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ORIGINAL ARTICLE

Disentangling eukaryotic biodiversity patterns from man-made environments (port and marina) and nearby coral reefs in the Red Sea: A focus on the surveillance of non-indigenous species

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Abstract

Man-made environments such as ports and marinas are gateways for many marine non-indigenous species (mNIS) transported via shipping worldwide. These habitats are often the focus of biosecurity programs for the surveillance of mNIS, but there have been few studies investigating the distribution of biofouling communities in natural habitats and nearby artificial habitats. Here, following a DNA metabarcoding approach, we tested for differences in spatio-temporal trends of biological pioneer communities established after one-week colonization between a port, a marina, and coral reefs using two different matrices (PVC panels and water samples) in the central Red Sea. In addition, we aimed to investigate differences in the prevalence of mNIS between man-made and natural habitats during the summer and winter seasons, and whether the number and patterns of mNIS differed between the port (an active historic port subjected to intense shipping traffic) and the marina (a small recently developed marina utilized by small and medium research vessels). The community structure from samples collected at the two coral reefs showed greater similarity to each other compared to that obtained from the port and the marina. A total of 29 mNIS were detected across all sites, of which 16 were reported in the Red Sea for the first time, with Jeddah port hosting 28 mNIS. By applying standardized methodological approaches, this study provides comparable data regarding the monitoring of introduced and invasive species, particularly within the data-poor Red Sea, a major shipping corridor. This study serves as the foundation for implementing a robust and rapid baseline monitoring system to assess native biodiversity and facilitate the early detection of NIS.

KEYWORDS

biosecurity, coral reefs, invasive species, man-made environments, metabarcoding

Eva Aylagas and Hailey Shchepanik should be considered joint first author.

1 | INTRODUCTION

Globalization and the associated international trade and transport have driven an increase in the spread of non-indigenous species in the marine environment via ship ballast water and hull fouling (Rey et al., 2019). Marine non-indigenous species (mNIS) can establish outside their native biogeographic range and become invasive, potentially threatening the health and function of marine ecosystems by disrupting native populations (Piola & Johnston, 2007). Once mNIS become invasive in a new environment, eradication efforts are not only costly but also of limited success (Carvalho et al., 2023; Ojaveer et al., 2015), highlighting the importance of early detection initiatives and robust scientific advice proactively safeguarding marine environments from biological invasion (Borrell et al., 2017; Leblanc et al., 2020; Miralles et al., 2016). Biosecurity action plans require comprehensive biodiversity assessments to provide a thorough description of the species present in an area and the subsequent surveillance of mNIS. Traditionally, the methods used for such assessments, including mNIS monitoring, are based on the morphological taxonomic identification of specimens manually isolated from environmental samples or detected during underwater visual surveys (von Ammon et al., 2018b). However, these methods may lack the resolution required to identify early life stages (e.g., eggs, larvae, and immature individuals), broken or degraded organisms, and cryptic or morphologically indistinguishable species, which are commonly found in ballast water and early biofouling assemblages (von Ammon et al., 2018a).

In recent years, methodologies based on the detection of DNA present in the environment, known as environmental DNA (eDNA), have been increasingly applied in biosecurity monitoring programs as complementary and cost-effective methods for the surveillance of invasive species (Bowers et al., 2021; Clarke et al., 2023; Rees et al., 2008). DNA-based methods are non-disruptive and do not rely on taxonomic expertise, which, when coupled with advancements in high-throughput sequencing techniques, can increase the speed, accuracy, and sensitivity of characterizing biological assemblages across different trophic levels and the early detection of mNIS. Molecular methodologies have been extensively used for the assessment of mNIS in different regions using a wide range of sampling approaches, in combination or alone, such as PVC panels (Rey et al., 2019), the collection of biofouling samples from permanent artificial structures (Pearman et al., 2021; von Ammon et al., 2018a), and water (Borrell et al., 2018) or sediment (Holman et al., 2019). Although these studies were successful at the detection of non-indigenous species, those that assessed the performance of different sampling matrices found significant differences among the communities detected (Holman et al., 2019; Rey et al., 2019).

Most efforts dedicated to investigating the presence of mNIS in coastal areas using DNA-based techniques have focused on man-made environments, such as ports and marinas. These environments may serve as a path for the secondary spread of mNIS, introduced via shipping activities, including ballast water exchange and biofouling (Molnar et al., 2008). For this reason, monitoring ports and marinas on a regular basis is essential for the early detection of potential mNIS and the timely implementation of management measures to prevent the further spread of NIS (Ricciardi et al., 2017). While mNIS have shown a competitive advantage over native species at artificial sites, which are usually subject to transient and/or persistent pollution and increased disturbance (Piola & Johnston, 2007), it has been suggested that the native biodiversity of recipient communities may confer resistance to invasions. Miralles et al. (2016) described this phenomenon as "biotic resistance," in which diverse and mature communities, such as those inhabiting coral reef ecosystems, might offer more resistance to invasions than impoverished communities, such as those present in degraded environments typical of ports. In this context, information regarding the occurrence of mNIS in natural ports and marinas may assist in improved risk assessment and mNIS management strategies (Zaiko et al., 2018). In addition to the differential habitat preference of mNIS, the timing of newly introduced substratum may strongly influence the pattern and rate of initial succession in biofouling communities. Based on earlier studies, the most accurate identification and quantification of mNIS occurs after approximately 5 days of colonization (Zaiko et al., 2016). Although these patterns are well understood in man-made environments, the spatial and temporal patterns of mNIS in natural habitats remain understudied.

To date, information on the presence of mNIS in the Red Sea is very limited. This is in part due to the lack of efforts for the surveillance of these species but also due to the limited biodiversity baseline information in the area. In this study, we aim to provide a baseline characterization of biofouling communities established in artificial substrates from a port, a marina, and nearby coral reefs and to identify potential mNIS in the Red Sea using molecular-based taxonomic analysis. For this purpose, we used PVC settlement panels as standard sampling structures, developed by the Smithsonian Environmental Research Center (SERC) to assess sessile fouling communities and used now by several countries (Tamburini et al., 2021), followed by DNA metabarcoding to assess: (i) community patterns in man-made environments and coral reef habitats using a multimethod approach, that is, two different sampling matrices (PVC settlement panels and water samples) and two different genetic markers (COI and 18S rRNA), (ii) the occurrence and diversity of potential mNIS in Red Sea natural and man-made habitats, and (iii) the influence of seasonality on the occurrence of mNIS. In addition, for the first time, we provided a database for marine organisms, including invertebrates, fish, and algae, with the potential to become invasive species in Red Sea waters. The database has been created based on existing literature, international databases, and technical reports addressing the status of mNIS. This study represents the foundation for future mNIS assessments in the Red Sea and establishes the first steps to inform future monitoring programs in the region.

2 | METHODS

2.1 | Study area

The study area is located in the Saudi Arabian central Red Sea, characterized by the presence of several anthropogenic activities, including a large industrial port and several marinas, and featuring rapid industrialization and urbanization in recent decades. The central Red Sea has an average monthly near-shore sea surface temperature ranging between 34°C and 37°C and salinity between 36 and 40 (Daqamseh et al., 2019). Tidal currents in the area are weak, with the most energetic tidal constituent (a semidiurnal tide) presenting a magnitude of 4 cm s^{-1} (Churchill et al., 2014).

