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Site-specific variation and non-indigenous species detection in Arabian Gulf biofouling communities using DNA metabarcoding and photographic surveys

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Ports and marinas are global hotspots for non-indigenous species (NIS), yet biofouling communities in the Arabian Gulf remain poorly documented. Here, we present the first spatially extensive assessment of biofouling communities along ~300 km of the Saudi Arabian Gulf coastline. Across 12 sites, we combined COI metabarcoding of scrapings from pontoons/docks and settlement panels with image-based analysis of same panels to characterize taxonomic composition and quantify native, cryptogenic, and non-indigenous taxa. Community analyses revealed a clear site-level differentiation across all datasets, while broader geographic locations and environmental risk categories explained little variation. DNA metabarcoding detected more taxa than visual analyses, including 57 cryptogenic and NIS, many of which could not be visually detected. Industrial and commercial ports harbored higher NIS richness and relative abundance than recreational marinas, indicating localized invasion risk. Despite the sensitivity of DNA-based approaches, species-level assignments must be interpreted cautiously due to sparse regional reference libraries and limited representation of Gulf taxa in global databases. By generating one of the first spatially explicit baselines of biofouling biodiversity and NIS in the northwestern Indian Ocean, our study underscores the urgent need to expand region-specific reference databases and to implement integrated, site-resolved monitoring frameworks to support emerging marine biosecurity programs across this understudied system.

Keywords Biofouling communities, Marine bioinvasion, DNA metabarcoding, Artificial marine habitats, Site-specific integrated monitoring, Arabian Gulf

Human activities have altered marine ecosystems through practices such as coastal development, industrialization, and resource exploitation^{1,2}. Among these, the introduction of artificial structures in the marine environment leads to biodiversity loss and disruption of critical ecosystem services through reduced habitat heterogeneity,

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altered natural hydrodynamic regimes, and the provision of simplified substrates that favor opportunistic colonization^{3–5}. The construction of artificial habitats destroys and replaces natural ecosystems and promotes the establishment and spread of non-indigenous species (NIS)^{5–8}. The widespread occurrence of NIS in artificial habitats is strongly linked to maritime trade, with shipping serving as a primary dispersal vector through ballast water discharge and hull fouling, facilitating the crossing of biogeographical barriers and establishment of NIS in new regions^{9,10}. The interaction between shipping and artificial habitats, particularly in ports and marinas, amplifies the risk of biological invasions. Consequently, port areas have become NIS hotspots and are among the most invaded coastal marine ecosystems worldwide^{11–14}. The high concentration of NIS in these modified environments underscores their function as conduits for the spread of NIS into surrounding natural systems^{15,16}, and the frequent movement of recreational craft between artificial and natural habitats may further facilitate the dispersal of NIS^{11,17–20}. Together, these dynamics not only accelerate the distribution of invasive species but also increase the ecological risks to nearby natural ecosystems, threatening biodiversity and disrupting ecological balance^{17,21}.

Marine NIS are increasingly recognized as a major source of environmental, ecological, and socioeconomic disruption^{22,23}. Examples include significant impacts on biodiversity and ecosystem functioning (e.g., disrupting food webs, modifying nutrient cycling processes, and outcompeting native species)^{23–25}. From an economic perspective, NIS contribute to extensive biofouling of aquaculture infrastructure (nets and water intake systems), resulting in reduced harvest yields and increased maintenance operational costs^{26–29}. These impacts are rarely isolated, with a single introduction potentially resulting in cascading consequences. For instance, the loss of critical coastal habitats such as seagrass beds or mangroves due to invasive species can compromise natural shoreline defenses, increasing coastal vulnerability to erosion, storm surges, and flooding^{30,31}. Despite increased global attention, significant knowledge gaps remain in the understanding of marine bioinvasions, particularly in geographically understudied regions, ecologically sensitive habitats, and poorly characterized taxa. Addressing these gaps is essential for developing effective mitigation and management strategies. A critical priority is the expansion of baseline data on genetic and taxonomic diversity in data-poor regions. This must be accompanied by the implementation of sustained monitoring programs that integrate traditional survey methods with molecular tools. Such integrated approaches can improve the accuracy of NIS inventories, support evidence-based decision-making, and facilitate early detection and rapid response through targeted stakeholders and public engagement^{23,32,33}.

The Indian Ocean region has consistently been highlighted as a major knowledge gap in marine invasion science³⁴. This is particularly concerning given the increasing pressures from intense maritime traffic, industrial expansion, and large-scale coastal reclamation projects, particularly in rapidly developing areas within the region, such as the Arabian Gulf shorelines³⁵. In the Arabian Gulf, research has primarily focused on documenting first records of NIS^{36–38}, investigating introduction pathways^{39,40}, and conducting risk assessments based on NIS lists⁴¹. While these studies provided valuable insights, they remain fragmented and limited in time and space. Therefore, further research is needed based on more comprehensive, standardized frameworks across the region to ensure effective long-term monitoring and proactive NIS management.

This study aims to provide a foundational step toward standardized, long-term monitoring of NIS in the Arabian Gulf, addressing the urgent need for proactive measures to understand NIS invasion dynamics and mitigate their impact on the region's ecologically and economically valuable coastal ecosystems. Specifically, the objectives of this research are to: (1) document biofouling taxa from three-month deployed PVC settlement panels and permanently submerged artificial structures (i.e., pontoons and dock seawalls); (2) quantify and compare the diversity, distribution, and relative abundance of NIS across sites under varying degrees of anthropogenic influence; and (3) characterize community structure and investigate patterns of invasion dynamics through complementary approaches, combining photographic assessments with DNA metabarcoding. By combining these complementary methods, our study aims to generate critical baseline data to inform regional biosecurity frameworks and ecosystem-based management strategies.

Methods

Study sites

The study was conducted in four locations along Saudi Arabia's Arabian Gulf coastline: Al-Jubail, Tarout Island, Dammam, and Al-Khobar (Fig. 1). Each location included three sites. The sites in the northernmost study location, Al-Jubail, were: JBCF (Jubail Commercial Fishing Harbor), a site supporting large-scale fishing operations; JBIP (Jubail Industrial Port), a major artificial facility handling petrochemical exports and raw material imports; and JBNO (Jubail Coast Guard Harbor), a smaller structure used for maritime security purposes. Tarout Island featured three sites: TINO (Tarout Island North), a recreational fishing harbor for small recreational vessels, and the commercial fishing harbors TAFM (Tarout Island Fishing Marina) and TISO (Tarout Island South), which support large-scale fishing operations. In Dammam, the study included DMCG (Dammam Coast Guard Marina), a small Coast Guard marina dedicated to maritime safety and search-and-rescue operations; and DMCN (Dammam Commercial Port) and DMSC (Dammam Shipping Control Harbor), located on concrete docks in two different wharfs within Dammam Commercial Port, one of the largest cargo-handling ports in the Arabian Gulf. The southernmost study location, Al-Khobar, comprised: KBCN (Al-Khobar Fishing Harbor), an artificial facility supporting artisanal fishing and preserving local fishing traditions; and two recreational marinas, KBGT (Khobar Golden Tulip Hotel Marina) and KBSB (Khobar Sunset Beach Marina), both catering to leisure activities such as boating and yachting, which contribute to the local tourism industry. The study was conducted on plastic floating pontoons at JBNO, TINO, TAFM, DMCG, KBCN, KBGT and KBSB, and on concrete dock seawalls at JBCF, JBIP, TISO, DMCN, DMSC and KBSB.

