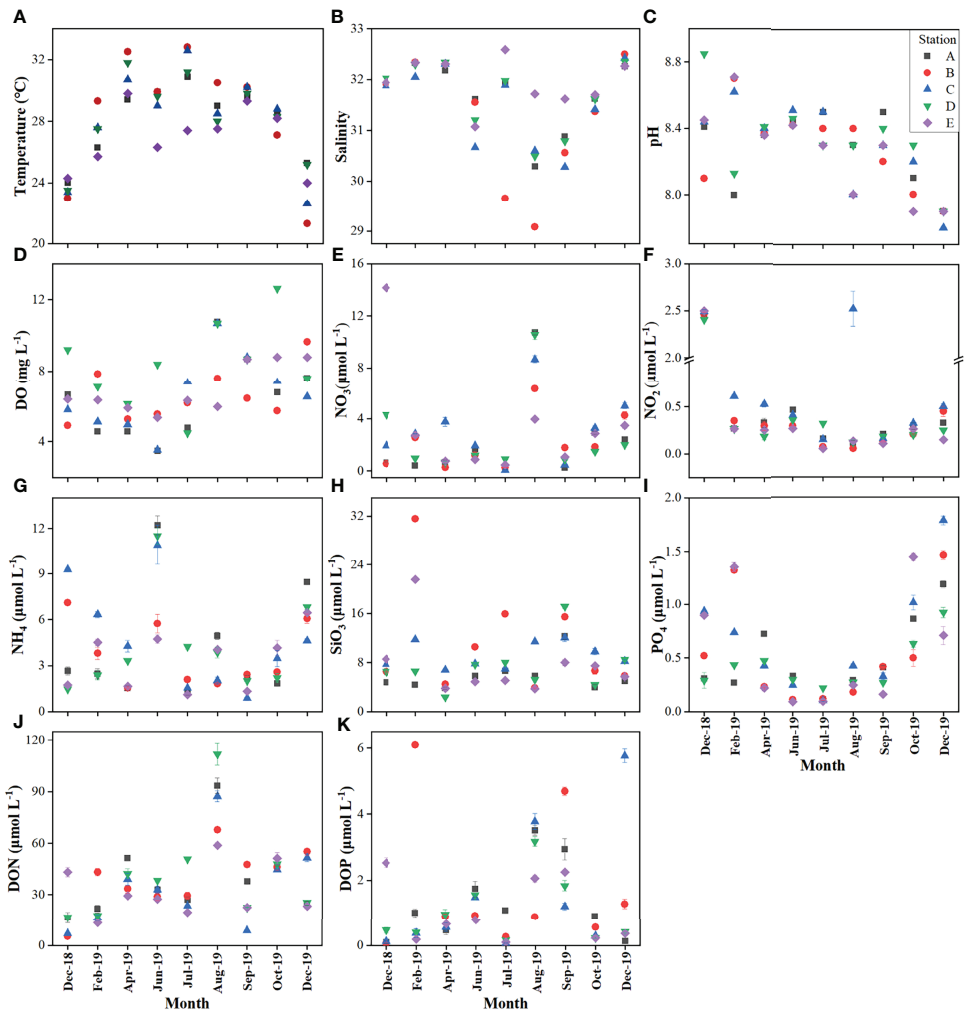

FIGURE 3 | Spatial-temporal variations of environmental parameters during the sampling period. **(A)** Temperature; **(B)** Salinity; **(C)** pH; **(D)** dissolved oxygen; **(E)** Nitrate; **(F)** Nitrite; **(G)** Ammonium; **(H)** Silicate; **(I)** phosphate; **(J)** dissolved organic nitrogen; **(K)** dissolved organic phosphorus.

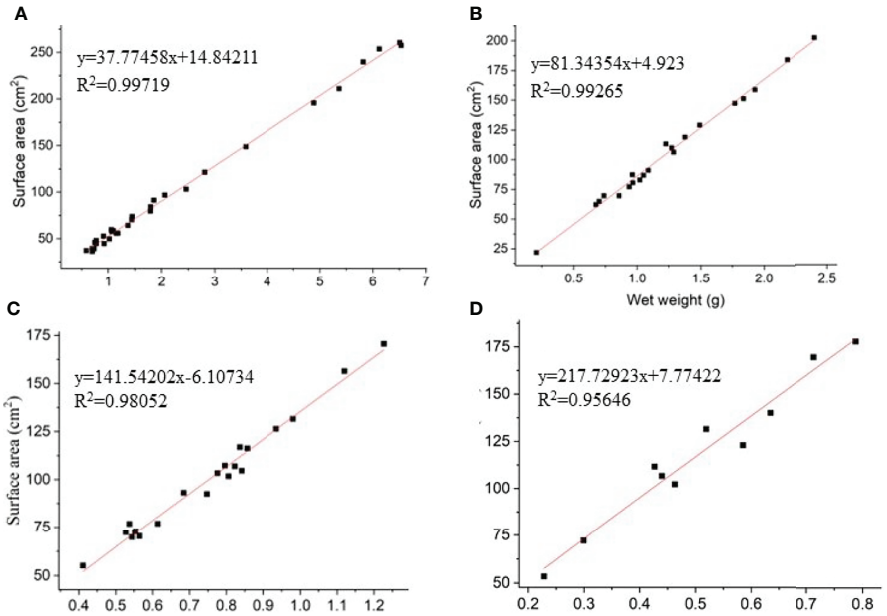

FIGURE 4 | The linear curves and equations between the surface area and weight of *Enhalus acoroides* **(A)**, *Thalassia hemperichii* **(B)**, *Cymodocea rotundata* **(C)** and *Ulva lactuca* **(D).**

lactuca) were smooth and correctly measured in the Xincun lagoon. The linear curves and equations between surface area and weight were presented in **Figure 4**.

3.2.2 Seasonal Variations in *P. concavum*

The seasonal distribution of *P. concavum* was shown based on the data from seagrasses, macroalgae, sediments, and the water column in the seagrass bed (stations A and D, **Figure 5**). *P. concavum* was found on seagrasses throughout the sampling period in the Xincun Bay, with abundances ranging from 468 ± 134 on *E. acoroides* in December 2018, to (1.7 ± 0.59) × 10^6 cells (100 cm^2)$^{-1}$ on *T. hemperichii* in July (**Figure 5**). Moreover, the epibenthic abundances on *U. lactuca* and sediments varied from 230 ± 109 in December 2019, to (2.1 ± 1.2) × 10^5 cells (100 cm^2)$^{-1}$ in August, and from (7.3 ± 4.5) × 10^3 in February to (6.7 ± 1.4) × 10^5 cells (100 cm^2)$^{-1}$ in September, respectively (**Figure 5**). The first *P. concavum* bloom occurred in February on *T. hemperichii* at station A, with up to (3.4 ± 0.27) × 10^5 cells (100 cm^2)$^{-1}$ at a temperature of 26.3°C (**Figures 3A**, **5**). Although no significant differences were found between the wet and dry seasons (ANOVA, p = 0.383, p = 0.252, and p = 0.864 for *P. concavum* on seagrasses, macroalgae, and sediments, respectively), the abundances of benthic *P. concavum* on seagrasses and macroalgae in the wet season were 2.52 and 2.8 times, respectively, greater than those in the dry season. Similarly, the presence of *P. concavum* was observed in the water column throughout the sampling period. The abundances of *P. concavum* ranged from 208 ± 295 in December to (2.0 ± 0.47) × 10^4 cells L^{-1} in June 2019 in the seagrass bed (**Figure 5**). At station A, the planktonic abundance of *P. concavum* increased from February to June (maximum abundance) and then declined continuously. At station D, the abundances were lower than those at Station A and the maximum abundance was observed in August (**Figure 5**). The average abundance of planktonic *P. concavum* based on the nine-month survey revealed marked differences between the two seasons (Mann Whitney U test, p = 0.02; abundance 2.3 times higher in the wet season than that in the dry season).

3.2.3 Spatial Distribution

High spatial heterogeneity was found among the four habitats (seagrass, macroalgae, mangrove, and coral reef) in Xincun Bay (**Figures 5**, **6**). The mean abundances of benthic *P. concavum* based on the nine-month survey showed significant differences among the five sampling stations (Kruskal-Wallis test, p < 0.01). The densities in the seagrass meadow (stations A and D) were markedly higher than those in the macroalgal bed (station B), mangrove (station C), and reference site (coral reef, station E) (**Figure 6A**; Kruskal-Wallis test, p < 0.01).

Although there was no statistically significant difference (Kruskal-Wallis test, p = 0.591), approximately 5.6 times more *P. concavum* was found at station A than at station D (**Figure 6**). Also, we identified other benthic dinoflagellates, including species of *Prorocentrum*, *Coolia*, *Ostreopsis*, *Gambierdiscus*, and *Amphidinium* in Xincun Bay during the sampling period. The ecological dominance of these five genera in the benthic dinoflagellate community could be seen in **Table 2**. Benthic *P. concavum* was the most abundant species in the seagrass meadow, with proportions ranging from 42.56% to 59.88%; at stations B and C, the proportions were only 7.82% and 0.25%, respectively, of the benthic dinoflagellate community. *P. concavum* also dominated in the detached epibenthic dinoflagellates in the water column, with ecological dominance fluctuating from 15.25% to 68.75% (**Table 2**). Statistically significant differences in planktonic *P.*

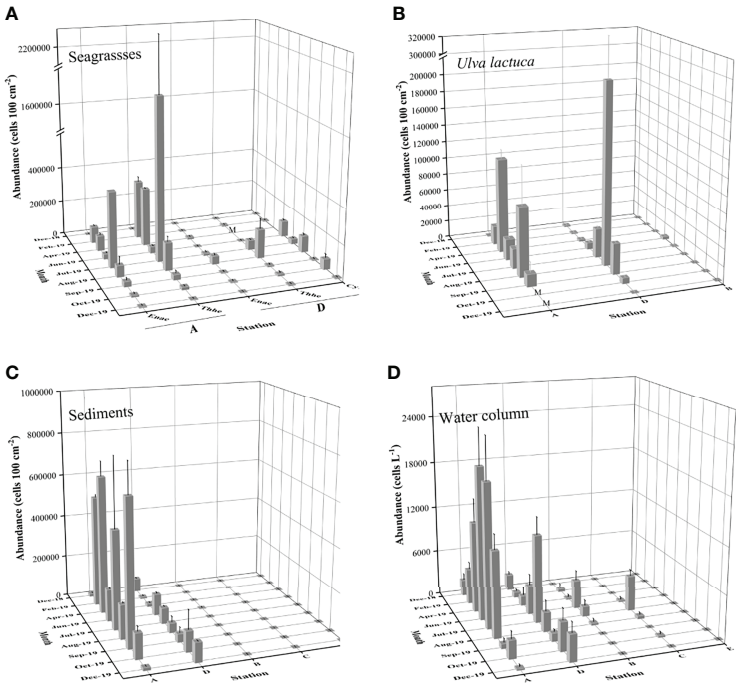

FIGURE 5 | Spatial-temporal variations of mean *P. concavum* abundance with standard bar on seagrasses, macroalgae, sediments and in the water column in the Xincun Bay. M represents the missing data Spatial-temporal variations of P. concavum abundance with standard bar on seagrasses **(A)**, macroalgae **(B)**, sediments **(C)** and in the water column **(D)** in the Xincun Bay. M represents the missing data.

concavum abundances were found among the five sites (**Figure 6**; Kruskal-Wallis test, $p < 0.01$). Similar to the benthic distribution pattern, proportions of planktonic *P. concavum* in waters of the seagrass bed were higher than those in the waters of the macroalgal bed (station B), mangrove area (station C), and coral reef (station E). No significant differences in planktonic cells were found between the two seagrass sites, but abundances at site A were 2.4 times greater than those at site D (**Figure 6**).

In addition to the differences among stations, high heterogeneity in *P. concavum* abundance on different substrates was also observed, as shown by the high standard errors calculated from the replicates (**Figures 5**, **7**). Mean abundances of *P. concavum* on seagrasses (*E. acoroides*, *T. hemperichii* and *C. rotundata*), macroalgae (*U. lactuca*), and sediments were $(4.6 \pm 2.4) \times 10^4$, $(1.7 \pm 1.1) \times 10^5$, $(3.6 \pm 0.14) \times 10^5$, $(2.4 \pm 0.96) \times 10^4$, and (7.3 ± 2.5) cells $(100\ cm^2)^{-1}$, respectively (**Figure 7**). Moreover, the differences in *P. concavum* on each substrate were compared based on the data collected for the seagrass bed. Mean abundances of *P. concavum* on these substrates showed significant variations (Kruskal-Wallis test, $p = 0.014$), and pairwise comparisons showed that the abundance of epiphytic *P. concavum* (on *Thalassia hemperichii*) was significantly higher than that of epipelic *P. concavum* ($p = 0.029$).

3.3 Relationship Between Benthic and Planktonic *P. Concavum*

P. concavum on seagrasses (*E. acoroides*, *T. hemperichii*, and *C. rotundata*), macroalgae (*U. lactuca*), and sediments were all positively correlated with the abundance of planktonic cells

TABLE 2 | Ecological dominance (%) of benthic dinoflagellates attached on the substrate and in the water column in Xincun Bay during sampling period.

Sites	Status	*P. concavum*	*Prorocentrum*	*Coolia*	*Amphidinium*	*Gambierdiscus*	*Ostreopsis*
A	epiphytic	59.88	64.36	34.56	0.99	0.09	0.00
	planktonic	68.72	76.76	22.43	0.42	0.39	0.00
B	epiphytic	7.82	90.30	6.13	3.51	0.07	0.00
	planktonic	34.18	72.09	18.12	0.00	9.79	0.00
C	epiphytic	0.25	48.68	5.98	45.25	0.00	0.09
	planktonic	21.57	82.61	14.21	0.00	3.18	0.00
D	epiphytic	42.56	86.65	11.09	1.81	0.09	0.36
	planktonic	15.25	62.72	36.45	0.62	0.21	0.00
E	epiphytic	4.76	53.57	38.28	3.07	0	0.32
	planktonic	0.00	82.32	17.78	0.00	0.00	0.00

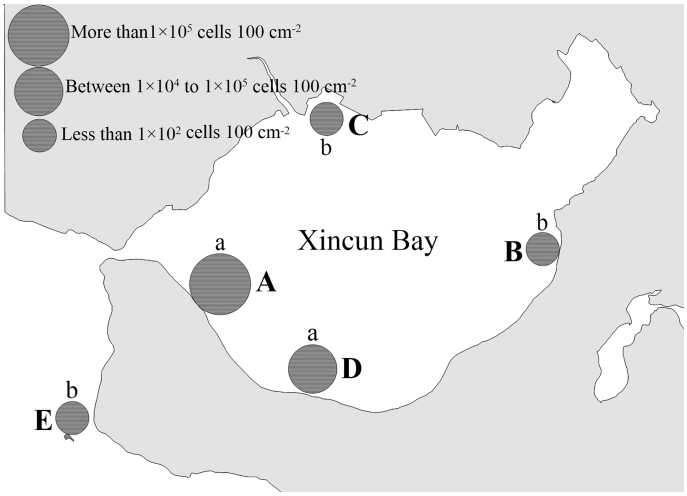

FIGURE 6 | Total mean density of benthic *P. concavum* presented in Xincun Bay from December 2018 to December 2019. Small letters (a and b) indicated statistically differences according to the non-parameters test ($p<0.01$). A, B, C, D and E represent sampling Site A,Site B, Site C, Site D and Site E, respectively.

(**Table 3**) and the cells on *T. hemperichii*, *U. lactuca*, and sediments were clearly related to floating *P. concavum* ($p < 0.01$).

3.4 Relationships Between Environmental Factors and *P. Concavum* Abundance

Over the sampling period, benthic and planktonic abundances of *P. concavum* were positively correlated with temperature, except the cells on *C. rotundata* and sediments (**Table 3**). The maximal abundances of *P. concavum* on *C. rotundata* and sediment occurred in August and April, with temperatures of 29.6°C and 29°C, respectively (**Figures 3A**, **5**) but no significant relationships were found. In addition, the Spearman test revealed that concentrations of DO were weakly negatively associated with planktonic *P. concavum*, whereas no significant relationship was found between DO and epiphytic cells (**Table 3**). Considering nutrients, the Spearman test showed that densities of *P. concavum* on *E. acoroides*, *T. hemperichii*, *C. rotundata* and *U. lactuca* were clearly positively related to DON. The abundances on *E. acoroides*, *U. lactuca* and sediment were positively correlated with DOP. Moreover, the abundances of *P. concavum* on *E. acoroides* and *T. hemperichii* were negatively correlated with nitrite and ammonium, and the densities of *P. concavum* on *U. lactuca* were negatively associated with nitrite and phosphate (**Table 3**). Finally, temperature was positively correlated with pH and DON, but negatively associated with salinity, nitrate, ammonium, and phosphate (**Table 3**).

TABLE 3 | Coefficients of Spearman correlation performed on the full datasets.

	Enac	Thhe	Cyro	Ulla	Sedi	Water	T	Sal	DO	pH	NO_3	NO_2	NH_4	PO_4	SiO_3	DON	DOP
Enac	1.000	**0.939**	0.478	**0.741**	**0.668**	**0.517**	**0.475**	-0.201	-0.301	0.167	-0.245	**-0.554**	**-0.54**	-0.437	-0.335	**0.525**	**0.505**
Thhe		1.000	0.571	**0.689**	**0.647**	**0.624**	**0.525**	-0.159	-0.266	0.191	-0.154	**-0.564**	**-0.63**	-0.38	-0.257	**0.542**	0.422
Cyro			1.000	1.000	0.262	**0.762**	0.619	-0.524	-0.024	0.12	0.429	-0.478	0.071	-0.238	0.119	**0.762**	0.69
Ulla				1.000	**0.789**	**0.791**	**0.421**	-0.329	-0.242	0.094	-0.215	**-0.632**	-0.197	**-0.51**	-0.344	**0.674**	**0.582**
Sedi					1.000	**0.857**	0.171	-0.132	-0.284	0.119	-0.272	0.052	-0.059	-0.139	-0.145	0.381	**0.47**
Water						1.000	**0.349**	-0.235	**-0.365**	0.119	-0.215	0.029	0.06	-0.242	-0.101	0.415	0.178
T							1.000	**-0.344**	-0.188	**0.335**	**-0.423**	**-0.393**	**-0.356**	**-0.52**	0.098	**0.482**	0.421
Sal								1.000	-0.22	-0.118	-0.055	0.279	0.06	**0.343**	-0.212	**-0.548**	-0.467
DO									1.000	-0.241	0.382	-0.251	-0.155	0.242	0.14	0.077	0.015
pH										1.000	-0.226	0.162	0.096	**-0.307**	0.24	0.017	0.274
NO_3											1.000	0.238	**0.33**	**0.472**	0.043	0.243	0.077
NO_2												1.000	**0.379**	**0.472**	0.125	**-0.600**	**-0.492**
NH_4													1.000	**0.372**	0.086	-0.439	-0.156
PO_4														1.000	0.186	-0.304	-0.412
SiO_3															1.000	-0.251	-0.044
DON																1.000	0.346
DOP																	1.000

Enac, Thhe, Cyro, Ulla, Sedi and water represent the abundances of P. concavum on Enhalus acoroides, Thalassia hemperichii, Cymodocea rotundata, Ulva lactuca, sediments and in the water column, respectively. Bold and underlined fonts represent p<0.05 and p<0.01, respectively.

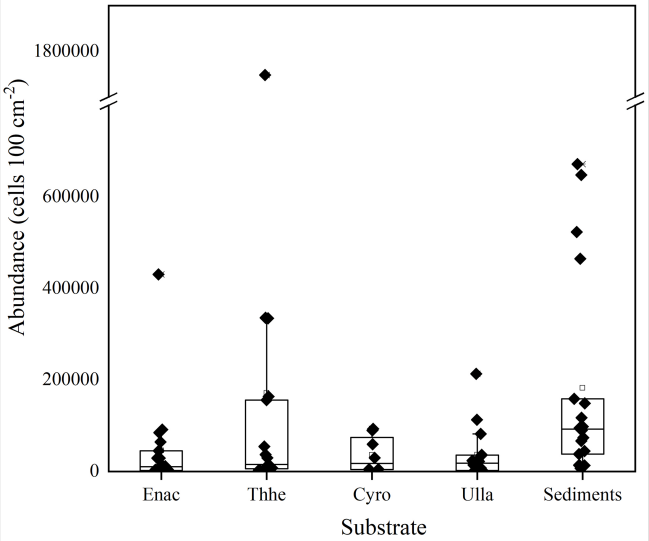

FIGURE 7 | The boxplot showing the epiphytic and epipelic abundances of *P. concavum*. *Enhalus acoroides* (Enac); *Thalassia hemperichii* (Thhe); *Cymodocea rotundata* (Cyro); *Ulva lactuca* (Ulla).

4 DISCUSSIONS

4.1 Quantitative Methodology for Benthic Dinoflagellates

Compared with planktonic microalgae, standard methods for the sampling and quantification of benthic dinoflagellates that provide researchers with comparable data among studies are lacking (Berdalet et al., 2017). To date, the universal unit expressing the abundance of benthic dinoflagellates is based on the wet or dry weight of substrates (macrophytes). However, this expressive method of abundance not only neglects the three-dimensional structure of host species but is also unsuitable for sampling stations with a lack of biotic substrates. Benthic dinoflagellates attach on the surface of biotic substrates based on the flagellum and/or produced mucus (Heil, 1993; Reynolds, 2007). The different biotic substrates have obviously various three dimensional structures, which provide different surface area for the attachments of benthic dinoflagellate (Berdalet et al., 2017). For example, surface area per gram were 53 and 225 cm^2 in *E. acoroides* and *U. lactuca* in Xincun Bay (**Figure 4**). In the present study, the area unit, cells $(100\ cm^2)^{-1}$, was employed and standard curves between the surface area of hosts and fresh weight were established. This method provides a convenient way to calculate a host's surface area and for comparison among studies. Tester et al. (2014) first used artificial substrates (fiberglass screen) to investigate the abundance of benthic dinoflagellates, which attaches great importance to the use of cells $(100\ cm^2)^{-1}$ in filed studies. However, this method is not applicable to some studies that explore the relationships (e.g., substrate preferences) between benthic cells and hosts (Boisnoir et al., 2019; Gharbia et al., 2019). In the present study, a field method was established to quantify the densities of epipelic dinoflagellates. This method provides researchers with a way to compare epiphytic and epipelic abundances. While this method has certain limitations, it provides new insight into surveys of benthic dinoflagellates.

4.2 Spatiotemporal Distribution of *P. concavum*

P. concavum, a harmful dinoflagellate, was first described by Fukuyo (1981) in French Polynesia, New Caledonia, and the Ryukyu Islands. To date, many studies have demonstrated that *P. concavum* is a tychoplanktonic dinoflagellate. This species has been reported in the Arabian Sea (Morton et al., 2002), Knight Key, Gulf of Mexico, northwestern Australia (Verma et al., 2019), northern Hainan Island (Luo et al., 2017), and the Xincun Bay of China (present study). Moreover, Morton et al. (2002) described a new species, *P. arabianum* [synonym of *P. concavum*; Mohammad-Noor et al. (2007)], and reported a planktonic *P. arabianum* bloom in the Gulf of Oman, Arabian Sea in May 1995. In spite of a lack of cell abundance data for this bloom, there was evidence that *P. concavum* was a bloom-causative species, which may be harmful to marine ecosystems and public health. A bloom of *P. concavum* in Xincun Bay presented high abundances on seagrasses, macroalgae (*U. lactuca*), and in the water column [3.9×10^5, 1.4×10^4 cells $(100\ cm^2)^{-1}$ and 1.7×10^4 cells L^{-1}, respectively]. The population showed extremely high dominance (more than 90% of the benthic dinoflagellate community) in August 2018, in Xincun Bay (Zou et al., 2020). In the present study, the maximal abundances of *P. concavum* [$(1.7 \pm 0.59) \times 10^6$ cells $(100\ cm^2)^{-1}$ on *T. hemperichii*, **Figure 5**] and high population dominance were observed in the same seagrass bed in July 2019 (**Table 2**). These findings suggest that *P. concavum* is a dominant species in benthic microalgal assemblages and periodically causes blooms in the summer in Xincun Bay, especially in shallow seagrass beds.

While no statistically significant differences between the wet and dry seasons were found (ANOVA, $p>0.05$), we identified that the abundances of *P. concavum* on seagrasses and macroalgae were 2.5 and 2.82 times higher in the wet season than those in the dry season. The maximal density was recorded at site A in July with a high temperature of 30.9°C (**Figures 3A**). Moreover, a significant difference was found in the abundance of planktonic *P. concavum* between the two seasons. In general, the abundance of *P. concavum* showed seasonal variation in the tropical Xincun Bay. A harmful benthic bloom of *P. concavum* occurred in August 2018, which provided further evidence for seasonality (Zou et al., 2020). In another tropical ecosystem, there was also apparent seasonality in benthic *Prorocentrum* (mainly *P. concavum* and *P. lima*), with the lowest abundances of these species recorded between January and May (dry season) (Tindall and Morton, 1998). Similarly, a recent survey investigated the spatial-temporal distributions of benthic dinoflagellate in the Caribbean Sea and demonstrated that the lowest abundances of benthic *Prorocentrum* appeared from October to January, corresponding with lower seawater temperatures (Boisnoir et al., 2019). Nishimura et al. (2019) reported that the density of benthic *Prorocentrum*, including *P. concavum*, was notably higher in subtropical areas than that in temperate areas in Japan. In addition, some studies in the temperate Mediterranean Sea showed a similar phenomenon, that benthic *Prorocentrum*

had a maximal density from July to October (Aissaoui et al., 2014). Hence, we conclude that *P. concavum*, like other benthic *Prorocentrum* species, shows high abundance, even blooms, in the summer, associated with higher seawater temperatures.

The abundances of both benthic and planktonic *P. concavum* were significantly higher in the seagrass bed than those in the macroalgal bed (station B), mangrove (station C), or coral reef (station E). Station A was close to the cage fish-culture area and showed maximal benthic *P. concavum* abundances 5.6 times higher than those at station D. *P. concavum* showed high spatial heterogeneity in Xincun Bay, which is consistent with the results of a number of previous field studies. For example, Boisnoir et al. (2019) suggested that the distributions of benthic dinoflagellates, including *Prorocentrum*, significantly differed between sampling sites and between islands (Guadeloupe and Martinique, Caribbean Sea). A survey of epiphytic dinoflagellates in the Gulf of Tunis, Mediterranean Sea, revealed that *P. lima* showed significant spatial patterns and higher abundance in seagrass beds (Hachani et al., 2018). Moreover, the spatial heterogeneity of *P. concavum* among sites in Xincun Bay can be, to some extent, explained by the differences in habitats and substrates. At station A, in addition to the relatively stable hydrometric conditions, the dense fish cages provide sufficient nutrients and suitable substrate for the proliferation of macroalgae. As mentioned by Zhang et al. (2014), the nutrient concentrations are high in cage cultures in Xincun Bay, which results in high macroalgal biomass in this area (Liu et al., 2019). Macroalgae and dense mature seagrasses (Huang et al., 2006) offer suitable environments for the growth of *P. concavum* (Glibert et al., 2012). Yong et al. (2018) found that microhabitat can be a key factor determining the abundance of benthic dinoflagellates, and *Prorocentrum* preferred microhabitats covered with high turf algae. In addition, many surveys have suggested that substrate preference, which is also reflected in the spatial heterogeneity, is common in benthic species of *Prorocentrum* (Boisnoir et al., 2019; Gharbia et al., 2019). In the present study, we found that benthic *P. concavum* showed maximal abundance on *T. hemperichii* and abundances were significantly higher on *T. hemperichii* than on other substrates. This finding suggests that benthic *P. concavum* has a preference for *Thalassia*, which is consistent with a previous description by (Delgado et al., 2006). Consequently, we conclude that *P. concavum* shows a clear spatial distribution pattern in ecosystems and on substrates in Xincun Bay. The differences between ecosystems can be explained by the fact that the abundant and dominant seagrasses *T. hemperichii* and *E. acoroides* (also showed high *P. concavum* density) act as a trap for harmful benthic *P. concavum* blooms (Huang et al., 2006; Zou et al., 2020). Moreover, the highest abundances of *P. concavum* at station A were associated with higher nutrient provision from dense caged-fish cultures.

4.3 Environmental Factors Related to the Distribution of *P. concavum*

4.3.1 Temperature

Seawater temperature was positively correlated with *P. concavum* on seagrasses, macroalgae, and in the water column, but not on sediments (**Table 3**). In addition, the significant correlations between temperature and other environmental factors (salinity, pH, nitrate, nitrite, phosphate, and DON) indicated that temperature might be the most important factor driving the spatiotemporal distribution of *P. concavum* (**Table 3**). The preference of *P. concavum* for higher temperatures is consistent with other benthic *Prorocentrum*, which have been identified as thermophilic in previous studies. Glibert et al. (2012) summarized previous studies and demonstrated that benthic *Prorocentrum* showed higher densities in tropical/subtropical areas than in temperate zones. Moreover, a two-year survey showed that the maximal abundance of *P. lima* occurred between July and October, and *P. lima* (positively correlated with temperature) was discovered when temperature ranged from 18 to 28.5°C, with a preference for 27–30°C (Aissaoui et al., 2014). Results from laboratory studies also demonstrated that higher temperatures are suitable for benthic *Prorocentrum*. For example, Accoroni et al. (2018) showed that *P. hoffmannianum* grew rapidly and had a larger maximum quantum yield of PSII at 27°C than at 21°C. We assume that the occurrence of a higher biomass of benthic *P. concavum* in the Xincun Bay was mainly induced by temperatures between 28 and 30°C.

4.3.2 Salinity, pH, and Dissolved Oxygen

Previous studies showed that salinity can influence the growth of *P. concavum* and this species had a maximum growth rate at a salinity of 30 (Morton et al., 1992). In the present study, salinity was not significantly related to either benthic or planktonic *P. concavum* abundances (**Table 3**). The insignificant effect of salinity on *P. concavum* in Xincun Bay may be a result of the small salinity range during the sampling period (from 29.08 to 32.59, **Figure 3B**). In addition, pH was not associated with the abundance of *P. concavum* in Xincun Bay. While no obvious correlations between benthic cells and dissolved oxygen were found, planktonic *P. concavum* was negatively associated with this factor (**Table 3**). Aissaoui et al. (2014) suggested that planktonic *P. lima* and *P. emarginatum* in the Mediterranean were also negatively associated with dissolved oxygen.

4.3.3 Nutrients

The abundance of benthic *P. concavum* was negatively associated with concentrations of nitrite, ammonium, and phosphate, but no correlation was found with nitrate. More importantly, *P. concavum* densities were positively correlated with concentrations of DOP and DON (**Table 3**). To date, available data on nutrient utilization by benthic *Prorocentrum* species is limited and mostly concentrated on *P. lima* (Glibert et al., 2012). Nitrate is always a primary source of nitrogen, but is rarely a limiting factor for microalgae (Cohu et al., 2013). Aissaoui et al. (2014) demonstrated that *P. lima* abundance in the Mediterranean was negatively correlated with ammonium, which is consistent with our findings. However, a laboratory study found that *P. lima* showed a preference for ammonium uptake rather than nitrate or nitrite (Pan et al., 1999). These contradictions could be explained by the descriptions of Aissaoui et al. (2014) that ammonium was taken up rapidly and showed low concentrations at the maximal abundances of *P. concavum* (Aissaoui et al., 2014). Pan et al.

(1999) suggested that the uptake of nitrite by *P. lima* occurred only when other nitrogen sources were exhausted. Finally, negative correlations between phosphate and *P. concavum*, coupled with the positive correlation between DOP and this dinoflagellate, indicate that DOP is an important factor driving spatiotemporal variation in *P. concavum* abundance. Also, *P. concavum* abundance was positively associated with concentrations of DON (**Table 3** and **Figure 8**). Ou et al. (2022) investigated the activities of extracellular enzymes, including leucine aminopeptidase (LAP) and alkaline phosphatase (AP) which hydrolyzed the DON and DOP, respectively, in the Xincun Bay from December 2018 to December 2019. The results showed that the activities of LAP and AP in the Xincun Bay were greater than other coasts of Chinese waters, even in a bloom period (Ou et al., 2018; Ou et al., 2022). These findings and high concentrations of DON and DOP increased the risk of harmful dinoflagellate blooms in the Xincun Bay. Therefore, concentrations of DON and DOP from aquaculture were the important factors in the occurrences of *P. concavum* bloom in the Xincun Bay. These findings also explain why the highest abundances were seen at station A (near the cage-culture area).