Biofouling and water samples were collected from three types of environments: a large commercial port (Jeddah Islamic Port), a small marina located in the King Abdullah University of Science and Technology (KAUST—marina), and two coral reefs (Tahla Reef and Al Horba Reef) throughout 2021 (Figure 1). The Jeddah Islamic Port (JP) is an open harbor of 10.5 km^2 located in the middle of the international shipping route between east and west. Established in 1947, Jeddah Port receives over 5000 vessels annually and processes 75% of the country's exports and inbound transshipment (Elentably, 2015). Located in a small, protected, shallow harbor 100 km north of Jeddah, the KAUST marina (KM) was established in 2009. With a single finger and 5 floating docks, it is utilized by 10 small and medium research vessels. Tahla Reef (TR), with a length of 4 km from north to south, is located 5 km southeast of KAUST marina and 3 km from shore, dominated by hard coral and shell rubble. Al Horba Reef (AR) is a patchy reef 2.6 km long from north to south and is 8.5 km northeast of KAUST marina and 3.5 km from shore.

2.2 | Sampling methodologies: PVC Settlement panels and water samples

Two different sampling methodologies were used to characterize marine communities: PVC settlement panels and water samples.

2.2.1 | PVC settlement panels

PVC settlement panels (14 × 14 cm, gray 4 mm polyvinyl chloride, PVC) were deployed in triplicate in individual vertical suspended arrays for a 7-day period (Figure 1) during three consecutive sampling events in the boreal winter and three consecutive sampling events in the boreal summer. After 1 week of the first deployment, the three panels were retrieved, followed by the deployment of a new set of panels for 1 week, except for the first deployment at Jeddah Port, which was 2 weeks due to permitting issues. This process was done three times in winter and three times in summer (see Table S1), with a total of 18 panels deployed at each site over the period of the study. In the port and the marina, the panels were secured to docks or floating pontoons using a rope, suspended 1–2 m below the surface (Tamburini et al., 2021). In the KAUST marina, panels were evenly spaced along the perimeter of the marina, 85 m apart from each other. In Jeddah port, settlement panles were deployed 1.5–2.5 km apart due to the comparatively larger size of the port. In coral reef habitats, PVC settlement panels were deployed at the exposed side of the reef 50 m apart from each other, attached to bricks using a rope connected to a floating buoy at the opposite end to ensure the vertical arrangement of the unit. Upon retrieval, each panel was placed into individually sealed plastic bags, transported on ice to the laboratory, and stored at −80°C until further processing. Individually packed sterilized swabs (Deltalab) were used to collect biofouling from each thawed panel by thoroughly and consistently rubbing material from the surface first, using a swab moistened with nuclease-free water, and second, using a dry swab to maximize the amount of sample retrieved and standardize yield (Brownlow et al., 2012; Pang and Cheung 2007). DNA extraction proceeded immediately after sample collection.

2.2.2 | Water samples

During each sampling event (at panel deployment and retrieval events), 3 replicate water samples (2 L) were collected at 1–2 m depth using a Niskin bottle (artificial habitats) or by submerging a 2 L Nalgene below the surface (natural habitats). When retrieval and deployment happened on the same day (i.e., settlement panels were deployed at the same time as previous ones were collected), only one water sample (in triplicate) was taken, representing both the retrieval and deployment for that sampling event (i.e., the winter 2 deployment sample would be the same as the winter 3 deployment sample) (see Table S1 for details).

Samples were transported on ice to the laboratory for immediate processing (3 h maximum). Water samples were filtered through one individually packaged sterile 0.45 μm (47 mm diameter) filter membrane (Millipore) placed on each of the adaptor holders of a 6-branch aluminum manifold Vacuum Filtration System. Each water sample replicate was transferred to a clean filtering cup until the entire sample was filtered. During each sampling event, negative control samples were obtained by filtering 500 mL of MilliQ water following the protocol for seawater samples. Filters were wrapped individually in aluminum foil and stored at −80°C. All equipment in contact with water samples and filter membranes was soaked in a 10% bleach solution for 5 min and rinsed three times with MilliQ water between each sample to prevent cross-contamination.

2.3 | DNA extraction, library preparation, and sequencing

Biofouling swabs and filters were cut into small pieces using sterile forceps and scissors and placed directly into 540 μL Buffer ATL and 60 μL proteinase K. Samples were incubated at 56°C for 3 h in a ThermoMixer (350 rpm) and vortexed hourly. Additionally,

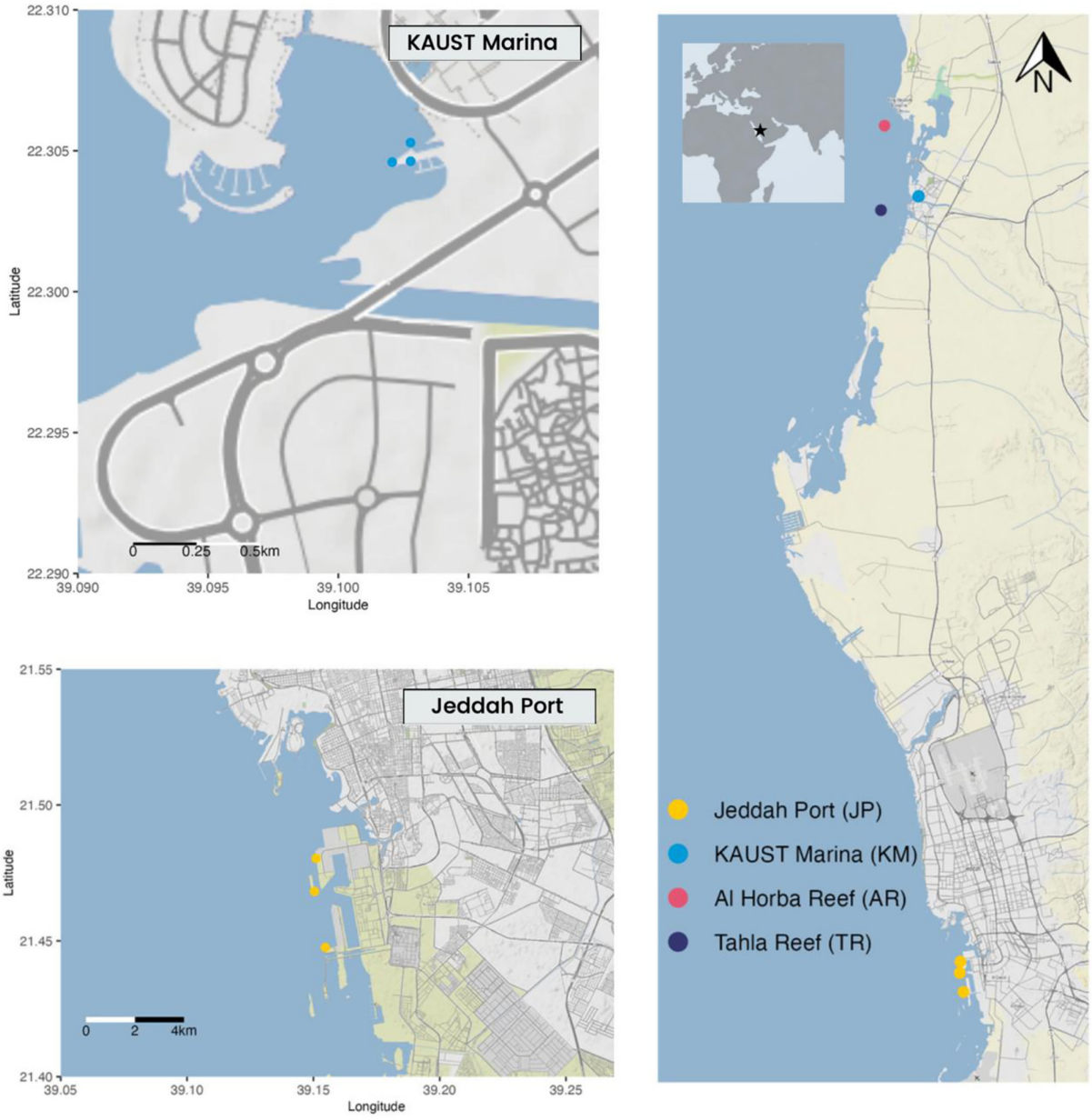

FIGURE 1 Map of the study area in the central Red Sea. Colored circles indicate the sampling sites (man-made environments: Jeddah Port; JP, and KAUST Marina; KM, coral reefs: Al Horba Reef (AR), and Tahla Reef (TR);) where PVC settlement panels were deployed during a 7-day period and water samples were collected. Maps of the man-made environments are depicted showing the placement of the replicates.