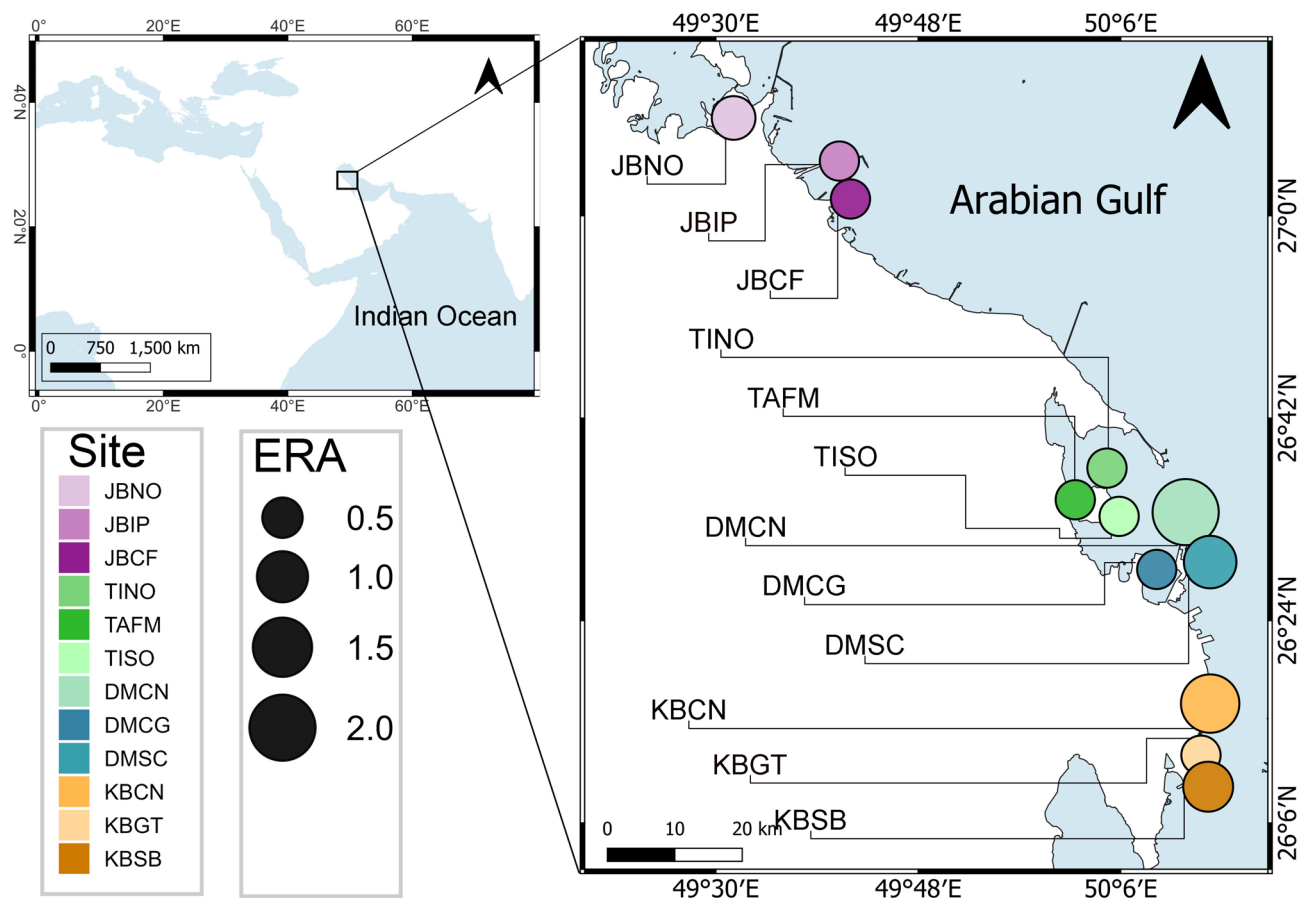


Fig. 1. Map of the study sites for the locations Al Jubail (shade of purple), Tarout Island (shade of green), Dammam (shade of blue), and Al Khobar (shade of brown), along Saudi Arabian Gulf coastline. Bubble diameter indicates the results for the Environmental Risk Assessment (ERA) calculated per site. The map was created by the authors using QGIS Desktop v3.40.2 (<https://qgis.org>).

Environmental risk assessment of study sites

An Environmental Risk Assessment (ERA) was conducted for each site using the formula: $ERA = Pr * St^{42}$, where: St is the Environmental State and Pr the Environmental Pressure.

Pr was calculated as $Pr = NV + PT + EX + DG$, where NV is the Navigation Activity, estimated as the port's capacity (berths/m²), PT the Port Activity, which reflects the presence or absence of dry docks and refueling facilities in the site (PT = 1 and PT = 0, respectively), EX the External activity, which depends on the main land use within a one km radius from the studied site, and DG the dredging activity. An EX value of 1 was given for industrial land uses and 0.5 for residential and agricultural uses. DG was estimated by considering the typology of sites: a high probability of dredging (DG = 1) for commercial and fishing ports with high vessel activity (JBIP, JBCF, TAFM, TISO, DMCG and DMCN), and a moderate probability (DG = 0.5) for the rest of harbors (marinas and docks).

St was calculated as $St = NA + EV + CTRI_i / CTRI_{max}$, where the Naturalness (NA) indicates the level of modification of the site's water body (see categories at⁴²), the Ecological Value (EV) is the number of ecological singular elements in a 1 km buffer distance around the water surface where the port activity takes place, and CTRI stands for the Complexity Tidal Range Index, as in⁴³, which is used to estimate the flushing capacity of each port's water body (CTRI_i), in relation to the highest CTRI among the studied ports (CTRI_{max}). For the CTRI, the medium tidal range was set at 0.6 m at all sites due to their geographical proximity.

The information to calculate NV, PT, EX and NA values was based on observations during sample collection at each site. Google Earth images from the year 2022 were used to confirm NV, EX and NA, and to measure each site's water body area, maximum width, and entrance width for the calculation of CTRI_i. The information on existing natural protected areas in the area used to calculate EV was gathered from governmental sources⁴⁴.

Study design and sample collection

Five unsanded polyvinyl chloride (PVC) settlement panels (14 × 14 cm) were deployed at each study site from December 2023 to March 2024, following standardized protocols^{33,45,46}. Each panel was attached horizontally to a brick secured to concrete docks or floating pontoons using a rope, with the experimental surfaces of the panels facing downward, and suspended at a depth of 1–2 m below the surface. After three months of deployment, the

panels were retrieved, detached from the brick and placed in trays or buckets containing local filtered seawater for transportation to the laboratory. High-resolution images of each panel were taken immediately upon retrieval using a Canon R5 camera equipped with external flashes to document the biofouling communities, prior to any specimen collection or scraping. Representatives of each observed taxon were then collected during panel retrieval as voucher material for conservative morphological screening by a team of taxonomists with expertise in different taxonomic groups, and taxa were recorded to the lowest reliable taxonomic level. Morphological screening was intended to support image interpretation and cross-reference taxa detected by metabarcoding, rather than to produce exhaustive species-level identifications for all benthic groups. Subsequently, biofouling assemblages attached to the panels were scraped off using a spatula, homogenized, and preserved in 96% ethanol at a ratio of 1:5 (biological material to ethanol). From each panel sampled, the homogenized material was aliquoted into up to two replicate 50 ml falcon tubes (A and B), depending on biomass availability, yielding a maximum of ten subsamples per site. Samples were stored at 4 °C for downstream processing.

In addition to the settlement panels, biofouling communities were sampled from vertical fixed artificial substrates, including pontoons and concrete docks (see Sect. 2.1), at the same sites during the panel retrieval period. Five random samples were collected from each site using a hand-held metal scraper with a 1 mm mesh size and a collection area of 20 × 25 cm. The scraping method targeted the biofouling from approximately 1 m depth to the surface, following established protocols used in Mediterranean biofouling studies (e.g.^{17,47}). This approach aims to provide a comparative dataset of the biofouling communities naturally occurring at the study sites. Biofouling samples scraped from pontoons and docks were not photographed but otherwise preserved and processed in an identical manner to panel scrapings to ensure comparability between sample types.