5 CONCLUSIONS

The spatiotemporal distribution of *P. concavum* was demonstrated over a 9-month period in Xincun Bay. Both benthic and planktonic *P. concavum* showed seasonal variation patterns, with higher abundances in the wet season and lower abundances in the dry season, although the benthic abundances were not significantly different. High spatial heterogeneity among different ecosystems and substrates was observed. The seagrass bed had a higher abundance of *P. concavum* than macroalgal beds, mangroves, or coral reefs. The abundance of *P. concavum* on the seagrass *T. hemperichii* was significantly higher than that on sediments. Temperature, DOP, and DON were the three important environmental factors driving the spatiotemporal variation in benthic *P. concavum* in Xincun Bay. Abundance of planktonic *P. concavum* was positively associated with benthic cells and negatively associated with dissolved oxygen, indicating that the abundance of *P. concavum* in the water column is primarily influenced by epiphytic cells and the concentration of dissolved oxygen. Overall, we found that the dense cage-fish culture in the Xincun Bay provided sufficient organic nutrients for the growth and reproduction of *P. concavum* and the seagrass bed in Xincun Bay may become a reservoir for harmful benthic dinoflagellates.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

SL and JZ designed the present study. HX and CZ carried out the sampling and experiments. JZ carried out sampling, conducted experiments, analyzed the data and wrote this manuscript. SL revised this manuscript and funded this study. All authors contributed to the article and approved the submitted version.

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SYSTEMATIC REVIEW
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Epibiotic Fauna on Cetaceans Worldwide: A Systematic Review of Records and Indicator Potential

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Each individual cetacean is an ecosystem itself, potentially harboring a great variety of animals that travel with it. Despite being often despised or overlooked, many of these epizoites have been proven to be suitable bio-indicators of their cetacean hosts, informing on health status, social interactions, migration patterns, population structure or phylogeography. Moreover, epizoites are advantageous over internal parasites in that many of them can be detected by direct observation (e.g., boat surveys), thus no capture or dissection of cetaceans are necessary. Previous reviews of epizoites of cetaceans have focused on specific geographical areas, cetacean species or epibiotic taxa, but fall short to include the increasing number of records and scientific findings about these animals. Here we present an updated review of all records of associations between cetaceans and their epibiotic fauna (i.e., commensals, ecto- or mesoparasites, and mutualists). We gathered nearly 500 publications and found a total of 58 facultative or obligate epibiotic taxa from 11 orders of arthropods, vertebrates, cnidarians, and a nematode that are associated to the external surface of 66 cetacean species around the globe. We also provide information on the use as an indicator species in the literature, if any, and about other relevant traits, such as geographic range, host specificity, genetic data, and life-cycle. We encourage researchers, not only to provide quantitative data (i.e., prevalence, abundance) on the epizoites they find on cetaceans, but also to inform on their absence. The inferences drawn from epizoites can greatly benefit conservation plans of both cetaceans and their epizoites.

Keywords: epibiotic, fauna, cetacean, indicator, systematic review, checklist

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INTRODUCTION

General Features of Epibiosis in Cetaceans

Cetaceans have developed a number of symbiotic associations (*sensu* Leung and Poulin, 2008) with other organisms, including endo-, meso- and ectoparasitism, commensalism, and mutualism (e.g., Arvy, 1982; Raga, 1994). Some of these organisms, the epibionts (also known as episymbionts or ectosymbionts), are associated to the external surface of cetaceans and can be classified into two basic types. On the one hand, ectoparasites live in/on the skin and cause a variable degree of harm by feeding on hosts' integument (e.g., Smyth, 1962; Geraci and St. Aubin, 1987; Hopla et al., 1994). On the other hand, commensals or phoronts do not trophically depend on the tissues of cetaceans (also

named basibionts in this case), thus they are generally harmless but benefit from epibiosis in multiple ways, e.g., *via* an improved feeding performance, reduction of predation, favored intraspecific contacts for reproduction, or offspring dispersion (Anderson, 1994; Seilacher, 2005; Carrillo et al., 2015). Not surprisingly, though, the limits of each type of interaction are not always clear-cut. For instance, whale-lice (fam. Cyamidae) are considered ectoparasites that primarily feed on hosts' skin, but it has been speculated that they may opportunistically feed on plankton, even helping whales to detect plankton blooms, leading to a potentially mutualistic relationship (Rowntree, 1996). Or, high loads of commensal epibionts could increase the swimming drag or damage the skin on the site of settlement, thus producing indirect harm to cetaceans (Tomilin, 1957).

Given the high variety of life cycles of the epibionts of cetaceans, it is perhaps not surprising that their specific interactions are similarly diverse. Some epibionts depend strictly on cetaceans during their whole life (e.g., whale lice; Leung, 1976), whereas others use them only at some stages (e.g., barnacles; Nogata and Matsumura, 2006). Among commensals, many species are obligate epibionts, settling exclusively on cetaceans (e.g., coronulid barnacles; Hayashi et al., 2013), but others can colonize also inanimate substrata such as vessels or floating debris (e.g., *Conchoderma* spp. and *Lepas* spp.; Frick and Pfaller, 2013). The degree of host/basibiont specificity is also variable. For instance, many whale lice are known only from single, or a few, host species (Iwasa-Arai and Serejo, 2018), but other epibionts have a very broad host spectrum (e.g., *Xenobalanus globicitipitis* Steenstrup, 1852 or *Pennella balaenoptera* Koren & Danielssen, 1877; Kane et al., 2008; Fraija-Fernández et al., 2018). Finally, there are examples of hyperepibiosis in which some epibionts, e.g., barnacles, can act as basibionts for other epibionts, e.g., *Conchoderma* spp. or cyamids (Cornwall, 1927; Matthews, 1937; Leung, 1970a).

Susceptibility and Health Impact of Cetacean Epibiosis

As many other symbionts, epibionts must succeed twice to live their associative life. This two-step process is mediated by the so-called encounter and compatibility filters (Combes, 2001). First, spatial and temporal overlap must take place for initial settlement. Second, whether the host is a suitable substratum will determine survival and/or reproduction on it. Epidermis renewal and hydrolytic substances of cetacean skin may prevent fouling, at least to some extent (Hicks 1985; Baum et al., 2000; Baum et al., 2001), but skin regeneration and immune functions are seemingly lower in debilitated dolphins (J. R. Geraci and S. H. Ridgway pers comm. in Aznar et al., 1994). Poor health can also result in slower swimming (Aznar et al., 1994; Lehnert et al., 2021), fostering better conditions for epibiotic settlement (e.g., providing more time for contact with blooms of free-living infective stages, or mild water flow over the host's body, thus reducing drag and facilitating initial colonization). For instance, striped dolphins, *Stenella coeruleoalba* (Meyen, 1833), infected by morbillivirus and in poor nutritional condition harbored high loads of parasitic and commensal epizoites (Aguilar and Raga, 1993; Aznar et al., 1994; Aznar et al., 2005). Also, higher prevalence of cyamids in porpoises could hint a higher incidence of disease-related skin injuries, where they attach (Lehnert et al., 2021). Another example is the massive infestation of cyamids on a stranded humpback whale, *Megaptera novaeangliae* (Borowski, 1781), that suffered from severe discospondylitis and, as a result, reduced mobility (Groch et al., 2018).

Once settled, the impact of epibionts on cetacean health varies among taxa (especially between ectoparasites and commensals; see above). For instance, the mesoparasite *Pennella balaenoptera* penetrates the skin and blubber of its hosts; this process has been related to both macro- and microscopic lesions such as abscesses, inflammation, and dermatitis (Cornaglia et al., 2000; Gomerčić et al., 2006; IJsseldijk et al., 2018). In contrast, no direct damage has been related to whale lice infections (e.g., Migaki, 1987; Lehnert et al., 2021), although it has been speculated that their occurrence may hinder skin healing processes (Lehnert et al., 2021). On the other hand, the possibility that some cetacean epibionts can act as viral or bacterial vectors is an open question, as it has been observed for ectoparasitic crustaceans parasitizing fish (Smit et al., 2019) or lice infecting seals (La Linn et al., 2001). Climate changes have shifted the geographical distribution of arthropod-borne viruses (Gould and Higgs, 2008) and whether these may emerge in cetaceans and even be transmitted by their epibonts (e.g., ectoparasitic lice, see Van Bressem et al., 2009) remains unknown.

Epibionts as Cetacean Indicators

Due to temporal or permanent association with their hosts/basibionts, both endoparasites and epibionts represent a cost-effective tool to study multiple facets of cetacean biology (e.g., Dailey and Vogelbein, 1991; Balbuena et al., 1995; Gomes et al., 2021). However, epibionts are advantageous over endoparasites in that many of them are detectable in the field (e.g., using boat-based photography; see Hermosilla et al., 2015; Siciliano et al., 2020; Flach et al., 2021), and can often be easily found and counted on stranded hosts, be alive or dead, with minimum dissection, if at all (Balbuena et al., 1995). Most studies using epibionts as markers only require basic data to be gathered, i.e. genus- or, preferably, species-level identification, and quantification of population size at host individual or population scales. More elaborated research may require additional information on (1) degree of host specificity, (2) size measurements as an estimate of time since attachment, (3) distribution patterns on hosts' body, (4) geographic range, and/or (5) selected molecular markers (e.g., Bushuev, 1990; Kaliszewska et al., 2005; Ten et al., 2019; Moreno-Colom et al., 2020; Lehnert et al., 2021).

At present, cetacean epibionts have been used, *inter alia*, as 'tags' to trace past (e.g., Collareta et al., 2018a; Taylor et al., 2019) or present-day (e.g., Pearson et al., 2020; Visser et al., 2020) migratory routes and habitat use; shed light on phylogeography, population structure, and ecological stock delimitation (e.g., Bushuev, 1990; Kaliszewska et al., 2005; Iwasa-Arai et al., 2018); give insight into hydrodynamics (e.g., Kasuya and Rice,

1970; Briggs and Morejohn, 1972; Fish and Battle, 1995; Carrillo et al., 2015; Moreno-Colom et al., 2020), assist in individual recognition (e.g., Visser et al., 2020); and act as sentinels of health status (Mackintosh and Wheeler, 1929; Van Waerebeek et al., 1993; Aznar et al., 1994; Aznar et al., 2005; Lehnert et al., 2007; Vecchione and Aznar, 2014; Lehnert et al., 2021; for more references see Results). Nonetheless, there is plently of further opportunities to exploit the full potential of these organisms as biological indicators.

Aims

Studies including information on cetacean epibionts have usually focused on particular geographical areas (e.g., Kane et al., 2008; Lehnert et al., 2019), host species (e.g., Rice, 1978; Stimmelmayr and Gulland, 2020) or epibiotic taxa (e.g., Kane et al., 2008; Iwasa-Arai and Serejo, 2018). Furthermore, in the last decades a number of nomenclatural changes, new associations, and geographical records have been accumulating, thus we think that the available comprehensive reviews and checklists on this subject (Beneden, 1870; Dailey and Brownell, 1972; Arvy, 1977; Arvy, 1982; Raga, 1994) should be updated. On the other hand, few articles have reviewed the use of marine mammal parasites as biological tags (Balbuena et al., 1995; Mackenzie, 2002), and none gathered information about the whole epibiotic fauna of cetaceans.

The present systematic review aims to compile and update all records of cetacean epibiotic fauna (= epizoites) to date as a thorough, handy catalogue for researchers. Other organisms, i.e. diatoms and cookie-cutter shark, *Isistius brasiliensis* (Quoy & Gaimard, 1824) are also included in a specific section of this review to provide a complete picture of other externally-associated organisms that have been proven to be valuable biological indicators for cetaceans. Finally, we identify information gaps and future research directions and highlight the value of cetacean epibionts as indicator tools, encouraging their application in cetacean research.

METHODS

Literature Search

A systematic literature review was performed following PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines (Moher et al., 2015; **Figure 1**). We conducted a thorough bibliographic search in the following databases: Google Scholar (https://scholar.google.com), Scopus (https://www.scopus.com), ScienceDirect (https://www.sciencedirect.com), Web of Science (https://www.webofscience.com), and Sage (https://journals.sagepub.com). The following search string was used for Scopus, ScienceDirect, Web of Science, and Sage: (*epibiont* OR *epibiotic* OR *epibiosis* OR *epizoite* OR *epizoic* OR *barnacle* OR *ectoparasite* OR *mesoparasite*) AND (*balaena* OR *eubalaena* OR *balaenoptera* OR *megaptera* OR *eschrichtius* OR *caperea* OR *cephalorhynchus* OR *delphinus* OR *feresa* OR *globicephala* OR *grampus* OR *lagenodelphis* OR *lagenorhynchus* OR *lissodelphis* OR *orcaella* OR *orcinus* OR *peponocephala* OR *pseudorca* OR *sotalia* OR "*Sousa chinensis*" OR "*Sousa plumbea*" OR "*Sousa sahulensis*" OR "*Sousa teuszii*" OR *stenella* OR "*Steno bredanensis*" OR *tursiops* OR "*Inia geoffrensis*" OR *kogia* OR *delphinapterus* OR "*Monodon monoceros*" OR *neophocaena* OR *phocoena* OR *phocoenoides* OR *physeter* OR *platanista* OR *pontoporia* OR *berardius* OR *hyperoodon* OR *mesoplodon* OR *tasmacetus* OR *ziphius* OR *indopacetus*)

Note that the use of genus name in some cetacean genera, i.e., *Monodon* Linnaeus, 1758, *Sousa* Gray, 1866, and *Steno* Gray, 1846 yielded many records of unrelated taxa, thus full species name was included in these cases. The output was exported and checked for duplicates and non-relevant papers with the open-source reference management software Zotero.

In the case of Google Scholar, only the first 100 result pages are available, thus we used the search strings "(*epibiont* OR *epibiotic* OR *epibiosis* OR *epizoite* OR *epizoic* OR *barnacle* OR *ectoparasite* OR *mesoparasite*) AND *i*", where *i* stands for a cetacean genus, to maximize the number of obtainable records. The output of each search was checked manually. In addition, we searched each epibiotic species in GBIF.org and included those associations and geographic locations that had not been reported in scientific publications. For all publications obtained, we looked up their references to search for potential missing records.

The final list includes the literature published until December 2021 that provides information on cetacean-epibiont(s) associations (**Figure 1**). These results are listed according to the epibiotic (see the Results) and the cetacean taxa (**Supplementary Table 1**). For each selected record, we extracted the following information, when available: cetacean species, epibiotic species, geographic area(s), prevalence (i.e., percent occurrence of the epibiont in each cetacean species of the sample), location on the cetacean, and any information related to indicator potential. Current species nomenclature and synonyms were checked in WoRMS (https://www.marinespecies.org/) and recent literature. Geographical locations were also classified at the scale of Large Marine Ecosystem (LME) (see e.g., Brotz et al., 2012).

For comparative purposes, we investigated research effort on each cetacean species using the number of results in Google Scholar as a proxy. For each species, we used the scientific name in quotation marks as search string. For the 6 species that previously constituted the *Lagenorynchus* genus (see Vollmer et al., 2019), we used the former nomenclature for the search to avoid understimation (i.e., "*Lagenorynchus*" followed by species name).

RESULTS

General Patterns

A total of 492 published documents, including 7 unpublished manuscripts, and 9 GBIF records were found. Three additional reliable records were serendipitously found in internet photo-catalogues and were also included in the final list (**Supplementary Table 1**). A roughly exponential trend in the

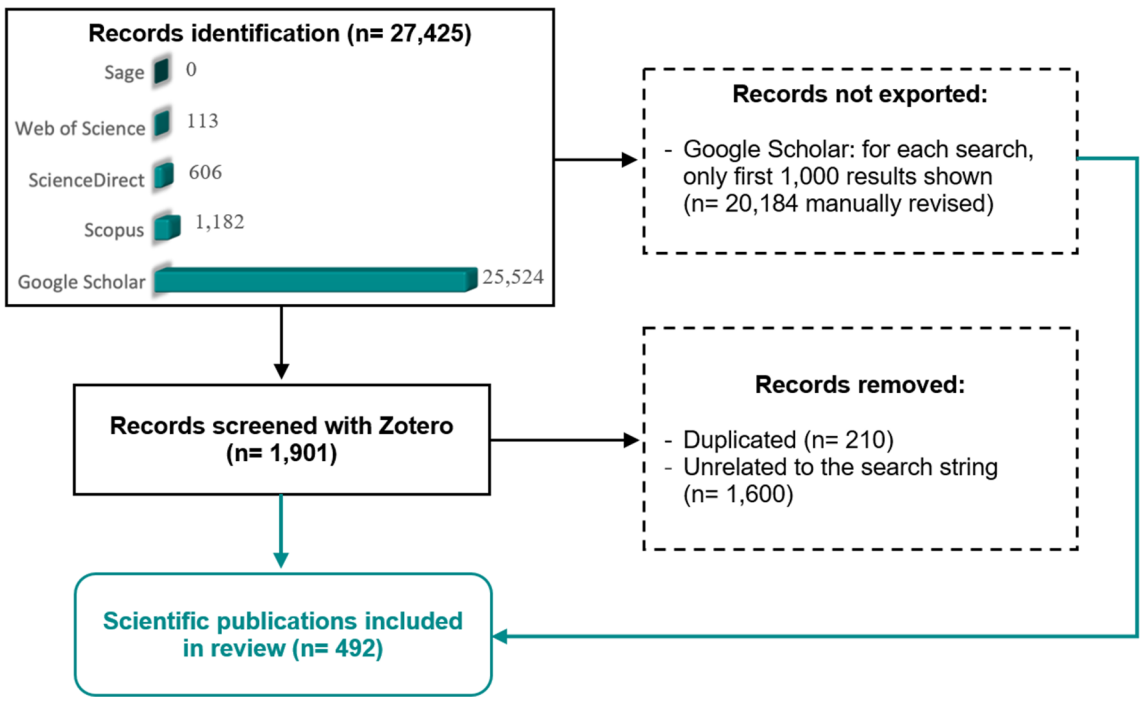

FIGURE 1 | Flow diagram of the methodology used in the literature search performed in this systematic review. Adaptation from PRISMA (Preferred Reporting Items for Systematic Reviews) template (Page et al., 2021).

number of publications was found throughout the period covered (1655-2021), with a peak in the 2010s decade (**Figure 2**); 2020 was the most productive year with 21 publications.

Baleen whales, and particularly *Megaptera novaeangliae* (Borowski, 1781), show the highest diversity of epibionts, followed by *Tursiops* spp. (**Figure 3**). However, it is difficult to ascertain the extent to which this pattern is affected by sampling effort (**Figure 3**). Likewise, 26 cetacean species from four genera have no published records of epibiotic fauna to date (**Supplementary Table 1**), but these hosts have also been generally little studied (< 4,000 publications in Google Scholar, **Figure 3**). Research effort varies also among geographic regions (**Figure 4**). The Mediterranean Sea and Antarctica are, by far, the geographic areas with the highest number of publications of cetacean epizoites, and some areas still lack such studies.

Systematic List

A systematic list of the 58 epizoic taxa (53 at species level) found to date on cetaceans follows. For each one, we provide information on (i) taxonomic synonyms; (ii) a subset of selected references that provide a complete overview of the species morphology; (iii) molecular sequences available on GenBank (https://www.ncbi.nlm.nih.gov/genbank/), with references or with Accession Number whenever no published manuscript was available; (iv) primary type of association, including parasitic (34 spp.), obligate commensal (8-9 spp.), facultative commensal (8 spp.), mutualistic (possibly 1 sp.), or unknown (2 spp.); (v) a list of cetacean hosts/basibionts; (vi) geographic range; (vii) life-cycle; and (viii) microhabitat, i.e., the location(s) on the cetacean body, with references; and (ix) indicator use or potential, with references. Any other relevant data are reported in the 'Remarks' section, and all records of association between epizoites and cetaceans are cited in the 'References' section.

Phylum Arthropoda von Siebold, 1848
Class Malacostraca Latreille, 1802
Subclass Eumalacostraca, Grobben, 1892
Order Amphipoda Latreille, 1816
Family Cyamidae Rafinesque, 1815

The Cyamidae ('whale lice') comprises a group of amphipods that are found exclusively on marine cetaceans (see, e.g., Iwasa-Arai and Serejo, 2018). These 3-30 mm creatures use their pereopods to cling to areas of reduced water flow (e.g., ventral grooves, blowhole, genital slit), where they spend their whole life feeding primarily on cetacean skin (Rowntree, 1983; Rowntree, 1996; Schell et al., 2000); thus, they are all considered ectoparasites. However, evidence that they cause any harm is rather scarce, so some authors support the use of the term 'ectocommensals' for them (Leung, 1976; Kenney, 2009). Rowntree (1996) discussed the possibility that some cyamids from whales may also feed on plankton, having perhaps developed mutualistic associations with their hosts. In particular, the cyamid species covering the

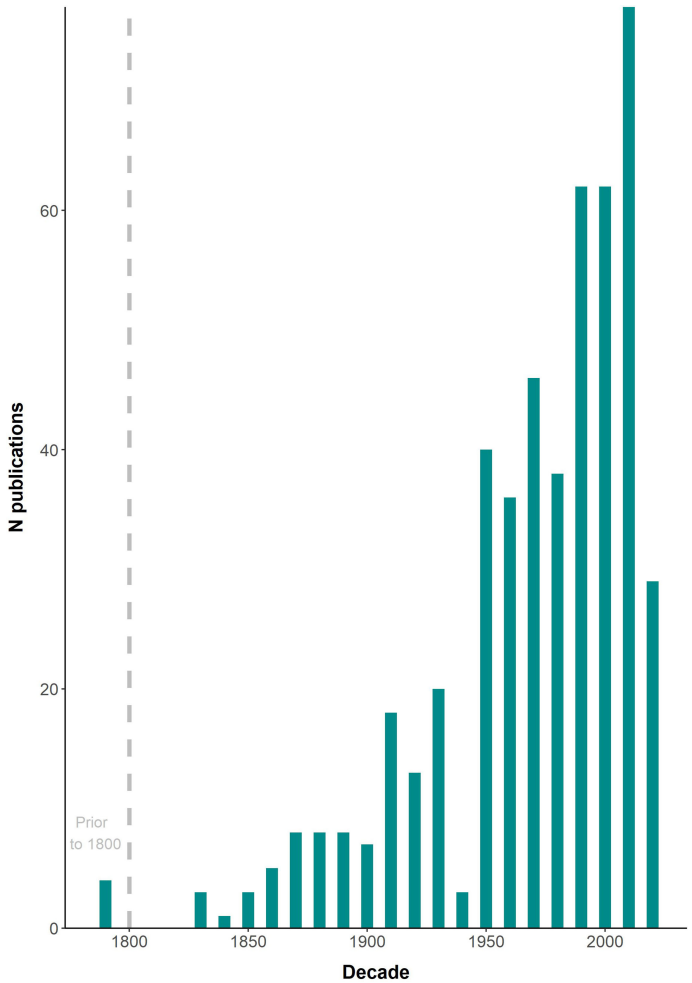

FIGURE 2 | Number (N) of publications including data on cetacean epibiotic fauna at a decadal scale from 1655 to 2021.

sensory hairs of whales could increase their activity during plankton blooms, amplifying the signal for prey detection by whales. In addition, it has also been suggested that cyamids could feed on cetaceans' dead skin and epibiotic algae, thus cleaning up wounds and speeding up healing (Williams and Bunkley-Williams, 2019). Lehnert et al. (2021), on the contrary, hypothesized that cyamids' feeding activity could actually hinder the healing of skin injuries, and some authors have suggested that heavy cyamid infections may contribute to the death of their hosts (Mignucci-Giannoni et al., 1998).

Since cyamids lack swimming stages, transmission must occur through bodily contacts (Fransen and Smeenk, 1991; Pfeiffer, 2009). Males are typically larger than females (but see Fraija-Fernández et al., 2017) and, at least in some species, have been observed to perfom pre-copulatory mate guarding (Rowntree, 1996; Oliver and Trilles, 2000). Females mate after molting (Conlan, 1991) and incubate eggs and protect the hatchling in a ventral brood pouch (Leung, 1976; Williams and Bunkley-Williams, 2019).

Balaenocyamus balaenopterae (Barnard K.H. 1931)

Synonyms

Cyamus balaenopterae Barnard K.H. 1931

Morphological Description

Barnard, 1932; Margolis, 1959; Leung, 1967; Iwasa-Arai and Serejo, 2018

Molecular Sequences

18S rRNA (Ito et al., 2011)

Association

Ectoparasite

Cetacean Hosts/Basibionts

Balaenoptera acutorostrata Lacépède, 1804, *B. bonaerensis* Burmeister, 1867, *B. musculus* (Linnaeus, 1758), *B. physalus* (Linnaeus, 1758)

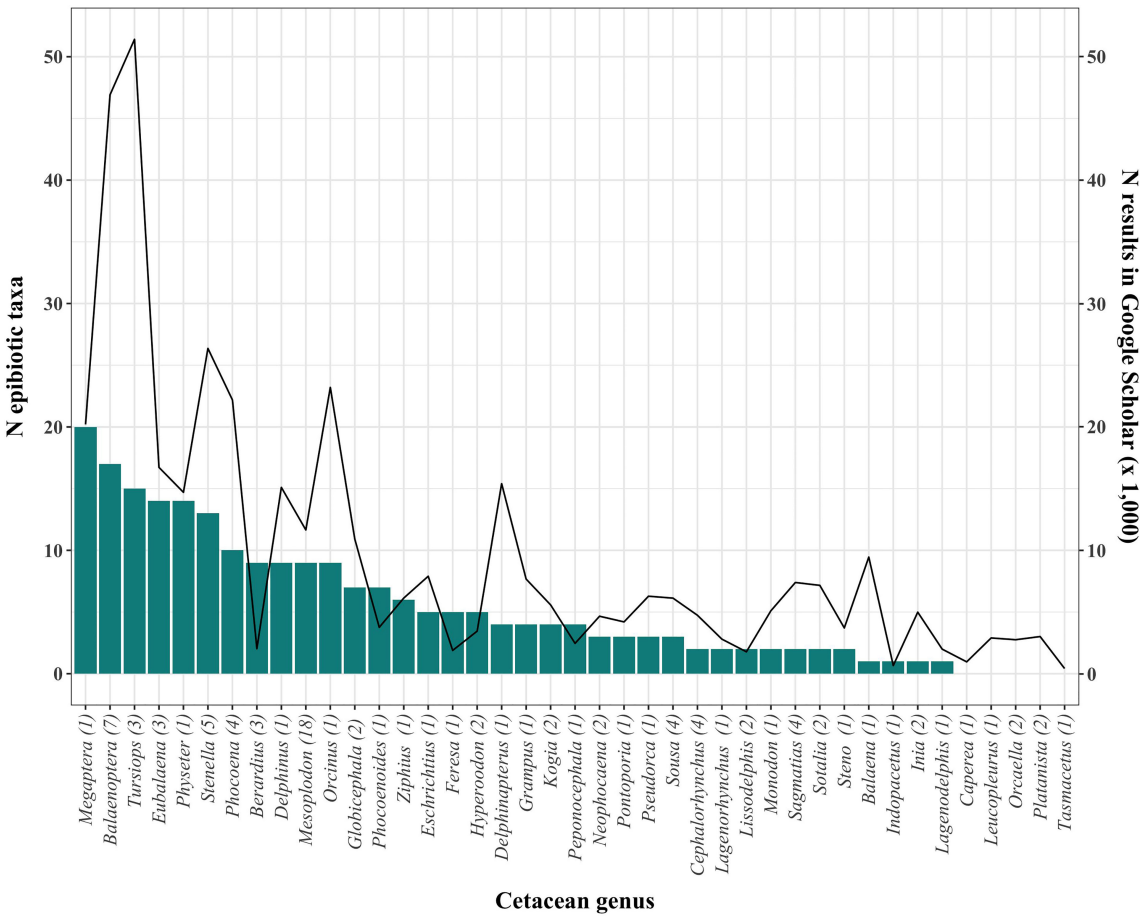

FIGURE 3 | Number of epibiotic species (bars, left y-axis) and total number of general results in Google Scholar (line, right y-axis) of each cetacean genus. The number of cetacean species in each genus is shown in parentheses.

Geographic Range
Atlantic, Pacific, Mediterranean, Indian Ocean, Antarctica

Life Cycle
In common minke whales, *Balaenoptera acutorostrata*, captured off Iceland, a one-year long life cycle is assumed; similar to other whale lice, hatching occurs in autumn, juveniles are released from the females' pouch in mid-winter, and they reach sexual maturity in spring or summer (Ólafsdóttir and Shinn, 2013). This life cycle may be synchronized with whales' seasonal migration (Raga and Sanpera, 1986).

Microhabitat
Natural orifices, i.e., ventral grooves, eyes, umbilicus, mammary slits, anus, and genital slit (Ohsumi et al., 1970; Ivashin, 1975; Raga and Sanpera, 1986)

Use as Indicator
Used to delineate ecological stocks and detect sex segregation in migrating cetaceans (Kawamura, 1969; Bushuev, 1990; Ólafsdóttir and Shinn, 2013).

Remarks
-

References
Mackintosh and Wheeler, 1929; Barnard, 1931; Barnard, 1932; Margolis, 1959; Leung, 1965; Kawamura, 1969; Ohsumi et al., 1970; Lincoln and Hurley, 1974a; Ivashin, 1975; Rice, 1978; Berzin and Vlasova, 1982; Best, 1982; Raga and Sanpera, 1986; Avdeev, 1989; Bushuev, 1990; Sedlak-Weinstein, 1990 (unpubl.); Dailey and Vogelbein, 1991; Kuramochi et al., 1996; Araki et al., 1997; Uchida, 1998; Kuramochi et al., 2000; Margolis et al., 2000; Uchida and Araki, 2000; Ólafsdóttir and Shinn, 2013; Iwasa-Arai and Serejo, 2018; Ten et al., unpubl.

Cyamus boopis (Lütken, 1870)

Synonyms
Cyamus elongatus Hiro, 1938, *C. pacificus* Lütken, 1873, *C. suffuses* Dall, 1872, *Paracyamus boopis* (Lütken, 1870)

Morphological Description
Sars, 1895; Barnard, 1932; Leung, 1967; Margolis et al., 2000; Iwasa-Arai et al., 2016

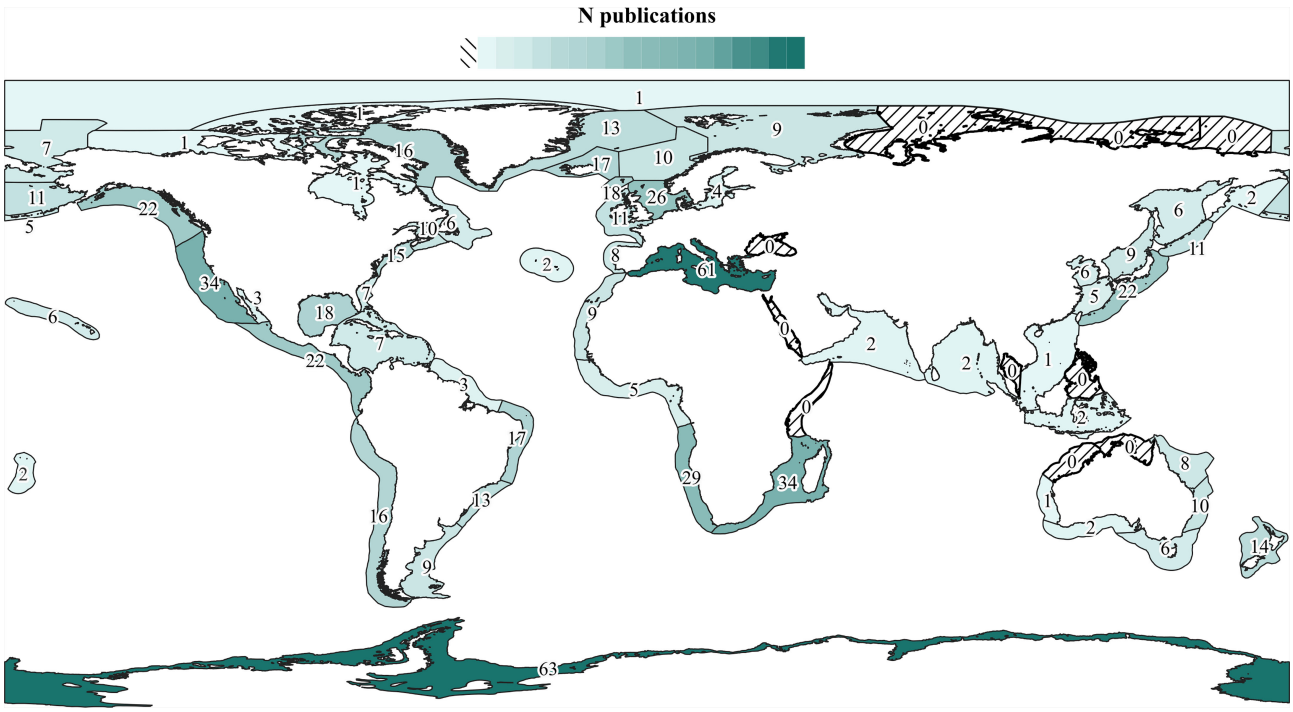

FIGURE 4 | Number of publications (indicated by numbers and color gradient) on cetaceans that contain data on their epibiotic fauna at least to genus level grouped by Large Marine Ecosystems (LME). When the same publication includes data for several LMEs, it is counted separately for each one. Azores (NE Atlantic) and Tonga (SW Pacific) are not in the LME system but were included as additional areas.