biofouling samples were centrifuged hourly at 10,000*g* for 1 min, and subsequently vortexed to encourage full penetration of the buffer into the swab material. After incubation, all liquid was collected and transferred into a new Eppendorf tube for total genomic DNA isolation using the DNeasy Blood and Tissue Kit (QIAGEN) with the following modifications: 400 µL of AL buffer and 400 µL of ethanol. Negative extraction controls (without filter or swab) were included in each DNA extraction and followed the same protocol. DNA concentration was measured with the Qubit dsDNA High Sensitivity assay Kit (Invitrogen, Thermo Fisher Scientific) using the Qubit 4 Fluorometer. DNA integrity was visualized using gel electrophoresis (1% agarose). Each DNA sample was normalized to 5 ng/µL using nuclease-free water and stored at −20°C until further processing. Three replicates from each sampling method were used for library preparation. For internal quality control, a mock community was prepared by pooling equal volumes of genomic DNA (normalized at 5 ng/µL) extracted from four previously isolated marine species (*Lauriea gardineri*, *Echinometra mathaei*, *Diadema setosum*, and *Drupella cornus*) and included in the COI DNA reference library.

A two-step PCR procedure was followed for cytochrome c oxidase subunit (COI) and nuclear small subunit ribosomal DNA (18S rRNA) genes in MiSeq Illumina library preparation. First-stage (amplicon) PCR was performed in triplicate, then pooled. Each sample was amplified in triplicate using the universal primers (Table 1) mlCOIint/jgHCO2198 and Uni18SF/Uni18SR, which were used to

TABLE 1 Primer sequences amplifying a fragment of the COI gene and 18S rRNA gene.

Target gene	Forward 5′–3′	Reverse 5′–3′
COI	mlCOIint: GGWACWGGWTGAACWGTWTAYCCYCC	jgHCO2198: TAIACYTCIGGRTGICCRAARAAYCA
18S rRNA	Uni18SF: AGGGCAAKYCTGGTGCCAGC	Uni18SR: GRCGGTATCTRATCGYCTT

amplify a 313 bp fragment of the COI gene (Leray et al., 2013) and the V4 region of the 18S rRNA gene (Zhan et al., 2013), respectively, with attached Illumina overhang adapters. Each amplicon PCR reaction contained a total volume of 25 μL (2.5 μL 10× PCR Rxn Buffer, 0.5 μL dNTPs (10 mM), 0.5 μL forward primer (10 μM), 0.5 μL reverse primer (10 μM), 1.25 μL BSA (50 μg L^{-1}), 1.25 μL $MgCl_2$ (0.25 mM), 0.25 μL *Taq* DNA Polymerase, 16 μL nuclease-free water, and 2 μL template DNA). For COI, reaction cycling conditions were: 98°C for 3 min, followed by 35 cycles of 98°C for 10 s, 46°C for 30 s, and 72°C for 45 s, and a final extension of 72°C for 5 min. For 18S, reaction cycling conditions were: 95°C for 5 min, followed by 25 cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 90 s, and a final extension of 72°C for 10 min.

A second PCR was used to attach a unique combination of MiSeq Illumina dual indexes (Nextera™ XT Index Kit) to the Illumina sequencing adapters of each pooled sample. Final quality control, including library quantification, normalization, and pooling, was done using a Bioanalyzer DNA 1000 chip and qPCR. In preparation for cluster generation and sequencing, the pooled library was denatured and loaded into the Illumina MiSeq sequencing platform (v3 chemistry) at KAUST (Bioscience CORE Labs) to determine sequence reads. Twenty percent phiX was included for internal control. Sequence data were automatically demultiplexed using MiSeq Reporter (v2), and forward and reverse reads were assigned to samples.

2.4 | Raw sequences analysis and ASVs taxonomic classification

For both the 18S rRNA and COI reads, identical bioinformatic pipelines were used unless otherwise stated. Primer sequences were removed from the raw reads using cutadapt (Martin, 2011), allowing for a single mismatch. Trimmed reads were then processed using DADA2 within R (Callahan et al., 2016). The 18S rRNA gene reads were truncated at 226 and 228 bp (forward and reverse, respectively), and at 165 and 160 bp for the COI gene reads. A maximum number of "expected errors" (maxEE) of 2 were allowed for the forward reads and 4 for the reverse reads. Reads not meeting this threshold were discarded. A parametric error matrix was constructed within DADA2 using the first 10^8 bp. Following sequence dereplication, this error matrix was used to infer amplicon sequence variants (ASVs). Paired-end reads were merged, allowing for a 1 bp mismatch and a minimum overlap of 10 bp. For the COI dataset, ASVs were aligned against the MIDORI (Leray et al., 2022) database, and anomalies in amino acid translations were detected using Multiple Alignment of Coding Sequences (MACSE) (Ranwez et al., 2011). This was undertaken in a two-step process, first translating the sequences using the invertebrate translation code and then using the vertebrate translation code. Any sequences with a stop code or possessing more than two frameshifts were classified as possible pseudogenes and removed from the analysis.

After chimera (and pseudogene) checking, ASVs were taxonomically classified. The 18S rRNA ASVs were classified against the PR2 (Guillou et al., 2013) database using the DADA2 assign Taxonomy script, which is based on the rdp classifier (Wang et al., 2007), with a bootstrap cutoff of 0.7 to allow higher classifications. For the COI dataset, assignment at higher taxonomic levels (genus and above) was undertaken using DADA2 with a cutoff of 0.5, while species identities were achieved based on a blastn search (percentage ID >0.95) against the NCBI nt database (Sayers et al., 2021). ASVs were only retained if they were assigned to Eukaryota. To remove possible contamination, we investigated the controls (field controls, DNA extraction controls, and PCR controls) and used a maximum sequence count as a removal threshold. Samples with less than 10,000 reads were removed from the dataset. The two mock communities used as a positive control were checked for the taxonomic identifications obtained based on blastn.

2.5 | Red Sea marine non-indigenous species (mNIS) database

A database containing 167 potential mNIS in the region was built (Table S2), including species previously identified as non-native in the Red Sea and well-known mNIS reported in nearby regions but not detected yet in the Red Sea. The species included in the local mNIS database were retrieved through a literature review and from public databases: The NEMESIS databases (https://invasions.si.edu/nemesis/), the Smithsonian Institutions' research repositories (https://repository.si.edu/), and the map of the number of recorded alien taxa for terrestrial obtained through the DASCO workflow (Seebens & Kaplan, 2022). Academic, peer-reviewed, and published papers were the main focus of the literature review. Sources on potential non-indigenous species in the Red Sea cited by the World Register of Marine Species (WoRMS) and the World Register of Invasive Marine Species (WRIMS) were used to gain a general understanding of the distribution of the relevant marine species. However, the lack of relevant literature for biodiversity records of the Red Sea, as well as the ever-changing nature of non-indigenous species presence in the Red Sea, led to the use of data from the GBIF (non-academic sightings of previously unknown non-indigenous species in the Red Sea). The Checklist of

Red Sea Fishes with delineation of the Gulf of Suez, Gulf of Aqaba, endemism, and Lessepsian migration (Golani & Fricke, 2018) was used to retrieve potential invasive fish species. Given the scarce information on the native species in the Red Sea, cryptogenic species have been included in the potential mNIS list.