Dataset preparation

Image-based dataset

Photographic images of the settlement panels were analyzed to quantify the relative abundance of biofouling species. Each image was processed to a standardized size and resolution, and a digital 10 × 10 cm quadrat was overlaid over the images, covering the center of the panel and discarding a 2 cm border. Species coverage within the quadrat was assessed using 50 random points stratified within a 5 × 5 grid. Points were classified based on species presence or as bare substrate if no biofouling was present. Points over two overlapping species (e.g., epibiosis) were counted as two points (basibiont and epibiont). Species present on panels and visible on images but not represented by point-counts were assigned 0.5% coverage. Species identification for the image analysis was supported by conservative morphological screening of voucher material and macro-photographs taken during panel retrieval, with taxa recorded to the lowest reliable taxonomic level. Identifications were further informed by cross-referencing with taxa detected by metabarcoding (See Supplementary Fig. S1). Photo-analyses were conducted using PhotoQuad v1_4 software⁴⁸.

DNA-based dataset

Genomic DNA was extracted from the biofouling material scraped from the settlement panels and from pontoons and docks. DNA extraction was performed using the DNeasy PowerLyzer PowerSoil Kit (QIAGEN) following the protocol described in³³. For each sample, 0.4–0.6 g of homogenized biofouling material were incubated overnight at 56 °C with proteinase K, and the extracted DNA was eluted in 100 µL of buffer. This subsampling range is commonly used in metabarcoding studies of complex biofouling and benthic assemblages, providing a balance between sufficient DNA yield and extraction efficiency. To account for potential heterogeneity within homogenized samples, up to two DNA extraction replicates (A and B; see Sect. 2.3) were processed per sample. When both replicates were available and passed quality filtering, read counts were averaged prior to downstream analyses^{49,50}. DNA concentration was quantified using the Qubit dsDNA High Sensitivity Assay Kit (Thermo Fisher Scientific). Samples were subsequently normalized to a concentration of 5 ng/µL prior Polymerase Chain Reaction (PCR).

A 313 bp fragment of the mitochondrial cytochrome c oxidase I (COI) gene was amplified using the universal primers mICOLint-jgHCO2198⁵¹ with overhang Illumina adaptors, following the PCR protocol and conditions described in³³. Amplicon libraries were indexed using a unique combination of Illumina dual indexes (Nextera XT Index Kit), pooled, and sequenced on an Illumina MiSeq sequencing platform (300 bp, paired-end) at KAUST Bioscience CORE labs. 20% phiX was included for internal control. A total of 214 sequencing libraries were submitted across three Illumina MiSeq runs, comprising 197 biological libraries, 15 DNA extraction blanks, and 2 PCR negative controls (see Supplementary Table S1). Among the biological replicates, some DNA extraction replicates (A or B) could not be generated due to insufficient biomass, and 19 extracted subsamples failed PCR amplification and were excluded prior to library preparation. In addition, eight settlement panels were lost during deployment, resulting in the absence of both extraction replicates for those panels (Supplementary Table S1). Following sequencing, 13 successfully amplified libraries yielding fewer than 1,000 sequence reads were excluded from further analyses. After quality filtering, 184 biological samples were retained for downstream analyses. Sequence data were automatically demultiplexed using MiSeq Reporter (v2), and forward and reverse reads were assigned to samples. Raw sequencing data generated in this study are publicly available in the European Nucleotide Archive (ENA) under study accession number PRJEB94117 (please see the Data Availability Statement for further details).

Bioinformatic processing Raw sequence reads were decontaminated of phiX and adapter-trimmed using the BBDuk tool from the BBMap suite (Bushnell B, <http://sourceforge.net/projects/bbmap/>). The cutadapt tool⁵² was used to remove primer sequences from the reads. The retained sequences were analyzed on DADA2⁵³ under the standard inference mode (as detailed in the DADA2 workflow for Big Data: https://benjjneb.github.io/dada2/bigdata_paired.html) to define the amplicon sequence variants (ASVs). Forward and reverse reads were merged

with the *mergePairs* function of DADA2. Sequence tables resulting from the individual sequencing runs were further merged via the *mergeSequenceTables* function. Chimeric ASVs were identified with the *removeBimeraDe-novo* function and removed. The ASVs were clustered into molecular operational taxonomic units (MOTUs) at 97% identity^{54–56} using the *IdClusters* function in the DECIPHER package⁵⁷. Potential contaminant MOTUs identified by the *decontam* tool⁵⁸ using default parameters were removed from the analysis based on their prevalence in DNA extraction blanks and control libraries. MOTUs representing putative pseudogenes or nuclear mitochondrial DNA segments (nuMTs) were identified and filtered out based on length as described in⁵⁹. Taxonomy was assigned to chimera-, contaminant- and nuMT-free MOTU sequences using BLASTn⁶⁰, based on up to five nucleotide hits per MOTU and an e-value threshold of 1e-10, in combination with the BOLDigger3 tool⁶¹ and the *lcaPident* function in the *biohelper* R package (<https://github.com/olar785/biohelper/>), referencing the curated BOLD database v5⁶² and the NCBI core nucleotide database (version 5; January, 2025). Singleton sequences or sequences that were detected in less than two libraries were excluded along with non-target MOTUs representing taxa that were outside of Eukaryota. Samples that failed PCR amplification were excluded prior to sequencing. Among successfully amplified samples, libraries yielding fewer than 1000 sequence reads ($n = 13$) after sequencing were excluded from further analyses. Following quality filtering, 1,497 COI MOTUs were retained and used to analyze species composition and diversity in biofouling assemblages from settlement panels and from permanent artificial structures (pontoon and docks).

Prior to statistical analyses, the COI-derived MOTU table ($n = 1,497$) was first filtered to include only MOTUs assigned to Kingdoms Animalia and Plantae. MOTUs were then manually screened to exclude taxa without representation in marine or brackish environments, according to World Register of Marine Species⁶³. During this screening, taxonomic names were standardized to current accepted nomenclature and inconsistencies among taxonomic ranks (e.g., family or order not matching the assigned genus) were corrected when supported by authoritative reference databases. After this taxonomic and ecological filtering, a total of 719 MOTUs were retained for downstream statistical analyses. For each sample, replicate DNA extractions (A and B), when available, were merged by averaging read counts to generate a single MOTU profile. The same procedure was applied to pontoon and dock samples.

Species-level status assignment Following taxonomic assignment, species-level names were interpreted conservatively for the purpose of ecological and biogeographical status assignment. Each species identified through photo-based analysis, and each MOTU assigned to species level in the COI metabarcoding dataset were individually screened for their native or non-native range. The status ‘non-indigenous’ was assigned to species not historically present in the Arabian Gulf and whose presence in the study region likely resulted from human-mediated introductions⁶⁴. Taxa with unknown or poorly known evolutionary and biogeographical origin and unresolved species complex non-historically present in the Arabian Gulf, which could potentially be non-native to the Arabian Gulf were classified as ‘cryptogenic’⁶⁴.

Statistical analyses

Some replicates were lost due to unforeseen circumstances during the 3-months deployment period and methodological constraints (see final number of samples per site and dataset at Supplementary Table S1). Eight settlement panels were lost after deployment and could not be retrieved: two in JBIP, four in TAFM, and one in each DMSC and KBCN. These samples were lost for photo analysis and panel metabarcoding. PCR amplification was unsuccessful for several settlement panel metabarcoding samples. All panel samples from KBCN (except the lost panel) and all panel samples from KBSB failed PCR amplification. Additional PCR failures occurred in JBNO (one panel). Despite testing template dilutions and the addition of bovine serum albumin (BSA); these samples were excluded from the dataset. No samples were lost from pontoon/docks vertical surface scrapings, although a small number of extraction replicates were excluded following sequencing due to low read counts (< 1,000 reads; see Supplementary Table S1).

For multivariate analyses and ordinations, the datasets (1) DNA-based MOTUs from pontoon/dock scrapings, (2) DNA-based MOTUs from settlement panel scrapings, and (3) photo-based percent-cover matrices from point-count analysis of panel images were converted by total-sum normalization in PRIMER v7 (“Standardize samples by total”) to provide quantitative estimates of species relative abundance. To reduce noise in the metabarcoding datasets, we kept only those variables (i.e., MOTUs) that contributed $\geq 5\%$ of MOTUs relative abundance to at least one sample. Datasets were then square-root-transformed and used to compute Bray–Curtis resemblance matrices. Prior to hypothesis testing, homogeneity of multivariate dispersions was tested separately for two factors: Location and Site using PERMDISP routine. A two-way permutational multivariate analysis of variance (PERMANOVA) was then carried out for each dataset, testing for differences in community composition with the environmental risk assessment (ERA) results as a covariate, testing the factors Location (fixed), and Site (random, nested within Location). Analyses used Type I sequential sums of squares and 9,999 permutations under a reduced-model framework. Additionally, resemblance matrixes were used to compute Principal Coordinates Analysis ordinations (PCoA) with taxon biplot vectors overlaid by fitting Spearman’s rank correlations to the ordination axes ($p \geq 0.6$) using classical multidimensional scaling (*cmdscale* in R).