Molecular Sequences

COI (Iwasa-Arai et al., 2017a, Iwasa-Arai et al., 2018; GenBank FJ751158; FJ751159; MT551876; OK562816-OK562832), COII, COIII, ATP6, ATP8, ND3 (Kaliszewska et al., 2005) and the complete mitochondrial genome (GenBank MT458501)

Association

Ectoparasite

Cetacean Hosts/Basibionts

Typically on *Megaptera novaeangliae*, but once reported on *Berardius bairdii* Duvernoy, 1851, *Eubalaena australis* (Desmoulins, 1822), and *Tursiops truncatus* (Montagu, 1821)

Geographic Range

Arctic, Atlantic, Pacific, Mediterranean, Indian Ocean, Antarctica

Life Cycle

Transmission may regularly occur during contacts between migrating hosts or at the feeding areas (Iwasa-Arai et al., 2018).

Microhabitat

Ubiquitous, i.e., head tubercles, eye, jaw, ventral grooves, genital slit, fins (Matthews, 1937; Cockrill, 1960; Ivashin, 1965; Rowntree, 1996). Sometimes attached to the epibiotic cirripedes *Coronula diadema* (Linnaeus, 1767) and *Conchoderma* spp. (Dall, 1872; Matthews, 1937; Stephensen, 1942; Angot, 1951; Cockrill, 1960).

Use as Indicator

Haplotype and nucleotide diversities have been used to assess inter-mixing between different breeding populations of humpback whales (Iwasa-Arai et al., 2018). Also, its presence on a southern right whale suggests an interspecific interaction with humpback whales in Brazilian waters (Iwasa-Arai et al., 2017a). The presence of an alive unidentified cyamid (likely *C. boopis*) on a humpback whale was used to infer that the stranding occurred less than three days before (Bortolotto et al., 2016).

Remarks

Some records of *C. boopis* on sperm whales (e.g., Barnard, 1932) were re-classified as *C. catodontis* by Margolis (1955) and later authors (e.g., Stock, 1973a; Iwasa-Arai and Serejo, 2018).

References

Lütken, 1870; Dall, 1872; Scammon, 1874; Pouchet, 1888; Pouchet, 1892; Sars, 1895; Collet, 1912; Chevreux, 1913a; Liouville, 1913; Ishi, 1915; Cornwall, 1928; Barnard, 1932; Matthews, 1937; Hiro, 1938; Scheffer, 1939; Angot, 1951; Hurley, 1952; Rees, 1953; Margolis, 1954a; Cockrill, 1960; Rice, 1963; Ivashin, 1965; Leung, 1965; Leung, 1970b; Lincoln and Hurley, 1974a; Berzin and Vlasova, 1982; Sedlak-Weinstein, 1991; Rowntree, 1996; Abollo et al., 1998; Osmond and Kaufman, 1998; Margolis et al.,

2000; Alonso de Pina and Giuffra, 2003; Carvalho et al., 2010; Iwasa-Arai et al., 2016; Iwasa-Arai et al., 2017b; Iwasa-Arai et al., 2018; Groch et al., 2018; Iwasa-Arai et al., 2021; Iwasa-Arai et al., 2018; Qiao et al., 2020

Cyamus catodontis (Margolis, 1954)

Synonyms
Cyamus bahamondei Buzeta, 1963

Morphological Description
Margolis, 1954a; Margolis, 1955; Buzeta, 1963; Leung, 1967; Stock, 1973a; Margolis et al., 2000

Molecular Sequences
-

Association
Ectoparasite

Cetacean Hosts/Basibionts
Typically on *Physeter macrocephalus* Linnaeus, 1758, but once reported on *Balaenoptera acutorostrata*, *B. bonaerensis*, *B. musculus*, *B. physalus*, and *Berardius bairdii*

Geographic Range
Eastern Atlantic, Pacific, Indian Ocean, Antarctica

Life Cycle
-

Microhabitat
One record on a sperm whale's deformed jaw (Buzeta, 1963)

Use as Indicator
Used to detect social segregation in sperm whales; large males, but not females nor male bachelors, were infected with *C. catodontis*, suggesting that the former leave their natal pods at puberty (Best, 1969a; Best, 1979).

Remarks
-

References
Barnard, 1932; Margolis, 1954a; Clarke, 1956; Buzeta, 1963; Rice, 1963; Leung, 1965; Best, 1969a; Best, 1969b; Best, 1979; Stock, 1973b; Lincoln and Hurley, 1974a; Berzin and Vlasova, 1982; Fransen and Smeenk, 1991; Iwasa-Arai and Serejo, 2018

Cyamus ceti (Linnaeus, 1758)

Synonyms
Oniscus ceti Linnaeus, 1758

Morphological Description
Krøyer, 1843; Leung, 1967; Margolis et al., 2000; Iwasa-Arai and Serejo, 2018

Molecular Sequences
COI (GenBank FJ751160-FJ751180)

Association
Ectoparasite

Cetacean Hosts/Basibionts
Typically on *Balaena mysticetus* Linnaeus, 1758, but once reported on *Eschrichtius robustus* (Lilljeborg, 1861) and *Eubalaena japonica* (Lacépède, 1818)

Geographic Range
Artic, North Pacific

Life Cycle
Similar to *C. scammoni* (see below), but juveniles reach maturity before whales' northern migration to summer grounds (Leung, 1976). Females carry 150-240 eggs in the brood pouch, of which about 75% are fertilized (Leung, 1976).

Microhabitat
Creases of the lips, flippers, flukes, and thin areas, e.g., armpit and genital slit (Stephensen, 1942; Leung, 1976)

Use as Indicator
-

Remarks
-

References
Linnaeus, 1758; Lütken, 1870; Dall, 1872; Scammon, 1874; Margolis, 1955; Omura, 1958; Rice, 1963; Lincoln and Hurley, 1974a; Leung, 1976; Berzin and Vlasova, 1982; Heckmann et al., 1987; Margolis et al., 2000; Kaliszewska et al., 2005; Von Duyke et al., 2016; Chernova et al., 2017; Iwasa-Arai and Serejo, 2018

Cyamus erraticus (Roussel de Vauzème, 1834)

Synonyms
Paracyamus erraticus Roussel de Vauzème, 1834

Morphological Description
Barnard, 1932; Iwasa, 1934; Margolis, 1955; Leung, 1967

Molecular Sequences
COI, COII, COIII, ATP6, ATP8, ND3 (Kaliszewska et al., 2005), EF1a (Seger et al., 2010)

Association
Ectoparasite

Cetacean Hosts/Basibionts
Typically on *Eubalaena australis*, *E. glacialis* (Müller, 1776), and *E. japonica*; also found on *Megaptera novaeangliae*

Geographic Range
Atlantic, Pacific, Indian Ocean, Antarctica

Life Cycle
-

Microhabitat
Genital, mammary, and anal slits, armpits, and opportunistically on wounds (Stephensen, 1942; Rowntree, 1996; see Remarks)

Use as Indicator
Sequence variation in mitochondrial DNA was used to investigate associations among right whale individuals and subpopulations, to estimate the time of past divergence of right whale populations, and to infer possible changes in their population sizes (Kaliszewska et al., 2005).

Remarks
Transmission probably occurs from mothers's genital slit to calves' head at birth. As callosity tissue develops, calves are colonized by the putative competitor *Cyamus ovalis* Roussel de Vauzème, 1834, likely by head-to-head contact with the mother; the distribution of *C. erraticus* is then restricted to skin folds and wounds (Rowntree, 1996).

References
Rossel de Vauzème, 1834; Lütken, 1873; Collet, 1912; Chevreux, 1913a; Liouville, 1913; Barnard, 1932; Iwasa, 1934; Margolis, 1955; Lincoln and Hurle7y, 1974a; Berzin and Vlasova, 1982; Rowntree, 1996; Margolis et al., 2000; Iwasa-Arai and Serejo, 2018

Cyamus eschrichtii (Margolis, McDonald & Bousfield, 2000)
Synonyms
-

Morphological Description
Margolis et al., 2000

Molecular Sequences
-

Association
Ectoparasite

Cetacean Hosts/Basibionts
Eschrichtius robustus

Geographic Range
California (eastern North Pacific)

Life Cycle
-

Microhabitat
-

Use as Indicator
-

Remarks
-

References
Margolis et al., 2000

Cyamus gracilis (Roussel de Vauzème, 1834)
Synonyms
Paracyamus gracilis (Roussel de Vauzème, 1834)

Morphological Description
Barnard, 1932; Leung, 1967; Iwasa-Arai and Serejo, 2018

Molecular Sequences
COI, COII, COIII, ATP6, ATP8, ND3 (Kaliszewska et al., 2005), EF1a (Seger et al., 2010)

Association
Ectoparasite

Cetacean Hosts/Basibionts
Eubalaena australis, *E. glacialis*, *E. japonica*

Geographic Range
Atlantic, Pacific, Antarctica

Life Cycle
-

Microhabitat
Head callosities (Barnard, 1932; Rowntree, 1996)

Use as Indicator
See *C. erraticus*.

Remarks
In a South African sample, *C. gracilis* co-occurred with *C. ovalis* Roussel de Vauzème, 1834 (Barnard, 1932).

References
Rossel de Vauzème, 1834; Lütken, 1873; Barnard, 1932; Margolis, 1955; Leung, 1965, Leung 1967; Lincoln and Hurley, 1974a; Berzin and Vlasova, 1982; Rowntree, 1996; Alonso de Pina and Giuffra, 2003; Iwasa-Arai and Serejo, 2018

Cyamus kessleri (A. Brandt, 1873)
Synonyms
-

Morphological Description
Brandt, 1872; Leung, 1967; Margolis et al., 2000; Iwasa-Arai and Serejo, 2018

Molecular Sequences
COI (GenBank FJ751215-FJ751224)

Association
Ectoparasite

Cetacean Hosts/Basibionts
Eschrichtius robustus

Geographic Range
From Chukchi Sea to California (eastern North Pacific)

Life Cycle
Similar to *C. scammoni* (see below), but juveniles reach maturity before whales' northern migration to summer grounds (Leung, 1976). Females carry up to 300 eggs in the brood pouch, of which 75-80% are fertilized (Leung, 1976).

Microhabitat
Umbilicus, genital slit, and anal aperture (Leung, 1976)

Use as Indicator
-

Remarks
-

References
Hurley and Mohr, 1957; Leung, 1976; Berzin and Vlasova, 1982; Margolis et al., 2000; Kaliszewska et al., 2005; Iwasa-Arai and Serejo, 2018

Cyamus mesorubraedon (Margolis, McDonald & Bousfield, 2000)
Synonyms
-

Morphological Description
Margolis et al., 2000

Molecular Sequences
-

Association
Ectoparasite

Cetacean Hosts/Basibionts
Physeter macrocephalus

Geographic Range
Vancouver Island (eastern North Pacific)

Life Cycle
-

Microhabitat
-

Use as Indicator
-

Remarks
-

References
Margolis et al., 2000

Cyamus monodontis (Lütken, 1870)
Synonyms
-

Morphological Description
Leung, 1967; Margolis et al., 2000; Iwasa-Arai et al., 2017b; Iwasa-Arai and Serejo, 2018

Molecular Sequences
-

Association
Ectoparasite

Cetacean Hosts/Basibionts
Delphinapterus leucas (Pallas, 1776), *Monodon monoceros* Linnaeus, 1758, *Ziphius cavirostris* Cuvier, 1823

Geographic Range
Arctic, western North Atlantic, eastern North Pacific

Life Cycle
-

Microhabitat
Tusk base, caudal fin along with *C. nodosus*, skin injuries (Porsild, 1922; Stephensen, 1942)

Use as Indicator
-

Remarks
-

References
Lütken, 1870; Porsild, 1922; Lincoln and Hurley, 1974a; Heyning and Dahlheim, 1988; Mignucci-Giannoni et al., 1998; Margolis et al., 2000; Iwasa-Arai et al., 2017a

Cyamus nodosus (Lütken, 1861)
Synonyms
Paracyamus nodosus (Lütken, 1861)

Morphological Description
Leung, 1967; Iwasa-Arai et al., 2017b; Iwasa-Arai and Serejo, 2018

Molecular Sequences
-

Association
Ectoparasite

Cetacean Hosts/Basibionts
Delphinapterus leucas, *Monodon monoceros*

Geographic Range
Greenland (Arctic, western North Atlantic)

Life Cycle
-

Microhabitat
Tusk base, caudal fin along with *C. monodontis*, skin injuries (Porsild, 1922; Stephensen, 1942)

Use as Indicator
-

Remarks
-

References
Lütken, 1870; Porsild, 1922; Margolis, 1954b; Margolis, 1955; Lincoln and Hurley, 1974a; Iwasa-Arai et al., 2017a

Cyamus orubraedon (Waller, 1989)
Synonyms
-

Morphological Description
Margolis et al., 2000

Molecular Sequences
-

Association
Ectoparasite

Cetacean Hosts/Basibionts
Berardius bairdii

Geographic Range
North Pacific

Life Cycle
-

Microhabitat
Lower jaw (Waller, 1989)

Use as Indicator
-

Remarks
-

References
Waller, 1989; Margolis et al., 2000; Iwasa-Arai and Serejo, 2018

Cyamus ovalis (Roussel de Vauzème, 1834)
Synonyms
-

Morphological Description
Roussel de Vauzème, 1834; Iwasa, 1934; Leung, 1967; Margolis et al., 2000; Iwasa-Arai and Serejo, 2018

Molecular Sequences
COI (Kaliszewska et al., 2005; Seger et al., 2010), COII, COIII, ATP6, ATP8, ND3 (Kaliszewska et al., 2005), EF1a (Seger et al., 2010)

Association
Ectoparasite

Cetacean Hosts/Basibionts
Eubalaena australis, *E. glacialis*, *E. japonica*, *Physeter macrocephalus*; once reported on *Megaptera novaeangliae*

Geographic Range
Atlantic, Pacific, Antarctica

Life Cycle
-

Microhabitat
Head callosities, sometimes with *C. erraticus* (Stephensen, 1942; Rowntree, 1996; see *C. erraticus*, above)

Use as Indicator
See *C. erraticus*.

Remarks
Once misidentified as *Cyamus rhytinae* (J. F. Brandt, 1846), ectoparasitic on the extinct Steller's sea cow, *Hydrodamalis gigas* (Zimmermann, 1780) Palmer, 1895 (see Leung, 1967; O'Clair and O'Clair, 1998).

References
Roussel de Vauzème 1834; Lütken, 1873; Collet, 1912; Liouville, 1913; Barnard, 1932; Iwasa, 1934; Margolis, 1955; Leung, 1967; Lincoln and Hurley, 1974a; Berzin and Vlasova, 1982; Rowntree, 1996; Margolis et al., 2000; Pettis et al., 2004; Iwasa-Arai and Serejo, 2018

Cyamus scammoni (Dall, 1872)
Synonyms
-

Morphological Description
Lütken, 1887; Leung, 1967; Margolis et al., 2000; Iwasa-Arai and Serejo, 2018

Molecular Sequences
COI (GenBank FJ751214), hemocyanin mRNA (Terwilliger and Ryan, 2006)

Association
Ectoparasite

Cetacean Hosts/Basibionts
Eschrichtius robustus

Geographic Range
North Pacific

Life Cycle
Females can carry about 1,000 eggs in the brood pouch, although only about a 60% are fertilized (Leung, 1976). Eggs hatch in autumn, when gray whales arrive in California, and the young remain in the female's pouch for 2-3 months and then find shelter in host's crevices (Leung, 1976). Juveniles reach maturity during the winter northward migration of whales, and have full-grown brood upon arrival to summer grounds. The whole cycle takes 8-9 months to complete and there is probably some overlap in the life cycle of different individuals, given that juveniles are present throughout the year (Leung, 1976). The number of instars is presumed to be at least 7 or 8, but the number of ecdysis was untraceable (Leung, 1976).

Microhabitat
Ventral grooves, i.e., jaw and belly; flukes; on the cirriped *Cryptolepas rachianecti* Dall, 1872 (Leung, 1976; Dailey et al., 2000)

Use as Indicator
-

Remarks
Chonotrichous ciliates can infest its ventral surface (Leung, 1976).

References
Dall, 1872; Scammon, 1874; Lütken, 1887; Margolis, 1954a; Rice, 1963; Leung, 1965; Lincoln and Hurley, 1974a; Leung, 1976; Sullivan and Houck, 1979; Berzin and Vlasova, 1982; Dailey et al., 2000; Margolis et al., 2000; Kaliszewska et al., 2005; Takeda and Ogino, 2005; Murase et al., 2014; Iwasa-Arai and Serejo, 2018

Isocyamus antarcticensis (Vlasova in Berzin & Vlasova, 1982)
Synonyms
Cyamus antarcticensis Vlasova in Berzin & Vlasova, 1982

Morphological Description
Berzin and Vlasova, 1982

Molecular Sequences
-

Association
Ectoparasite

Cetacean Hosts/Basibionts
Orcinus orca (Linnaeus, 1758)

Geographic Range
Antarctica

Life Cycle
-

Microhabitat
Pectoral fins, umbilicus (Berzin and Vlasova, 1982)

Use as Indicator
-

Remarks
-

References
Berzin and Vlasova, 1982

Isocyamus delphinii (Guérin-Méneville, 1836)
Synonyms
Cyamus delphinii Guérin-Méneville, 1836, *C. globicipitis* Lütken, 1870

Morphological Description
Barnard, 1932; Leung, 1967; Stock, 1973a; Stock, 1973b; Stock, 1977; Sedlak-Weinstein, 1991; Margolis et al., 2000; Lehnert et al., 2007; Lehnert et al., 2021

Molecular Sequences
COI (Lehnert et al., 2021)

Association
Ectoparasite

Cetacean Hosts/Basibionts
Typically found on *Globicephala melas* (Traill, 1809); some records on *Delphinus delphis* Linnaeus, 1758, *Grampus griseus* (G. Cuvier, 1812), *Lagenorhynchus albirostris* (Gray, 1846), *Phocoena phocoena* (Linnaeus, 1758), and *Pseudorca crassidens* (Owen, 1846); once reported on *Globicephala macrorhynchus* Gray, 1846, *Megaptera novaeangliae*, *Mesoplodon europaeus* (Gervais, 1855), *Peponocephala electra* (Gray, 1846), *Phocoena dioptrica* Lahille, 1912, *Steno bredanensis* (G. Cuvier in Lesson, 1828), and *Tursiops truncatus*

Geographic Range
Arctic, Atlantic, Pacific, Mediterranean, Indian Ocean

Life Cycle
-

Microhabitat
Ubiquitous; i.e., blowhole, eyes, jaw, insertion of pectoral fin, wounds (Stock, 1973a; Stock, 1977; Greenwood et al., 1979; Raga et al., 1988; Balbuena et al., 1989; Balbuena and Raga, 1991; Raga and Balbuena, 1993; Jauniaux et al., 2002; Lehnert et al., 2007; Batista et al., 2012; Lehnert et al., 2021)

Use as Indicator
The higher prevalence and intensity of *I. delphinii* on mature long-finned pilot whale males (*vs.* females and immature males) may identify the males that are dominant in sexual fights, given that the resulting wounds serve as shelter for this cyamid species (Balbuena and Raga, 1991; Raga and Balbuena, 1993).

Remarks
Lehnert et al. (2021) pose that some records around the 1970-90s misidentified this species and refer to *Isocyamus deltobranchium* Sedlak-Weinstein, 1992, which has triangular accessory gills (*vs.* cylindrical in *I. delphinii*).

References
Lütken, 1870; Lütken, 1893; Collet, 1912; Chevreux, 1913b; Hiro, 1938; Bowman, 1955; Sergeant, 1962; Leung, 1965; Stock, 1973a; Stock, 1973b; Lincoln and Hurley, 1974a; Stock, 1977; Van Bree and Smeenk, 1978; Greenwood et al., 1979; Berzin and Vlasova, 1982; Raga et al., 1983a; Rappé, 1985; Raga et al., 1988; Balbuena et al., 1989; Mead, 1989; Rappé, 1991; Balbuena and Raga, 1991; Fransen and Smeenk, 1991; Sedlak-Weinstein, 1991; Raga and Balbuena, 1993; Abollo et al., 1998; Gibson et al., 1998; Margolis et al., 2000; Wardle et al., 2000; Haelters, 2001; Jauniaux et al., 2002; Haney et al., 2004; Lehnert et al., 2007; Batista et al., 2012; Lehnert et al., 2021; Iwasa-Arai and Serejo, 2018

Isocyamus deltobranchium (Sedlak-Weinstein, 1992)
Synonyms
-

Morphological Description
Sedlak-Weinstein, 1992a; Martínez et al., 2008; Lehnert et al., 2021

Molecular Sequences
COI (Lehnert et al., 2021)

Association
Ectoparasite

Cetacean Hosts/Basibionts
Phocoena phocoena; once reported on *Delphinus delphis*, *Globicephala macrorhynchus*, *G. melas*, *Mesoplodon mirus* True, 1913, and *Orcinus orca*

Geographic Range
Eastern North Atlantic, western north Pacific, Indian Ocean

Life Cycle
-

Microhabitat
Skin wounds (Sedlak-Weinstein, 1992a; Martínez et al., 2008; Lehnert et al., 2021)

Use as Indicator
Higher prevalence in some harbor porpoise populations may reveal more interspecific contacts than in other areas (Lehnert et al., 2021). Also, temporal changes in prevalence could trace trends in the health status of cetacean hosts, given that it has been suggested that poor nutritional status may increase the susceptibility of porpoises to whale lice infections (Lehnert et al., 2021).

Remarks
Diatoms have been reported between *I. deltobranchium* forearms (Lehnert et al., 2021).

References
Sedlak-Weinstein, 1992a; Martínez et al., 2008; Iwasa-Arai and Serejo, 2018; Lehnert et al., 2021

Isocyamus indopacetus (Iwasa-Arai & Serejo, 2017)
Synonyms
-

Morphological Description
Iwasa-Arai et al., 2017b; Iwasa-Arai and Serejo, 2018; Kobayashi et al., 2021

Molecular Sequences
-

Association
Ectoparasite

Cetacean Hosts/Basibionts
Indopacetus pacificus (Longman, 1926)

Geographic Range
Japan, New Caledonia (western Pacific)

Life Cycle
-

Microhabitat
Mouth, mammary slits, and scars provoked by *Isistius* sp. (Kobayashi et al., 2021)

Use as Indicator
-

Remarks
-

References
Iwasa-Arai et al., 2017a; Kobayashi et al., 2021

Isocyamus kogiae (Sedlak-Weinstein, 1992)
Synonyms
-

Morphological Description
Sedlak-Weinstein, 1992b

Molecular Sequences
-

Association
Ectoparasite

Cetacean Hosts/Basibionts
Kogia breviceps (de Blainville, 1838)

Geographic Range
Australia (western South Pacific)

Life Cycle
-

Microhabitat
Skin wounds (Sedlak-Weinstein, 1992b)

Use as Indicator
-

Remarks
-

References
Sedlak-Weinstein, 1992b

Neocyamus physeteris (Pouchet, 1888)
Synonyms
Cyamus fascicularis Verrill, 1901, *C. physeteris* Pouchet, 1888, *Paracyamus physeteris* (Pouchet, 1888)

Morphological Description
Pouchet, 1892; Leung, 1967; Margolis et al., 2000; Iwasa-Arai and Serejo, 2018

Molecular Sequences
-

Association
Ectoparasite

Cetacean Hosts/Basibionts
Typically on *Physeter macrocephalus*; single record on *Phocoenoides dalli* (True, 1885)

Geographic Range
Eastern Pacific, Atlantic

Life Cycle
-

Microhabitat
-

Use as Indicator
Used to detect social segregation in sperm whales: females and male bachelors, but not large males, harbour *N. physeteris*, suggesting that the later leave their natal pods at puberty (Best, 1969a; Best, 1979).

Remarks
-

References
Pouchet, 1888; Pouchet, 1892; Verrill, 1902; Clarke, 1956; Margolis, 1959; Buzeta, 1963; Leung, 1965; Leung, 1967; Best, 1969a; Lincoln and Hurley, 1974a; Best, 1979; Berzin and Vlasova, 1982; Mignucci-Giannoni et al., 1998; Margolis et al., 2000; Iwasa-Arai and Serejo, 2018

Orcinocyamus orcini (Leung, 1970)
Synonyms
Cyamus orcini Leung, 1970b

Morphological Description
Leung, 1970b; Margolis et al., 2000

Molecular Sequences
-

Association
Ectoparasite

Cetacean Hosts/Basibionts
Orcinus orca

Geographic Range
Senegal (eastern South Atlantic)

Microhabitat
-

Use as Indicator
-

Remarks
-

References
Leung, 1970b

Platycyamus flaviscutatus (Waller, 1989)

Synonyms
-

Morphological Description
Margolis et al., 2000

Molecular Sequences
-

Association
Ectoparasite

Cetacean Hosts/Basibionts
Berardius bairdii

Geographic Range
North Pacific

Life Cycle
-

Microhabitat
Head, back, flanks, flukes (Waller, 1989)

Use as Indicator
-

Remarks
-

References
Waller, 1989; Margolis et al., 2000

Platycyamus thompsoni (Gosse, 1855)

Synonyms
Cyamus thompsoni Gosse, 1855

Morphological Description
Gosse, 1855; Lütken, 1873; Wolff, 1958; Leung, 1967; Sedlak-Weinstein, 1991; Iwasa-Arai and Serejo, 2018

Molecular Sequences
-

Association
Ectoparasite

Cetacean Hosts/Basibionts
Typically on *Hyperoodon ampullatus* (Forster, 1770); once reported on *H. planifrons* Flower, 1882 and *Mesoplodon grayi* von Haast, 1876

Geographic Range
North Atlantic, Pacific, Antarctica

Life Cycle
At least four instars have been distinguished in females (Wolff, 1958). Males are more difficult to classify by morphological features and could die and fall off the whale after copulation (Wolff, 1958).

Microhabitat
Ubiquitous on skin, i.e., eyes, beak, corners of the mouth (Tomilin, 1957; Wolff, 1958; Lincoln and Hurley, 1974a; Sedlak-Weinstein, 1991)

Use as Indicator
-

Remarks
-

References
Gosse, 1855; Lütken, 1870; Vosseler, 1889; Collet, 1912; Liouville, 1913; Tomilin, 1957; Wolff, 1958; Stock, 1973b; Lincoln and Hurley, 1974a; Berzin and Vlasova, 1982; Fransen and Smeenk, 1991; Sedlak-Weinstein, 1991; Iwasa-Arai and Serejo, 2018

Scutocyamus antipodensis (Lincoln & Hurley, 1980)

Synonyms
-

Morphological Description
Lincoln and Hurley, 1980

Molecular Sequences
-

Association
Ectoparasite

Cetacean Hosts/Basibionts
Cephalorhynchus hectori (Lacépède, 1804), *Phocoena dioptrica*, *Sagmatias obscurus* (Gray, 1828)

Geographic Range
Off Namibia (eastern South Atlantic) and New Zealand (western South Pacific)

Life Cycle
-

Microhabitat
Ubiquitous on skin (Lincoln and Hurley, 1980; Best and Meÿer, 2010; Lehnert et al., 2017)

Use as Indicator
-

Remarks
-

References
Lincoln and Hurley, 1980; Best and Meÿer, 2010; Lehnert et al., 2017

Scutocyamus parvus (Lincoln & Hurley, 1974)
Synonyms
-

Morphological Description
Lincoln and Hurley, 1974b

Molecular Sequences
-

Association
Ectoparasite

Cetacean Hosts/Basibionts
Lagenorhynchus albirostris

Geographic Range
North Sea

Life Cycle
-

Microhabitat
-

Use as Indicator
-

Remarks
-

References
Lincoln and Hurley, 1974a, Lincoln and Hurley, 1974b; Stock, 1977; Fransen and Smeenk, 1991

Syncyamus aequus (Lincoln & Hurley, 1981)
Synonyms
See Remarks.

Morphological Description
Lincoln and Hurley, 1981; Raga, 1988; Sedlak-Weinstein, 1991

Molecular Sequences
-

Association
Ectoparasite

Cetacean Hosts/Basibionts
Delphinus delphis, *Stenella coeruleoalba*; once reported on *Sousa chinensis* (Osbeck, 1765), *Stenella longirostris* (Gray, 1828), *Tursiops aduncus* (Ehrenberg, 1832 [1833]), and *T. truncatus*

Geographic Range
Mediterranean, western South Pacific, Indian Ocean

Life Cycle
-

Microhabitat
Blowhole, eyes, corner of mouth, snout, jaw, axilla (Lincoln and Hurley, 1981; Raga and Raduan, 1982; Aznar et al., 1994; Cerioni and Mariniello, 1996; Haney, 1999; Haney et al., 2004; Fraija-Fernández et al., 2017)

Use as Indicator
-

Remarks
On the one hand, Mediterranean striped dolphins, *Stenella coeruleoalba*, harbored low prevalence and intensity of *S. aequus* (27% and 3 ind./host, respectively; Fraija-Fernández et al., 2017). Since striped dolphins are highly social animals (Carlucci et al., 2015), transmission success would be hardly hampered by the scarcity of contacts, but rather by the low sizes of source populations. These small populations may result from the extreme limitation of suitable microhabitats to shelter on these fast-swimming dolphins (Fraija-Fernández et al., 2017). This phenomenon seems also to impact the reproductive strategy of this species (Fraija-Fernández et al., 2017). On the other hand, the species *Cyamus chelipes* was first described by Costa (1866) and later re-classified in the genus *Syncyamus* by Bowman (1958). It is considered a nomen dubium (Haney, 1999), the type series is lost (Bowman, 1958), and it was not included in later reviews of the Cyamidae (Leung, 1965; Iwasa-Arai and Serejo, 2018|). Thus, it is possible that *S. chelipes* is a synonym of *S. aequus*, later described and common in the Mediterranean Sea (see above, **Supplementary Table 1**).

References
Lincoln and Hurley, 1981; Raga and Raduan, 1982; Raga et al., 1983; Raga and Carbonell, 1985; Raga, 1988; Sedlak-Weinstein, 1991; Aznar et al., 1994; Mariniello et al., 1994; Ross et al., 1994; Cerioni and Mariniello, 1996; Margolis et al., 2000; Fraija-Fernández et al., 2017

Syncyamus ilheusensis (Haney, de Almeida & Reid, 2004)
Synonyms
-

Morphological Description
Haney et al., 2004; Iwasa-Arai et al., 2017b; Iwasa-Arai and Serejo, 2018

Molecular Sequences
-

Association
Ectoparasite

Cetacean Hosts/Basibionts
Globicephala macrorhynchus, *Peponocephala electra*, *Stenella clymene* (Gray, 1850)

Geographic Range
Brazil (western South Atlantic)

Life Cycle
-

Microhabitat
Eyes, blowhole (Haney et al., 2004; Batista et al., 2012)

Use as Indicator
-

Remarks
-

References
Haney et al., 2004; Batista et al., 2012; Iwasa-Arai et al., 2017a; Iwasa-Arai et al., 2018

Syncyamus pseudorcae (Bowman, 1955)
Synonyms
-

Morphological Description
Bowman, 1955; Leung, 1967

Molecular Sequences
-

Association
Ectoparasite

Cetacean Hosts/Basibionts
Delphinus delphis, *Pseudorca crassidens*, *Stenella clymene*

Geographic Range
North Atlantic, Pacific

Life Cycle
-

Microhabitat
Blowhole, mouth, snout, jaw (Carvalho et al., 2010)

Use as Indicator
-

Remarks
-

References
Bowman, 1955; Leung, 1970a; Sedlak-Weinstein, 1991; Jefferson et al., 1995; Carvalho et al., 2010

Order Isopoda Latreille, 1817
Family Cymothoidae Leach, 1818
Representatives from the family Cymothoidae are obligate parasites of mainly marine but also freshwater fish (Smit et al., 2014). Identification of cymothoid isopods is often difficult because species often show high morphological variation (Trilles et al., 2013). Many species of *Nerocila* Leach, 1818 require taxonomic revision (Aneesh et al., 2019).

