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The Tree of Life eDNA metabarcoding reveals a similar taxonomic richness but dissimilar evolutionary lineages between seaports and marine reserves

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Abstract

Coastal areas host a major part of marine biodiversity but are seriously threatened by ever-increasing human pressures. Transforming natural coastlines into urban seascapes through habitat artificialization may result in loss of biodiversity and key ecosystem functions. Yet, the extent to which seaports differ from nearby natural habitats and marine reserves across the whole Tree of Life is still unknown. This study aimed to assess the level of α and β-diversity between seaports and reserves, and whether these biodiversity patterns are conserved across taxa and evolutionary lineages. For that, we used environmental DNA (eDNA) metabarcoding to survey six seaports on the French Mediterranean coast and four strictly no-take marine reserves nearby. By targeting four different groups—prokaryotes, eukaryotes, metazoans and fish—with appropriate markers, we provide a holistic view of biodiversity on contrasted habitats. In the absence of comprehensive reference databases, we used bioinformatic pipelines to gather similar sequences into molecular operational taxonomic units (MOTUs). In contrast to our expectations, we obtained no difference in MOTU richness (α-diversity) between habitats except for prokaryotes and threatened fishes with higher diversity in reserves than in seaports. However, we observed a marked dissimilarity (β-diversity) between seaports and reserves for all taxa. Surprisingly, this biodiversity signature of seaports was preserved across the Tree of Life, up to the order. This result reveals that seaports and nearby marine reserves share few taxa and evolutionary lineages along urbanized coasts and suggests major differences in terms of ecosystem functioning between both habitats.

KEYWORDS
coastal biodiversity, environmental DNA, marine protected area, marine urbanization, Mediterranean Sea, operational taxonomic unit

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1 | INTRODUCTION

Marine coasts are among the most diverse ecosystems on the planet (Ray, 1991). They encompass a wide variety of habitats, from soft substrates and rock bottoms to seagrass meadows, kelp forests and living reefs, that are vital in many steps of organisms' life cycle. This diversity of environments and life forms promotes a vast spectrum of ecological functions and associated ecosystem services (Barbier, 2017). Yet, home to more than a third of humanity, coastal regions are under a widespread and ever-increasing human pressure, with very few intact areas remaining (Williams et al., 2022). Fast and global expansion of marine urbanization, the 'ocean sprawl' (Duarte et al., 2013), is mainly characterized by the building of seaports and the elevation of coastal defences to support and shield human activities (Todd et al., 2019). Such artificialization destroys native habitats and imposes new physical barriers to natural dispersion patterns, impeding tidal and diadromous movements but also migrations in the marine realm along the coast and beyond (Bishop et al., 2017; Dafforn et al., 2015; Perkins et al., 2015). Seaports are considered as the uttermost urbanized systems in seascapes (Sahavacharin et al., 2022). They are usually enclosed by breakwaters, reducing water flow and increasing local turbidity inside (Bulleri & Chapman, 2010; Dafforn et al., 2015), and their vertical and smooth artificial structures offer less microhabitats like crevices or rock pools, generally associated with high species richness (Aguilera et al., 2014; Firth et al., 2013; Lam et al., 2009; Perkins et al., 2015). Consequently, communities of sessile organisms attached on artificial structures are impoverished or differ from those living on adjacent natural habitats (Aguilera et al., 2014; Bulleri et al., 2005; Bulleri & Chapman, 2004; Firth et al., 2013; Martins et al., 2012; Scherner et al., 2013). However, artificial structures may also act as corridors for opportunistic species offering new dispersal pathways for previously separated populations to extend their distribution and facilitate gene flow (Alter et al., 2021; Sammarco et al., 2004; Touchard et al., 2023). Additionally, seaports are stepping stones for non-indigenous species (NIS) introductions and expansions, spreading from one seaport to another (Aglieri et al., 2023; Andrés et al., 2023; Darling et al., 2020; Rey et al., 2020).