For univariate analyses, untransformed (3) percent-cover data from point-count analysis were used to obtain percent coverage of biofouling per panel (biofouling coverage dataset). Additionally, the standardized datasets (1) MOTUs from pontoon/docks, (2) MOTUs from settlement panels, and (3) taxa percent-cover point-count analysis were used to obtain the number of MOTUs (1 and 2) and taxa (3) per sample (taxonomic richness datasets), the number of non-indigenous cryptogenic species per sample (cryptogenic and NIS richness datasets), and the proportion of readings (1 and 2) and coverage on panels (3) occupied by cryptogenic and NIS to total taxa (relative abundance of cryptogenic and NIS datasets). All these datasets were used to compute Euclidean distances resemblance matrices, which were analyzed using two-way univariate PERMANOVAs following the

same design as multivariate analyses. All univariate and multivariate PERMANOVAs were performed with 9,999 permutations, and results were considered statistically significant at $P \leq 0.05$.

Ordinations and plots were carried out in R version 4.2.1 (R Core Team, 2022) with primary analytical functions and graphical packages including *dplyr*, *tidyr*, *ggplot2*, *vegan*, *patchwork*, *ggridges* and *ggvenn*^{65–67}. Multivariate and univariate PERMANOVA were carried out using Primer v7. + PERMANOVA^{68,69}.

Ethical approval and permits

All field sampling and handling of environmental biological material were conducted in compliance with institutional biosafety and bioethics regulations. The study was reviewed and approved by the Institutional Biosafety and Bioethics Committee (IBEC) at King Abdullah University of Science and Technology (KAUST) under protocol IBEC #24IACUC020. Fieldwork and sample collection were conducted under permits issued by the National Center for Wildlife (NCW), Saudi Arabia. All procedures complied with relevant institutional guidelines and national regulations.

Results

Community dispersion, richness, and taxonomic composition

Patterns of community dispersion, diversity, and taxonomic composition differed significantly across sites, while broader location-level or environmental risk factors played more limited roles. Multivariate dispersion, as a proxy for community heterogeneity, varied significantly across locations for both pontoon/dock ($p < 0.05$, $F = 3.42$) and settlement panel DNA datasets ($p < 0.05$, $F = 4.81$) (Supplementary Table S2). This suggests the presence of broader-scale differences in beta diversity among locations. However, site-level effects were consistently stronger: multivariate dispersion varied significantly among individual sites for both pontoon/dock DNA metabarcoding ($p < 0.001$, $F = 6.13$) and settlement panel photo analysis ($p < 0.001$, $F = 7.90$), indicating pronounced within-location heterogeneity (Supplementary Fig. S2). In contrast, no significant differences in dispersion were detected among sites for the panel DNA dataset ($p = 0.2432$), despite visible variation in violin plots (Supplementary Fig. S2). This discrepancy likely reflects limited replication due to sample loss during DNA extraction or amplification failures. As shown in Supplementary Table S1, complete panel DNA data were unavailable for several sites (e.g., only two samples at JBNO, three at JBIP, and none at KBCN and KBSB), substantially reducing statistical power. In some locations, panel photos were also unavailable due to panels being removed or tampered with by port users. Sites such as JBNO and DMCG repeatedly exhibited broad dispersion across methods, suggesting patchy or unstable fouling communities. In contrast, sites like KBGT and TISO showed more compact dispersion, potentially reflecting more stable or environmentally uniform assemblages. Building on these patterns, PERMANOVA analysis of community structure (Table 1A) confirmed that site-level differences were the dominant factor shaping biofouling assemblages. Across all three datasets (pontoon/dock DNA, panel DNA, and panel photo) assemblage composition differed significantly among sites ($p < 0.001$ for all), while neither location nor the Environmental Risk Assessment (ERA) explained a meaningful portion of variance. Only the photographic dataset showed a marginal ERA effect ($p = 0.045$), potentially reflecting visible changes in panel cover with increasing anthropogenic pressure. These results are further supported by PCoA ordinations (Fig. 2), which showed clear site-level clustering, especially in high-ERA sites like JBCE, JBNO, and DMCN. Vectors identifying key taxa ($p \geq 0.6$) consistently pointed to the same suite of species across the employed methods, including some non-indigenous or cryptogenic species such as *Amathia verticillata*, *Botrylloides niger*, and *Jassa falcata*.

Species richness followed similar spatial trends as community structure, with consistent differences observed across sites (Table 1B). The pontoon/dock DNA dataset revealed a total of 639 unique MOTUs, compared to the 362 unique MOTUs identified from the settlement panels (Fig. 3), reflecting a richer and likely more mature and established biofouling community on permanent vertical structures. MOTU richness varied significantly among sites but not among locations (PERMANOVA, Table 1B), with the highest richness observed at industrial and commercial sites such as JBIP, JBCE, JBNO, and DMCN (up to 78 MOTUs per sample), and consistently lower values recorded at recreational sites such as KBGT and KBSB (Fig. 3a and c). DNA-based panel results showed similar site-level patterns despite overall lower richness, suggesting that communities developing on the settlement panels are shaped by the same spatial filters influencing biofouling assemblages on permanently submerged structures. Comparisons between metabarcoding and photo-based assessments of settlement panels further highlighted the limitations of image-based approaches in detecting full taxonomic diversity. Only 37 morphologically distinguishable taxa were identified in photo analyses, with richness peaking at JBCE and sharply declining at sites with panels either dominated by bare space or covered by a few fast-growing species (e.g., hydroids or colonial ascidians; Fig. 3e). While the absolute richness captured by photo analysis was substantially lower, likely due to difficulties in resolving small, cryptic, or encrusting taxa such as sponges, polychaetes, and red algae, the relative richness rankings across sites were broadly consistent with those obtained via metabarcoding. This alignment suggests that photo-based assessments can still reflect dominant site-level biodiversity patterns, even if they underestimate total diversity.

Taxonomic composition at the phylum level revealed consistent differences across substrates and methods, reflecting both biological variation and methodological constraints (Fig. 3). DNA metabarcoding of pontoons/docks indicated high relative read abundance of Arthropoda, Porifera, and Annelida, detected consistently across sites, followed by Mollusca and Bryozoa, with a high relative abundance at some sites (Fig. 3b). Assemblages on pontoons appeared taxonomically richer and more even in composition than those detected on panels, consistent with the longer establishment time and likely greater habitat complexity on these permanent structures. On settlement panels, DNA-based profiles were mainly dominated by Arthropoda and Bryozoa, but showed a reduced representation of Porifera and Annelida, compared to pontoon/docks communities, potentially due to early successional dynamics or differing settlement preferences (Fig. 2d). Phylum-level trends