Nerocila sp.
Synonyms
-

Morphological Description
A general account of the genus *Nerocila* and of some of its species can be found Hai-yan and Xin-zheng (2002) and Trilles et al. (2013).

Molecular Sequences
COI, LSU rRNA, 16S rRNA, and 18S rRNA of nine *Nerocila* spp. (see GenBank)

Association
Unknown

Cetacean Hosts/Basibionts
Pontoporia blainvillei (Gervais & d'Orbigny, 1844)

Geographic Range
-

Life Cycle
See Brusca (1978) and Smit et al. (2014) for a description of the cymothoid cycle.

Microhabitat
Neck region (Brownell, 1975)

Use as Indicator
-

Remarks
Brownell (1975) reported this ectoparasite on some La Plata dolphins that had been captured accidentally in gillnets, and interpreted that it could have been transmitted from sharks or other fish while all were trapped in the gillnet. Thus, the association with cetaceans should be viewed as accidental.

References
Brownell, 1975

Class Thecostraca Gruvel, 1905
Subclass Copepoda Milne Edwards, 1840
Order Harpacticoida Sars G.O., 1903
Family Balaenophilidae Sars G.O., 1910

The genus *Balaenophilus* Aurivillius P.O.C., 1879 contains two species that live in close association with marine vertebrates. *B. unisetus* Aurivillius P.O.C., 1879 is considered an obligate commensal of baleen whales that feeds on algae and/or baleen tissue (Vervoort and Tranter, 1961; Fernandez-Leborans, 2001; Badillo et al., 2007), causing no harm to hosts (Ogawa et al., 1997; Badillo et al., 2007). In contrast, *B. manatorum* (Ortiz et al., 1992) infects manatees and sea turtles; in the latter they can feed on healthy skin (Badillo et al., 2007; Domènech et al., 2017), sometimes producing extensive lesions (Crespo-Picazo et al., 2017). Thus, this species is considered an ectoparasite.

Balaenophilus unisetus (Aurivillius P.O.C., 1879)

Synonyms

-

Morphological Description

Aurivillius, 1879; Vervoort and Tranter, 1961; Bannister and Grindley, 1966

Molecular Sequences

-

Association

Obligate commensal

Cetacean Hosts/Basibionts

Balaenoptera borealis Lesson, 1828, *B. edeni* Anderson, 1878, *B. musculus*, *B. physalus*

Geographic Range

Arctic, Atlantic, eastern Pacific, Indian Ocean, Antarctica

Life Cycle

Aurivillius (1879) describes a nauplius and five copepodite stages preceding the adult phase. In the allied species *B. manatorum* nauplii and early copepodite stages are unable to swim, and copepodite V and adults can perform only short swimming excursions (Domènech et al., 2017). Thus, host bodily contact or closeness is likely necessary for transmission in both species.

Microhabitat

Baleen plates (Aurivillius, 1879; Cocks, 1885; Lillie, 1910; Scharff, 1913; Matthews, 1938b; Vervoort and Tranter, 1961; Rice, 1963; Gambell, 1964; Bannister and Grindley, 1966; Ichihara, 1966; Ichihara, 1978; Collet, 1986; Raga and Sanpera, 1986; Dalla Rosa and Secchi, 1997; Esteves et al., 2020), corner of the mouth (Raga and Sanpera, 1986)

Use as Indicator

-

Remarks

The presence of this species is likely underestimated since it can be easily overlooked without exhaustive inspection of baleen plates (Aurivillius, 1879; Vervoort and Tranter, 1961). It can sometimes be colonized by chonotrichous ciliates, acting as basibiont (Fernandez-Leborans, 2001).

References

Cocks, 1885; Aurivillius, 1879; Lillie, 1910; Collet, 1912; Scharff, 1913; Allen, 1916; Cornwall, 1927; Cornwall, 1928; Matthews, 1938b; Vervoort and Tranter, 1961; Rice, 1963; Gambell, 1964; Bannister and Grindley, 1966; Ichihara, 1966; Kawamura, 1969; Rice, 1977; Ichihara, 1978; Collet, 1986; Raga and Sanpera, 1986; Dalla Rosa and Secchi, 1997; Esteves et al., 2020

Family Harpacticidae Dana, 1846

Members of this family are mostly marine or brackishwater macroalgal associates, with a few freshwater species (Joon and Young, 1993).

Harpacticus pulex (Humes, 1964)

Synonyms

-

Morphological Description

Humes, 1964

Molecular Sequences

-

Association

Unknown

Cetacean Hosts/Basibionts

Tursiops truncatus

Geographic Range

-

Life Cycle

Unknown for this species, but naupliar and copepodite stages have been described for other *Harpacticus* spp. (e.g., Itô, 1976; Walker, 1981; Choi and Kim, 1994). Harpacticoids generally lack planktonic larval stages, but adults are active swimmers (e.g., Hicks, 1985; Palmer, 1988). It is thus plausible that transmission to bottlenose dolphin occurred during the adult phase.

Microhabitat

On ulcerated and sloughed skin (Humes, 1964)

Use as Indicator

-

Remarks

This species was described by Humes (1964) on captive marine mammals and has never been reported again. Species of *Harpacticus* Milne Edwards H., 1840 typically colonize seagrass, algal clumps or sandy and muddy bottoms (Ólafsson, 2001 and references therein), thus the occurrence of *H. pulex* on cetaceans is intriguing and perhaps forced by confinement

conditions (Humes, 1964). Future re-examination of the taxonomic status of *H. pulex* is advisable.

References
Humes, 1964

Order Siphonostomatoida Burmeister, 1835
Family Caligidae Burmeister, 1835

The family Caligidae ("sea lice") contains 30 genera (Walter and Boxshall, 2020); species of *Caligus* Müller O. F., 1785 and *Lepeophtheirus* Nordmann, 1832 have great economic relevance due to their impact on salmonid fish mariculture (Costello, 2006; Hemmingsen et al., 2020). Caligids use their siphon and a pair of mandibles to feed on fish skin (Kabata, 1974), causing ulcerations and even death to their hosts (Tørud and Håstein, 2008), but their impact on cetaceans has not yet been reported.

Caligus elongatus (Nordmann, 1832)
Synonyms
Caligus arcticus Brandes, 1956, *C. kroyeri* Milne Edwards, 1840, *C. latifrons* Wilson C.B., 1905, *C. leptochilus* Leuckart in Frey & Leuckart, 1847, *C. lumpi* Krøyer, 1863, *C. rabidus* Leigh-Sharpe, 1936, *C. rissoanus* Milne Edwards, 1840, *C. trachypteri* Krøyer, 1863

Morphological Description
Hemmingsen et al., 2020 and references therein

Molecular Sequences
COI (Øines and Heuch, 2005; Raupach et al., 2015; GenBank AY386272; AY386273; EF452647), 16S rRNA (Øines and Schram, 2008; GenBank AY660020), 18S rRNA (Huys et al., 2006; Øines and Schram, 2008; Mohrbeck et al., 2015; Khodami et al., 2017; GenBank JX845119-JX845131), 28S rRNA (Khodami et al., 2017; GenBank DQ180336; DQ180337; EU118301; EU118302)

Association
Ectoparasite

Cetacean Hosts/Basibionts
Balaenoptera acutorostrata, *Hyperodoon ampullatus*

Geographic Range
North Atlantic (Hemmingsen et al., 2020)

Life Cycle
Two free-living planktonic nauplius stages, one free-swimming infective copepodid stage, and four chalimus stages and one adult stage attached to the host (Maran et al., 2013).

Microhabitat
Skin (O'Reilly, 1998; Ólafsdóttir and Shinn, 2013)

Use as Indicator
-

Remarks
This is a typical fish ectoparasite that has been reported on more than 80 species (Kabata, 1979; Agusti-Ridaura et al., 2019). Infections in cetaceans are exceptional and likely related to their occurrence close to cage farms (Ólafsdóttir and Shinn, 2013). The hyperparasitic monogenean *Udonella caligorum* Johnston, 1835, which typically attaches to fish copepods (Freeman and Ogawa, 2010), has been found on *C. elongatus* infecting common minke whales (Ólafsdóttir and Shinn, 2013).

References
O'Reilly, 1998; Ólafsdóttir and Shinn, 2013

Caligus rufimaculatus (Wilson C.B., 1905)
Synonyms
-

Morphological Description
Wilson, 1905; Takemoto and Luque, 2002; Kim et al., 2019

Molecular Sequences
-

Association
Ectoparasite

Cetacean Hosts/Basibionts
Tursiops truncatus

Geographic Range
Western Atlantic (Benz et al., 2011)

Life Cycle
See *C. elongatus* (above).

Microhabitat
Skin (Benz et al., 2011)

Use as Indicator
-

Remarks
This species typically infects fish, but there is an exceptional record of adult individuals, including ovigerous females, on a carcass of bottlenose dolphin (Benz et al., 2011).

References
Benz et al., 2011

Lepeophtheirus crassus (Wilson & Bere, 1936)
Synonyms
Gloiopotes crassus Wilson & Bere, 1936

Morphological Description
Lewis, 1967

Molecular Sequences
-

Association
Ectoparasite

Cetacean Hosts/Basibionts
Delphinus delphis

Geographic Range
Western Atlantic, North Pacific, Indian Ocean (Lewis, 1967)

Life Cycle
Species of *Lepeophtheirus* have 2-4 chalimus stages and two preadult stages. The latter can be distinguished by their ability to detach and move over the surface of the host (Krøyer, 1834; see Hamre et al., 2013).

Microhabitat
Hyperparasitic on *Remora australis* (Bennett, 1840; Radford and Klawe, 1965)

Use as Indicator
-

Remarks
-

References
Radford and Klawe, 1965

Family Pennellidae Burmeister, 1835

Unlike other families of the order Siphonostomatoida, members of the family Pennellidae do have intermediate hosts, usually a fish or invertebrate (Kabata, 1979; Nagasawa et al., 1985; Suyama et al., 2021a and references therein). Mating seemingly occurs in the intermediate host and fertilized females attach to the final host in which they produce and release the eggs (Arroyo et al., 2002).

Pennella balaenoptera (Koren & Danielssen, 1877)

Synonyms
Pennella antarctica Quidor, 1913, *P. anthonyi* Quidor, 1913, *P. balaenopterae* Koren & Danielssen, 1877, *P. cettei* Quidor, 1913, *P. charcoti* Quidor, 1913

Morphological Description
Koren and Danielssen, 1877; Turner, 1905; Hogans, 1987, Hogans, 2017; Abaunza et al., 2001; Vecchione and Aznar, 2014; Suyama et al., 2021b

Molecular Sequences
COI (Fraija-Fernández et al., 2018)

Association
Mesoparasite. The head penetrates the blubber and musculature to feed on blood and expands as 2-3 cephalic horns in host's tissue to enable attachment, whereas the trunk, genital complex, and abdominal plumes protrude and hang on the host body (Hogans, 1987; Abaunza et al., 2001; Schmidt and Roberts, 2009; Hogans, 2017).

Cetacean Hosts/Basibionts
Balaenoptera acutorostrata, *B. bonaerensis*, *B. borealis*, *B. edeni*, *B. musculus*, *B. physalus*, *Delphinus delphis*, *Eubalaena australis*, *Feresa attenuata* Gray, 1874, *Globicephala melas*, *Grampus griseus*, *Hyperoodon ampullatus*, *Kogia breviceps*, *Lissodelphis borealis* (Peale, 1848), *Megaptera novaeangliae*, *Mesoplodon bidens* (Sowerby, 1804), *M. carlhubbsi* Moore, 1963, *M. mirus*, *Orcinus orca*, *Phocoena phocoena*, *Physeter macrocephalus*, *Stenella coeruleoalba*, *Tursiops truncatus*, *Ziphius cavirostris*

Geographic Range
Atlantic, Pacific, Mediterranean, Indian Ocean, Antarctica

Life Cycle
Based on information from other penellids, its life cycle is believed to include a pelagic naupliar stage and several copepodid and chalimus instars on the intermediate (squid) hosts; females are fertilized as late chalimi and undergo a pelagic phase to search out the definitive host, where they metamorphose into the adult stage (Schmidt and Roberts, 2009). In the case of *P. balaenoptera*, only adult females and the first naupliar stage are known (Arroyo et al., 2002). However, the copepodid and chalimus stages have been described for *P. filosa* (Linnaeus, 1758) collected from squids (Rose and Hamon, 1953; see also Arroyo et al., 2002), and *P. filosa* is now considered conspecific with *P. balaenoptera* (Fraija-Fernández et al., 2018; see also the Discussion). The life cycle of *P. balaenoptera* could be primarily oceanic because this species is more prevalent on pelagic versus coastal cetaceans (Fraija-Fernández et al., 2018).

Microhabitat
Commonly on the flanks (Raga and Sanpera, 1986; Aznar et al., 1994; Gomerčić et al., 2006; Souza et al., 2005; Çiçek et al., 2007; Foskolos et al., 2017), but occasionally reported on the head (Pouchet and Beauregard, 1889; Foskolos et al., 2017) and flukes (Foskolos et al., 2017). A single record on a whale sucker, *Remora australis* (Bennett, 1840) attached to a dolphin (Radford and Klawe, 1965).

Use as Indicator
It may be an indicator of compromised health in cetacean hosts (Mackintosh and Wheeler, 1929; Aznar et al., 2005; Vecchione and Aznar, 2014).

Remarks
Since *P. balaenoptera* is the only recognized species of *Pennella* Oken, 1815 parasitizing cetaceans, we consider that the published records of *Pennella* sp. in cetaceans could be

assigned to this species, unless proven otherwise. Dailey et al. (2002) reported *P. balaenoptera* in one northern elephant seal, *Mirounga angustirostris* (Gill, 1866). Recently, molecular analyses revealed that specimens of *P. balaenoptera* collected from several cetaceans in western Mediterranean could be conspecific with *P. filosa* from swordfish, *Xiphias gladius* Linnaeus, 1758, collected in the same area (Fraija-Fernández et al., 2018). This finding begs further attention (see the Discussion).

References
Steenstrup and Lütken, 1861; Sars, 1866; Pouchet and Beauregard, 1889; Anthony and Calvet, 1905; Turner, 1905; Bouvier, 1910; Japha, 1910; Mörch, 1911; Collet, 1912; Quidor, 1912; Liouville, 1913; Olsen, 1913; Scharff, 1913; Cornwall, 1927; Cornwall, 1928; Mackintosh and Wheeler, 1929; Van Oorde-de Lint and Schuurmans-Stekhoven, 1936; Matthews, 1938b; Allen, 1941; Stephensen, 1942; Mizue, 1950; Nishiwaki and Hayashi, 1950; Mizue and Murata, 1951; Nishiwaki and Oye, 1951; Ohno and Fujino, 1952; Kakuwa et al., 1953; Barnard, 1955; Chapman and Santler, 1955; Clarke, 1956; Zenkovich, 1956; Tomilin, 1957; Rice, 1963; Radford and Klawe, 1965; Kawamura, 1969; Berzin, 1972; Rice, 1977; Rice, 1978; Dailey and Stroud, 1978; Dailey and Walker, 1978; Ivashin and Golubovsky, 1978; Greenwood et al., 1979; Best, 1982; Raga and Carbonell, 1985; Raga and Sanpera, 1986; Smiddy, 1986; Mead, 1989; Bushuev, 1990; Dorsey et al., 1990; Sedlak-Weinstein, 1990 (unpubl.); Dailey and Vogelbein, 1991; Raga and Balbuena, 1993; Aznar et al., 1994; Aznar et al., 2005, unpubl.; Raga, 1994; Vecchione, 1994; Cerioni and Mariniello, 1996; Kuramochi et al., 1996; Araki et al., 1997; Kuramochi et al., 2000; McAlpine et al., 1997; Terasawa et al., 1997; Uchida, 1998; Walker and Hanson, 1999; Cornaglia et al., 2000; Uchida and Araki, 2000; Abaunza et al., 2001; Arroyo et al., 2002; Brzica, 2004; Gomerčić et al., 2006; Souza et al., 2005; Ciçek et al., 2007; Kautek et al., 2008; Martín et al., 2011; Rosso et al., 2011; Bertulli et al., 2012; Ólafsdóttir and Shinn, 2013; Tonay and Dede, 2013; Danyer et al., 2014; Öztürk et al., 2015; Delaney et al., 2016; Birincioğlu et al., 2017; Foskolos et al., 2017; Hogans, 2017; Fraija-Fernández et al., 2018; IJsseldijk et al., 2018; Marcer et al., 2019; Methion and Díaz López, 2019; Herr et al., 2020; Orrell, 2020; Ten et al., unpubl.

Subclass Cirripedia Burmeister, 1834
Order Balanomorpha Pilsbry, 1916
Family Balanidae Leach, 1817

Thoracic barnacles (Infraclass Thoracica) are sessile, hermaphroditic crustaceans that attach to diverse substrata and have specialized cirri to filter organic particles from water for feeding (Anderson, 1994). The life cycle typically includes a free-swimming nauplius larva that undergoes several (usually 6) moults, and a non-feeding cypris larva that searchs out, and attaches to, an appropriate substratum. Subsequent metamorphosis leads to a juvenile filter-feeding version of the adult (Darwin, 1854; Cornwall, 1955; Maruzzo et al., 2012). The cyprid stage is unique to barnacles and shows little morphological variability across species, even though they can attach to strikingly different substrata (Maruzzo et al., 2012; Dreyer et al., 2020).

This family originally encompassed all sessile barnacles (Leach, 1817), but whale barnacles and most sea turtles were later re-classified (Pitombo, 2004; see below). Most members of Balanidae are intertidal, although some species are facultative epibionts, e.g., those found on sea turtles, such as *Balanus trigonus* (Ten et al., 2019).

Balanus trigonus (Darwin, 1854)
Synonyms
-

Morphological Description
Darwin, 1854

Molecular Sequences
COI (Chen et al., 2013; Ashton et al., 2016; GenBank JQ035523; JQ035524; MF974362; MK308152; MK308163; MK308322; MK496572; MT258956; MW277718; MW277822), EF1a (Chan et al., 2017), RPII (Chan et al., 2017), 12S rRNA (Endo et al., 2010; Kamiya et al., 2012; Pérez-Losada et al., 2014; Chan et al., 2017; GenBank GU983669; GU983670), 16S rRNA (Chan et al., 2017; GenBank JQ035491; JQ035492), 18S rRNA (Pérez-Losada et al., 2014; Chan et al., 2017), 28S rRNA (Pérez-Losada et al., 2014), and the complete mitochondrial genome (GenBank MW646099; MZ049958; NC_056392)

Association
Facultative commensal

Cetacean Hosts/Basibionts
Megaptera novaeangliae

Geographic Range
Cosmopolitan (Werner, 1967)

Life Cycle
Metamorphosis from nauplius to cyprid stage is speeded up at higher water temperature, i.e., 4-11 days (Thiyagarajan et al., 2003). Recruitment is seasonal and takes place at approximately 24°C (Lam, 2000).

Microhabitat
As a hyperepibiont on the barnacle *Coronula diadema* (Cornwall, 1928)

Use as Indicator
-

Remarks
-

References
Cornwall, 1928

Balanus spp.

Synonyms
-

Morphological Description
A general account of *Balanus* spp. can be found in Darwin (1854); Newman and Ross (1976), and Pitombo (2004).

Molecular Sequences
> 5,000 results in GenBank

Association
Presumably facultative commensal

Cetacean Hosts/Basibionts
Megaptera novaeangliae

Geographic Range
-

Life Cycle
Information for *Balanus* spp. is available from Brown and Roughgarden (1985) and Maruzzo et al. (2012).

Microhabitat
As a hyperepibiont on the barnacle *Coronula* spp. (Rice, 1963)

Use as Indicator
-

Remarks
Balanus spp., as in Rice (1963), may correspond to a single or several species.

References
Rice, 1963

Megabalanus tintinnabulum (Linnaeus, 1758)

Synonyms
Balanus tintinnabulum (Linnaeus, 1758), *Lepas tintinnabulum* Linnaeus, 1758

Morphological Description
Darwin, 1854; Barnard, 1924

Molecular Sequences
COI (Chen et al., 2013; Ashton et al., 2016; GenBank JQ035525-JQ035527), H3 (Pérez-Losada et al., 2004), 12S rRNA (Pérez-Losada et al., 2004), 16S rRNA (Pérez-Losada et al., 2004; GenBank JQ035505-JQ035508), 18S rRNA, 28S rRNA (Pérez-Losada et al., 2004), and the complete mitochondrial genome (Che et al., 2019; GenBank MW281857; NC_056162)

Association
Facultative commensal

Cetacean Hosts/Basibionts
Unidentified whale

Geographic Range
Tropical or sub-tropical to warm temperate waters (Otani et al., 2007)

Life Cycle
In the Arabian Sea, barnacles breed at lower temperatures, i.e., less than 24 °C in winter *vs.* > 28 °C in summer; and grow at a rate of 0.44-0.63 mm/year (Ali and Ayub, 2021).

Microhabitat
As a hyperepibiont on the barnacle *Coronula diadema* (Barnard, 1924)

Use as Indicator
-

Remarks
-

References
Barnard, 1924

Family Coronulidae Leach, 1817
Coronulids are typically obligate epibionts of sea turtles, sirenians or cetaceans (Marlow, 1962; Hayashi et al., 2013). One species, *Chelonibia testudinaria* (Linnaeus, 1758), can also be found on crustaceans and sea snakes, and even on inanimate substrata (Frazier and Margaritoulis, 1990; Cheang et al., 2013).

Cetopirus complanatus (Mörch, 1852)

Synonyms
Coronula balaenaris (Gmelin, 1791), *C. complanata* (Mörch, 1852)

Morphological Description
Darwin, 1854; Pilsbry, 1916; Scarff, 1986; Pastorino and Griffin, 1996; Seilacher, 2005

Molecular Sequences
-

Association
Obligate commensal

Cetacean Hosts/Basibionts
Eubalaena australis, *E. glacialis*

Geographic Range
Arctic, Atlantic, eastern North Pacific, Antarctica

Life Cycle
-

Microhabitat
Lips, fins (Guiler, 1956; Best, 1991)

Use as Indicator
Shell plate remains of *C. complanatus* in Nerja Cave (Málaga, southern Spain) were used as indirect evidence of whale consumption by humans in the Upper Magdalenian (Álvarez-Fernández et al., 2013) and of the presence and migration of right whales (Balaenidae) in the Mediterranean during the Early Pleistocene (Collareta et al., 2016; Bosselaers et al., 2017).

Remarks
There is a single record on *Megaptera novaeangliae* (Guiler, 1956), but it was probably confused with *Coronula reginae* (Holthuis et al., 1998).

References
Chemnitz, 1785; Chemnitz and Martini, 1790; Darwin, 1854; Gruvel, 1903; Pilsbry, 1916; Nilsson-Cantell, 1931; Best, 1991

Coronula diadema (Linnaeus, 1767)
Synonyms
-

Morphological Description
Darwin, 1854; Dall, 1872; Cornwall, 1955; Scarff, 1986; Anderson, 1994

Molecular Sequences
H3, 12S rRNA, 16S rRNA, 18S rRNA, 28S rRNA (Hayashi et al., 2013)

Association
Obligate commensal

Cetacean Hosts/Basibionts
Typical from *Megaptera novaeangliae* but some records on *Balaenoptera bonaerensis*, *B. borealis*, *B. musculus*, *B. physalus*, *Eubalaena glacialis*, *Hyperoodon ampullatus* and *Physeter macrocephalus*

Geographic Range
Atlantic, Pacific, Indian Ocean, Antarctica

Life Cycle
A one-year life cycle has been proposed (Angot, 1951; Newman and Abbott, 1980). Larval release and settlement seem to occur in warm waters (20-25°C in September-October off Madagascar), whereas adult development may take place during whale migration to the poles (Angot, 1951). Details of development from the embryo to the juvenile stage have been studied *in vitro* (Nogata and Matsumura, 2006). Larval settlement is likely induced by chemical cues from whale skin, such as alpha-2-macroglobulin (Nogata and Matsumura, 2006).

Microhabitat
Rostrum, lips, lower jaw, fins (Dall, 1872; Pilsbry, 1916; Nilsson-Cantell, 1930a, Nilsson-Cantell, 1930c; Stephensen, 1938; Scheffer, 1939; Tomilin, 1957; Scarff, 1986)

Use as Indicator
Isotope analyses ($\delta^{18}O$) of shells of *C. diadema* and its direct ancestor *C. bifida* (Dominici et al., 2011) accurately trace current and Pleistocene-Miocene whale migration routes (Buckeridge et al., 2018; Collareta et al., 2018a; Collareta et al., 2018b; Buckeridge et al., 2019; Taylor et al., 2019). Fossil remains have also been used to infer humpback whale migration routes and breeding areas in the Late Pliocene-Pleistocene (Bianucci et al., 2006a; Bianucci et al., 2006b). Present-day observations of *Coronula* sp. (Olsen, 1913; Angot, 1951) helped to elucidate right whales' migration from warmer waters (Best, 1991). The co-occurrence of *C. bifida* with *Cetopirus complanatus* may indicate that whales belonging to Balaenopteridae and Balaenidae shared breeding grounds during the Early Pleistocene (Collareta et al., 2016). Interestingly, the presence of *C. diadema* on cetaceans other than humpback whales could also indicate some geographical overlap between species (see the Discussion). *Coronula* spp. have been suggested as natural marks for individual photo-identification (Franklin et al., 2020). The pattern of attachment of barnacles (presumably *C. diadema*) indicates non-uniform water flow over humpback whale flippers and has shed light on the function of leading-edge tubercles (Fish and Battle, 1995). Rubbing against rocks and the sea bottom has been observed in humpback whales, which may be an attempt to remove these barnacles (Tomilin, 1957) and could limit its application as an indicator.

Remarks
This species serves as a basibiont of the facultative epibionts *Balanus* spp., *Conchoderma auritum* (Linnaeus, 1767), and *Megabalanus tintinnabulum*, and of the hydroid *Obelia dichotoma* (Linnaeus, 1758) (Liouville, 1913; Barnard, 1924; Cornwall, 1928; Stephensen, 1938; Rice, 1963; Kim et al., 2020).

References
Dall, 1872; Scammon, 1874; Fischer, 1884; Sars, 1890-1895; Borradaile, 1903; Liouville, 1913; Pilsbry, 1916; Cornwall, 1924; Cornwall, 1927; Cornwall, 1928; Nilsson-Cantell, 1930a; Nilsson-Cantell, 1930c, Hiro, 1935; Hiro, 1938; Stephensen, 1938; Nilsson-Cantell, 1939; Scheffer, 1939; Mizue and Murata, 1951; Rees, 1953; Tomilin, 1957; Nishiwaki, 1959; Cockrill, 1960; Wolff, 1960; Rice, 1963; Nilsson-Cantell, 1978; O'Riordan, 1979; Scarff, 1986; Paterson and Van Dyck, 1991; Young, 1991; Holthuis and Fransen, 2004; Félix et al., 2006; Nogata and Matsumura, 2006; Wirtz et al., 2006; Jones, 2010; Ávila et al., 2011; Jiménez et al., 2011; Hayashi, 2012; Angeletti et al., 2014; Kim et al., 2020; Minton et al., 2020 (*in press.*); Tasmanian Museum and Art Gallery, 2020; Ueda, 2020; Ten et al., unpubl.

Coronula reginae (Darwin, 1854)
Synonyms
-

Morphological Description
Darwin, 1854; Scarff, 1986

Molecular Sequences
-

Association
Obligate commensal

Cetacean Hosts/Basibionts
Balaenoptera bonaerensis, *B. borealis*, *B. musculus*, *B. physalus*, *Eubalaena glacialis, Megaptera novaeangliae*; single report on *Delphinapterus leucas* and *Physeter macrocephalus*

Geographic Range
Arctic, Atlantic, North Pacific, Indian Ocean, Antarctica

Life Cycle
-

Microhabitat
Lower jaw, flukes (Cockrill, 1960; Scarff, 1986)

Use as Indicator
See *Coronula diadema* (above).

Remarks
-

References
Collet, 1912; Pilsbry, 1916; Cornwall, 1927; Cornwall, 1928; Mackintosh and Wheeler, 1929; Nilsson-Cantell, 1930a; Nilsson-Cantell, 1930b; Hiro, 1938; Stephensen, 1938; Scheffer, 1939; Rees, 1953; Guiler, 1956; Tomilin, 1957; Cockrill, 1960; Rice, 1963; Klinkhart, 1966; Kawamura, 1969; Rice, 1977; Nilsson-Cantell, 1978; Silva-Brum, 1985; Scarff, 1986; Bushuev, 1990; Smiddy and Berrow, 1992; Holthuis and Fransen, 2004; Ten et al., unpubl.

Cryptolepas rhachianecti (Dall, 1872)
Synonyms
-

Morphological Description
Dall, 1872; Cornwall, 1955; Achituv, 1998; Seilacher, 2005

Molecular Sequences
H3, 12S rRNA, 16S rRNA, 18S rRNA, 28S rRNA (Hayashi et al., 2013)

Association
Obligate commensal, although Tomilin (1957) considered this species to be potentially harmful because it can impede whales' movement and damage their skin.

Cetacean Hosts/Basibionts
Eschrichtius robustus; once reported on *Delphinapterus leucas* and *Orcinus orca*

Geographic Range
North Pacific; one record in the Gulf of Mexico (eastern North Atlantic)

Life Cycle
Gray whales wintering in waters off California and Mexico bear large and small specimens of *C. rhachianecti* when migrating northward, but only large barnacles when sighted during the southbound migration (Rice and Wolman, 1971). This would suggest that larval settlement occurs in wintering areas. This interpretation is supported by the observation that belugas held captive in San Diego Bay have *C. rhachianecti* in synchrony with gray whale northward migration (Rice and Wolman, 1971; Ridgway et al., 1997). Vertical shell growth is 0.12 mm/day (Killingley, 1980).

Microhabitat
Rostrum, lips, throat, peduncle, fins (Kasuya and Rice, 1970; Briggs and Morejohn, 1972)

Use as Indicator
Isotope analysis ($\delta^{18}O$) and geographical patterns of occurrence of fossilized remains have helped to reveal gray whale migration routes (Killingley, 1980; Bosselaers and Collareta, 2016; Taylor et al., 2019). Small size of barnacles and other features (appearance and associated scarring) have been used to identify calves of gray whale in photo-identification studies (Bradford et al., 2011). Barnacle orientation reflects waterflow patterns on gray whales (Kasuya and Rice, 1970; Briggs and Morejohn, 1972). Greater abundance of *C. rachianecti* on the left side of the head of gray whales may indicate that the right side is used predominantly for benthic feeding (Kasuya and Rice, 1970). In fact, right-sided feeding bias has been observed in some cetaceans (e.g., Clapham et al., 1995; Marino and Stowe, 1997; Karenina et al., 2016), including gray whales (e.g., Woodward and Winn, 2006).

Remarks
-

References
Dall, 1872; Pilsbry, 1916; Rice, 1963; Roest, 1970; Rice and Wolman, 1971; Briggs and Morejohn, 1972; Leung, 1976; Wellington and Anderson, 1978; Sullivan and Houck, 1979; Achituv, 1998; Weller et al., 1999; Takeda and Ogino, 2005; Sokolov and Arsen'ev, 2006; Murase et al., 2014; Scordino et al., 2017; Kasuya and Rice, 1970; Killingley, 1980; Swartz, 1981; Samaras, 1989; Ridgway et al., 1997; Findley and Vidal, 2002

Tubicinella major (Lamarck, 1802)
Synonyms
-

Morphological Description
Darwin, 1854; Seilacher, 2005

Molecular Sequences
-

Association
Obligate commensal, although Tomilin (1957) considered this species to be potentially harmful because it can impede whales' movement and damage their skin.