Surprisingly, the level of biodiversity in seaports and the extent to which this biodiversity differs from that of nearby natural habitats and marine reserves is still unknown since literature on this subject is very scarce. According to a recent review, only 0.01 and 0.03% of world commercial and recreational seaports respectively have been surveyed (Madon et al., 2023). Additionally, the vast majority of seaport studies on biodiversity have focused on the detection of NIS and sessile organisms while many taxa have been overlooked. In particular, there has been little interest in highly mobile organisms like fish and specific groups of invertebrates such as crustaceans or sponges. Mediterranean seaports are no exception (see the literature review in Appendix S1: Methods and associated Table S1) with 50% of the studies reporting new NIS or monitoring their spread, and all of them focusing on specific taxonomic groups without providing a holistic view of seaport ecosystems. This lack of baseline knowledge is even more critical given that coastal artificialization may certainly intensify in the next decades (Hanson & Nicholls, 2020). This deficit of biomonitoring may partly come from the inherent difficulty to sample or detect species in seaports given regulations and water turbidity preventing both fishing and visual surveys. As an alternative, environmental DNA (eDNA) metabarcoding has the potential to bypass accessibility and visibility issues (Cheang et al., 2020; Ip et al., 2021; Sigsgaard et al., 2016) and is a promising tool to monitor seaport biodiversity (Aglieri et al., 2023; Rey et al., 2020). Defined as free or adsorbed DNA molecules released by organisms, eDNA can be extracted from environmental samples and then sequenced with appropriate markers (Miya, 2022; Taberlet et al., 2012). Its low persistence in seawater, from hours to a few days, can provide an accurate snapshot of local biodiversity (Collins et al., 2018; Mauvisseau et al., 2022; Wood et al., 2020). This technique allows non-invasive, standardized and comprehensive assessments of marine biodiversity across all taxa and habitats, outperforming traditional fishing, visual or video surveys (Miya, 2022; Polanco Fernández et al., 2021; Rey et al., 2023). By combining different genetic markers to target a wide variety of organisms, the metabarcoding of eDNA provides high-resolution sights on habitats sampled (Compson et al., 2020; Rey et al., 2020), and even holistic views when combining primer pairs that together target all living taxa, from bacteria to mammals. This method called Tree of Life (ToL) metabarcoding has yet poorly been explored (see the literature review in Appendix S1: Methods and associated Table S2) with only two studies conducted in marine ecosystems (Stat et al., 2017; Zhang et al., 2020). When associated with taxonomic independent approaches like clustering of molecular operational taxonomic units (MOTUs), ToL-metabarcoding provides information on ecosystem-wide biodiversity patterns (Stat et al., 2017). Yet, this ToL-metabarcoding approach has not revealed its potential in highly anthropized marine habitats like seaports and to perform comparative analyses of biodiversity among coastal habitats.

In a previous eDNA metabarcoding study focusing on fish, similar levels of diversity were found between seaports and outside areas (Manel et al., 2024). Here, we extend the scope to other taxa by targeting the whole Tree of Life with MOTU clustering. The objective of this study is to characterize the biodiversity of seaports in comparison with that of nearby marine reserves across the Tree of Life using multi-marker eDNA metabarcoding. More specifically, we assessed whether (i) the local or α-diversity is similar or higher inside marine reserves than in seaports, (ii) the turnover or β-diversity is high or not between seaports and marine reserves, reflecting differences between communities, and (iii) these biodiversity patterns are conserved or not across evolutionary lineages. We expect that seaports host a lower α-diversity in terms of species richness or MOTUs when compared to natural sites (e.g., reserves) but would contain more non-indigenous species. We also anticipate that β-diversity is somewhat high between seaports and marine reserves owing to biotic homogenization promoted by artificialization. We finally make the hypothesis that these biodiversity patterns differ across the Tree of Life since some lineages may be more sensitive to artificialization or protection than others. We applied a multi-marker eDNA metabarcoding approach associated with a MOTU clustering bioinformatic pipeline to compare communities between six recreational seaports and four marine reserves along the French Mediterranean coast. We investigated the effect of habitat (seaport versus reserve) and of season within seaports (autumn versus summer) on α- and β-diversity for prokaryotes, eukaryotes, metazoans and fish. We identified taxa involved in the observed patterns, uncovering seaports biodiversity signature. Finally, we evaluated the taxonomic level up to which these biodiversity patterns were conserved.