Source	Pontoon/dock (DNA metabarcoding)				Settlement panels (DNA metabarcoding)				Settlement panels (photo-analysis)			
	df	MS	Pseudo-F	P(perm)	df	MS	Pseudo-F	P(perm)	df	MS	Pseudo-F	P(perm)
A: Assemblage structure (multivariate)												
Covariate (ERA)	1	5,803	0.5036	0.848	1	8081.8	0.70026	0.7168	1	17,555	1.8084	0.057
Location	3	11,646	1.0108	0.4583	3	8933.3	0.88064	0.6208	3	9571.9	0.99369	0.505
Site (Location)	7	11,522	11.867	0.0001	5	8308.9	8.018	0.0001	7	8500.8	7.2452	0.001
Residual	48	970.9			28	1036.3			40	1173.3		
B: Taxonomic richness												
Covariate (ERA)	1	12.51	1.3492	0.2842	1	3.0938	0.36317	0.8009	1	2.0411	1.2139	0.3138
Location	3	8.2284	0.88746	0.4927	3	0.23689	0.058012	0.9795	3	1.6993	1.0116	0.4474
Site (Location)	7	9.2719	8.7298	0.0001	5	6.74	6.7446	0.0004	7	1.4703	12.529	0.0001
Residual	48	1.0621			28	0.99932			40	0.11735		
C: Relative abundance of NIS and cryptogenic												
Covariate (ERA)	1	8281.7	0.67519	0.862	1	11,047	0.90735	0.5702	1	17,358	1.8659	0.045
Location	3	11,987	0.97729	0.4767	3	9401.2	0.88092	0.6324	3	8234.8	0.89783	0.62
Site (Location)	7	12,266	4.3348	0.0001	5	8916.4	4.8341	0.0001	7	8173.3	5.305	0.001
Residual	48	2829.6			28	1844.5			40	1540.7		
D: NIS and cryptogenic richness												
Covariate (ERA)	1	47.1	0.61639	0.4594	1	11.681	0.1258	0.985	1	3.573	5.0654	0.0573
Location	3	61.398	0.80351	0.5225	3	12.031	0.14061	0.8948	3	0.5927	0.85903	0.4991
Site (Location)	7	76.413	12.915	0.0001	5	74.015	18.509	0.0001	7	0.61421	5.751	0.0002
Residual	48	5.9167			28	3.9988			40	0.1068		

Table 1. Nested two-way PERMANOVA results for Location and Site for the biofouling datasets: pontoon/dock scrapings (DNA metabarcoding), settlement-panel scrapings (DNA metabarcoding), and photographic point-counts of the same settlement panels (photo-analysis). Response variables were (A) multivariate analysis of taxa relative abundances (structure of assemblages), (B) number of MOTUs (DNA metabarcoding datasets) and taxa (photo-analysis dataset), (C) proportion of cryptogenic and non-indigenous species to total number of taxa per sample in terms of number of readings (DNA metabarcoding datasets) or space occupied on panels (photo-analysis dataset), and (D) number of cryptogenic and non-indigenous species. ERA = Environmental Risk Assessment; df = degrees of freedom; MS = mean square sum; P(perm) = permutation-based p-values (9,999 permutations).

in photo-based panel analysis were broadly aligned with DNA findings but were more limited in scope. Visually identifiable taxa included colonial ascidians (Chordata), barnacles (Arthropoda), bryozoans, and mollusks, with frequent underrepresentation of soft-bodied or encrusting groups such as sponges and polychaetes (Fig. 3f). Notably, photo-based community composition also captured contributions from abiotic categories such as bare space and sediment, which were especially prominent at sites like KBSB, KBCN, and DMCG, reflecting either physical disturbance or delayed colonization. These patterns reinforce the value of integrating high-resolution DNA approaches with photo-based visual surveys, which, while taxonomically limited, still provide insights into spatial variation in dominant taxa and substratum use.

Distribution and detection of non-indigenous and cryptogenic species across sites and methods

Across all methods, a total of 57 species as non-indigenous or cryptogenic were identified (Supplementary Fig. S4), with taxonomic assignments spanning Arthropoda, Bryozoa, Chordata, Mollusca, Porifera, Annelida, and Rhodophyta. DNA metabarcoding of pontoons/docks yielded the highest richness, detecting 42 NIS/cryptogenic taxa, followed by 37 species detected in DNA-based panel samples, and 17 visually recognizable taxa identified from photo-based panel analysis (Fig. 4a, c and e). PERMANOVA results confirmed that site-level effects were the primary drivers of both the relative abundance (Table 1C) and richness (Table 1D) of NIS and cryptogenic taxa across all three datasets ($p < 0.001$). No significant differences were found at the location level, and the Environmental Risk Assessment (ERA) levels explained only a small and marginally significant portion of variation in photo-based NIS dominance ($p = 0.045$), with no effect detected in DNA-based datasets.

The highest richness of NIS and cryptogenic species was observed at commercial and industrial sites such as JBCF, JBNO, DMCN, and DMSC, where samples contained up to 15 taxa (Fig. 4a and c). In contrast, recreational sites such as KBSB, KBCN, and KBGT consistently showed lower richness and lower relative abundance of NIS, especially in DNA datasets. These patterns were mirrored in the visual data: panels at KBSB and KBCN often exhibited substantial proportions of bare space or sediment (Fig. 4f), indicating delayed colonization or physical disturbance that may limit the establishment of NIS. Relative abundance of NIS and cryptogenic taxa in DNA reads also varied among sites, with proportions exceeding 50% at JBCF and JBNO in both panel and pontoon samples (Fig. 4b and d). Despite overall lower richness, photo-based analysis aligned with DNA trends,