Cetacean Hosts/Basibionts
Eubalaena australis; once reported on *Balaenoptera borealis* and *E. glacialis*

Geographic Range
Atlantic, western South Pacific, Antarctica

Life Cycle
-

Microhabitat
Upper jaw, callosities, forehead, over the eye (Pilsbry, 1916; Scarff, 1986)

Use as Indicator
Shell plate remains of *T. major* found in Nerja Cave (Málaga, southern Spain) were used as indirect evidence of whale consumption by humans in the Upper Magdalenian (Álvarez-Fernández et al., 2013).

Remarks
Reported as a basibiont of facultative epibionts of the genus *Conchoderma* (Liouville, 1913).

References
Worm, 1655; Marloth, 1900; Gruvel, 1903; Liouville, 1913; Pilsbry, 1916; Reeb et al., 2007

Xenobalanus globicipitis (Steenstrup, 1852)
Synonyms
-

Morphological Description
Darwin, 1854; Cornwall, 1955; Rajaguru and Shantha, 1992; Anderson, 1994; Seilacher, 2005

Molecular Sequences
COI (Pérez-Losada et al., 2014), H3, 12S rRNA, 16S rRNA, 18S rRNA, 28S rRNA (Hayashi et al., 2013)

Association
Obligate commensal

Cetacean Hosts/Basibionts
Balaenoptera acutorostrata, *B. bonaerensis*, *B. borealis*, *B. edeni*, *B. musculus*, *B. physalus*, *Delphinus delphis*, *Feresa attenuata*, *Globicephala macrorhynchus*, *G. melas*, *Grampus griseus*, *Kogia* sp., *Lagenodelphis hosei* Fraser, 1956, *Lissodelphis borealis*, *Megaptera novaeangliae*, *Mesoplodon bidens*, *M. mirus*, *Neophocaena asiaeorientalis* Pilleri & Gihr, 1972, *N. phocaenoides* (Cuvier, 1829), *Orcinus orca*, *Peponocephala electra*, *Phocoena phocoena*, *P. sinus* Norris & McFarland, 1958, *P. spinnipinnis* (Burmeister, 1865), *Physeter macrocephalus*, *Pontoporia blainvillei*, *Pseudorca crassidens*, *Sagmatias obliquidens* (Gill, 1865), *S. obscurus*, *Sotalia fluviatilis* (Gervais & Deville in Gervais, 1853), *S. guianensis* (Van Beneden, 1864), *Sousa plumbea* (G. Cuvier, 1829), *Stenella attenuata*, *S. clymene*, *S. coeruleoalba*, *S. frontalis* (Cuvier, 1829), *S. longirostris*, *Steno bredanensis*, *Tursiops aduncus*, *T. truncatus*, *Ziphius cavirostris*

Geographic Range
Cosmopolitan (Arctic, Atlantic, Pacific, Mediterranean, South China Sea, Indian Ocean, Antarctica)

Life Cycle
Under experimental conditions at 28°C, the nauplii develop into cyprids in c. 8 days of hatching (Dreyer et al., 2020). Cyprids are similar to those of other barnacles but show variation in the structures that contact the substratum (Dreyer et al., 2020). In Guiana dolphins, *Sotalia fluviatilis*, off southern Brazil, field observations suggest that barnacle growth rate is initially fast and slows down after c. 30 days; sexual maturity seems to be reached in 40-45 days, and life span does not exceed one year (Flach et al., 2021).

Microhabitat
Trailing edge of dorsal fin, pectoral flippers, and mostly tail flukes (Calman, 1920; Barnard, 1924; Cornwall, 1927; Cornwall, 1928; Pope, 1958; Caldwell et al., 1971; Devaraj and Bennet, 1974; Bryden, 1976; Rice, 1978; Greenwood et al., 1979; Bane and Zullo, 1980; Spivey, 1980; Raga et al., 1983b; Ross, 1984; Raga and Sanpera, 1986; Brownell et al., 1987; Mead and Potter, 1990; Rajaguru and Shantha, 1992; Van Waerebeek et al., 1993; Watson et al., 1994; Jefferson et al., 1995; Reyes and Van Waerebeek, 1995; Araki et al., 1997; Orams and Schuetze, 1998; Rittmaster et al., 1999; Vidal et al., 1999; Barros and Stolen, 2001; Parsons et al., 2001; Resendes et al., 2002; Berland et al., 2003; Di Beneditto and Ramos, 2004; Palacios et al., 2004; Kane et al., 2008; Bearzi and Patonai, 2010; Best and Meÿer, 2010; Carvalho et al., 2010; Ribeiro et al., 2010; Foote et al., 2011; Karaa et al., 2011; Martín et al., 2011; Oliveira et al., 2011; Rosso et al., 2011; Díaz-Aguirre et al., 2012; González et al., 2012; Ólafsdóttir and Shinn, 2013; Towers et al., 2013; Whitehead et al., 2014; Díaz-Gamboa, 2015; Kim and Sohn, 2016; Methion and Díaz López, 2019; Pacheco et al., 2019; Herr et al., 2020; Matthews et al., 2020; Siciliano et al., 2020; Visser et al., 2020; Flach et al., 2021); also reported on the head (Samaras, 1989; Engel, 1994) and on a facial lesion (Alves-Motta et al., 2020).

Use as Indicator
The high detectability of *X. globicipitis* from visual surveys makes it applicable for individual marking of cetaceans (Visser et al., 2020) and as a multifaceted indicator. First, differences in its prevalence have been used to trace cetacean long-distance

migrations (Best, 1982; Bushuev, 1990; Matthews et al., 2020; Ten et al., unpubl.) and to discriminate ecological stocks (Kawamura, 1969; Bushuev, 1990; Toth et al., 2012; Towers et al., 2013; Urian et al., 2019; Silva et al., 2020) and climate change-derived shifts in cetacean distribution (Visser et al., 2020). Second, its settlement patterns on hosts, which seem mainly driven by water flow, have been used to investigate cetacean swimming and hydrodynamics (Carrillo et al., 2015; Moreno-Colom et al., 2020). Lastly, the higher prevalence on immunosuppressed hosts highlights its potential as an indicator of health status in cetacean populations (Aznar et al., 1994; Aznar et al., 2005).

Remarks

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References

Steenstrup, 1852; Darwin, 1854; Hoek, 1883; True, 1890; Richard and Neuville, 1897; Weltner, 1897; Gruvel, 1905; Gruvel, 1912; Collet, 1912; Liouville, 1913; Gruvel, 1920; Calman, 1920; Nilsson-Cantell, 1921; Barnard, 1924; Broch, 1924; Cornwall, 1927; Cornwall, 1928; Mackintosh and Wheeler, 1929; Nilsson-Cantell, 1930a; Richard, 1936; Matthews, 1938b; Heldt, 1950; Cornwall, 1955; Pope, 1958; Rice, 1963; Zullo, 1963; Stubbings, 1965; Pilleri, 1967; Dollfus, 1968; Kawamura, 1969; Pilleri and Gihr, 1969; Pilleri and Knuckey, 1969; Pilleri, 1970; Rice, 1977; Rice, 1978; Caldwell et al., 1971a; Devaraj and Bennet, 1974; Brownell, 1975; Mead, 1975; Bryden, 1976; Spivey, 1977; Dailey and Walker, 1978; Greenwood et al., 1979; Bane and Zullo, 1980; Spivey, 1980; Raga et al., 1982; Raga et al., 1983b; Ross, 1984; Raga and Carbonell, 1985; Gittings et al., 1986; Raga and Sanpera, 1986; Brownell et al., 1987; Rappé, 1988; Rappé and Van Waerebeek, 1988; Pinedo et al., 1989; Samaras, 1989; Bushuev, 1990; Mead and Potter, 1990; Van Waerebeek et al., 1990; Young, 1991; Duignan et al., 1992; Rajaguru and Shantha, 1992; Aguilar and Raga, 1993; Raga and Balbuena, 1993; Van Waerebeek et al., 1993; Aznar et al., 1994; Aznar et al., 2005; Aznar et al., 2016, unpubl.; Engel, 1994; Fertl, 1994; Watson et al., 1994; Jefferson et al., 1995; Reyes and Van Waerebeek, 1995; Azevedo et al., 1996; Fertl et al., 1996; Araki et al., 1997; Orams and Schuetze, 1998; Uchida, 1998; Rittmaster et al., 1999; Vidal et al., 1999; Di Beneditto and Ramos, 2001; Guerrero-Ruiz and Urbán, 2000; Kuramochi et al., 2000; Uchida and Araki, 2000; Addink and Smeenk, 2001; Barros and Stolen, 2001; Parsons et al., 2001; Danilewicz et al., 2002; Louella and Dolar, 2002; Resendes et al., 2002; Berland et al., 2003; Di Beneditto and Ramos, 2004; Karuppiah et al., 2004; Palacios et al., 2004; Watson and Gee, 2005; Bellido et al., 2006; Sakai et al., 2006; Best, 2007; Pitman et al., 2007; Toth-Brown and Hohn, 2007; Kane et al., 2008; Kautek et al., 2008; Rotstein et al., 2009; Sakai et al., 2009; Bearzi and Patonai, 2010; Best and Meÿer, 2010; Carvalho et al., 2010; Ribeiro et al., 2010; Weir, 2010; Foote et al., 2011; Karaa et al., 2011; Martín et al., 2011; Oliveira et al., 2011; Rosso et al., 2011; Bertulli et al., 2012; Díaz-Aguirre et al., 2012; González et al., 2012; Hayashi, 2012; Pugliese et al., 2012; Toth et al., 2012; Ólafsdóttir and Shinn, 2013; Towers et al., 2013; Lane et al., 2014; Whitehead et al., 2014; Díaz-Gamboa, 2015; Carrillo et al., 2015; Blum and Fong, 2016; Prestridge, 2016; Kim and Sohn, 2016; Denkinger and Alarcon, 2017; Donnelly et al., 2018; Ronje et al., 2018; Cortés-Peña, 2019; Methion and Díaz López, 2019; Pacheco et al., 2019; Urian et al., 2019; Alves-Motta et al., 2020; Gagnon and Torgersen, 2020; Gómez-Hernández et al., 2020; Herr et al., 2020; Matthews et al., 2020; Minton et al., 2020 (in press); Minussi, 2020; Moreno-Colom et al., 2020; Natural History Museum, 2020; Orrell, 2020; Siciliano et al., 2020; Silva et al., 2020; Ueda, 2020; Vargas-Bravo et al., 2020; Visser et al., 2020; CW Azores, 2021; Flach et al., 2021; iNaturalist, 2021; Ten et al., unpubl.

Order Scalpellomorpha Buckeridge & Newman, 2006
Family Lepadidae Darwin, 1852

Lepadids are oceanic fugitive species with relatively rapid growth and require a hard substratum to settle (e.g., Skerman, 1958; Patel, 1959; Southward, 1987; Harper, 1995; Hinojosa et al., 2006; Fraser et al., 2011; Wegner and Cartamil, 2012; Frick and Pfaller, 2013; Schiffer and Herbig, 2016). Overall, they are generalistic settlers on floating objects, be living or inanimate. This feature makes it often difficult to ascertain whether settlement on putative basibionts is *pre-* or *postmortem* (e.g., Magni et al., 2015; Ten et al., 2019). However, some degree of specialization for living cetaceans seems to be apparent especially for *Conchoderma auritum* (see below). Apart from cetaceans, other basibionts for species of *Lepas* and *Conchoderma* are, *inter alia*, bull kelps (Fraser et al., 2011; López et al., 2017), sea turtles (Ten et al., 2019), and even human corpses (Magni et al., 2015). Extensive description of the metamorphosis for species of this family is provided by Darwin (1854).

Conchoderma auritum (Linnaeus, 1767)

Synonyms

Conchoderma leporinum Olfers, 1814, *Lepas aurita* Linnaeus, 1767, *Otion stimpsoni* Dall, 1872

Morphological Description

Darwin, 1854; Dall, 1872; Monod, 1938; Cornwall, 1955

Molecular Sequences

COI (Ashton et al., 2016; GenBank MT563423; MT563438; MT563441), H3 (Pérez-Losada et al., 2008), 12S rRNA (Endo et al., 2010), 16S rRNA (Tomioka et al., 2020), 18S rRNA, 28S rRNA (Pérez-Losada et al., 2008)

Association

Facultative commensal. However, Newman and Abbott (1980) considered that this species might actually be an obligate commensal on cetaceans because most records of this species involve, as substrata, the shells of coronulid barnacles and/or on exposed hard surfaces of these mammals, e.g., baleens or tusks of ziphids. Rasmussen (1980) postulated that *C. auritum* prefers hard substrates in motion, although this species has also been reported on animate objects (see below).

Cetacean Hosts/Basibionts

Balaenoptera acutorostrata, *B. bonaerensis*, *B. borealis*, *B. musculus*, *B. physalus*, *Berardius bairdii*, *Eschrichtius robustus*, *Eubalaena glacialis*, *Feresa attenuata*, *Globicephala macrorhynchus*, *G. melas*, *Hiperoodon ampullatus*, *H. planifrons*, *Megaptera novaeangliae*, *Mesoplodon bidens*, *M. densirostris* (de Blainville, 1817), *M. europaeus*, *M. hectori* (Gray, 1871), *M. layardii* (Gray, 1865), *M. mirus*, *M. stejnegeri* True, 1885, *Neophocaena phocaenoides*, *Peponocephala electra*, *Physeter macrocephalus*, *Pontoporia blainvillei*, *Stenella attenuata*, *S. frontalis*, *S. longirostris*, *Tursiops aduncus*, *T. truncatus*, *Ziphius cavirostris*

Geographic Range

Cosmopolitan

Life Cycle

Growth rate of metamorphosed individuals are available only from inanimate substrata (0.1-1.0 mm/day; Il'in et al., 1978; Rasmussen, 1980; Dalley and Crisp, 1981). At a mean temperature of 23°C, the capitulum of newly recruited individuals can reach 1 mm long in just two days, and 6 mm in 9 days; in older individuals, growth rate estabilizes at 0.55 mm/day (Dalley and Crisp, 1981). Cyprids of *C. auritum* sampled along the Atlantic Ocean were found in low concentration between 25° N and 34 °S (Dalley and Crisp, 1981).

Microhabitat

On baleen plates (Nilsson-Cantell, 1930a; Nilsson-Cantell, 1939; Omura, 1950a; Christensen, 1985; Raga and Sanpera, 1986; Ólafsdóttir and Shinn, 2013); odontocete teeth (Beneden, 1870; Ohlin, 1893; Lillie, 1910; Hamilton, 1914; Broch, 1924; Nansen, 1925; Nilsson-Cantell, 1930a; Nilsson-Cantell, 1930c; Gauthier, 1938; Monod, 1938; Nilsson-Cantell, 1939; Scheffer, 1939; Fabian, 1950; Mizue, 1950; Omura, 1950a; Omura et al., 1955; Sergeant and Fisher, 1957; Tomilin, 1957; Wolff, 1960; Marlow, 1963; Rice, 1963; Morris and Mowbray, 1966; Pilleri, 1969a; Pilleri, 1969b; Caldwell et al., 1971b; Van Bree, 1971; Fordyce et al., 1979; Dixon, 1980; Baker, 1983; Pastene et al., 1990; Balbuena, 1991; Debrot, 1992; Rodríguez-López and Mignucci-Giannoni, 1999; Soto, 2001; O'Connor and Franco, 2003; Bermúdez-Villapol et al., 2006; Van Waerebeek et al., 2008; Holmes and Franco, 2010; Martín et al., 2011; Bachara and Gullan, 2016; Foskolos et al., 2017; Tomioka et al., 2020), and on the coronulid barnacles *C. diadema* (Beneden, 1870; Dall, 1872; Sars, 1880; Gruvel, 1911; Mörch, 1911; Liouville, 1913; Borradaile, 1916; Pilsbry, 1916; Broch, 1924; Cornwall, 1924; Cornwall, 1927; Cornwall, 1928; Nilsson-Cantell, 1930a; Nilsson-Cantell, 1930c; Hiro, 1935; Stephensen, 1938; Nilsson-Cantell, 1939; Scheffer, 1939; Tomilin, 1957; Rice, 1963; Clarke, 1966; Newman and Ross, 1971; Holthuis and Fransen, 2004; Kim et al., 2020), *C. reginae* (Nilsson-Cantell, 1930a; Nilsson-Cantell, 1939; Wolff, 1960; Rice, 1963; Clarke, 1966; Newman and Ross, 1971), and *X. globicipitis* (Ten et al., unpubl.). Also, on deformed or injured jaws that leave the teeth exposed (Davis, 1874; Mörch, 1911; Matthews, 1938c; Chapman and Santler, 1955; Clarke, 1956; Nasu, 1958; Cockrill, 1960; Wolff, 1960; Slijper, 1962; Spaul, 1964; Clarke, 1966; Pilleri, 1969b; Beach, 2015). Once recorded as an hyperepibiont on *P. balaenoptera* (Nilsson-Cantell, 1930a).

Use as Indicator

Holmes and Franco (2010) observed several individuals of *C. auritum* on the left tooth of Sowerby's beaked whale, *Mesoplodon bidens*, but none on the right tooth. These authors speculated that the barnacles could indicate some type of chirality during feeding, which may hinder barnacle development on the right side (see *Cryptolepas rachianecti* above). On the other hand, the presence of *C. auritum* has been suggested as an indicator of previous interaction of cetaceans with fisheries since these barnacles can attach on scarred mouth injuries (Beach, 2015; Welch, 2017). Finally, knowledge of growth rates of *C. auritum* makes this species potentially suitable to make temporal calibrations of time since settlement. This could inform on basibiont movements or interaction with fisheries (see, e.g., Dalley and Crisp, 1981; Wegner and Cartamil, 2012; Zettler, 2021), although this application has not been used yet in cetaceans.

Remarks

Also recorded on inanimate substrata (e.g., ship hulls, moorings, ropes; Foster and Willan, 1979; Rasmussen, 1980; Farrapeira et al., 2007) and elephant seals, *Mirounga* spp. (Best, 1971; Joseph et al., 1986).

References

Bennet, 1837; Bennett, 1840; Hallas, 1868; Beneden, 1870; Dall, 1872; Davis, 1874; Sars, 1880; Ohlin, 1893; Lillie, 1910; Gruvel, 1911; Mörch, 1911; Collet, 1912; Liouville, 1913; Hamilton, 1914; Allen, 1916; Borradaile, 1916; Pilsbry, 1916; Broch, 1924; Cornwall, 1924; Hinton, 1925; Nansen, 1925; Cornwall, 1927; Cornwall, 1928; Mackintosh and Wheeler, 1929; Nilsson-Cantell, 1930a; Nilsson-Cantell, 1930c; Matthews, 1937; Matthews, 1938c; Gauthier, 1938; Hiro, 1938; Monod, 1938; Stephensen, 1938; Nilsson-Cantell, 1939; Scheffer, 1939; Fabian, 1950; Mizue, 1950; Omura, 1950a; Omura, et al., 1955; Angot, 1951; Ohno and Fujino, 1952; Kakuwa et al., 1953; Rees, 1953; Chapman and Santler, 1955; Clarke, 1956; Sergeant and Fisher, 1957; Tomilin, 1957; Nasu, 1958; Symons and Weston, 1958; Cockrill, 1960; Wolff, 1960; Sergeant, 1962; Slijper, 1962; Marlow, 1963; Rice, 1963; Spaul, 1964; Clarke, 1966; Morris and Mowbray, 1966; Perrin, 1969; Pilleri, 1969b; Newman and Ross, 1971; Van Bree, 1971; Monod and Serene, 1976; Fordyce et al., 1979; Dixon, 1980; Baker, 1983; Christensen, 1985; Raga and Sanpera, 1986; Mead, 1989; Bushuev, 1990; Pastene et al., 1990; Bordino and González, 1992; Debrot, 1992; García-Godos, 1992; Raga and Balbuena, 1993; Mignucci-Giannoni et al., 1998; Rodríguez-López and Mignucci-Giannoni, 1999; Huang et al., 2000; Soto, 2001; O'Connor and Franco, 2003; Holthuis and Fransen, 2004; Bermúdez-Villapol et al., 2006; Van Waerebeek et al., 2008; Holmes and Franco, 2010; Ávila et al., 2011; Martín et al., 2011; Ólafsdóttir and Shinn, 2013; Angeletti et al., 2014; Insacco et al., 2014; Beach, 2015; Elorriaga-Verplancken et al., 2015; Bachara and Gullan, 2016; Foskolos et al., 2017; Iwasa-Arai

et al., 2017b; Wheeler and McIntosh, 2018; Kim et al., 2020; Natural History Museum, 2020; Tomioka et al., 2020; Ueda, 2020; Ten et al., unpubl.

Conchoderma virgatum (Spengler, 1789)

Synonyms
Conchoderma virgata (Spengler, 1790), *Lepas virgata* Spengler, 1790

Morphological Description
Darwin, 1854; Nilsson-Cantell, 1928

Molecular Sequences
COI (Chen et al., 2013), H3 (Pérez-Losada et al., 2008), 12S rRNA (Endo et al., 2010), 18S rRNA (Pérez-Losada et al., 2008; Yusa et al., 2012), 28S rRNA (Pérez-Losada et al., 2008)

Association
Facultative commensal

Cetacean Hosts/Basibionts
Balaenoptera acutorostrata, *B. bonaerensis*, *B. borealis*, *B. musculus*, *B. physalus*, *Delphinus delphis*, *Feresa attenuata*, *Megaptera novaeangliae*, *Neophocaena phocaenoides*, *Physeter macrocephalus*, *Stenella coeruleoalba*

Geographic Range
Cosmopolitan

Life Cycle
Most growth rate estimates of this species have been studied on inanimate substrata (0.1-1.5 mm/day; Darwin, 1851; Annandale, 1909; MacIntyre, 1966; Tsikhon-Lukanina et al., 1977; Il'in et al., 1978; Dalley and Crisp, 1981). For instance, at a mean temperature of 23°C and 14 days after metamorphosis, individuals grew 0.66 mm/day on an experimental torpedo (Dalley and Crisp, 1981). Eckert and Eckert (1987) provide a von Bertalanffy's growth equation obtained from *C. virgatum* measurements on nesting sea turtles, which shows an asymptotic trend comparable to that of previous studies. Differences in growth rate estimates and maximum size between studies suggest an effect of the ecological conditions (Eckert and Eckert, 1987).

Microhabitat
Mostly as a hyperepibiont of *Pennella balaenoptera* (Sars, 1866; Koren and Danielssen, 1877; Turner, 1905; Nilsson-Cantell, 1930a; Clarke, 1956; Clarke, 1966; Raga and Sanpera, 1986; Araki et al., 1997; Terasawa et al., 1997; Uchida, 1998; Ólafsdóttir and Shinn, 2013), but it can also attach directly to odontocete teeth (Lillie, 1910; Aznar et al., 1994). Once reported on *C. auritum* (Clarke, 1966), *Neocyamus physeteris* (Oliver and Trilles, 2000), and on the shell of *Xenobalanus globicipitis* (Ten et al., unpubl.).

Use as Indicator
Knowledge of growth rates of *C. virgatum* makes this species potentially suitable to make temporal calibrations of time since settlement (see *C. auritum*). Indeed, unusual attachment of *C. virgatum* and *Lepas* spp. on dolphin teeth may have occurred after dolphin death, when teeth remain exposed (Aznar et al., 1994). This provides the opportunity to infer the approximate time of death, as it has been done in sea turtles (Ten et al., 2019). The finding of *Conchoderma* sp. (presumably *C. virgatum*) attached to a marlin spear that was inserted into the jaw of an Antarctic minke whale suggested that spearing occurred a few months before the finding (Ohsumi, 1973). Lastly, its presence and size has been used as an indicator of oceanic habitat use by sea turtles (Casale et al., 2004; Casale et al., 2012; Ten et al., 2019) and of interaction with pelagic fisheries (Wegner and Cartamil, 2012; Ten et al., 2019).

Remarks
It is typical settler of inanimate substrata, e.g., ship vessels, buoys (Foster and Willan, 1979; Farrapeira et al., 2007; González et al., 2012; Wegner and Cartamil, 2012), but also attaches to multiple marine animals, including fish (e.g., Crozier, 1916; Hastings, 1972; Ohsumi, 1973), sea turtles (e.g., Eckert and Eckert, 1987; Alonso et al., 2010), elephant seals (Joseph et al., 1986), sea snakes (Annandale, 1909; Yamato et al., 1996), and pelagic crabs (Jerde, 1967; Moazzam and Rizvi, 1979). It has also been reported as a hyperepibiont of fish copepods (e.g., Williams, 1978; Williams and Williams, 1986).

References
Sars, 1866; Koren and Danielssen, 1877; Turner, 1905; Lillie, 1910; Collet, 1912; Liouville, 1913; Mackintosh and Wheeler, 1929; Nilsson-Cantell, 1930a; Clarke, 1956; Clarke, 1966; Kawamura, 1969; Berzin, 1972; Rice, 1977; Greenwood et al., 1979; Raga and Carbonell, 1985; Raga and Sanpera, 1986; Bushuev, 1990; Aguilar and Raga, 1993; Aznar et al., 1994, unpubl.; Araki et al., 1997; Terasawa et al., 1997; Uchida, 1998; Huang et al., 2000; Kuramochi et al., 2000; Oliver and Trilles, 2000; Uchida and Araki, 2000; Ólafsdóttir and Shinn, 2013; Ten et al., unpubl.

Lepas (Anatifa) hillii (Leach, 1818)

Synonyms
Lepas hillii (Leach, 1818)

Morphological Description
Darwin, 1854; Cornwall, 1955

Molecular Sequences
-

Association
Facultative commensal

Cetacean Hosts/Basibionts
Once reported on *Stenella coeruleoalba*

Geographic Range
Pantropical (González et al., 2012)

Life Cycle
At temperatures *ca.* 25 °C, individuals attached to a ship in central Atlantic Ocean reached maturity after 30-43 days for a capitulum 13-17 mm long (i.e., a growth rate of 0.5 mm/day; Evans, 1958). Similarly as in *Conchoderma* spp. (see above), growth was asymptotic and fell to 0.03 mm/day after maturity (Evans, 1958).

Microhabitat
Teeth (Aznar et al., 1994)

Use as Indicator
Deeper knowledge of growth rates of *L. hillii* would refine estimates of time since settlement (see *C. virgatum*). Some applications include the estimation of the time of death of basibionts (Aznar et al., 1994; Ten et al., 2019), interaction with fisheries (Wegner and Cartamil, 2012; Ten et al., 2019), and oceanic habitat use (Casale et al., 2004; Casale et al., 2012; Ten et al., 2019).

Remarks
On inanimate substrata, e.g., buoys, ship hulls, a rope (Il'in et al., 1978; Dalley and Crisp, 1981; Farrapeira et al., 2007; Wegner and Cartamil, 2012) and on marine vertebrates, including fish (Dulčić et al., 2015), sea turtles (Domènech et al., 2015; Ten et al., 2019), and elephant seals (Joseph et al., 1986).

References
Aznar et al., 1994

Lepas (Anatifa) pectinata (Spengler, 1793)
Synonyms
Lepas pectinata Spengler, 1793

Morphological Description
Darwin, 1854; Cornwall, 1955

Molecular Sequences
COI (Chen et al., 2013; Schiffer and Herbig, 2016; Aguilar et al., 2018; Rech et al., 2018; GenBank KY639421-KY639424; MF974366-MF974369), H3 (Pérez-Losada et al., 2008), 18S rRNA (Pérez-Losada et al., 2008; Schiffer and Herbig, 2016), 28S rRNA (Pérez-Losada et al., 2008)

Association
Facultative commensal

Cetacean Hosts/Basibionts
Stenella coeruleoalba

Geographic Range
Cosmopolitan (González et al., 2012)

Life Cycle
This is the most abundant lepadid in the Northeast Atlantic, where its development has been studied (Ellis et al., 1983; Conway et al., 1990). Interestingly, *L. pectinata* presumably performs ontogenetic depth migrations, i.e., nauplii feed in the upper 150 m and the non-feeding cyprids distribute at 300-400 m (Conway et al., 1990). Nauplii show similar feeding and swimming features as other barnacle larvae (Moyse, 1984).

Microhabitat
Teeth (Aznar et al., 1994)

Use as Indicator
See *L. hillii* (above).

Remarks
Closely associated to *Sargassum* spp. weed (Fine, 1970; Conway et al., 1990); also found on inanimate substrata (e.g., floating crude oil, plastic debris; Horn et al., 1970; Minchin, 1996; Bergami et al., 2021) and on sea turtles (Domènech et al., 2015; Ten et al., 2019).

References
Aguilar and Raga, 1993; Aznar et al., 1994

Phylum Chordata Haeckel, 1874
Class Actinopteri Cope, 1871
Subclass Teleostei Müller, 1846
Order Carangiformes Jordan, 1963
Family Echeneidae Rafinesque, 1810

Remoras or diskfishes include 8 species of specialized teleosts that use their dorsal fin as an adhesive disc to attach to a great variety of marine vertebrates from which they benefit through, e.g., ventilation, protection from predators, and increased contact with conspecifics (Fertl and Landry, 1999a; Fertl and Landry, 1999b). The fact that remoras live in association with elasmobranchs, teleosts, sea turtles, and cetaceans (Cressey and Lachner, 1970) has hampered research on basic biological features such as growth and reproduction for most species (Battaglia et al., 2016).

Echeneis naucrates (Linnaeus, 1758)
Synonyms
Echeneis chiromacer Duméril, 1858, *E. fasciata* Gronow, 1854, *E. fusca* Gronow, 1854, *E. guaican* Poey, 1860, *E. lunata* Bancroft, 1831, *E. metallica* Poey, 1860, *E. naucratus* Linnaeus, 1758, *E. neucrates* Linnaeus, 1758, *E. scaphecrates* Duméril, 1858, *E. vittate* Rüppell, 1838, *Echensis naucrates* Linnaeus, 1758, *Echneis naucrates* Linnaeus, 1758, *Leptecheneis flaviventris* Seale, 1906, *L. naucrates* (Linnaeus, 1758)

Morphological Description
Collette, 2003; Skaramuca et al., 2009

Molecular Sequences
> 40,000 results in GenBank

Association
Facultative commensal

Cetacean Hosts/Basibionts
Sotalia guianensis, *Tursiops truncatus*

Geographic Range
Cosmopolitan (Collette et al., 2015)

Life Cycle
In the eastern Gulf of Mexico females show slower growth but achieve larger size than males; spawning takes place in August (Bachman et al., 2018).

Microhabitat
Flanks and both dorsal and ventral sides

Use as Indicator
-

Remarks
It can free swim in the water column while feeding on small fishes and plankton (O'Toole, 2002), but also attach to a broad spectrum of basibionts, including reef teleosts, sharks, and sea turtles (O'Toole, 2002; Sazima and Grossman, 2006; Gray et al., 2009), nearshore dolphins (above), and even to conspecifics (Brunnschweiler and Sazima, 2006). It is considered a sister-species of *E. neucratoides* (O'Toole, 2002).

References
Fertl and Landry, 1999b; Fertl et al., 2002; Noke, 2004; Santos and Sazima, 2005

Echeneis neucratoides (Zuiew, 1789)
Synonyms
-

Morphological Description
-

Molecular Sequences
COI (GenBank KF461171), EGR1, EGR2B, EGR3 (Campbell et al., 2013), ITS1 (Gray et al., 2009), ND2 (Gray et al., 2009), RAG1, RH1 (Campbell et al., 2013), VCPIP, ZIC1 (Betancur et al., 2013), 5.8S rRNA, 12S rRNA, 16S rRNA, 18S rRNA (Gray et al., 2009)

Association
Presumably facultative commensal

Cetacean Hosts/Basibionts
Two unidentified cetaceans

Geographic Range
Western Atlantic Ocean (Fertl and Landry, 1999a; Fertl and Landry, 1999b)

Life Cycle
-

Microhabitat
-

Use as Indicator
-

Remarks
Typical commensal of sharks and once observed on a West Indian manatee captured in Puerto Rico (Mignucci-Giannoni et al., 1999).

References
O'Toole, 2002

Remora australis (Bennett, 1840)
Synonyms
Echeneis australis Bennett, 1840, *E. scutata* Günther, 1860, *Remilegia australis* (Bennett, 1840), *Remora australia* (Bennett, 1840), *R. scutata* (Günther, 1860)

Morphological Description
Rice and Caldwell, 1961

Molecular Sequences
COI (GenBank GU440495; OK030822), CYTB (Sanciangco et al., 2016), ITS1, ND2 (Gray et al., 2009), RAG1 (GenBank EU167871), 5.8S rRNA, 12S rRNA, 16S rRNA, 18S rRNA (Gray et al., 2009)

Association
Obligate commensal/mutualist. Although previously considered as an obligate commensal (Rice and Caldwell, 1961), later evidence has shown that this species can feed on host's ectoparasites (O'Toole, 2002). However, remoras may potentially disrupt the flow over cetaceans' body, increasing drag, and their sucking disk may produce irritation (Fish et al., 2006).