The criteria for inclusion in the list were:

A mNIS, whose status in the Red Sea was well documented in publications and/or written reports;

B Species of fish and invertebrates imported to the Red Sea for aquaculture that are non-native to the Red Sea with the risk of becoming invasive;

C Fish species present in the Red Sea that originated from the Mediterranean Sea (anti-Lessepsian migrant) are well documented in publications and/or written reports;

D mNIS reported in nearby regions (e.g., Arabian Sea, Western Indian Ocean, Eastern Mediterranean) that are non-native in the Red Sea but have not been detected yet.

2.6 | Community composition analysis and potential mNIS detection

All statistical analyses were conducted in R version 4.3.0 (R Core Team, 2019). To assess the number of ASVs per habitat and sampling method using each marker (COI and 18S), the datasets were rarefied using the Phyloseq package to the lowest sample read depth for each marker (13,200 reads for COI and 14,400 for 18S). Venn diagrams to investigate the number of shared ASVs were done using ggvenn. Differences between the number of ASVs obtained were tested using Kruskal-Wallis as the data were not normally distributed. Analysis was undertaken for habitat (2 levels: Natural, Artificial) and season (2 levels: Winter, Summer) separately. Multivariate analysis was undertaken on both datasets (COI and 18S) and sampling methods (biofouling and water samples). Principal coordinate analysis was used to visualize the 2D representation of the ASV structure. Permanova was run to test for statistical differences in the community structure between habitats and seasons using the adonis function in vegans (Oksanen et al., 2015) using Bray–Curtis distance matrices on the abundance data after rarefaction (two factors: Habitat; 2 levels: Natural, Artificial. Season; 2 levels: Winter, Summer). Figures were made in R using the package ggplot (Wickham, 2016).

A custom database for the mNIS species of the Red Sea was created from publicly available sequences present in the NCBI (Sayers et al., 2021) and BOLD (Ratnasingham & Hebert, 2007) databases (BOLD was only used for COI) using CRABS v0.1.7 (Jeunen et al., 2019). Sequences were obtained for the Red Sea mNIS as well as those for other species within the same genus, and a blast database was constructed. ASV sequences were blasted against this database and classified as mNIS if they had >99% similarity with a minimum length of 250 bp.

3 | RESULTS

3.1 | Community patterns observed by each sampling method (PVC settlement panels and water samples) and genetic marker (COI and 18S)

The four species used as a positive control accounted for >99% of the mock community in both samples. The relative contribution of each species to mock communities one and two were 34.09% and 38.95% for *Lauriea gardineri*, 14.15% and 6.47% for *Drupella cornus*, 21.11% and 16.04% for *Echinometra mathaei*, and 29.77% and 38.49% for *Diadema setosum*. Overall, there were 32,083 ASVs in the rarefied COI dataset, with 11,381 ASVs only found on the settlement panels and 17,530 unique to the water samples. For the 18S rarefied dataset, there were a total of 20,567, with 6262 ASVs only found on the settlement panels and 11,280 unique to the water samples. The proportion of shared ASVs between water samples and panels was relatively low (~10% for COI and ~14% for 18S) compared to the ASVs that were unique to each sampling method. The ASV and taxonomy tables for COI and 18S can be found in Tables S3 and S4.

For COI, a large proportion of the reads, especially in the water samples (75%), could not be assigned to a phylum (Figure 2). For the reads that could be assigned to a phylum, clear differences in community composition were observed between water samples and panels. Arthropoda dominated the water samples (16%), while Cnidaria (28%) had the highest relative abundance on the panels, followed by Bacillariophyta (20%) and Arthropoda (11%). In the 18S rRNA gene dataset, the proportion of reads belonging to ASVs that could not be assigned a phylum was lower than those for COI (only reaching 5% of the overall reads for both water and biofouling samples; Figure 2). In the water samples, Dinoflagellata had the highest relative proportion of reads (26%), followed by Arthropoda (21%). Arthropoda was the most dominant phyla (18%), followed by Cnidaria (12%), Chlorophyta (12%), and Dinoflagellata (11%) in the biofouling pioneer communities collected from settlement panels. The differences between community composition obtained using COI and 18S markers were significant (p-value < 0.005), with Bacillariophyta and Mollusca only detected using COI, and Dinoflagellata, Nematoda, and Chlorophyta detected only using 18S.

3.2 | Effect of seasonality (winter vs. wummer) and habitats (coral reefs vs. man-made) on community composition using COI and 18S

Clear differences in the biological communities retrieved across sampling seasons and habitats were observed in the ordination plots based on Bray-Curtis dissimilarities (Figure 3) and confirmed by the adonis test, which showed a significant interaction between habitat and season for both the panels (COI: $F = 4.55$ $p < 0.001$; 18S: $F = 3.45$; $p < 0.001$) and water samples (COI: $F = 8.03$, $p < 0.001$; 18S: $F = 8.56$,

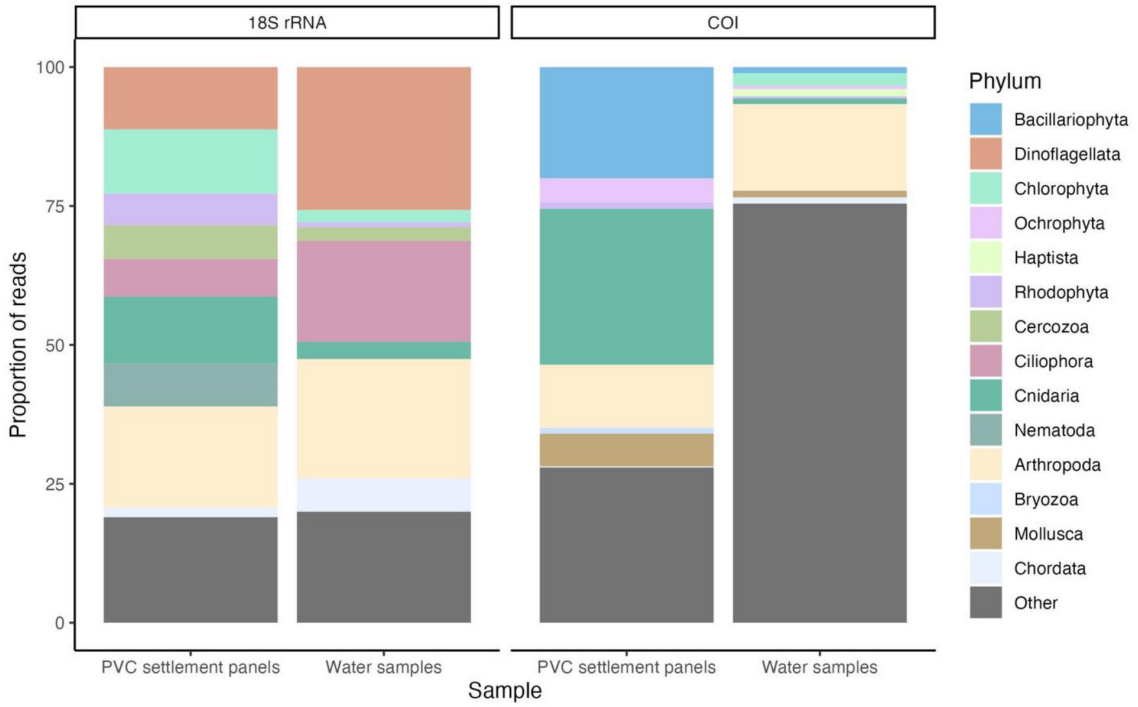

FIGURE 2 Proportions of taxa at the Phylum level retrieved from water samples and panels using COI (left) and 18S (right) markers.