2 | MATERIALS AND METHODS

2.1 | Study area and sampling design

Six Mediterranean recreational seaports (i.e., marinas) as well as four adjacent no-take marine reserves were sampled in the Western Mediterranean Sea for this study (Figure 1). Seaport sampling, also described in Manel et al. (2024), was performed at two different seasons in autumn 2021 and summer 2022. Samples from marine reserves were collected along coasts in strictly no-take marine protected areas (MPAs) in summer 2020 for a previous study (Boulanger et al., 2021) and were specifically re-analysed here. Seaports and marine reserves were chosen to compare biodiversity between highly urbanized areas (i.e., seaports) and areas preserved by protective measures (i.e,. marine reserves). Since the protection status of several MPAs in the Mediterranean is questionable (Claudet et al., 2020), we selected MPAs where fishing is forbidden—strictly 'no-take' areas referred as 'reserves' here—to ensure a minimal human impact in comparison with seaports. Sampling consisted of collecting 30 L of seawater 1 m below the sea surface using a disposable sterile tubing and a peristaltic pump (1.0 L/min nominal flow) and filtering through a VigiDNA 0.2 μm crossflow filtration capsule (SPYGEN, Le Bourget-du-Lac, France) during 30 min. Immediately after filtration, capsules were emptied from the remaining seawater, filled with 80 mL of CL1 conservation buffer (SPYGEN), and then stored at room temperature until DNA extraction. Two replicates were collected at each site, providing a total of 24 samples from the six seaports (12 in autumn and 12 in summer) and eight from the four marine reserves. In seaports, transects were realized from a kayak covering the largest area possible within the seaport boundaries. In both habitats, transects covered different types of substrates to capture the broadest and most representative ecological communities possible. Filters used for amplification with the metazoa primer pair in reserves were different from filters used with other markers because no DNA was left for those analyses, but they were all collected at the same time and location. Further details are given in Tables S3 and S4.