Cetacean Hosts/Basibionts
Balaenoptera borealis, *B. edeni*, *B. musculus*, *Delphinus delphis*, *Orcinus orca*, *Physeter macrocephalus*, *Stenella attenuata*, *S. frontalis*, *S. longirostris*, *Tursiops truncatus*

Geographic Range
eastern Pacific, Atlantic, Indian Ocean, Indonesian Sea

Life Cycle
Off Brazil, remoras of the smallest size class (i.e., < 10 cm) were the most abundant size class in May and their frequency fell until none were reported in October (Wingert et al., 2021).

Microhabitat
Ubiquitous on skin (Wingert et al., 2021)

Use as Indicator
Remoras on blue whales preferentially attach to regions with reduced drag. Therefore, they could evince patterns of water flow over swimming whales, which could optimize tag deployment for extended ecological monitoring (Flammang et al., 2020).

Remarks
The records from *B. edeni, O. orca, S. attenuata*, and *T. truncatus* above provide only identification to genus level, but are here assigned to *R. australis* since it is the only species of *Remora* associated to cetaceans (O'Toole, 2002). Individuals of *R. australis* appear to disengage from whales during whaling (Pike, 1951; Rice and Caldwell, 1961), which might result in gross underestimations of actual prevalence in nature. Prior to towing, the prevalence of *R. australis* on blue whales, *Balaenoptera musculus*, captured in California and Peru was close to 100 percent (Rice and Caldwell, 1961). Attachment marks of this species on the host's epidermis are superficial, and scarring is not typically observed (Rice and Caldwell, 1961; Visser, pers. obs.). There is a single record of two copepod hyperparasites on *R. australis*, namely *Pennella balaenoptera* and *Lepeophtheirus crassus* (Radford and Klawe, 1965).

References
Carl and Wilby, 1945; Cadenat, 1953; Krefft, 1953; Follet and Dempster, 1960; Mahnken and Gilmore, 1960; Rice and Caldwell, 1961; Rice, 1963; Radford and Klawe, 1965; Rice, 1977; Rice, 1978; Notarbartolo di Sciara and Watkins, 1979; Fertl and Landry, 1999a; Fertl and Landry, 1999b; Wingert et al., 2021

Order Siluriformes -
Family Trichomycteridae Bleeker, 1858

Catfishes (Siluriformes) are widely distributed in freshwater, estuarine, and marine habitats of continental shelves (de Pinna, 1998). Members of the family Trichomycteridae, known as pencil or parasitic catfishes (de Pinna and Wosiacki, 2003), inhabit continental freshwaters from Costa Rica to Patagonia (de Pinna and Wosiacki, 2003; Eschmeyer et al., 2017).

Ochmacanthus sp.
Synonyms
-

Morphological Description
Araújo-Wang et al., 2019

Molecular Sequences
COI, CYTB, H3, ND4, MYH6, RAG1, RAG2, 12S rRNA, and 16S rRNA of three *Ochmacanthus* spp. (see GenBank)

Association
Presumably obligate commensal

Cetacean Hosts/Basibionts
Inia geoffrensis (Blainville, 1817)

Geographic Range
South American rivers (Koch, 2002)

Life Cycle
-

Microhabitat
On lateral and ventral surfaces (Araújo-Wang et al., 2019)

Use as Indicator
-

Remarks
Candirus are generally commensal on various freshwater fishes (Adriaens et al., 2010), but Araújo-Wang et al. (2019) reported year-round observations on *Inia geoffrensis*.

References
Araújo-Wang et al., 2019

Class Hyperoartia Müller, 1844
Order Petromyzontiformes Berg, 1940
Family Petromyzontidae Bonaparte, 1831

Anadromous lampreys (Petromyzontiformes) are jawless fishes distributed antitropically around the world. They develop in estuaries and oceans, where they parasitize large vertebrates consuming their blood, fluids, and flesh, and then migrate into freshwater streams to spawn and die (Renaud, 2011; Johnson et al., 2015; Clemens et al., 2019). Species of Petromyzontidae are exclusively found in the Northern Hemisphere (Renaud, 2019; Miller et al., 2021). The family Petromyzontidae is described in Renaud (2019).

Entosphenus tridentatus (Richardson, 1836)
Synonyms
Entosphenus epihexodon Gill, 1862, *E. tridentatus tridentatus* (Richardson, 1836), *Lampetra tridentatus* (Richardson, 1836), *Petromyzon astori* Girard, 1858, *P. ciliatus* Ayres, 1855, *P. epihexodon* (Gill, 1862), *P. lividus* Girard, 1858, *P. tridentatus* Richardson, 1836

Morphological Description
Creaser and Hubbs, 1922

Molecular Sequences
COI (Yamazaki et al., 2006; April et al., 2011; Carim et al., 2017; GenBank GU440367; KF918874; KF918875; KF929845; KY570333), CR (GenBank AY205567), CYTB (Docker et al., 1999; Lorion et al., 2000; Yamazaki et al., 2006; Boguski et al., 2012; GenBank DW022992; GQ206157; KR422618; KR422619; KU672473-KU672485), ETR-1, ETR-2, ETR-3, ETR-4, ETR-5, ETR-6 (Spice et al., 2011), GnRH-III (Silver et al., 2004), ND1, ND2, ND4, ND5 (Docker et al., 2007), RT (GenBank AJ244558), 12S rRNA (GenBank LC091545; LC091546), 16S rRNA (GenBank KJ010762), and the whole genome (Hess et al., 2020)

Association
Ectoparasite

Cetacean Hosts/Basibionts
Balaenoptera borealis, *B. musculus*, *B. physalus*, *Berardius bairdii*, *Megaptera novaeangliae*, *Physeter macrocephalus*

Geographic Range
North Pacific, from Baja California north to the Bering and Chukchi seas and westward into Russia and Japan, showing the greatest latitudinal range of any lamprey (Renaud, 2011)

Life Cycle
Laboratory observations hypothesized that the time of residence in the ocean is ≤ 3.5 years (Beamish, 1980). Movements in the ocean are poorly understood, but they are typically caught between the surface and 500 m (see Clemens et al., 2019).

Microhabitat
-

Use as Indicator
Based on the degree of healing of the marks of Pacific lampreys on several species of whales, Pike (1951) inferred that lamprey attacks took place during the northward migration in the North Pacific. Therefore, marks could be used to trace whale's migration.

Remarks
Typically parasitizes fish (Clemens et al., 2019).

References
Carl, 1950; Pike, 1951; Nemoto, 1955; Rice, 1963; Rice, 1977; Rice, 1978

Petromyzon marinus (Linnaeus, 1758)
Synonyms
Ammocoetes bicolor Lesueur, 1818, *Batymyzon bairdii* (Gill, 1883), *Lampetra marina* (Linnaeus, 1758), *Oceanomyzon wilsoni* Fowler, 1908, *Petromyzon adriaticus* Nardo, 1847, *P. americanus* Lesueur, 1818, *P. bairdii* Gill, 1883, *P. concolor* Wright, 1892, *P. lampetra* Pallas, 1814, *P. maculosus* Gronow, 1854, *P. marinus dorsatus* Wilder, 1883, *P. marinus unicolor* Gage, 1928, *P. maximus* Cuvier, 1816, *P. nigricans* Lesueur, 1818, *P. ruber* Lacepède, 1800

Morphological Description
Creaser and Hubbs, 1922

Molecular Sequences
> 193,000 results in GenBank

Association
Ectoparasite, inferred from resulting wounds and scars (Silva et al., 2014)

Cetacean Hosts/Basibionts
Balaenoptera acutorostrata, *B. borealis*, *B. physalus*, *Eubalaena glacialis*, *Grampus griseus*, *Megaptera novaeangliae*, *Mesoplodon bidens*, *Orcinus orca*, *Physeter macrocephalus*, *Tursiops truncatus*, *Ziphius cavirostris*

Geographic Range
Atlantic coast of North America and Europe, including the central Mediterranean Sea (Holčík et al., 2004; Kottelat and Freyhof, 2007)

Life Cycle
This hematophagous species grows to adult size in 1 year; the complete metamorphosis and reproduction takes 1.5 years (Silva et al., 2013).

Microhabitat
Flanks of middle and posterior body areas (Bertulli et al., 2012; Ólafsdóttir and Shinn, 2013)

Use as Indicator
In some cases, the individuals are still attached to the host when found, being easier to detect (Nichols and Hamilton, 2004; Nichols and Tscherter, 2011; Samarra et al., 2012; Miočić-Stošić et al., 2020). In others, however, only the remaining marks are visible. The applicability of these marks is still to be determined. Samarra et al. (2012) stated that they apparently disappear within 1 year, whereas Miočić-Stošić et al. (2020) claim that they are seemingly short-lived, thus not being suitable markings in photo-identification. In the past years, it has been more commonly found in Icelandic waters, and this change in distribution seems to be due to a gradual increase in water temperatures around Iceland (Astthorsson and Palsson, 2006).

Remarks
This species is often found on freshwater and marine fishes (Collette and Klein-MacPhee, 2002).

References
Japha, 1910; Collet, 1912; Nichols and Hamilton, 2004; Nichols and Tscherter, 2011; Rosso et al., 2011; Bertulli et al., 2012; Samarra et al., 2012; Ólafsdóttir and Shinn, 2013; Silva et al., 2014; Bertulli et al., 2016; Miočić-Stošić et al., 2020

Phylum Cnidaria Hatschek, 1888
Class Hydrozoa Owen, 1843
Subclass Hydroidolina Collins, 2000
Order Leptothecata Cornelius, 1992
Family Campanulariidae Johnston, 1836

Members of this family of thecate hydroids are ubiquitous in marine benthic communities. Given that the morphology of colonies and polips are highly variable within species, it is difficult to find diagnostic morphological characters to separate congeneric species (Cunha et al., 2015), which may hinder correct identification to species level.

Obelia dichotoma (Linnaeus, 1758)
Synonyms
Multiple; see Schuchert (2021).

Morphological Description
Orejas et al., 2012

Molecular Sequences
COI (Govindarajan et al., 2006; Cunha et al., 2015; Cunha et al., 2017; GenBank MG791815; MW277711; MW277730;

MZ580517; MZ580890), calmodulin (Govindarajan et al., 2006), LSU rRNA (Pruski and Miglietta, 2019; Penney and Rawlings, 2021; GenBank MG786561; MG786562), SSU rRNA (MG792325), 5.8S rRNA (Cunha et al., 2015), 16S rRNA (Bridge et al., 1995; Govindarajan et al., 2006; Cunha et al., 2015; Cunha et al., 2017; Rech et al., 2018), 18S rRNA, 28S rRNA (Bridge et al., 1995; Govindarajan et al., 2006; Cunha et al., 2015; Maronna et al., 2016; Cunha et al., 2017)

Association
Unknown, although a commensalist or even mutualistic association cannot be ruled out since newly released medusae of this species are bacteriophagous (Boero et al., 2007).

Cetacean Hosts/Basibionts
Once reported on *Megaptera novaeangliae*

Geographic Range
Nearly cosmopolitan (Orejas et al., 2012)

Life Cycle
Kubota (1999) reported the complete life cycle of *O. dichotoma* in Northern Japan.

Microhabitat
As a hyperepibiont on the barnacle *Coronula diadema* (Cornwall, 1928)

Use as Indicator
-

Remarks
It can be found on hard substrata, such as floats, pilings, rocks, and shells (Orejas et al., 2012).

References
Cornwall, 1928

Obelia sp.
Synonyms
-

Morphological Description
Cornelius (1990) provides extensive descriptions of European *Obelia* spp.

Molecular Sequences
> 400 results in GenBank

Association
Unknown

Cetacean Hosts/Basibionts
Once reported on *Megaptera novaeangliae*

Geographic Range
-

Life Cycle
See Cornelius (1990).

Microhabitat
As a hyperepibiont on *Coronula* spp. (Rice, 1963)

Use as Indicator
-

Remarks
-

References
Rice, 1963

Phylum Nematoda Cobb, 1932
Class Chromadorea Inglis, 1983
Subclass Chromadoria Pearse, 1942
Order Monhysterida Filipjev, 1929
Family Monhysteridae de Man, 1876

This family is composed of terrestrial, freshwater, and marine forms. Some species are free-living in the sediment (e.g., Fonseca and Decraemer, 2008), bacterivorous on plants (Alkemade et al., 1992), associated to pack ice (Blome and Riemann, 1999) or living epibiotically on crustaceans in marine, limnetic, and terrestrial habitats (Lorenzen, 1986).

Odontobius ceti (Roussel de Vauzème, 1834)
Synonyms
-

Morphological Description
Roussel de Vauzème, 1834; Baylis, 1923; Lorenzen, 1986

Molecular Sequences
-

Association
Obligate commensal; it probably feeds primarily on organic particles from whales' diet (Baylis, 1923).

Cetacean Hosts/Basibionts
Balaenoptera borealis, *B. musculus*, *B. physalus*, *Eubalaena australis*, *Megaptera novaeangliae*

Geographic Range
Atlantic, North Pacific, Antarctica

Life Cycle
Eggs are laid on the baleen plates but, since no larval stages have been found on cetaceans, further development may take place in the sea (Baylis, 1923).

Microhabitat
Baleen plates (Roussel de Vauzème, 1834; Baylis, 1923; Skrjabin, 1959; Rice, 1963; Lorenzen, 1986), in association with the ciliated

protozoon *Haematophagus megaptere* Woodcock & Lodge, 1921 (Baylis, 1923).

Use as Indicator

-

Remarks

Considered a taxon inquirendum (WoRMS, 2021).

References

Roussel de Vauzème, 1834; Baylis, 1923; Skrjabin, 1959; Rice, 1963; Lorenzen, 1986

Other Taxa With Indicator Value

Some organisms have been reported on cetaceans but cannot be considered epibiotic animals (i.e., they belong to another kingdom or are not intimately associated to cetaceans). For instance, the cirolanid isopods *Natatolana* spp. or the hagfish *Myxine glutinosa* Linnaeus, 1758 are scavengers (Hale, 1926; Bowman, 1971; Pinedo et al., 1989; Martini, 1998; Keable, 2006; Zintzen et al., 2011) and records on living cetaceans are unusual (Pace et al., 2016). The following taxa, despite not being intimate associates or not belonging to the animal kingdom, can provide valuable information on cetacean biology.

At least 14 genera of diatoms (Chromista: Bacillariophyceae) have been recorded on over a dozen cetacean species (e.g., Hart, 1935; Matthews, 1938b; Hustedt, 1952; Nemoto, 1958; Nemoto et al., 1977; Heckman et al., 1987; Ferrario et al., 2018). Several species belonging to genera such as *Bennettella* Holmes, 1985, *Epipellis* Holmes, 1985, *Epiphalaina* Holmes, Nagasawa & Takano, 1993, *Plumosigma* Nemoto, 1956, and *Tursiocola* Holmes, Nagasawa & Takano, 1993 are believed to be exclusive to cetaceans. It has been proposed that these animal-specific diatoms settle on cetaceans in polar waters and take approximately one month to develop into a yellowish-brown film visible to the naked eye (Omura, 1950b). Therefore, it can be inferred that whales in polar areas that are covered by diatom films are at least one-month visitors, whereas those at lower latitudes and still showing skin colouration returned recently from polar regions (Hart, 1935; Matthews, 1938b; Omura, 1950b; Cockrill, 1960; Bannister, 1968; Sekiguchi et al., 1993). In South Africa, diatom films were detected more frequently as the Antarctic whaling season advanced (Cockrill, 1960) *vs*. at the beginning of the season (Best, 1969b). Diatom films have also been used to investigate population segregation, i.e., they were almost absent on sperm whale females and young males, which coincides with inferences of social segregation based in cyamid infections (see *C. catodontis* and *N. physeteris*; Best, 1969a; Best, 1969b).

The cookie-cutter shark *Isistius brasiliensis* (Quoy & Gaimard, 1824) preys on multiple marine organisms, including finfish (Papastamatiou et al., 2010), elasmobranchs (Yamaguchi and Nakaya, 1997), pinnipeds (Gallo-Reynoso and Figueroa-Carranza, 1992; Hiruki et al., 1993), sirenians (Reddacliff, 1988), and cetaceans (Dwyer and Visser, 2011). About 25% of stomach content consists of marine mammal remains, i.e., tissue plugs, skin, blubber (Carlisle et al., 2021), thus being considered a cetacean (micro)predator (Barros and Stolen, 2001). It has been hypothesized that cookie-cutter sharks use an ambush style of hunting; when potential preys are close enough, they latch and remove large plugs of tissue (Widder, 1998). This feeding mode has been catalogued as ectoparasitic (Carlisle et al., 2021). Despite its widespread distribution (Dwyer and Visser, 2011), its common range lies within equatorial and tropical waters (Nakano and Tabuchi, 1990; Yamaguchi and Nakaya, 1997). Accordingly, marks of *I. brasiliensis* on cetaceans at higher latitudes have been used as a migration tag (Tomilin, 1957 -who refers to them as 'light spots'; Renner and Bell 2009; Foote et al., 2011; Bertulli et al., 2016). Interestingly, this species has not been reported on the southern right whale, *Eubalaena australis* (Matthews, 1938a), which is found only further south than 13°S (Peters and Barendse, 2016). Also, due to the long duration of the marks it leaves on cetaceans, it has been suggested as a tool for individual recognition and marking (Dorsey et al., 1990; Visser, 1999; Gill et al., 2000; McSweeney et al., 2007; Visser et al., 2010; Rosso et al., 2011; Bertulli et al., 2016; Visser et al., 2020; Franklin et al., 2020). Other applications of this biological tag include distinguishing cetacean age classes (McSweeney et al., 2007), populations (Sherchenko, 1970; Best, 1977; Moore et al., 2003), and orca ecotypes (Dwyer and Visser, 2011; Visser et al., 2020); characterizing whale wintering grounds (Bushuev, 1990); and as an indicator of swimming in deep waters (Baird et al., 2006) and of emaciation (Gasparini and Sazima, 1996). Its congeneric member, the largetooth cookiecutter shark, *Isistius plutodus* Garrick & Springer, 1964, once observed on a Cuvier's beaked whale, *Ziphius cavirostris* (Pérez-Zayas et al., 2002), has a poorly known distribution (Moore et al., 2003). It leaves larger flesh "plugs" different from the wounds produced by *I. brasiliensis* (Compagno, 1984). Scars of *Isistius* spp. can harbor high loads of cyamids (Kobayashi et al., 2021).

As a final anecdotal remark, Ohsumi (1973) found the broken spear of a marlin, *Makaira* sp., stuck in the jaw of an Antarctic minke whale, *Balaenoptera bonaerensis*, which this author used to infer migration of this whale from tropical and sub-tropical waters, where marlins are distributed.

DISCUSSION

Gaps and Biases

The present review includes records covering over three and a half centuries, a fact that attests to the curiosity that cetacean epibionts have sparked among naturalists, probably due to their often bizarre appearance and conspicuousness. As a result, a reasonable account of the associations between cetaceans and their metazoan epibionts has been achieved. However, important biases and gaps still remain. First of all, the vast majority of studies has not primarily focused on epibiosis and thus provides little quantitative information on these associations. For instance, less than a quarter (110 out of 493) of the publications in this review include data on prevalence. This 'quantitative gap' problem is worsened by the selective 'picking' of positive records, i.e., there is a tendency to report

on the occurrence, but not on the absence, of epibionts in descriptive surveys on cetaceans. Consequently, it can be difficult to draw accurate pictures of the degree of specificity and, especially, geographic distribution of epizoic taxa. Another source of bias concerns epibiont size. Studies on large, visible barnacles such as *Xenobalanus globicipitis* are far more numerous than those focusing on minute creatures such as *Balaenophilus unisetus* (Badillo et al., 2007) or species of Cyamidae infecting dolphins (Fraija-Fernández et al., 2017). The genetic information available also varies among epibiotic taxa: 28 out of 54 species lack sequenced genetic material. Among these, some are poorly known species, but others have a long study history and numerous records (e.g., *Odontobius ceti vs. Coronula reginae*).

There is also an uneven coverage and research effort on cetaceans as basibionts, which can result in somewhat biased impressions on epibiont diversity among cetaceans. For instance, baleen whales as a group exhibit the greatest epibiont diversity most likely because they are large, slow-swimming hosts with a number of skin folds and callosities that provide suitable microhabitats for epibiont settlement (Berzin and Vlasova, 1982; see Fraija-Fernández et al., 2017). Moreover, the occurrence of certain epibionts on whales (e.g., coronulids) promotes the settlement and/or population growth of others (e.g., lepadids, cyamids), acting as pioneers (e.g., Matthews, 1937; Rice, 1963). However, mysticetes also are a well-studied cetacean group and, not surprinsingly, only the pygmy right whale, *Caperea marginata* (Gray, 1846), and Omura's whale, *Balaenoptera omurai* Wada et al., 2003, described in 2003 (Wada et al., 2003), still lack records of epibiotic fauna. Conversely, odontocetes may exhibit relatively poor epizoic fauna because many of them (e.g., delphinids) are fast-swimming hosts with small, smooth surfaces. Moreover, there are riverine dolphins, i.e. species of *Inia*, *Neophocaena*, *Orcaella*, *Platanista*, and *Sotalia* that can seldom, if at all, be exposed to epibiotic taxa of marine origin. Research effort is also low for many odontocetes, and no studies are indeed available from species of *Orcaella* Gray, 1866, *Platanista* Wagler, 1830, and *Tasmacetus* Oliver, 1937. The overall point is, therefore, that epibiotic richness in the less studied cetaceans likely has an unassessed degree of underestimation.

The spatial distribution of data is also heterogeneous. First, records from oceanic waters are far less common than those from coastal areas. Second, most geographic records concentrate in the Southern Ocean, Mediterranean Sea, off South Africa, and California, followed by other Northern Pacific regions (Eastern waters and Japan) and the North Sea. However, other vast areas have few surveys, or even none, including the Arctic, Black Sea, Red Sea, Indian Ocean (except South African waters), and the Southwestern Pacific and adjacent seas (e.g., Sulu-Celebes Sea). In this context, it is worth noting that the higher number of records in particular regions does not necessarily result from higher epizoite diversity or abundance, but rather from higher sampling effort. Whaling was a fundamental source of data but focused mainly on areas and seasons where the target species occurred at higher densities, e.g., Antarctic whaling during the austral summer or Saldanha and Durban whaling stations in South Africa (see Findlay and Best, 2016; IWC, 2021). Also, the Mediterranean and U.S. stranding networks have been working for several decades (Becker et al., 1994), while other areas have recently started to gather data on cetaceans (e.g., the Western Indian Ocean region; Plön et al., 2020) or lack active stranding or research programs (i.e., eastern Russian Arctic).

Finally, we still know very little about biology of the epibiotic fauna of cetaceans; a problem which results, at least in part, from the difficulties of dealing with organisms that depend on marine hosts whose accessibility is often limited due to economical, logistic, and legal constraints for sampling. We call this the 'association gap'; we often do not know basic aspects of many epibiont taxa, such as the complete life cycle or the actual nature of the interactions (commensal, parasitic or mutualistic).

The Nature of Epibiotic Associations and Their Indicator Potential

The origin of epibiotic associations of some animal groups with cetaceans is an exciting evolutionary issue since this epibiont fauna was acquired after the ancestors of these mammals colonized the sea (Aznar et al., 2001). Thus, there are instances of a simple use of cetaceans as additional substrata for facultative epibionts such as the Lepadidae (Newman and Abbott, 1980); host-switching events from prior obligate associations with other marine vertebrates, resulting in co-speciation, e.g., the Coronulidae (Frick et al., 2011; Hayashi et al., 2013; Buckeridge et al., 2019) and, perhaps, *B. unisetus* (Badillo et al., 2007); or putative colonization without speciation, e.g., in the case of *Pennella balaenoptera* (Fraija-Fernández et al., 2018). As far as we are aware, the Cyamidae could represent the only case of a potential primary adaptation to parasitism on cetaceans from a putative marine free-living ancestor (see Lowry and Myers, 2013). The nature of each type association brought about a variable degree of modifications in morphology, dependency of host/basibiont, and life history traits yet to be investigated in detail (see, e.g., Pugliese et al., 2012; Dreyer et al., 2020, for the case of *X. globicipitis*). These features define the potential of each epibiont as a tool to uncover aspects of cetaceans' biology. In what follows, we condense the key biological data shown above for the main epibiotic groups, i.e., amphipods, cirripeds, and copepods; we also summarize their use as indicators. Other members of the epibiotic fauna of cetaceans are certainly interesting from ecological and evolutionary points of view, e.g., the roundworm *Odontobius ceti* or the whalesucker *Remora australis*. However, their usefulness as indicators are, in principle, more limited, and will not be further discussed here.

The level of host specificity varies greatly among whale lice species; some species have been reported only, or preferentially, on single cetacean species (e.g., *Cyamus boopis*, *C. catodontis*, *C. ceti*, *C. eschrichtii*, *Neocyamus physeteris*) or clades (e.g., *Balaenocyamus balaenopterae*, *C. erraticus*, *C. gracilis*), whereas others appear to be more generalist (e.g., *Syncyamus aequus*).

The combination of bodily transmission and high specificity makes cyamids especially useful to shed light on phylogeography and social interactions of cetaceans (see references above). Moreover, cyamids can outlive their host for several days, thus providing a rough proxy of the time of death of cetaceans (Leung, 1976; Lehnert et al., 2007). However, when dealing with stranded cetaceans (a common scenario nowadays), these parasites can readily dislodge from hosts, which represents a potential drawback if quantitative infection data are to be used (Fraija-Fernández et al., 2017).

All epibiotic barnacles of cetaceans are filter-feeders whose life cycle includes a series of planktotrophic naupliar stages followed by a non-feeding cyprid, which permanently attaches to the basibiont (Darwin, 1851; Anderson, 1994; Høeg et al., 2003). Coronulids typical from whales tend to be selective and preferentially settle on single host species. For instance, *Coronula diadema* is associated to humpback whales (*ca.* 70% of records of *C. diadema*) and occurs on nearly all whales examined in surveys (Nishiwaki, 1959; Rice, 1963). In contrast, the basal representative of coronulids colonizing cetaceans, namely, *Xenobalanus globicipitis*, has been found on a total of 41 odontocete and mysticete species worldwide. The actual and potential indicator value of coronulids are thus defined by the commensal mode of feeding, the strict dependence on cetacean epidermis for attachment, and the variable degree of basibiont specificity. Species of this family have been used to unveil hydrodynamic features of cetaceans (Kasuya and Rice, 1970; Fish and Battle, 1995; Carrillo et al., 2015; Moreno-Colom et al., 2020) or systemic disease (Aznar et al., 1994; Aznar et al., 2005; Flach et al., 2021). However, their utility to inform on other aspects of cetacean biology, particularly movements and stock identification, are still far from full exploitation. For instance, Bushuev (1990) found significant differences of prevalence of *X. globicipitis* on Antartic minke whales from different Antarctic sectors, and interpreted them as evidence that whales used different wintering areas and did not mix in the Southern Ocean. However, this interpretation relies on the untested assumption that barnacle recruitment can only occur at low latitudes.

The second group of barnacles occurring on cetaceans, i.e., members of the Lepadidae, includes generalist dwellers on any type of hard substrata available in oceanic waters (e.g., Farrapeira et al., 2007; Wegner and Cartamil, 2012). Perhaps the most interesting species in this respect is *Conchoderma auritum* because, as noted above, it tends to be associated to cetaceans, either directly (on teeth) or indirectly (via the shell of coronulids, or the body of the mesoparasite *P. balaenoptera*). This raises the interesting question over the extent to which individuals of *C. auritum* recognize cetaceans as preferential substrata, and whether their populations depend on these basibionts for long-term stability. In any event, lepadids are fast-growing organisms that can be amenable for observational and experimental studies to determine their growth rate at different temperatures (Evans, 1958; Rasmussen, 1980; Dalley and Crisp, 1981; Eckert and Eckert, 1987; Inatsuchi et al., 2010). This makes them suitable as indicators of drifting time of their 'living platforms' (Fraser et al., 2011; Magni et al., 2015; López et al., 2017; Ten et al., 2019), and other aspects yet to be explored in cetaceans.

Two copepods have also developed intimate associations with cetaceans. *Balaenophilus unisetus* occurs on the baleen plates of four *Balaenoptera* spp. and is believed to feed on baleen's keratin (or the associated microfilm) as an obligate commensal (Vervoort and Tranter, 1961; Ogawa et al., 1997; Fernandez-Leborans, 2001; Badillo et al., 2007). Interestingly, available evidence for the congeneric species *B. manatorum* suggests that direct contact is necessary for transmission of *Balaenophilus* spp. (Domènech et al., 2017). This feature has allowed to draw striking inferences on unexpected contacts between otherwise solitary juveniles of marine turtles (Domènech et al., 2017). However, the indicator value of *B. unisetus* seems much more limited because accessibility to whale samples is very restricted.

Females of the world-largest known copepod, *Pennella balaenoptera*, act as mesoparasite of at least 24 cetacean species, penetrating the blubber and musculature to feed on blood (Schmidt and Roberts, 2009; Hogans, 2017). Recent evidence has shown that there are not clear diagnostic morphological traits to differentiate this species from its congener *P. filosa* except for the use of different hosts (Abaunza et al., 2001; Hogans, 2017). Moreover, molecular data do not support segregation between specimens collected from cetaceans and the swordfish in the western Mediterranean (Fraija-Fernández et al., 2018). *Pennella filosa* parasitizes a broad spectrum of large marine fishes in the oceanic realm (Román-Reyes et al., 2019 and references herein). Apparently, then, the occurrence of *P. balaenoptera* (= *filosa*) in oceanic cetaceans could have resulted from and co-accommodation of the parasite on further hosts sharing the same habitat. However, this conclusion should be confirmed by analyzing more specimens of *P. balaenoptera* collected from other fish and cetaceans in other geographical regions. This is paramount because both *P. balaenoptera* and *P. filosa* exhibit low host specificity, contrary to other members of the family Pennellidae, which infect one or two hosts (Hogans, 2017). Thus, the possibility that cryptic speciation have occurred in *P. balaenoptera* (= *filosa*) cannot be ruled out. This taxonomic issue is also relevant to assess the usefulness of *P. balaenoptera* as an indicator species. So far, the species has been used as an indicator of host's health status, i.e., heavy loads of this parasite could reflect poor health of the affected cetacean (Vecchione and Aznar, 2014). However, population inferences are more dependent on whether or not fishes should be included as part of the actual host community supporting the local population of *P. balaenoptera* (= *filosa*).

CONCLUDING REMARKS

Every epibiotic organism must first contact a potential basibiont, attach, and then successfully thrive on it (Crisp and Barnes, 1954; Crisp, 1955; Mullineaux and Butman, 1991). Accordingly, its presence on a vagile animal implies prior coincidence in time and

space between both organisms and the suitability of the basibiont/host as a habitat. In addition, since epibionts essentially live in the ecotone between the basibiont/host surface and the marine environment, abiotic conditions (e.g., temperature, salinity) must also fit the auto-ecological requirements of the epibionts during all their life-span, regardless of the migratory activity of the basibiont/host (see Moreno-Colom et al., 2020, and references therein). All these features are the ones that potentially allow to draw inferences on hosts' biology and ecology at individual, population, or community levels. However, the absence of epibionts is also informative, particularly at population level. For instance, investigating marine-mammal breeding and feeding grounds, and migratory routes, is especially important for conservation (Pompa et al., 2011), and can be elucidated, not only by the presence of selected epibionts, but also by their absence. We therefore encourage cetologists to report on both the presence or absence of epibionts whenever possible. Also, quantitative data (e.g., prevalence, mean number of individuals per host) would be most welcome.

Lastly, it is not an overstatement to claim that cetacean epibionts bear intrinsic value, thus should benefit from explicit consideration in conservation policies (see Whiteman and Parker, 2005; Aznar et al., 2011; Kwak et al., 2020). This becomes highly relevant for specific taxa associated to threatened cetaceans (e.g., whale lice), which are also on the verge of unnoticed extinction (see Buckeridge, 2012).