$p < 0.001$). Kruskal-Wallis tests showed that for water samples, there was a significant difference in the number of ASVs per season for both the COI (chi-sq = 53.214; $p < 0.001$) and 18S (chi-sq =47.811; $p < 0.001$), with winter having a higher number of ASVs (COI: 1803 ASVs; 18S: 1005 ASVs) compared with summer (COI: 1163 ASVs; 18S: 674 ASVs). A similar pattern was observed for the panels (COI: chi-sq = 12.462; $p < 0.001$; 18S: chi-sq = 33.121; $p < 0.001$), with winter (COI: 833 ASVs; 18S: 629 ASVs) having a higher number of ASVs than summer (COI: 603 ASVs; 18S: 345 ASVs). There was a significantly higher number of ASVs in the water samples compared to the panels in both seasons for the COI (winter: chi-sq = 55.39; $p < 0.001$. summer: chi-sq = 52.195; $p < 0.001$) and the 18S (winter: chi-sq = 36.659; $p < 0.001$. summer: chi-sq = 63.187; $p < 0.001$). The biological communities analyzed from KAUST marina, the most recent man-made habitat, were more dissimilar compared to natural habitats than those present in Jeddah Port, the oldest man-made habitat.

In winter, based on the COI marker, Arthropoda (predominantly Calanoida) were the dominant taxa retrieved from water samples for all sites, with an average of 46.7% (Figure 4). Chordata, predominantly belonging to the order Phlebobranchia (class Ascidiacea), had higher relative abundances in the marina/port sites (12% and 10% for KM and JP, respectively), with lower relative abundances in the reef sites (2% and 4% for TR and AR, respectively). This group was only detected in the water samples. In contrast, in both water samples and settlement panels, Ochrophyta were present in the reef sites at higher relative abundances (>10%) compared to the port and marina sites (<2%). Representatives of the order Bacillariales dominated the community from the settlement panels in the reefs, accounting for 47% and 43% of the reads for TR and AR, respectively. On the other hand, Cnidaria (predominantly Leptothecata) dominated the settlement panels of the port and the marina sites (KM—34%; JP—74%). In all sites, in the 18S rRNA dataset, Arthropoda (mean = 17%) contributed to a lower relative abundance compared to the values in the COI dataset in winter. Dinoflagellata (mean = 31%) and Ciliophora (mean = 24%; the dominant class in all sites was Strombidiida) were the dominant phyla in the water samples of all sites.

Similar to the winter, in the COI dataset, Arthropoda (mean = 66%) was the dominant group retrieved from water samples at all sites (Figure 4). Mollusca were observed to have a higher relative abundance in the summer (reaching 13% at KR). In the PVC panel samples, Bacillariophyta dominated the community in the winter at the reefs, while Cnidaria (TR—72%; AR—46%; mainly order Leptothecata) accounted for the highest proportion of reads in the summer. The opposite trend was observed in the artificial sites, with the relative abundance of Cnidaria decreasing and being replaced by Bacillariophyta (32%; mainly order Bacillariales) in JP and Mollusca (42%; mainly Aplysiida) in KM. In the water samples of the 18S dataset, the dominant phyla in the summer were Dinoflagellata, Arthropoda, and Ciliophora at all sites. Ciliophora in general showed a decline from an average of 24% in winter to only 12% in summer across sites. In the 18S dataset, Cnidaria accounted for a higher relative proportion of reads compared to winter (reaching 39% in TR) from panels. Arthropoda, especially the order Harpacticoida, were the other dominant taxa retrieved from panels at reef sites and accounted for 27% and 21% of reads in TR and AR, respectively.

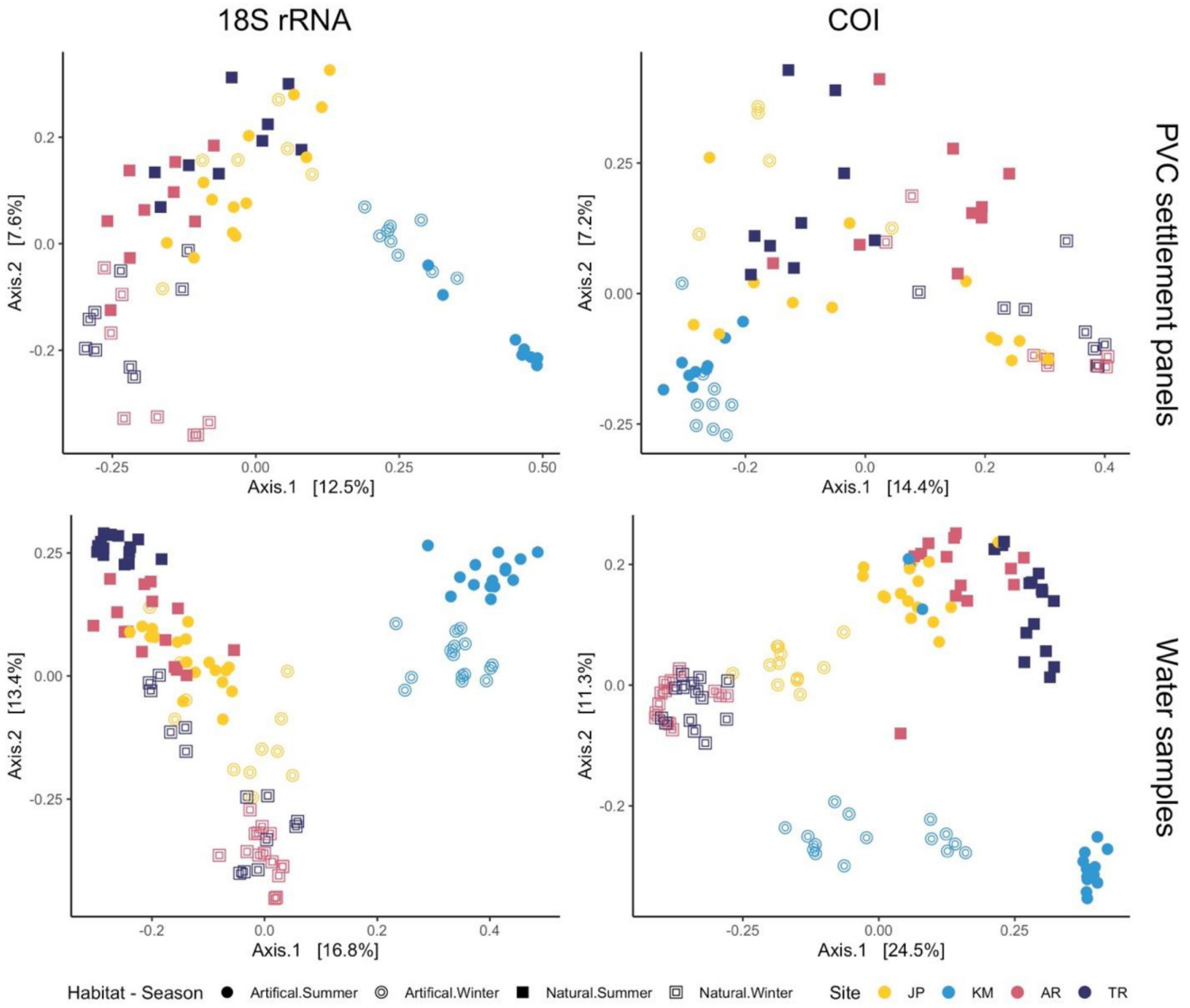

FIGURE 3 Principal coordinate analysis plot based on Bray–Curtis dissimilarities for PVC settlement panels using the 18S rRNA gene (a) and COI (b) and water samples with the 18S rRNA gene (c) and COI (d). Man-made sites are JP (Jeddah Port) and KM (KAUST Marina). Reef sites are AR (Al Horba Reef) and TR (Tahla Reef). ■, Artificial or man-made habitat in summer season; ●, natural habitats in summer season; □, artificial or man-made habitat in winter season; ○, natural habitats in winter season.