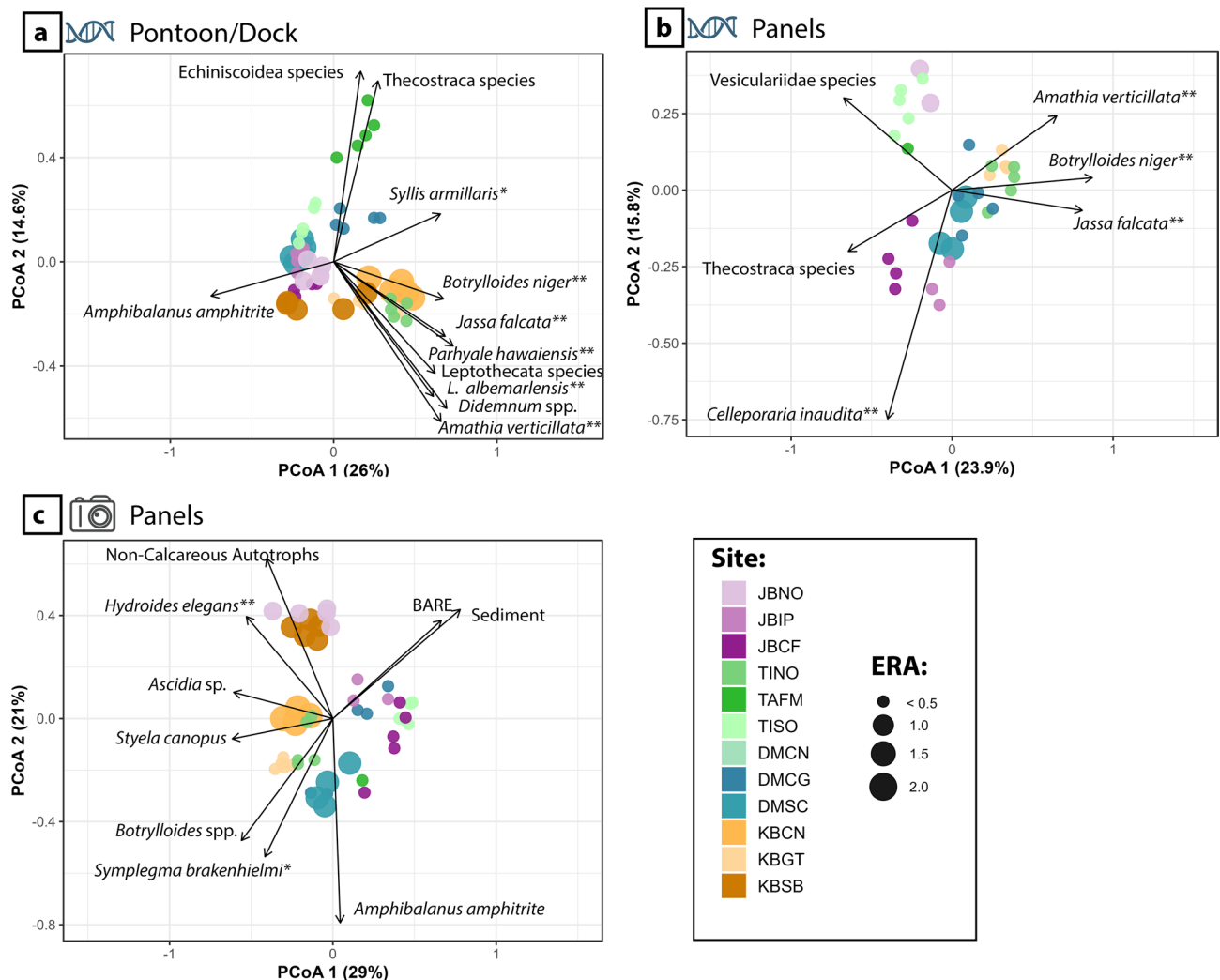


Fig. 2. Principal coordinates analysis (PCoA) of biofouling assemblages from (a) pontoons/docks (DNA metabarcoding), (b) settlement panels (DNA metabarcoding), and (c) settlement panels (photo analysis). Points are colored by site and scaled by environmental risk assessment (ERA). Vectors represent taxa with strong correlations ($p \geq 0.6$) to ordination axes. Double asterisks (**) denote non-indigenous species (NIS), single asterisks (*) indicate cryptogenic species. *L. albemarlensis* refers to *Lissodendoryx* (*Lissodendoryx*) *albemarlensis*.

detecting similar site-level patterns in dominance, particularly at high-ERA locations like JBCF and TISO (Fig. 4f, Table 1C).

PCoA ordinations further illustrated the influence of NIS and cryptogenic species in shaping community structure. In both pontoon and panel DNA datasets, vectors associated with key non-indigenous taxa, including *Amathia verticillata*, *Botrylloides niger*, *Jassa falcata*, and *Lissodendoryx albemarlensis*, explained much of the observed variation and were tightly linked to high-ERA sites (Figs. 2 and 4a and b). Similar taxa were identified in the photo-based dataset, along with visual categories such as sediment and bare space, which also contributed strongly to community divergence (Fig. 4c). Integration of detection results across methods (Supplementary Fig. S3) confirmed the complementarity of DNA metabarcoding and visual analysis. Only seven species were shared across all three methods, while 23 were detected exclusively in both DNA datasets and not observed in photos. Eleven species were unique to pontoons; six were exclusive to panel DNA, and eight were identified only in photo analysis. Method-specific taxonomic biases were further evident in the presence/absence matrix (Supplementary Fig. S3). Photo analysis disproportionately captured conspicuous colonial taxa such as ascidians and barnacles, while DNA metabarcoding more consistently detected soft-bodied, encrusting, or cryptic taxa including sponges, red algae, and polychaetes. Several phyla, especially Porifera, Rhodophyta, and Tardigrada, were completely absent from photo data but consistently recovered in metabarcoding, underscoring the limitations of morphology-based identification. These findings emphasize the need for multi-method approaches to adequately document the breadth and spatial distribution of NIS and cryptogenic species in marine fouling communities.

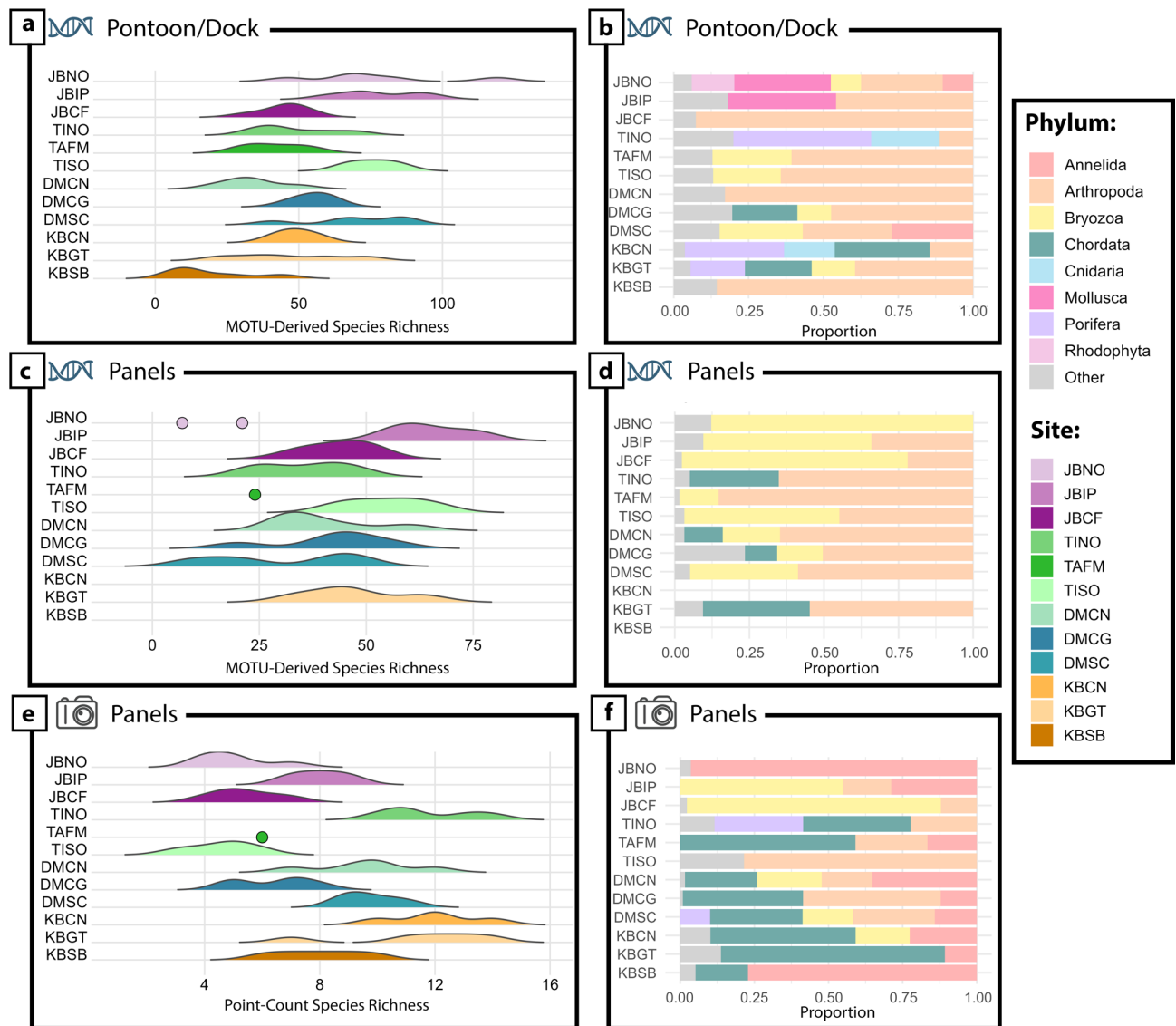


Fig. 3. Molecular operational taxonomic units (MOTUs) and species richness, and phylum-level taxonomic structure of biofouling assemblages from pontoon/dock vertical surfaces (metabarcoding) and horizontal settlement panels (image analysis and metabarcoding) across artificial marine habitats along Saudi Arabia's Arabian Gulf coast. Ridge plots (a,c,e) represent site-level distributions and stacked bar plots (b,d,f) display proportional composition at the phylum level for each site for MOTUs (DNA strain icon) from pontoon/docks (a,b) and panels (c,d), and species richness from image analysis (camera icon) of panels (e,f). For visualization clarity, phyla contributing <10% relative abundance within a site were pooled into an "Other" category in stacked bar plots. Ridge plots summarize the distribution of per-sample richness values for each site; sites with one or two samples are represented by individual points.