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

ST carried out literature search, wrote the initial version of the manuscript, and prepared the figures. FJA participated in developing the ideas and organizing and writing the manuscript, and JAR revised the text. All authors read manuscript drafts and contributed content to the developing paper. 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.2022.846558/full#supplementary-material

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Surface microbiota of Mediterranean loggerhead sea turtles unraveled by 16S and 18S amplicon sequencing

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The loggerhead sea turtle is considered a keystone species with a major ecological role in Mediterranean marine environment. As is the case with other wild reptiles, their outer microbiome is rarely studied. Although there are several studies on sea turtle's macro-epibionts and endo-microbiota, there has been little research on epibiotic microbiota associated with turtle skin and carapace. Therefore we aimed to provide the identification of combined epibiotic eukaryotic, bacterial and archaeal microbiota on Mediterranean loggerhead sea turtles. In this study, we sampled skins and carapaces of 26 loggerheads from the Mediterranean Sea during 2018 and 2019. To investigate the overall microbial diversity and composition, amplicon sequencing of 16S and 18S rRNA genes was performed. We found that the Mediterranean loggerhead sea turtle epibiotic microbiota is a reservoir of a vast variety of microbial species. Microbial communities mostly varied by different locations and seas, while within bacterial communities' significant difference was observed between sampled body sites (carapace vs. skin). In terms of relative abundance, Proteobacteria and Bacteroidota were the most represented phyla within prokaryotes, while Alveolata and Stramenopiles thrived among eukaryotes. This study, besides providing a first survey of microbial eukaryotes on loggerheads via metabarcoding, identifies fine differences within both bacterial and eukaryotic microbial communities that seem to reflect the

host anatomy and habitat. Multi-domain epi-microbiome surveys provide additional layers of information that are complementary with previous morphological studies and enable better understanding of the biology and ecology of these vulnerable marine reptiles.

KEYWORDS

epizoic, microbiome, reptile, *Caretta caretta*, skin, carapace, high throughput sequencing, metabarcoding

Introduction

Microbial communities associated with the external surfaces of animals represent an important part of the animal microbiome. Animal integument is a physical barrier that protects animal's internal environment while interacting with their external environment. In vertebrates, one of the most extensively studied epimicrobiomes is the human skin (Turnbaugh et al., 2007; Byrd et al., 2018). The epidermal microbes enhance the skin barrier performance by modulating innate immunity and developing adaptive immunity (Sanford and Gallo, 2013), therefore helping to battle skin pathogens (Belkaid and Segre, 2014; Belkaid and Tamoutounour, 2016). A shift in the host's health can alter the composition and functions of the skin microbiota that can lead to various diseases (Sanford and Gallo, 2013). Similarly, the native microbial communities of the epidermis can be affected by sub-optimal environmental conditions, negatively influencing their protective properties (Scharschmidt and Fischbach, 2013; Byrd et al., 2018).

The skin and other external body surfaces (e.g., horns, carapaces, hair and other keratinous hard tissues) of vertebrates differ between taxonomic groups and provide fairly diversified habitats for various animal-associated microbes (Ross et al., 2019). Since both intrinsic (e.g., species, sex, age) and extrinsic (e.g., geographic location, biotic and abiotic environmental conditions, captivity affecting the natural behavior, and diet) factors shape the community composition of the epimicrobiome, differences between even closely related host species or individuals are to be expected (Ross et al., 2019; Woodhams et al., 2020). Nevertheless, a certain degree of phylosymbiosis, in which the microbiota mirrors the phylogeny of the host (Brooks et al., 2016), is also observed (Ross et al., 2018). This may be due to changes in host traits, or host microbial co-evolution. Apart from humans, much of the epimicrobiome research has focused on captive animals and pets as well as amphibians whose skin is more permeable and thus more susceptible to pollution and novel pathogens, potentially threatening the survival of entire populations and species (Ross et al., 2019). However, very little is known about the epimicrobiomes of reptiles, especially turtles, including terrestrial, freshwater, and marine species. Recently, there have been studies on external microbial communities of freshwater turtles (*Trachemys scripta*, *Pseudemys concinna*, and *Emydura macquarii krefftii*) that identified major microbial components of eukaryotic, bacterial and archaeal surface communities and showed that turtles' microbiotas differ between body parts and between animals and their environment (McKnight et al., 2020; Parks et al., 2020). New knowledge about the functional and phylogenetic composition of epimicrobiomes of different species improves our understanding of the relationships between the host, its microbial flora, and the environment. Such advances in knowledge may contribute to a more efficient conservation of endangered and threatened macroorganisms. Skin microbiome research has a lot of potential in conservation biology of marine animals because of its accessibility and non-invasive sampling procedures. The potential of microbiome as bioindicator of ecosystem's health has been recognized and effort is being put into the standardization of the methodology – e.g., from sampling to correct index calculations (Lau et al., 2015; Aylagas et al., 2017; Keeley et al., 2018; Cordier et al., 2019). It is possible that surface-associated microbiomes exhibit a stronger link with variations in the environment, while the internal microbial communities are more affected by the host's intrinsic factors (Woodhams et al., 2020).

Although loggerheads are the most abundant sea turtle species in the Mediterranean Sea, they are threatened by coastal development, fishing bycatch, tourism, pollution and climate change (Casale et al., 2018). Skin and carapace of loggerheads provide habitats for a surprising variety of unique and taxonomically diverse macro-epibionts, including barnacles, amphipods and red algae (Hollenberg, 1971; Broderick et al., 2002; Frick and Pfaller, 2013). Some of these organisms require the sea turtle substratum to attach and thrive, and thus their survival is inextricably linked to the wellbeing and fitness of their hosts. The existing body of literature on loggerhead and other sea turtle microbiomes includes mainly studies investigating the internal microbiota, such as those living in the gut, cloaca, faces, and oral cavities (Abdelrhman et al., 2016; Arizza et al., 2019; Biagi et al., 2019; Scheelings et al., 2020a,b; Filek et al., 2021). The epimicrobiomes of sea turtles, in turn, have received far less attention. Recent years brought increased interest in

micro-eukaryotic surface assemblages of sea turtles largely due to a series of projects exploring the diversity of sea turtle-associated diatoms (Majewska et al., 2015; Robinson et al., 2016; Rivera et al., 2018; Azari et al., 2020; Kanjer et al., 2020; Van de Vijver et al., 2020). Those studies identified a group of the diatom core taxa typical of sea turtles but also showed some biogeographic differences between diatom epizoic assemblages (Van de Vijver et al., 2020). Besides inventorial and ecological interest in diversity of epi-microbiome, there is a possible benefit for sea turtles' health that could arise from these kinds of studies. For example, *Fusarium* spp. fungal infection of loggerhead eggs is considered a global threat (Bailey et al., 2018) and its detection on carapace and skin could be beneficial (Cafarchia et al., 2020). Further, evidence of antibiotic resistant bacteria found on sea turtles highlight the direct effect of antibiotic pollution in the seas (Pace et al., 2019; Alduina et al., 2020; Trotta et al., 2021). However, reports on bacterial, archaeal or micro-eukaryotic non-diatom communities associated with the skin and carapace of sea turtles are extremely scarce and include only a recent study by Blasi et al. (2022) that reported the composition of bacterial microbial community based on 16S rRNA gene profiling from the carapaces of three juvenile loggerheads from the Tyrrhenian Sea.

The aim of our study was to investigate the micro-eukaryotic, bacterial and archaeal diversity found on the external surfaces of loggerhead sea turtles from the Mediterranean Sea using the 18S and 16S rRNA genes amplicon sequencing approach, respectively. Furthermore, we aimed to describe both loggerhead skin and carapace microbial communities to allow for comparison between these two biochemically, micro-topographically, and physiologically different substrata. Detailed observation and statistical analyses of microbial assemblages' taxonomic composition were addressed in accordance to our large and diverse loggerhead dataset. The roles of potential factors that could influence the microbial communities are considered and additional approaches in studies of this type are discussed.

Materials and methods

Sampling

Twenty-six loggerhead sea turtles were sampled from four different Mediterranean areas: Adriatic (n = 14), Ionian (n = 9), Tyrrhenian (n = 1) and Aegean Sea (n = 2; **Figure 1**) following recommendations from Pinou et al. (2019). Two separate samples were collected from each turtle, one from the carapace and one from the skin (**Supplementary Figure 1**). Biofilm scrapings were taken using clean toothbrushes and/or a sterilized scalpel and were resuspended in 96% ethanol in sterile 50 ml conical tubes immediately after collection. Carapace samples were collected randomly from an entire carapace, whereas skin samples were taken from the animal head, neck,

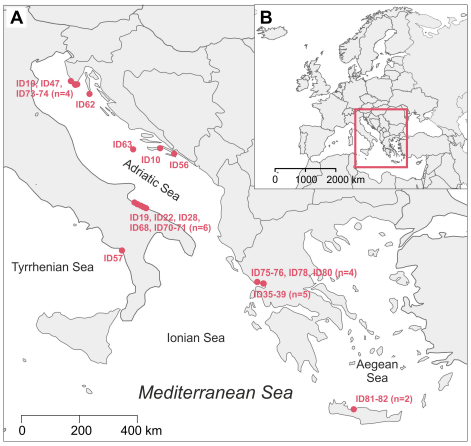

FIGURE 1
Map of the origin localities of sampled loggerhead turtles with indicated Turtle ID code **(A)**; position of our study area in map of Europe **(B)**. The map was made using R packages maps (Becker et al., 2021; RRID:SCR_019296) and mapdata (Becker et al., 2018).

and flippers. All samples were stored at −20°C until further processing. One turtle (ID010) was sampled twice: immediately upon arrival to the rescue center and after approx. one year in rehabilitation. In total, 54 samples were collected from August 2018 until November 2019 (**Table 1**). Due to the heterogeneity of sampled turtles, we differentiate a turtle's origin localities from "sampling locality" (**Table 1** and **Supplementary Table 1**). Origin locality is the location where a turtle was found in the sea or on the beach and the sampling locality refers to the place where samples were obtained. Origin locality and sampling locality is identical for the turtles sampled where they were found but differs for the turtles that were being brought to rehabilitation centers. The turtles were sampled in three rescue centers (Marine Turtle Rescue Center Aquarium Pula and Blue World Institute Lošinj in Croatia, and The Archelon Sea Turtle Protection Society in Greece) and one veterinary clinic (The Sea Turtle Clinic, STC, Department of Veterinary Medicine, University of Bari "Aldo Moro" in Bari, Italy). The sea turtle status was designated as "wild" if the turtle was sampled immediately after capturing without being immersed into the rehabilitation pool, and "admitted" if the animal was admitted to a rehabilitation center and was immersed in the rehabilitation pool prior to sampling. Time between the turtle admission and the sampling of its biofilm spans between 1 and 10 days (except for ID010).

DNA analysis

The DNA isolation and sequencing were performed in two batches, in 2019 (20 samples from ID10, ID19-39) and in 2020 (34 samples from ID10, ID47-82). The DNA was extracted from 0.25 g of an ethanol-free sample in duplicates

using the DNeasy PowerSoil kit (QIAGEN, RRID:SCR_008539). The extraction protocol followed the manufacturer's guidelines with several modifications (as described below). Samples were transferred into the PowerBead tubes and incubated in a sonicator at 50°C at 35 kHz for 15 min. The incubation times for C1, C2, and C3 solutions were extended (30 min at 65°C for C1 and 15 min at 4°C), and bead-beating was replaced with horizontal vortexing on IKA VXR basic Vibrax shaker (10 min at maximum speed of 2,200 rpm). The DNA was eluted with 50 µl of DNase-free molecular grade water (incubated at room temperature for 2 min). The quantity and purity of extracted DNA were measured by NanoDrop ND-1000 V3.8 spectrophotometer (Thermo Fisher). The extracted DNA samples were sent for 2 × 250 bp paired-end sequencing (Illumina MiSeq System, RRID:SCR_016379) of the 16S rRNA gene V4 region by 515F (5′-GTGYCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACNVGGGTWTCTAAT-3′) primers (Apprill et al., 2015; Parada et al., 2016), and the 18S rRNA gene V4 region by eukV4F (5′-CCAGCASCYGCGGTAATTCC-3′) and zigeukV4R (5′-ACTTTCGTTCTTGATYRATGA-3′) primers (Stoeck et al., 2010; Piredda et al., 2017) at Molecular Research MrDNA (Shallowater, TX, United States).

Sequence data processing and analysis

Sequences obtained from MrDNA were processed by FASTqProcessor (MrDNA), and all non-biological sequences were removed prior to exporting the data in QIIME2-readable format ("EMP protocol" multiplexed paired-end fastq format). The sequences were then imported to the QIIME2 (RRID:SCR_021258) environment, versions 2020.6 for 16S and 2021.4 for 18S (Bolyen et al., 2019). Demultiplexing of sequences was done by q2-demux plugin. DADA2 (q2-dada2 plugin) was used for sequence denoising (Callahan et al., 2016). A 18S rRNA sequences were truncated at 220 bp for forward and reverse sequences. Sequence alignment was performed with MAFFT (Katoh et al., 2002) and a phylogenetic tree was constructed with fasttree2 using q2-phylogeny (Price et al., 2010). Taxonomy was assigned to amplicon sequence variants (ASVs) via q2-feature-classifier (Bokulich et al., 2018) classify-sklearn naïve Bayes taxonomy classifier against the SILVA v.138 (99% 505F-806R nb classifier) (Quast et al., 2013) and PR2 4.13.0 (Guillou et al., 2013; del Campo et al., 2018) databases for 16S and 18S datasets, respectively. Prior to downstream analyses, mitochondria and chloroplast sequences were filtered from the 16S dataset, and metazoan and macroalgal sequences were filtered from the 18S dataset. For alpha and beta diversity analyses, we rarefied the 16S dataset to the sampling depth of 34 000 and the 18S dataset to 10 000 based on rarefaction curves (q2-diversity plugin). We calculated two alpha diversity indices via q2-diversity: observed ASVs (features) and Faith's phylogenetic diversity (PD) index (Faith, 1992) for both 16S and 18S datasets, and made visualizations using boxplots. Beta diversity was estimated using three distance matrices via q2-diversity: Bray-Curtis, weighted UniFrac (Lozupone et al., 2007) and robust Aitchison's distance (Aitchison and Shen, 1980; Aitchison and Ho, 1989; Martino et al., 2019). Principal Coordinates Analysis (PCoA) plots for Bray-Curtis and weighted UniFrac distance were produced using the q2-diversity plugin, and Robust Aitchison Principal Components Analysis (rPCA) was performed on non-rarefied data via DEICODE plugin (Martino et al., 2019). To compare the 16S and 18S datasets, we plotted the first principal coordinate (PC1) of each dataset's robust Aitchison's distance and performed the Procrustes analysis via q2-diversity. The permutational multivariate analysis of variance (PERMANOVA) with the q2-diversity plugin was used to test for significant differences between sample groups. Turtles from the Aegean and Tyrrhenian Seas were excluded from PERMANOVA calculations for "Origin Sea" due to the low number of samples in these two groups. For PERMANOVA statistic, turtles from Adriatic Sea were divided on East Adriatic (Croatian samples) and West Adriatic (Italian samples). PERMANOVA tested factor "Season" was obtained as following: samples obtained in spring and summer are put into "warm" category, while samples from autumn and winter are put into "cold" category. Data visualizations were made using ggplot2 (RRID:SCR_014601) (Wickham, 2016), phyloseq (RRID:SCR_013080) (McMurdie and Holmes, 2013), vegan (RRID:SCR_011950) (Oksanen et al., 2020), and pheatmap (RRID:SCR_016418) (Kolde, 2019) within R Studio (R Project for Statistical Computing, RRID:SCR_001905). The relative abundance of different groups of samples was calculated as a sum of the ASV count of selected taxon and then divided by the total sequence number in that sample group. Community composition was summarized by heatmaps (**Figure 2**) produced based on centered log-ratio (clr) transformed data from ASV counts.

Results

High throughput sequencing of 54 samples yielded 6,242,910 high quality 16S and 1,675,191 18S sequences. Median frequency per sample was 102,920.0 (min. 34,669.0; max. 257,399.0) for 16S while median frequency per sample for 18S was 20,048.5 (min. 1,634.0; max. 123,842.0). Sequences obtained for 16S and 18S were denoised to 17,636 ASVs and 1,917 ASVs, respectively (**Supplementary Tables 2–4**).

Community composition

Bacterial and archaeal microbiota

The microbial community showed the dominance of bacterial over archaeal taxa. The most abundant

TABLE 1 Turtle and sample information.

Turtle ID	Carapace sample ID	Skin sample ID	Origin sea	Origin locality	Sampling locality	Sampling date (DD.MM.YYYY.)	CCL (cm)	Turtle state
ID010	TB31	TB32	Adriatic Sea	CRO, Korčula	Pula Aquarium	11.12.2018.	n.a.	Admitted
	TB139	TB140	Adriatic Sea	CRO, Brijuni	Brijuni	04.11.2019.	69.7	Wild
ID019	TB49	TB50	Adriatic Sea	ITA, Barletta	Bari	09.01.2019.	50.7	Wild
ID022	TB55	TB56	Adriatic Sea	ITA, Barletta-Trani	Bari	10.01.2019.	72	Wild
ID028	TB73	TB74	Adriatic Sea	ITA, Barletta	Bari	17.01.2019.	74.5	Wild
ID034	TB89	TB90	Adriatic Sea	ITA, Bisceglie	Bari	22.01.2019.	72	Wild
ID035	GTB11	GTB12	Ionian Sea	GRE, Amvrakikos	Amvrakikos bay	01.08.2018.	78.6	Wild
ID036	GTB21	GTB22	Ionian Sea	GRE, Amvrakikos	Amvrakikos bay	01.08.2018.	51	Wild
ID037	GTB31	GTB32	Ionian Sea	GRE, Amvrakikos	Amvrakikos bay	01.08.2018.	69.6	Wild
ID038	GTB41	GTB42	Ionian Sea	GRE, Amvrakikos	Amvrakikos bay	01.08.2018.	58.5	Wild
ID039	GTB51	GTB52	Ionian Sea	GRE, Amvrakikos	Amvrakikos bay	01.08.2018.	53.2	Wild
ID047	TB115	TB116	Adriatic Sea	CRO, Kamenjak	Pula Aquarium	08.05.2019.	53.5*	Admitted
ID056	TB117	TB118	Adriatic Sea	CRO, Ston	Pula Aquarium	09.06.2019.	74.0*	Admitted
ID057	TB119	TB120	Tyrrhenian Sea	ITA, Maratea	Bari	24.06.2019.	77	Admitted
ID062	TB129	TB130	Adriatic Sea	CRO, Mali Lošinj	Lošinj	30.07.2019.	54	Wild
ID063	TB131	TB132	Adriatic Sea	CRO, Vis	Vis	10.06.2019.	24	Wild
ID068	TB145	TB146	Adriatic Sea	ITA, Molfetta	Bari	25.07.2019.	46.5	Admitted
ID070	TB149	TB150	Adriatic Sea	ITA, Molfetta	Bari	24.07.2019.	43	Admitted
ID071	TB151	TB152	Adriatic Sea	ITA, Margherita di Savoia	Bari	23.10.2019.	65.2	Wild
ID073	TB155	TB156	Adriatic Sea	CRO, Premantura	Pula Aquarium	20.11.2019.	32.2.	Admitted
ID074	TB157	TB158	Adriatic Sea	CRO, Ližnjan	Pula Aquarium	20.11.2019.	n.a.	Admitted
ID075	GTB61	GTB62	Ionian Sea	GRE, Amvrakikos	Amvrakikos bay	02.07.2019.	64.5	Wild
ID076	GTB71	GTB72	Ionian Sea	GRE, Amvrakikos	Amvrakikos bay	01.07.2019.	62.9	Wild
ID078	GTB91	GTB92	Ionian Sea	GRE, Amvrakikos	Amvrakikos bay	01.07.2019.	55.3	Wild
ID080	GTB111	GTB112	Ionian Sea	GRE, Amvrakikos	Amvrakikos bay	01.07.2019.	66.5	Wild
ID081	GTB121	GTB122	Aegean Sea	GRE, Rethimno	Rethimno bay	10.07.2019.	n.a.	Wild
ID082	GTB131	GTB132	Aegean Sea	GRE, Rethimno	Rethimno bay	14.07.2019.	n.a.	Wild

n.a., Indicates information not available; CCL, curved carapace length; asterisk (*) marks the straight carapace length (SCL) instead of curved carapace length (CCL).

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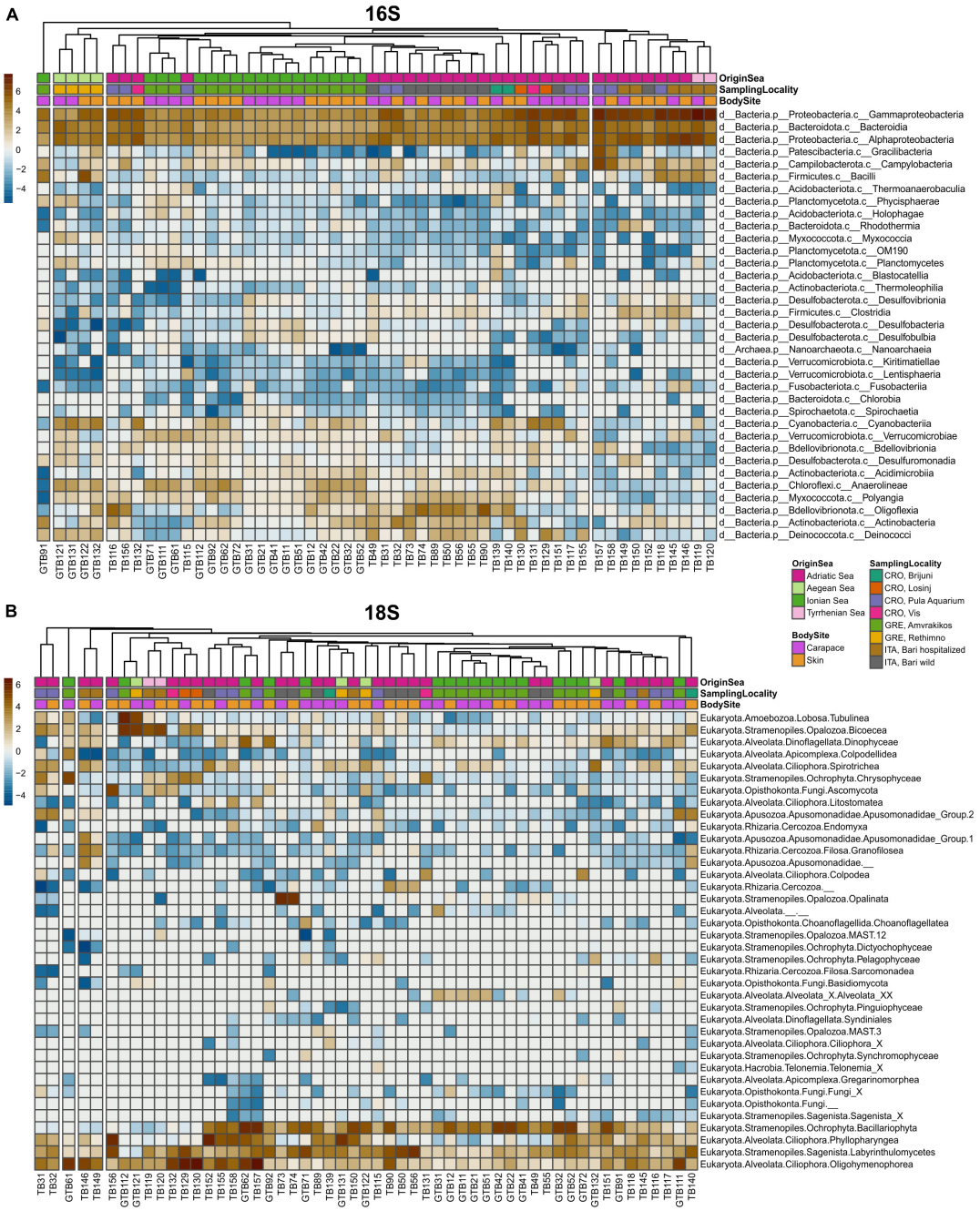

FIGURE 2
Heatmaps of surface microbiota of loggerhead sea turtles' skin and carapace samples for 16S **(A)** and 18S dataset **(B)**. Each column represents individual sample. Samples are color-coded by categories (Origin sea, Body site and Sampling locality) in the first three rows of each heatmap.

bacterial phylum in all samples was Proteobacteria, followed by Bacteriodota, Bdellovibrionota and Cyanobacteria (Figures 2, 3). Classes Gammaproteobacteria, Alphaproteobacteria and Bacteroidia dominated in all samples (Figure 2). Family *Rhodobacteraceae* was more abundant overall than any other bacterial family, followed by *Moraxellaceae* and *Pseudoalteromonadaceae* (Supplementary Table 5).

There were five core features (ASVs) identified in 100% of samples in the 16S dataset, four belonging to class Gammaproteobacteria and one to Oligoflexia (Supplementary Table 6). These features are classified as an uncultured bacterium from order Oligoflexales, genus *Pseudoalteromonas*, an unidentified ASV from class Gammaproteobacteria, an uncultured bacterium from

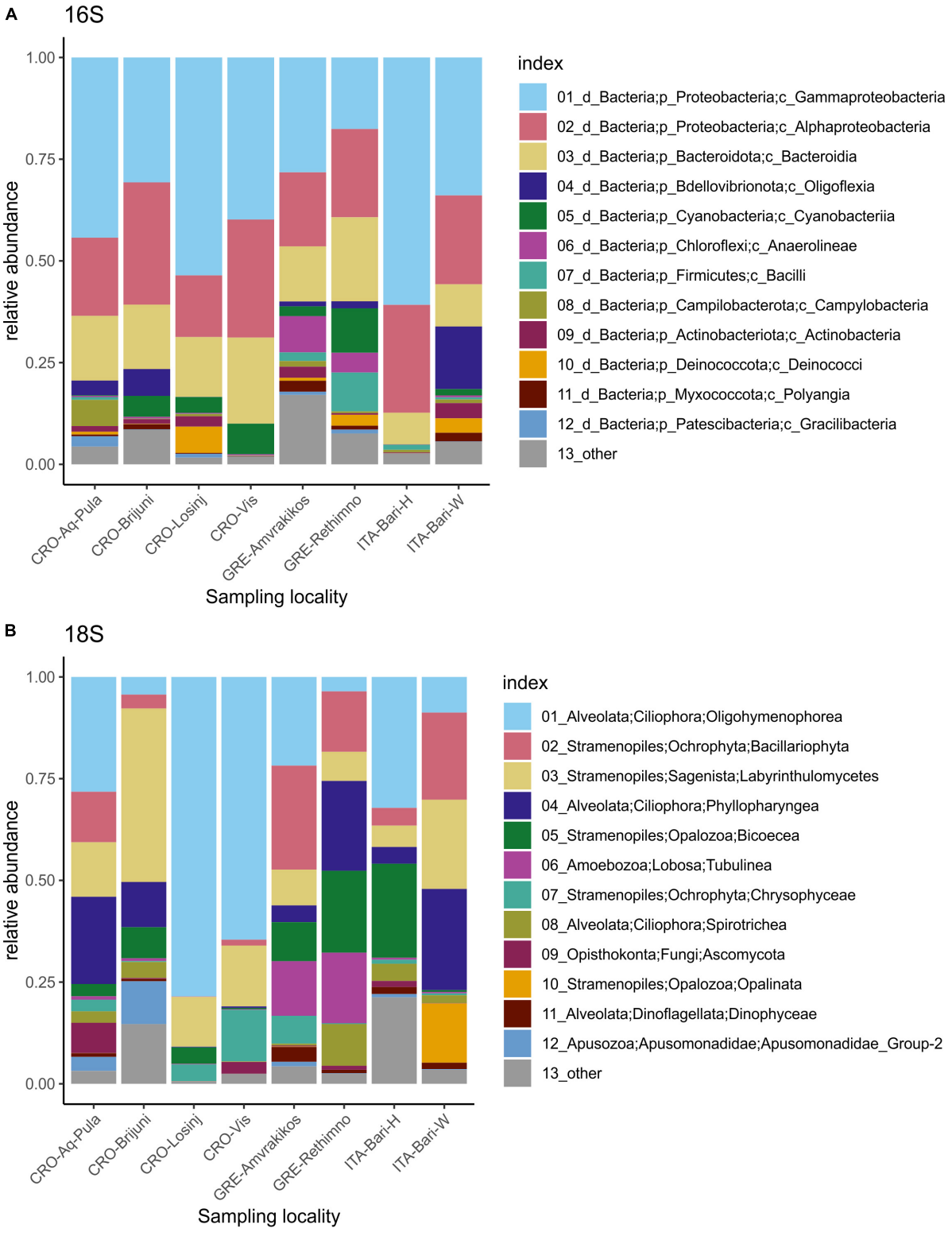

FIGURE 3
Relative abundances of 12 most abundant microbial taxa present on loggerhead sea turtles from the same sampling locality 16S dataset **(A)**, 18S dataset **(B)**.

family *Sedimenticolaceae*, and an uncultured bacterium from family *Saccharospirillaceae*. In carapace samples, additional five core features were identified in 100% of the samples classified as genus *Vibrio* (Gammaproteobacteria), BD1-7 clade (family *Spongiibacteraceae*, Gammaproteobacteria), an uncultured bacterium from family *Arcobacteraceae* (Campylobacteria), genus *Deinococcus* (Deinococci), and genus *Halarcobacter* (Campylobacteria). In skin samples, additional seven core features were identified and classified as an uncultured bacterium from family *Nannocystaceae* (Polyangia, Myxococcota), family *Rhodobacteraceae* (Alphaproteobacteria), an uncultured bacterium from genus *Psychrobacter* (Gammaproteobacteria), an uncultured bacterium from genus *Ahniella* (Gammaproteobacteria), genus *Tenacibaculum* (Bacteroidia), family *Stappiaceae* (Alphaproteobacteria), and genus *Poseidonibacter* (Campylobacteria).

Within the 16S dataset, Cyanobacteria were the most abundant photoautotrophic prokaryotes, with *Phormidesmiaceae* and *Paraspirulinaceae* being the most abundant families (**Figure 4**). *Phormidesmiaceae* and *Xenococcaceae* dominated in carapace samples, whereas *Paraspirulinaceae* and *Phormidesmiaceae* were most abundant in skin samples. Many of the detected cyanobacterial sequences remained unclassified (**Figure 4A**, pink bars). On average, Cyanobacteria comprised 3% of all ASV sequences. In individual samples, this group accounted for 0.01–19.77% of all sequences (**Figure 4B**).

Eukaryotic microbiota

The most abundant supergroups of micro-eukaryotes in the dataset were Alveolata and Stramenopiles. The dominant classes included Oligohymenophorea, Bacillariophyta, Labyrinthulomycetes, and Phyllopharyngea. The class Opalinata was highly prevalent in samples TB73 and TB74 (turtle ID28). Samples TB119 (carapace) and TB120 (skin) (from turtle ID57, the only animal sampled in the Tyrrhenian Sea) were dominated by Biocoeca. A high abundance of Ascomycota (Fungi) was recorded in the skin sample TB156 (Adriatic Sea), while *Chrysophyceae* were particularly abundant in the carapace sample GTB61 (Ionian Sea).

One core feature, belonging to the genus *Zoothamnium* (Oligohymenophorea, Ciliophora), was identified in all samples within the 18S dataset. An additional core feature of the carapace samples was found to be *Nitzschia communis* (Bacillariophyta). No additional core features were shared by all skin samples (**Supplementary Table 6**).

At the genus level (level 7 in the PR2 database), apart from the above-mentioned *Zoothamnium*, a taxon assigned to the level of "Raphid-pennate" group (Bacillariophyta) was found in all biofilm samples. In all carapace samples, *Nitzschia* (Bacillariophyta) and *Labyrinthula* (Labyrinthulomycetes) were identified as additional core genera. In 95% of all biofilm samples, the following core features were identified at the genus level: "Raphid-pennate" group (Bacillariophyta), *Zoothamnium* (Oligohymenophorea, Ciliophora), *Nitzschia* (Bacillariophyta) and *Labyrinthula* (Labyrinthulomycetes). In 95% of carapace samples, an additional core genus, *Caecitellus* (Opalozoa), was found. Four additional core genera were detected in 95% of skin samples: *Thraustochytrium* (Labyrinthulomycetes), *Uronema* (Oligohymenophorea), *Labyrinthulaceae* X (Labyrinthulomycetes), and *Fistulifera* (Bacillariophyta).