3.3 | Presence of potential marine non-native species

Our baseline study detected a total of 29 potential mNIS across the four sites (Jeddah Port, KAUST Marina, Tahla Reef, and Al Horba Reef) in the central Red Sea (Figure 5). It is to our knowledge the first time that 16 out of the 29 mNIS detected (*Bougainvillia muscus*, *Clava multicornis*, *Crepidula fornicata*, *Rapana venosa*, *Amphibalanus amphitrite*, *Amphibalanus reticulatus*, *Eriocheir sinensis*, *Paradella dianae*, *Cryptosula pallasiana*, *Schizoporella errata*, *Botrylloides niger*, *Ecteinascidia turbinata*, *Polyandrocarpa zorritensis*, *Polyclinum constellatum*, *Grateloupia filicina*, and *Ulva rigida*) are reported in the Red Sea (Table S3). From the other 13 mNIS detected, six species had been previously detected, but none of them were reported in the central Red Sea, and one (*Carcinus maenas*) was reported as mNIS in the southern Red Sea but failed to become established so far, according to the nemesis database (see https://invasions.si.edu/nemesis/). The status of seven mNIS detected in this study is cryptogenic as their origin is uncertain and therefore cannot be ascribed as native or invasive. Only three species (*Hydroides elegans*, *Bougainvillia muscus*, and *Pennaria disticha*) were detected using both 18S and COI markers, with 18S retrieving more mNIS (18 species) than COI (13 species). Only one species (*Oreochromis mossambicus*, Tilapia) was detected in all sites and both seasons (winter and summer) using 18S, while using COI, the barnacle *Amphibalanus amphitrite* was detected at all sites in winter and summer except for Al Horba Reef during winter. A total of 28 species were detected in the marina and port locality samples, but only 15 mNIS at the two reef habitats. Thirteen mNIS were exclusively found in the marina and port samples. Jeddah Port was the site with the highest number of mNIS reported (28

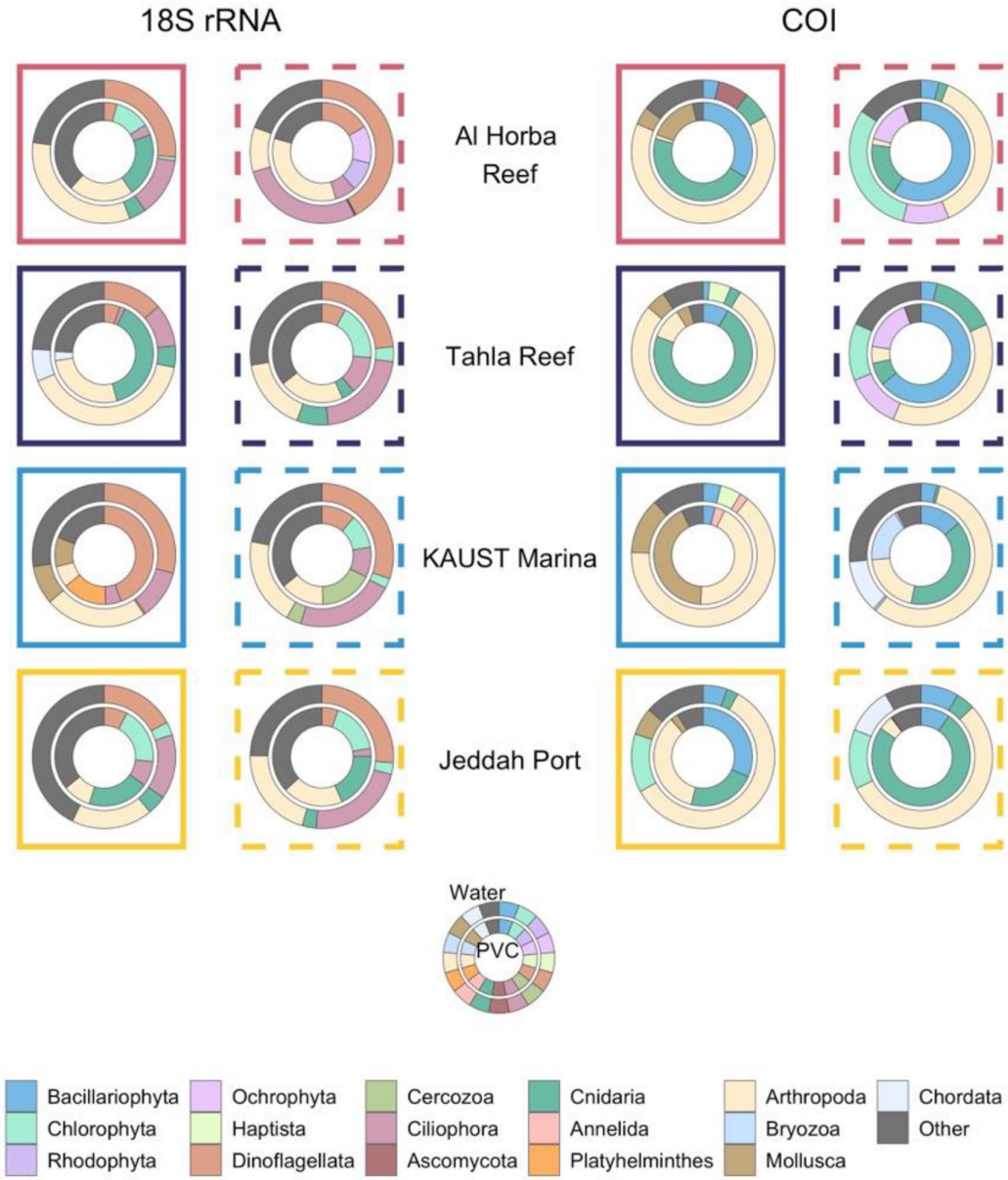

FIGURE 4 Community composition at the coral reefs (Al Horba Reef and Tahla Reef) and man-made environments (Jeddah Port and KAUST Marina) in winter (continuous line) and summer (dashed line). Community composition from the different sampling methods (PVC settlement panels and water samples) using COI and 18S markers is depicted using a donut chart with PVC panels in the inner ring and water samples in the outer ring. Note, "Other" in the taxonomy list defines various phyla with low relative abundances.

species), followed by KAUST Marina, Al Horba Reef (both 14 mNIS), and Tahla Reef (13 mNIS).

4 | DISCUSSION

By applying DNA metabarcoding to DNA extracted from filtered seawater and biofouling samples (PVC settlement panels) across habitats and seasons, we provided a baseline of the spatio-temporal trends of the eukaryotic community associated with man-made structures (i.e., a port and a marina) and natural habitats (i.e., coral reefs) in the central Red Sea and inferred the presence of potential marine NIS. Patterns in community structure differed among genetic markers (COI and 18S rRNA), sampling methods, and seasons, aligning with the results previously reported for port facilities and natural reefs (Dafforn et al., 2012; Rey et al., 2020). In addition, we reported for the first time the presence of 16 mNIS in the central Red Sea. Our study reinforces that, although molecular-based tools require further validation in the context of biosecurity monitoring (Zaiko et al., 2018), they hold great promise to facilitate early detection and subsequently foster prompt intervention at early stages of the invasion process (Duarte et al., 2021).