Discussion

This study represents one of the most spatially extensive assessments of biofouling communities along the Saudi Arabian Gulf coastline, spanning approximately 300 km and encompassing 12 sites across four ecologically and operationally distinct coastal regions. By integrating DNA metabarcoding and image-based surveys across pontoons, docks, and settlement panels, we documented a wide range of native, cryptogenic, and non-indigenous species (NIS) in a region that remains underrepresented in global marine biodiversity databases. Consistent with our statistical results, community structure was primarily shaped at the site level rather than by broader geographic location or the Environmental Risk Assessment (ERA) categories, in line with previous studies showing that biofouling assemblages are often shaped by hyper-local factors, including microhabitat complexity, water circulation, shading, infrastructure design, and proximity to pollution sources^{70–72}. In our study, sites such as JBNO and DMCG exhibited high multivariate dispersion across methods, suggesting patchy or unstable assemblages, whereas more compact assemblages at KBGT and TISO likely reflect more environmentally uniform and stable conditions. Together, these results emphasize the dominance of site-specific

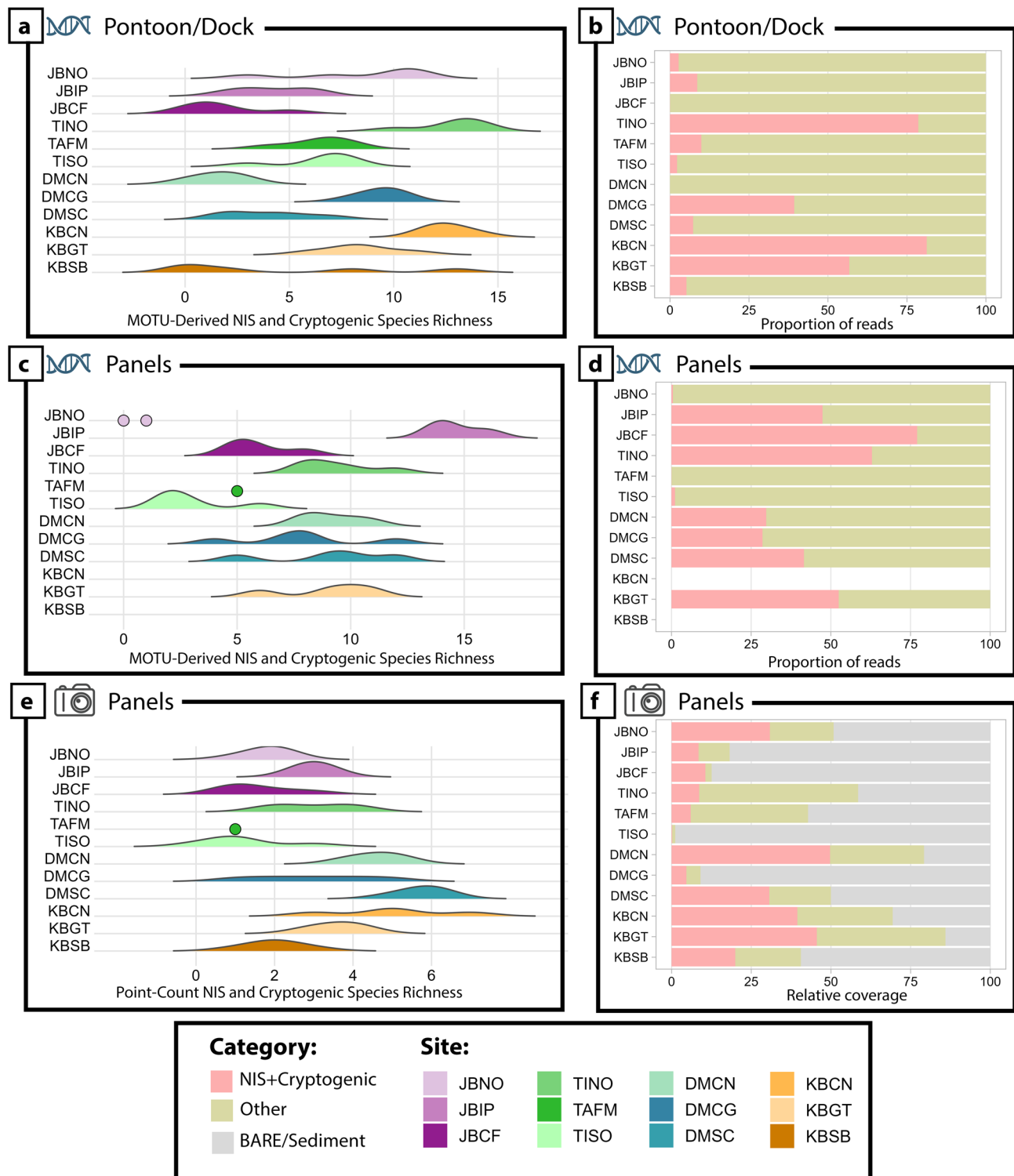


Fig. 4. Non-indigenous and cryptogenic species richness (a, c and e), abundance as proportion of reads (b and d) and relative cover on panels (f) for DNA metabarcoding datasets (DNA strain symbol) from dock/pontoon and settlement panels, and photo analysis (camera symbol) from settlement panel samples. Ridge plots summarize the distribution of per-sample richness values for each site; sites with one or two samples are represented by individual points.

environmental filtering in shaping biofouling communities and highlight the importance of localized ecological surveillance in artificial marine systems.

Substrate type and orientation emerged as key factors shaping biofouling community structure across study sites. Assemblages on pontoon and dock vertical surfaces were taxonomically distinct from those developing

on the downward-facing surfaces of horizontal settlement panels. This pattern is consistent with previous studies demonstrating that substrate orientation and physical configuration can influence larval settlement, hydrodynamic exposure, sedimentation rates, and access to light or predators^{73–77}. In our results, pontoons/docks consistently supported higher richness (639 MOTUs) and more even taxonomic representation than settlement panels (362 MOTUs), reflecting the difference between permanent vertical structures and short-term experimental substrates.

Biofouling communities on pontoons and docks supported higher taxonomic diversity and more compositionally even assemblages, with greater representation of Porifera and Annelida. These phyla were less abundant on settlement panels, likely reflecting short-term colonization dynamics and reduced microhabitat complexity. Horizontal, downward-facing panels are also more prone to sedimentation and shading, conditions that favor encrusting taxa such as bryozoans and bivalves over more structurally complex or filter-feeding organisms^{78,79}. Structural differences between floating pontoons and fixed docks may further influence biofouling development through predator access and hydrodynamic exposure. For example, pilings connected to the seafloor can facilitate access by benthic predators such as sea urchins or gastropods, suppressing sessile taxa⁷⁹. Floating pontoons, by contrast, are typically isolated from such pressures and may thus support assemblages dominated by opportunistic taxa such as ascidians, hydroids, and polychaetes. The consistent differentiation between pontoon/dock and panel assemblages across methods emphasizes the importance of accounting for substrate orientation, permanence, and infrastructure design in biofouling assessments. These substrate-driven differences also suggest distinct ecological trajectories, with panels representing early colonization stages and pontoons/docks reflecting more established fouling assemblages. Even when temporal dynamics are not the focus, orientation and structure alone can drive strong taxonomic divergence, reinforcing the need to include multiple substrate types in monitoring protocols. Our findings support previous recommendations that effective biofouling and NIS surveillance should incorporate a variety of substratum types and orientations to capture the full range of colonization outcomes and successional stages^{80–82}.

Our study revealed a high number of non-indigenous and cryptogenic species (NIS), with DNA metabarcoding detecting 42 taxa on pontoons and docks, and 37 on settlement panels, substantially more than the 17 taxa detected through photo-based surveys. These findings align with global observations that DNA-based methods substantially outperform traditional techniques in detecting soft-bodied, cryptic, or early-stage invaders^{83–85}. Importantly, the three datasets (pontoon/dock DNA, panel DNA, and photo analysis) each recovered unique species, with only seven taxa shared across all methods. This limited overlap underscores the complementarity of molecular and visual approaches and highlights the necessity of using integrated, multi-methods frameworks for robust NIS detection.