Alpha diversity

Alpha diversity indices for community richness (observed ASVs) and diversity [Faith's Phylogenetic Diversity (PD) index] were highly variable and ranged from 127 to 2,833 (richness) and from 12.68 to 135.65 (diversity) for the bacterial community (**Figures 5A,B**). Bacterial communities from turtles in different seas and from different body sites ("Origin Sea" and "Body Site" categories as shown in **Table 1**) showed significant differences (Kruskal-Wallis H test, $p < 0.05$). Within the 16S dataset, carapace samples showed higher median values of richness [1.032; interquartile range (IQR) = 750.0] and diversity (52.23; IQR = 40.11) than skin samples (richness 811; IQR = 386.5 and diversity 46.47; IQR = 20.64). The highest median values of bacterial Faith's PD were observed for samples from the Ionian Sea (75.36; IQR = 23.37), followed by the Aegean (59.39; IQR = 16.10) and Adriatic Seas (42.17; IQR = 18.92). The lowest Faith's PD values were recorded for the hospitalized turtle ID057 from the Tyrrhenian Sea (20.69; IQR = 1.77).

Microbial eukaryotes' community ASVs richness ranged from 44 to 197, and diversity values ranged from 9.47 to 25.53 (**Figures 5C,D**) which is considerably lower comparing to the prokaryotes. The highest median value of micro-eukaryotic ASV richness was observed for the Adriatic Sea (124.5; IQR = 50.25), followed by Ionian Sea (112; IQR = 42.00), Aegean Sea (89.5; IQR = 13.00), and Tyrrhenian Sea (82; IQR = 11.00). The highest median value of micro-eukaryotic Faith's PD was observed for Ionian Sea (17.11; IQR = 6.75), followed by Adriatic Sea (16.91; IQR = 6.00), Tyrrhenian Sea (15.76; IQR = 1.33) and Aegean Sea (15.35; IQR = 2.36), similar to the bacterial communities. Carapace microbial eukaryotes showed higher median values of ASVs richness (118; IQR = 44) and diversity (17.13; IQR = 3.61) than skin samples (richness 93; IQR = 58 and diversity 14.75; IQR = 6.75); however, no significant differences between the different seas or body sites were observed.

Beta diversity

Principal Components Analyses of robust Aitchison distance (rPCA) indicate groupings based on sampling locality and body site for prokaryotes (**Figure 6A**) and eukaryotes

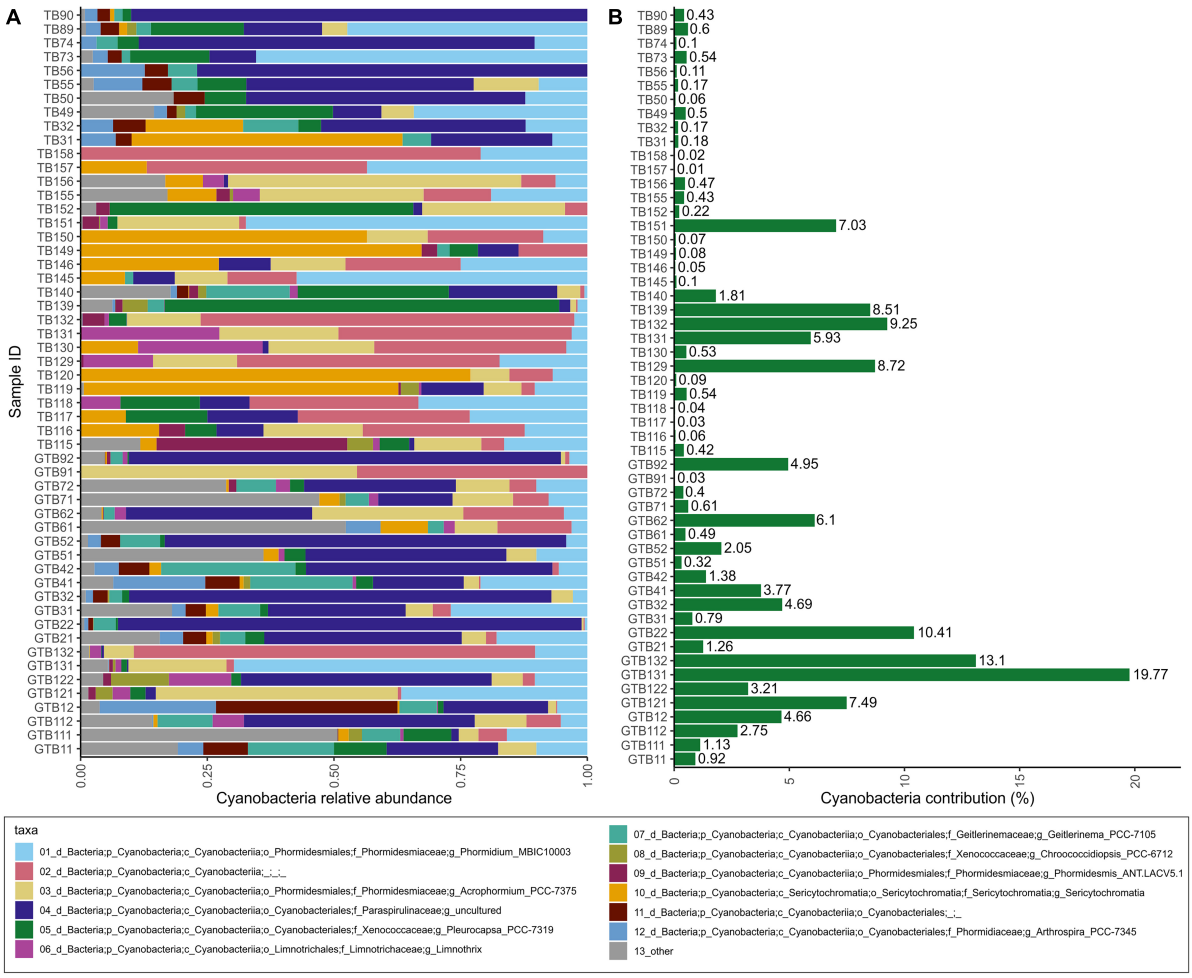

FIGURE 4
Relative abundances within the cyanobacterial group **(A)**; contribution of Cyanobacteria to total bacterial community within a sample based on relative abundance **(B)**.

(Figure 6B). For the 16S dataset (Figure 6A) we can observe groupings based on sampled body site (carapace on the right and skin on the left) and sampling locality. ASVs that drive those groupings belong to uncultured Oligoflexales, Nannocystales, *Rhodobacteraceae*, *Saccharospirillaceae*, *Pseudoalteromonas*, and *Vibrio*. PERMANOVA results (Table 2) show that there is a significant difference ($p < 0.05$) between the bacterial and archaeal communities of skin and carapace body sites the seas of origin (only Adriatic and Ionian), turtle state (wild vs. admitted), and sampling season (warm vs. cold). The only non-significant value was detected between body site groups for unweighted UniFrac. The highest pseudo-F values for all distance matrices were observed between "Origin Sea" categories.

For the 18S dataset (Figure 6B) we cannot observe clear groupings based on body site but there is an indication of samples grouping based on sampling locality. ASVs that drive the sample distribution for micro-eukaryotic communities (Figure 6B) belong to Ciliophora (*Zoothamnium* sp., Sessilida, *Uronema marinum*, *Uronema nigricans*, *Ephelota gigantea*, *Aspidiscida steini*), *Nitzschia communis* (Bacillariophyta) and *Cafeteria roenbergensis* (Bicoecea). PERMANOVA of eukaryotic communities showed no significant differences between sampled body sites. Significant differences between origin seas and turtle states were observed for Robust Aitchison and Bray-Curtis distances. According to all but one distance metrics tested, PERMANOVA showed a significant difference between sampling seasons (Table 2).

To gain insight into the whole epi-microbiome (bacterial, archaeal and eukaryotic) we combined the principal components (PC1s) of the rPCA for 16S and 18S datasets where clear groupings based on sampling locality can be distinguished (Figure 7). To compare and detect any congruence between the bacterial and eukaryotic communities the rPCA ordinates of both datasets were compared by the Procrustes analysis (Supplementary Figure 2) which showed the bacterial, archaeal and eukaryotic dataset congruence is low ($m^2 = 0.93095$, $p = 0.043$). Additional PCoA and rPCA ordinations are performed in order to visualize grouping of samples based on categories "Sampling locality"

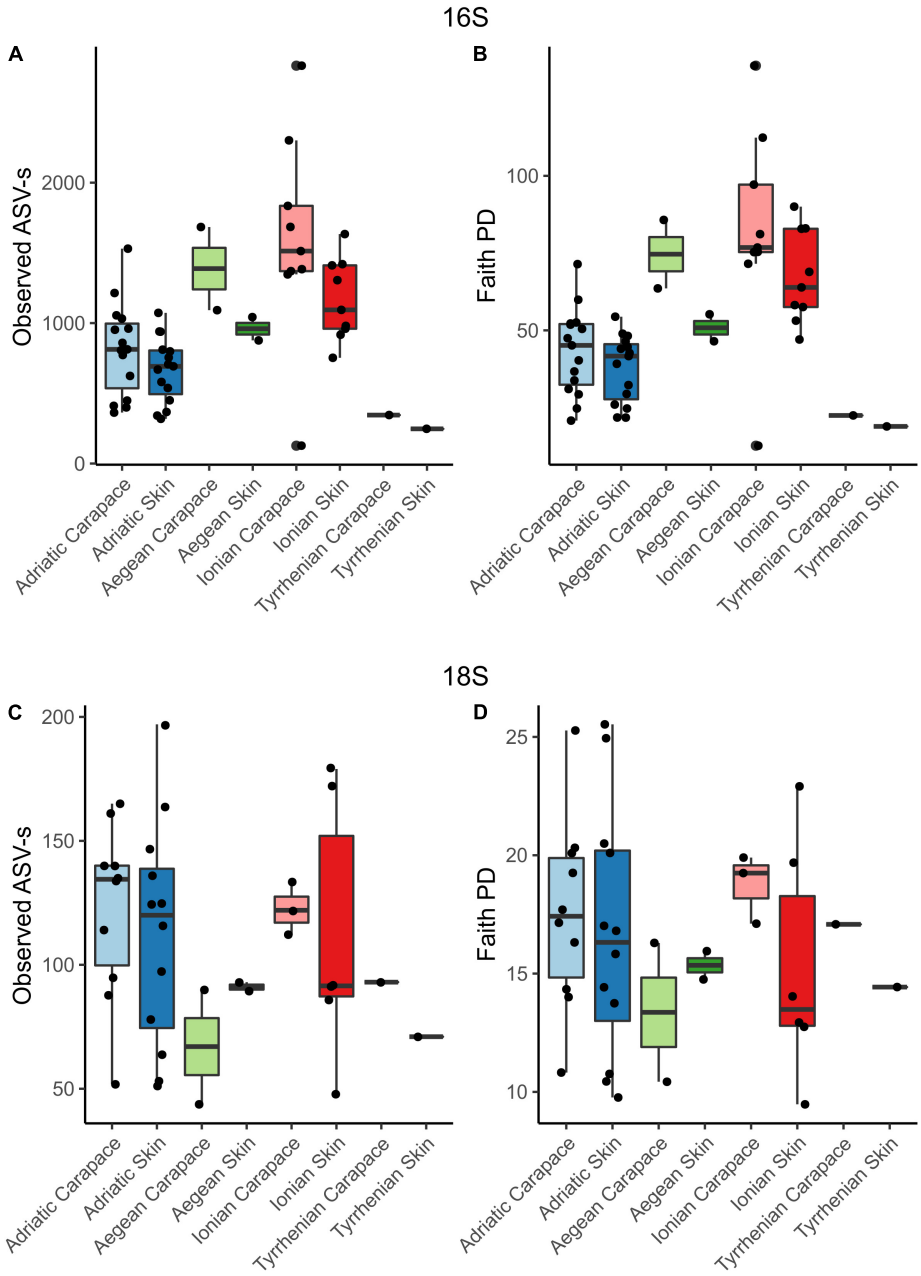

FIGURE 5
Observed ASV richness and Faith's phylogenetic diversity index for 16S and 18S datasets for skin and carapace microbiome of loggerhead sea turtles sampled at four locations in the Mediterranean. Bar colors are paired and represent locations: blues, Adriatic; greens, Aegean; reds, Ionian; and no color, Tyrrhenian.

(**Supplementary Figure 3**), "Origin sea" (**Supplementary Figures 4, 5**), "Season" (**Supplementary Figures 6, 7**), and "Turtle state" (**Supplementary Figures 8, 9**).

Discussion

In this study we provide insights into the epi-microbiota of loggerhead sea turtles using a combined 16S and 18S metabarcoding approach. Our results show that overall bacterial microbiota is dominated by a few classes of bacteria (Gammaproteobacteria, Alphaproteobacteria and Bacteroidia) and that the communities may differ depending on multiple extrinsic and intrinsic factors, which has been previously described in studies on other aquatic animals (as reviewed in Apprill, 2017). On the other hand, in spite of eukaryotic microbiota showing high heterogeneity, core taxa such as Oligohymenophorea, Bacillariophyta, Labyrinthulomycetes, and Phyllopharyngea were commonly present in the majority of samples. Despite the sampled turtles

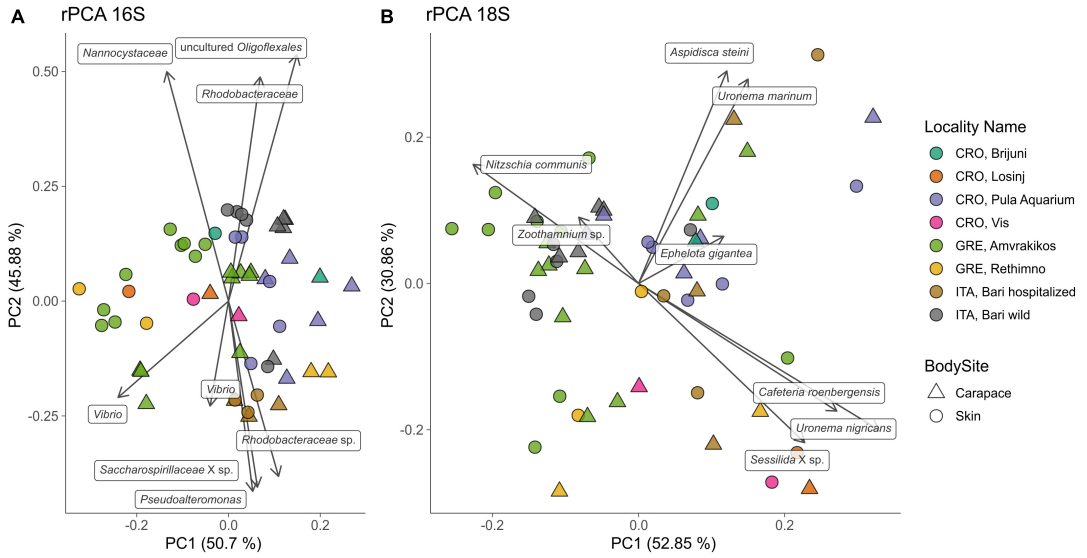

FIGURE 6
Principal component analysis (PCA) biplot of robust Aitchison distance for bacterial and archaeal (16S, **A**) and eukaryotic diversity (18S, **B**); arrows indicate individual highly ranked ASVs that contribute to the displayed positions of the samples; lowest taxonomic assignment of each ASV is written in textboxes at the end of each arrow. Sampling locality are indicated by color, body sites are indicated by shape.

TABLE 2 Permutational multivariate analysis of variance (PERMANOVA) for Bray-Curtis, Robust Aitchison, unweighted and weighted UniFrac distance metrics.

16S	**Bray-Curtis**		**Robust Aitchison**		**Unweighted UniFrac**		**Weighted UniFrac**	
	Pseudo-F	***P*-value**	**Pseudo-F**	***P*-value**	**Pseudo-F**	***P*-value**	**Pseudo-F**	***P*-value**
Body site (carapace vs. skin)	2.542	**0.002****	7.64	**0.002****	1.472	0.066	2.617	**0.009****
Sea (E Adriatic vs. W Adriatic vs. Ionian Sea)	4.212	**0.001****	13.269	**0.001****	4.320	**0.001****	6.326	**0.001****
Season (warm vs. cold)	3.106	**0.001****	12.987	**0.001****	2.364	**0.005****	3.717	**0.003****
Turtle state (wild vs. admitted)	4.486	**0.001****	9.583	**0.001****	4.65	**0.001****	9.918	**0.001****
18S	**Bray-Curtis**		**Robust Aitchison**		**Unweighted UniFrac**		**Weighted UniFrac**	
	Pseudo-F	***P*-value**	**Pseudo-F**	***P*-value**	**Pseudo-F**	***P*-value**	**Pseudo-F**	***P*-value**
Body site (carapace vs. skin)	0.745	0.849	0.61	0.617	0.738	0.806	0.919	0.469
Sea (E Adriatic vs. W Adriatic vs. Ionian Sea)	1.466	**0.036***	5.746	**0.001****	1.240	0.163	1.456	0.099
Season (warm vs. cold)	1.721	**0.013***	0.688	0.568	1.978	**0.022***	2.280	**0.018***
Turtle state (wild vs. admitted)	1.822	**0.011***	6.723	**0.002****	1.472	0.099	1.647	0.088

Significance levels are indicated by an asterisk: *$p \leq 0.05$, **$p \leq 0.01$ with all significant values bolded.

coming from different locations in the Mediterranean Sea, varying in age and health conditions, and being sampled in different seasons forming a diverse dataset, it is clear that several of the tested factors influenced their surface microbial community composition. Bacterial communities seem to be affected by the locality of origin, body site, turtle state, and sampling season while the eukaryotic microbiota followed a similar pattern, although to a lesser extent, and without detected differences between body sites.

The highest microbial diversity was observed on Ionian turtles from the lagoonal complex of the Amvrakikos Gulf, that is one of the most important and productive lagoonal complexes in Greece (Rees et al., 2013). The lagoonal shallow coastal aquatic systems, with a maximum depth of 65 m, are separated from the sea by sediment barriers and connected to it through channels, often characterized by salinity fluctuations and development of low dissolved oxygen conditions (Kapsimalis et al., 2005; Ferentinos et al., 2010). While the Amvrakikos Gulf offers a rich neritic foraging ground for subadult and adult

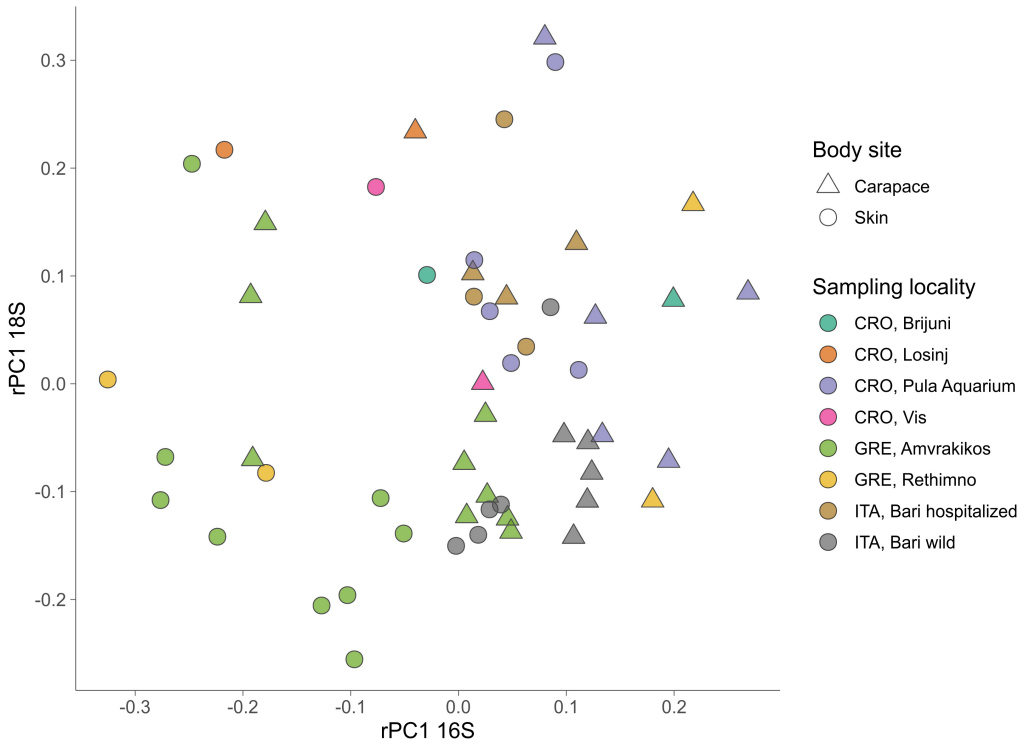

FIGURE 7
Combination plot of the first principal component (PC1) of PCAs based on robust Aitchison distance matrix for 16S (*x*-axis) and 18S (*y*-axis). Sampling locality are indicated by color, body sites are indicated by shape.

loggerheads (Rees et al., 2013), the second locality with highest diversity is the Rethimno bay in northern Crete (Greece) that is an important nesting site for adult females (Margaritoulis and Rees, 2011). The Tyrrhenian Sea has shorter continental shelf than the Adriatic Sea (Cognetti et al., 2000) and possibly lower availability of the rich benthic environment as a source of microbes which could colonize the loggerhead's body. That could explain lower diversity and richness of turtle-associated microbial communities from the Tyrrhenian Sea.

Bacterial diversity and richness of carapace samples was consistently higher than those of skin samples which could be explained by the large and rigid surface of the carapace covered by keratinous scutes that could allow for easier attachment and colonization of diverse microbes. Compared to the carapace, the skin of the neck and flippers (sampled in this study) is prone to higher mechanical disturbance caused by the turtle's movements. Parks et al. (2020) reported a higher diversity and richness of microbial communities on the freshwater turtles' carapace in comparison to the plastron, and provide the movement of the turtles as one of the possible explanations. Furthermore, Blasi et al. (2022) reported significant differences between microbial communities of differently positioned carapace scutes. The difference in bacterial community composition of anterior and posterior scutes of the sea turtle carapace might have been caused by different abiotic (hydrodynamics or sun exposure) and biotic factors (uneven distribution of macroorganisms across the carapace) affecting those areas (Blasi et al., 2022). The epimicrobiota of three juvenile loggerheads from the Tyrrhenian Sea harbored Firmicutes and Proteobacteria as the most prevalent phyla (Blasi et al., 2022). Contrastingly, in our dataset Proteobacteria were found to be the most abundant while Firmicutes were not among the highly abundant phyla. The most abundant bacterial family was *Rhodobacteraceae* which is known to be widely distributed in marine benthic habitats (Pohlner et al., 2019). Although the metabolic diversity within *Rhodobacteraceae* is great, they are mainly aerobic photo- and chemoheterotrophs, and purple non-sulfur bacteria that are known for anaerobic photosynthesis (Pujalte et al., 2014). Interestingly, we observed uncultured members of *Psychrobacter* and *Tenacibaculum* genera on all of the skin samples which were also reported as a part of the core microbiome on the humpback whales (Bierlich et al., 2018). This raises a question about *Psychrobacter* and *Tenacibaculum* genera members' dependence on animal skin metabolites, possibly making them mutualistic or commensal to marine animals. It is worth mentioning that some species of *Tenacibaculum* are known as pathogens on fish skin (Nowlan et al., 2020), however, we cannot be certain of the exact ecological role on the turtle skin without further research.

The micro-eukaryotic taxa that were dominant in the majority of samples, ciliates Oligohymenophorea and Phyllopharyngea (Alveolata), are mainly free-living heterotrophs that could possibly graze on the other microbes colonizing the turtles' surfaces. Commonly found Stramenopiles were mostly represented by diatoms (Bacillariophyta) and Labyrinthulomycetes (marine fungus-like organisms that produce filamentous webs for nutrient absorption). Diatoms are photosynthesizing microalgae with characteristic silica shells (Round et al., 1990) known for being among the first colonizers of submerged surfaces including marine vertebrates (Hooper et al., 2019). Our results show that diatoms are one of the major micro-eukaryotic groups present on sea turtles' bodies and the most dominant phototrophs in those communities. Contrary to bacterial community, differences between carapace and skin community were not detected for micro-eukaryotes. This is also not in congruence with the morphological study on diatoms from loggerheads of where they reported higher diversity and richness of carapace than in skin diatom community. Common epiphytic and epipelic diatom genera were found in abundance on carapace while putatively epizoic taxa were dominating in skin diatom samples (Van de Vijver et al., 2020).

Light availability on the sea turtle surfaces enables the development of phototrophic microbes that cannot be found as a part of the endozoic microbiome. Moreover, unlike endozoic microbial communities which are dependent on nutrient inflow from the host, epizoic communities are probably dependent mostly on the nutrients available in the surrounding environment and from the primary producers in those communities. The microalgae and cyanobacteria, i.e., main phototrophic taxa in the epizoic biofilms are usually firmly attached and embedded in thick extracellular organic matrix. However, protozoans and metazoan grazers successfully adapted to feed on biofilm-dwelling microalgae and cyanobacteria that forms a strong trophic intra-biofilm link between primary and secondary producers (Weitere et al., 2018). The most studied primary producers associated with sea turtles are diatoms (Majewska et al., 2015). Recent morphology-based studies on turtle-associated diatoms revealed that they are highly abundant, diverse, and that there are several putative obligate epizoic diatom taxa (Robinson et al., 2016; Rivera et al., 2018; Van de Vijver et al., 2020). Besides diatoms, photoautotrophic *Chrysophyceae* and *Dinophyceae* were detected in noticeable abundances, and Chrysophyte stomatocysts of unknown species were previously reported on the sea turtle carapace (Pang et al., 2021). Additionally, Labyrinthulomycetes that were the third most abundant taxon in our samples are known to be mainly decomposers or, rarely, parasitic (Tsui et al., 2009) with recently emphasized importance in carbon sequestration (Bai et al., 2021). Either turtle- or microbe-derived particulate carbon (photoautotrophs or heterotrophs) could provide Labyrinthulomycetes with significant amounts of energy sources leading to their high relative abundance across samples.

Bacterial photoautotrophic communities are dominated by Cyanobacteria with the most common in our samples being filamentous cyanobacteria like *Phormidium* and *Leptolyngbya* (*Acrophorium*), both known for cyanotoxin production (Frazão et al., 2010; McAllister et al., 2016; Li et al., 2019). It has been observed that cyanobacterial toxic compounds can interfere with composition and function of animal intestinal microbiome (Duperron et al., 2019; Li et al., 2019; Sehnal et al., 2021). Blasi et al. (2022) also highlighted the presence of Cyanobacteria on anterior scutes, specifically families *Pseudanabenaceae* and *Rivulariaceae*. However, *Phormidesmiaceae*, *Paraspirulinaceae* and *Xenococcaceae* were prevalent in our dataset. All reported cyanobacterial genera in our study are commonly found in marine benthic habitats forming colonies and cyanobacterial mats (Komárek et al., 2014). Sea turtles seem to provide additional surfaces for cyanobacterial colonization and could act as a highly mobile reservoir with unknown implications for the host's health and effects on the environment.

It should be noted, however, that observed significant differences in multiple groupings of microbial communities in this study could be explained by overlapping metadata categories (e.g., wild animals being sampled mostly in Greece and during summer months) that could not be controlled for within our study design due to the unpredictability/stochasticity of opportunistic sampling. Additionally, reference databases play an important role in investigating microbial eukaryotes, as we cannot grasp the full diversity of micro-eukaryotes through metabarcoding alone because of a lack of sequenced representatives and eukaryotes often being overlooked as a part of microbial communities (Lind and Pollard, 2021 and references therein). In our study, a major portion of the cyanobacterial ASVs could not be properly identified via metabarcoding as the current version of SILVA reference database taxonomy is based on Bergey's Manual of Systematic Bacteriology (Boone et al., 2001) in which cyanobacterial taxonomy higher than genus is not defined. Therefore, SILVA and Genome Taxonomy Database (GTDB) (Quast et al., 2013) proposed their own names for some taxa based on 16S rRNA phylogeny that is not in agreement with the currently valid cyanobacterial taxonomy in the CyanoDB database (Komárek et al., 2014). As microbial eukaryotes and cyanobacteria are an important part of microbial communities associated with Mediterranean loggerhead sea turtles, further efforts in their characterization are needed to reconcile multiple taxonomy databases and better understand the turtle-associated taxa and their possible effects on the host.

The Mediterranean loggerheads are widely distributed large hard-shelled top predators, and a highly migratory species which occupies different marine habitats at different life stages. Their major ecological role in bioturbation, energy flow, trophic status, mineral cycling, soil dynamics and connectivity between habitats makes it a keystone species in Mediterranean marine environment (Casale et al., 2018). This research brings us one

step closer to much needed understanding of the complexity of microbial communities associated with loggerheads and wild animals in general. We show in this study that microbial communities of loggerhead sea turtles are rich and highly diverse with reservoirs of microbial taxa potentially important both for turtles' and the ecosystem's state. Moreover, DNA-based surveys focusing on epizoic bacterial, archaeal and eukaryotic microbiota could prove to be a valuable addition to non-invasive methods for monitoring the status of endangered marine species and their environment.

Data availability statement

The amplicon sequence data are deposited in the European Nucleotide Archive (ENA) under accession numbers PRJEB51458 for 16S and PRJEB51472 for 18S.

Ethics statement

Sampling was performed in accordance with the 1975 Declaration of Helsinki, as revised in 2013 and the applicable national laws. The sampling at the Sea Turtle Clinic (Bari, Italy) was conducted with the permission of the Department of Veterinary Medicine Animal Ethic Committee (Authorization # 4/19), while sampling in Croatia was done in accordance with the authorization of the Marine Turtle Rescue Centre by the Ministry of Environment and Energy of the Republic of Croatia. Sampling activities in Greece were carried out with permission from the Hellenic Ministry of Agriculture and Environment.

Author contributions

SB, RG, and RM designed the study. LK, KF, AT, MC, AD, AP, and SB collected the samples. KF and MM carried out the laboratory work. LK and KF conducted the bioinformatics, statistical analyses, and data visualization and interpretation. LK wrote the first draft of the manuscript. SB conceived project and obtained the funding. All authors revised the manuscript and approved the final version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.907368/full#supplementary-material

SUPPLEMENTARY TABLE 1
Metadata table adapted for input in QIIME2, with information about samples and sampled sea turtles.

SUPPLEMENTARY TABLE 2
Sequencing results (raw counts) and taxonomy assignments of 16S dataset per ASV for all samples in this study.

SUPPLEMENTARY TABLE 3
Sequencing results (raw counts) and taxonomy assignments of 18S dataset per ASV for all samples in this study.

SUPPLEMENTARY TABLE 4
Sequencing results (raw counts) and taxonomy assignments of filtered cyanobacterial sequences of 16S dataset per ASV for all samples in this study.

SUPPLEMENTARY TABLE 5
Relative abundance and absolute sequence count of bacterial and archaeal families found on skin and carapace of loggerhead sea turtles.

SUPPLEMENTARY TABLE 6
Core features of loggerhead sea turtles from 16S to 18S amplicon sequencing dataset.

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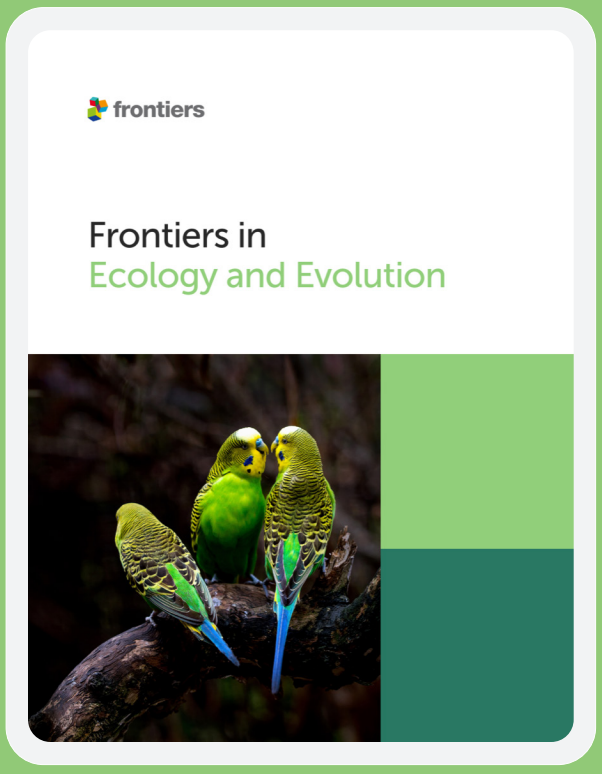

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