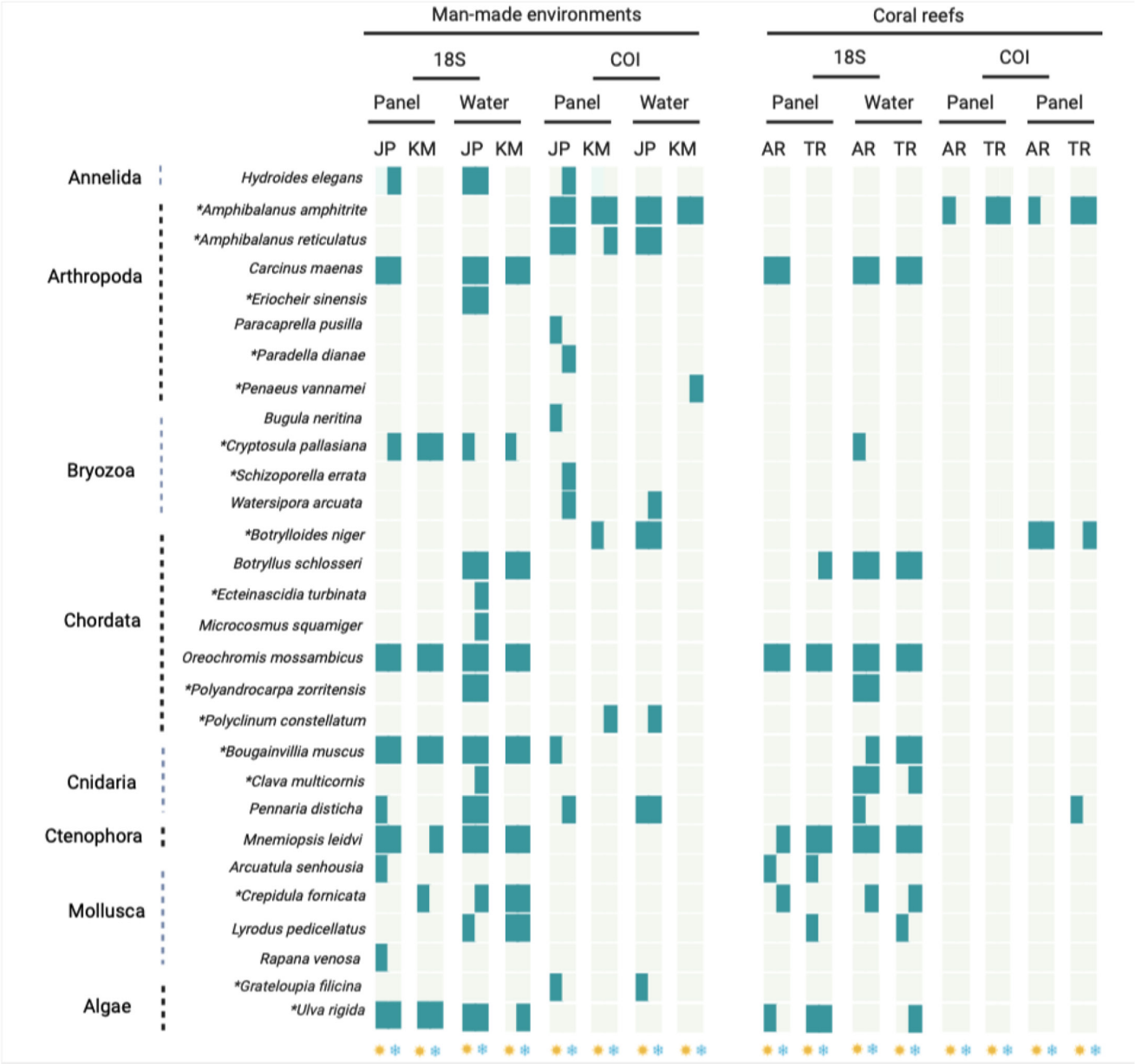

FIGURE 5 Chart of the 29 marine non-indigenous species found across the man-made environments (JP: Jeddah Port; and KM: KAUST Marina) and coral reefs (AR: Al Horba Reef; and TR: Tahla Reef). For each species, the detection success (positive dark green, negative light green) is shown for each marker (COI and 18S), sampling method (Panel: PVC settlement panels, and Water: Water samples), and season (boreal winter and boreal summer). Potential mNIS are organized by major groups from top to bottom. Summer and winter are depicted at the bottom of each column. The first records in the Red Sea are marked with an asterisk.

4.1 | Multimethod approaches are needed for reliable assessments of species distributions using molecular methods

Reliable biodiversity studies require the use of sampling methodologies that enable a comprehensive description of target communities. When the focus is the surveillance of mNIS, the methodology must be suited to capture the signals of a wide range of organisms representing different taxonomic groups, from biofouling invertebrates to fish inhabiting the water column (Bowers et al., 2021; Pochon et al., 2015). Recent studies have compared the performance of different matrices, including deployed PVC panels, underwater permanent artificial structures, sediment, and water, for biodiversity assessments in general and the detection of mNIS in particular, finding important differences among them (Holman et al., 2019; Pearman et al., 2021; von Ammon et al., 2018a). Here, using a multimethod approach combining two matrices (PVC settlement panels and water samples) and two genetic markers (COI and 18S rRNA), we showed that sampling methods influence community patterns. The relative proportion of certain taxonomic

groups varied significantly between biofouling and water samples. For example, cnidarians had a higher proportion of richness in biofouling samples compared to that in water samples. This pattern might be explained by the rapid larval settlement of cnidarians from the planktonic to the benthic stages (Fernandez et al., 2014). Cnidarians have been reported as one of the most common taxa found on recruitment panels (Migotto et al., 2001), and their opportunistic settlement allows them to colonize a variety of substrates. Thus, it is not surprising that their presence in the samples collected from PVC settlement panels was comparatively higher than that in water samples. The two genetic markers used also showed discrepancies between the taxonomic groups retrieved. While representatives of the phylum Mollusca were only retrieved from biofouling samples using COI, nematodes were only detected on the 18S rRNA dataset. This might be due to the underrepresentation of species belonging to these phyla in the current versions of the PR2 and BOLD databases. The lack of corresponding reference sequences is likely to result in biased results using DNA metabarcoding approaches (Dowle et al., 2015). However, PCR primer biases (i.e., different PCR efficiencies of the species in the bulk DNA sample) can also limit reliable estimates of species in high-throughput-based biodiversity studies (Wu et al., 2023). Taken together and in agreement with previous studies (Holman et al., 2019; Pearman et al., 2021), our findings revealed that biodiversity surveys based on DNA metabarcoding require the use of complementary methodologies to better describe marine communities. Relying on a single approach may exclude specific taxonomic groups or provide inaccurate descriptions of biodiversity. Here, using 1-week PVC panel deployments (except for the first deployment in Jeddah Port due to logistic issues), we found a high proportion of ASVs unique to biofouling samples that were not detected in water samples. This result reinforces previous findings revealing that water samples alone only retrieved a fraction of the metazoan taxa detected from biofouling samples collected from settlement panels deployed over 3 months (Rey et al., 2019). Although the time of exposure (i.e., duration of the deployment) influences the composition of biofouling communities (Zaiko et al., 2016), our findings confirm that while water sampling has been suggested as a promising approach to record the presence of invasive species (Sahu et al., 2023), bulk samples containing whole organisms, such as those obtained from biofouling samples, are needed for accurate descriptions of biodiversity, in particular mNIS.