The spatial patterns of NIS richness and relative abundance were strongly site-specific. Industrial and commercial ports, including JBCF, JBNO, DMCN, and DMSC, consistently hosted the highest richness and abundance of NIS, often with more than 50% of DNA reads corresponding to NIS or cryptogenic taxa. These patterns are consistent with existing literature indicating that high operational intensity, maritime traffic, structural complexity, and pollution increase propagule pressure and create environmental conditions conducive to colonization by opportunistic invaders^{72,86,87}. In contrast, recreational marinas such as KBSB and KBCN exhibited lower NIS presence, greater proportions of bare substratum or sediment cover, and in some cases, evidence of recent disturbance, conditions potentially limiting long-term colonization or promoting native assemblages. Several NIS, including *Amathia verticillata*, *Jassa falcata*, *Botrylloides niger*, and *Lissodendoryx albemarlensis*, were among the most influential taxa driving community differences across sites, particularly in high-ERA harbors. These species are known for their rapid colonization, tolerance of disturbed or polluted environments, and strong competitive abilities, often displacing native fauna and altering ecosystem functioning^{26,88}. Together, these results suggest that while NIS were consistently concentrated in high-ERA ports, their distribution did not follow broader regional gradients across locations. The absence of consistent NIS patterns among regions such as between Al-Jubail, Dammam, and Tarout Island suggests that invasion risk in artificial marine habitats is not driven by regional proximity alone, but is instead governed by localized environmental and operational factors. This distinction highlights a scale-dependent process, whereby overall community structure and broad-scale NIS presence are shaped by site-specific ecological conditions, while propagule pressure and anthropogenic disturbance in large ports disproportionately facilitated NIS establishment. Such patterns are consistent with broader ecological evidence showing that NIS thrive in disturbed and urbanized habitats^{89,90}, exhibit greater tolerance to pollutants such as copper than native taxa⁹¹, and are frequently introduced via ballast water or aquaculture, representing a form of biological pollution⁹². These findings help reconcile our results with invasion ecology theory, emphasizing the dominant role of fine-scale ecological filters and propagule supply in shaping NIS distributions^{86–88}.

The dominance of these taxa on artificial structures in the Arabian Gulf raises concerns about potential long-term impacts on native biodiversity, trophic dynamics, and ecosystem resilience. Functionally, many of the NIS identified in this study belong to encrusting filter-feeder groups, including ascidians, bryozoans, and serpulid polychaetes, which can modify habitat structure, facilitate further invasions by altering settlement surfaces, and influence energy flow through intense filter-feeding activity^{29,93}. Their prevalence in industrial ports with high shipping activity aligns with previous studies highlighting harbors and marinas as hotspots and stepping-stones for marine bioinvasions^{17,94,95} and supports the view that artificial coastal infrastructures can function as regional corridors promoting the spread of NIS across urbanized coastlines⁹⁵.

While DNA metabarcoding provides high taxonomic resolution and sensitivity, species-level assignments must nevertheless be interpreted with caution. The reliability of MOTU identifications depends strongly on the completeness and accuracy of reference databases, which remain limited for many marine invertebrate groups, particularly in understudied regions such as the Arabian Gulf. Although we screened all assignments against curated reference datasets and prioritized sequences published by taxonomic experts, some MOTUs

may correspond to undescribed native taxa or may be assigned to the closest available reference species from outside the region. Accordingly, species lists derived from DNA data should be interpreted as potential NIS identifications rather than confirmed presences, highlighting the urgent need for region-specific DNA reference libraries generated through voucher-based sequencing and taxonomic integration.

Methodological constraints also influenced our results. PCR amplification failures were site-dependent and persisted despite template dilutions and the adding of bovine serum albumin (BSA), consistent with evidence that hydrocarbons and humic compounds can inhibit PCR in contaminated marine environment^{96–98}. In addition, incomplete detection of some ecologically important taxa likely reflects a combination of primer bias, biological complexity, and reference database limitations rather than a general failure of DNA metabarcoding approaches. For example, some taxa, including ascidians, exhibit high levels of cryptic diversity and mitochondrial variability, which can complicate species-level assignments when reference libraries are incomplete or contain misidentified sequences^{99,100}. Consequently, the development of group-specific primers and improved reference databases remains essential. These constraints underscore the need to combine molecular and visual approaches, which together enhance species detectability, provide functional and successional context, and allow cross-validation of molecular identifications.

The absence of consistent NIS patterns across broader geographic locations (e.g., Al-Jubail, Dammam, and Tarout Island) reinforces the conclusion that invasion risk in artificial marine habitats is governed more by localized environmental and operational factors rather than spatial proximity alone. This finding is consistent with studies showing that large-scale environmental risk models have limited predictive power when site-specific ecological filters and disturbance regimes are not explicitly considered^{101–103}. In our study, Environmental Risk Assessment (ERA) scores, showed only marginal associations with NIS metrics in photo-based datasets and no significant effects in DNA-based analyses, suggesting that broad-scale risk classifications may fail to capture the fine-scale invasion dynamic.

Overall, our results highlight the importance of integrated, multi-method approaches for detecting and monitoring biofouling assemblages and NIS in complex, human-altered marine environments. DNA metabarcoding was particularly effective in detecting cryptic, soft-bodied, or early-stage settlers, revealing diverse taxa such as sponges, polychaetes, and bryozoans that are often overlooked in visual surveys. Conversely, image-based approaches provided complementary information on dominant taxa, visible cover, and substratum occupation. Together, molecular and visual methods enhance species detectability, provide functional and successional context, and enable cross-validation of molecular identifications. Integrating these approaches strengthens biodiversity surveillance and improves early detection capacity for NIS in coastal infrastructure settings.

Conclusions

Ports and marinas are widely recognized as global hotspots for non-indigenous species (NIS), acting as critical entry points and stepping-stones for biological invasions in coastal ecosystems^{17,104}. As such, they are ideal targets for establishing biodiversity baselines in regions with limited ecological data, including the Arabian Gulf. This study provides a foundational inventory of native, cryptogenic, and non-indigenous taxa across 12 artificial sites along the Saudi coastline, spanning diverse infrastructure types and environmental contexts. However, these efforts should be extended to ecologically important natural habitats, such as mangroves, coral reefs, oyster beds, and seagrass meadows, which are also vulnerable to NIS incursions and may act as reservoirs or recipients of fouling biota^{88,89}. Sustained monitoring of both artificial and natural systems is essential within a broader biosecurity and conservation framework, particularly under accelerating climate change and habitat transformation scenarios^{26,29}. The Arabian Gulf is a region of exceptional ecological and geopolitical significance, characterized by extreme salinity, high temperatures, and intense anthropogenic pressures from eight rapidly developing coastal nations^{105,106}. These compounding stressors heighten the vulnerability of its ecosystems to biological invasions, especially in degraded or recovering habitats. In this context, the integration of DNA metabarcoding and image-based assessments, as demonstrated here, provides a powerful approach for detecting emerging invasions and tracking biofouling dynamics with high resolution. This study not only updates the known list of cryptogenic and non-indigenous species associated with artificial structures in the Gulf but also establishes a spatially explicit baseline for long-term monitoring. It highlights the need for site-specific, scalable, and methodologically integrated surveillance strategies in the ROPME Sea Area. Such efforts will be vital to support evidence-based management, mitigate invasion risks, and preserve the ecological integrity of marine ecosystems in one of the world's most environmentally and economically strategic coastal regions.

Data availability

The data supporting the findings of this study are available within the article and its Supplementary Information. The raw read data from the sequencing of COI gene-based amplicons are available at the European Nucleotide Archive (ENA) under the study accession number PRJEB94117 (<https://www.ebi.ac.uk/ena/browser/view/PRJEB94117>).

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S.Ch. writing the original draft, investigation, data analysis, visualization; S.Car., E.A. supervision; investigation; E.A., A.A., M.D., R.C., L.J.R., M.Q., S.Car., project administration, logistics, and resources; E.A., A.A., J.S.V., A.P., D.C., V.S., I.M.B., L.X.I.V.S., M.C., R.C., C.P.A., M.A.L.T., C.B.C., J.C., A.S., B.A., investigation (laboratory and fieldwork), data analysis; S.Car., L.J.R., M.Q. conceptualization, funding acquisition. All the co-authors read and approved the final version of the manuscript.

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Declarations

Competing interests

Susana Carvalho served as an Associate Editor and is currently a guest Editor for Scientific Reports (Seascape Ecology collection). All other authors declare no competing interests.

Additional information

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