4.2 | Spatial and temporal variability influence the patterns of early biofouling species

Recent studies focused on the surveillance of mNIS in coastal areas have dedicated special attention to the assessment of biofouling samples collected from artificial substrates present at ports and marinas (Holman et al., 2019; Pearman et al., 2021). However, only a few studies have investigated the distribution of pioneer communities associated with man-made and natural habitats (Dafforn et al., 2012; Ferrario et al., 2020). Here, by analyzing samples collected from PVC settlement panels deployed at a port, a marina, and coral reefs, along with water samples, we contributed to developing baseline information to be used in future marine biosecurity survey invasions (Lehtiniemi et al., 2015) and enabling successful management of marine bioinvasions, especially in those regions lacking baseline data (Carvalho et al., 2023). We observed that spatial variability influenced the patterns of biofouling communities on artificial substrates from man-made environments (Zaiko et al., 2016). The community structure from samples collected at the two coral reefs showed greater similarity to each other compared to that obtained from the port and the marina. This similarity between coral reef samples could be attributed to the comparable environmental conditions present in these natural habitats. Interestingly, the structure of the communities retrieved from samples collected from KAUST Marina was the most distinct one.

Notably, the period that the port and marina have been active differs for over several decades, with Jeddah port being the oldest man-made habitat, suggesting that mature biological communities associated with coral reefs nearby have established in Jeddah port waters and have colonized the newly introduced panels. Some taxonomic groups were only detected at KAUST Marina, such as representatives of the phylum Bryozoan retrieved from the settlement panels in winter or a higher relative abundance of Mollusca, detected only in summer, compared to the other three sites analyzed. The distinct seasonality in recruitment patterns found in this study aligns with the findings reported by Zaiko et al. (2016) in New Zealand. In their study, they observed a clear difference in recruitment patterns between winter and summer, with peak recruitment occurring in summer. As recently reported for the Red Sea (Webb et al., 2021), many marine invertebrates spawn in the summer, which might explain the higher prevalence of metazoan taxa during the summer period, as reported by Zaiko et al. (2016).

These findings highlight the importance of performing comprehensive biodiversity assessments, including the collection of representative samples at different spatio-temporal (i.e., habitats and seasons) scales, to gain a thorough understanding of the existing biological communities (Bowers et al., 2021).

4.3 | Comprehensive local DNA reference libraries are essential for the surveillance of non-indigenous species

In this study, a total of 29 potential mNIS were detected, with Jeddah Port showing the greatest number of mNIS across the four sites. Invasive marine species have proven to have the capability to cause massive ecological and economic damage worldwide (Sunarto et al., 2022). For example, the commercial fish *Oreochromis mossambicus* (Mozambique tilapia), detected in this study across the

four sites and previously reported in the northern Red Sea, presents the capacity to reproduce rapidly and the potential to outcompete native fishes for space and food (Lowe et al., 2000). The dietary overlap between native and non-native species might lead to a loss of biodiversity. Hence, *O. mossambicus*, together with other non-native commercial species that are expected to be introduced in Saudi Arabia as a response to increased aquaculture production (Young & Shaikhi, 2022), may represent a potential threat to the populations of indigenous species. As well as *Rapana venosa*, a predatory sea snail that could put heavy predatory pressure on indigenous bivalves and damage bivalve aquaculture harvests, as it has in its other invasive ranges worldwide (Harding, 2003). *R. venosa* was detected only in Jeddah Port, serving as an early warning indicator to monitor the potential spread of this species to other sites before it becomes established. Most of the mNIS detected in KAUST Marina, the recent man-made habitat, have been reported as mNIS in other areas, but no significant threats or important economic impacts have been associated with them. KAUST Marina can be used as an example to begin the implementation of biosecurity frameworks to prevent the colonization and establishment of mNIS on high-risk infrastructure (Carvalho et al., 2023). Yet, as shipping traffic increases in the region, small boats from commercial ports, such as Jeddah port, might become a vector of connectivity for mNIS in nearby habitats (Zabin et al., 2014). Therefore, given the constant visits of research boats from KAUST marina to nearby coral reefs, ongoing monitoring efforts are critical to ensuring the preservation of the natural habitats.

Our study used DNA metabarcoding to assess biodiversity and detect mNIS from water and biofouling samples based on a local database of mNIS created for the Red Sea. Yet, the results rely on publicly available DNA barcodes associated with the species present in the database. The comprehensiveness of reference databases and the representation of species in these databases can influence the ability to accurately detect and identify species using eDNA (Duarte et al., 2021; Ip et al., 2021). For the Red Sea in particular, reference databases are still in their infancy and the synergistic use of visual morphological taxonomic identification (through the use of microscopes and high-resolution photographs) and DNA barcoding may enable the development of a more comprehensive DNA reference library and improve the performance of DNA metabarcoding to characterize biodiversity and detect mNIS. We strongly recommend that future mNIS studies in the region consider the collection of biofouling organisms followed by their identification by taxonomist experts. When possible, efforts to catalog biodiversity and create a more robust sequence library should be extended to other data-poor regions (Carvalho et al., 2023). Bioblitzes (intensive biodiversity screenings with taxonomic experts) are an efficient approach to combine taxonomic expertise and DNA barcoding within a limited timeframe (Meeus et al., 2023). Taken together, our study shows that DNA metabarcoding can effectively detect the presence of mNIS in natural and man-made habitats and sets the foundation for the development of robust and rapid baseline monitoring systems to assess native biodiversity and facilitate early detection of mNIS.

4.4 | Future considerations and policy context

Although the advent of eDNA for the rapid detection of potential invasive species poses clear advantages (Duarte et al., 2021; Holman et al., 2019), there are important considerations to account for reliable eDNA-based species surveillance. For example, it is of critical importance to understand and address the factors that influence the detection probability of species using eDNA. Variations in environmental conditions such as UV exposure, temperature, and flow rate (Furlan et al., 2016), or water movement (i.e., mixing or transportation), sedimentation, turbidity, and water quality (Ip et al., 2021) can impact eDNA concentration and therefore influence detection probability. Sample size and replication should be adjusted to the particularities of the study (Coston-Guarini et al., 2023). By acknowledging and mitigating these sources of uncertainty, particularly in the context of biosecurity and early warning detection, we can enhance the effectiveness of eDNA surveillance efforts.

Improvements in our ability to monitor and manage invasive species and emerging threats should be coupled with the implementation of national policies on mNIS. However, most policy-based efforts to prevent the spread of mNIS are focused on developed countries and temperate regions (Carvalho et al., 2023). Although actions to implement biosecurity policies in Saudi Arabia have started, national laws are not yet enforced. Initial biosecurity programs were implemented by the Saudi Aquaculture Society (SAS), under the supervision of the Ministry of Environment, Water, and Agriculture, to ensure that all national aquaculture facilities obtain a Best Aquaculture Practices (BAP) certification; however, it is unclear whether this goal has been achieved. Current efforts are being coordinated by KAUST, through a national project funded by the Government (National Center for Wildlife) to characterize Red Sea biodiversity and foster the understanding of mNIS in the area. One of the main goals of this project is to develop a Biosecurity Framework in the country following cutting-edge frameworks worldwide (e.g., US, Australia, and New Zealand).

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CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest.

DATA AVAILABILITY STATEMENT

Raw sequences reads have been deposited in the NCBI short read archive with accession numbers PRJNA1103945 and PRJNA1103941.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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