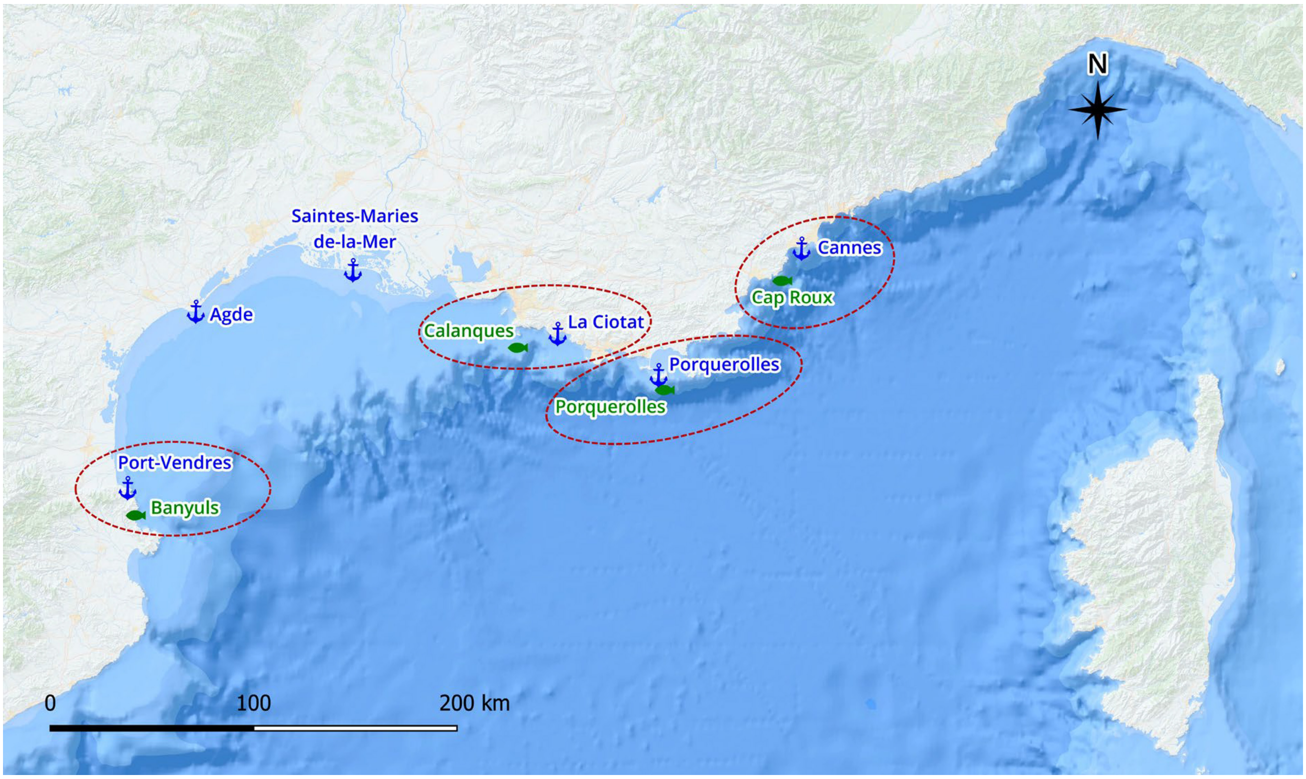

FIGURE 1 Sampling locations in the Western Mediterranean Sea. Location of seaports (in blue) and marine reserves (in green) sampled. Adjacent seaports and strictly no-take marine reserves (distance <20 km) are encircled with red dashed ellipses. Corresponding metadata can be found at Tables S3 and S4. SMM, Saintes-Maries-de-la-Mer.

2.2 | Environmental DNA extraction, amplification and next-generation sequencing

eDNA extractions were performed in a BSL-2 laboratory dedicated for eDNA samples following the protocol described in Polanco Fernández et al. (2021). Four PCR amplifications were conducted with different assays covering the whole Tree of Life. The teleo primer pair (Valentini et al., 2016) targets a 12S mitochondrial DNA marker from teleosts and elasmobranchs; the metazoa primer pair (Kelly et al., 2016) targets a 16S mitochondrial DNA marker from metazoans; the euka2 primer pair (Guardiola et al., 2015) targets a marker from eukaryotes located on the V7 region of the 18S ribosomal RNA; and the bact2 primer pair (Taberlet et al., 2018) targets a marker from prokaryotes located on the V4 region of the 16S ribosomal RNA. This experimental design aims to provide a holistic overview of communities, with a nested hierarchy euka2-metazoa-teleo to obtain a finer taxonomic resolution over animal communities, and particularly fish. Twelve PCR replicates per sample were run, with negative extractions and PCR positive and negative controls analysed in parallel. Unique tags were used for each PCR replicate amplified with the teleo primers only, allowing to differentiate them in the bioinformatic analysis (see after). NGS library preparation and MiSeq paired-end sequencing (2 × 150 bp) were performed at DNA Gensee (Le Bourget-du-Lac, France).

2.3 | Clustering of molecular operational taxonomic units

The bioinformatic pipeline used in the present study (Figure 2) is adapted from the workflow originally designed by Marques et al. (2020). It was run independently for each of the four markers to consider their differences at specific points (essentially barcode range size, reference databases and target taxa). This pipeline is built with classic pre-processing steps, a MOTU clustering main stage followed by a taxonomic assignment algorithm, and post-processing steps including MOTU curation.

Paired-end sequencing reads were first assembled by VSEARCH v2.13.4 (Rognes et al., 2016), and resulting amplicons demultiplexed and primers trimmed with CUTADAPT v3.4 (Martin, 2011). Amplicons were filtered with marker-dependent parameters, based on evaluations of the barcode range size. Thus, sequences amplified with teleo primers were kept if ranging from 20 to 150 bp (Taberlet et al., 2018); 20–200 bp for metazoa (Kelly et al., 2016); 20–950 bp for euka2 (Taberlet et al., 2018); and 20–400 bp for bact2 (Taberlet et al., 2018). Sequences with IUPAC ambiguities were discarded, and amplicons dereplicated.

MOTU clustering was performed using SWARM v3.1.0 (Mahé et al., 2021), with the default parameter of one nucleotide as the minimum distance between each cluster and considering virtual amplicons to avoid generating erroneous low abundant MOTUs ('fastidious' option, Mahé et al., 2015). Chimeras, that is, amplicons originating from different parent templates during PCR (Edgar et al., 2011), were detected with VSEARCH and rejected. The most abundant sequence from each MOTU was compared against a reference database built from in silico PCRs with ECOPCR v1.0.1 and taxonomic assignment executed with ECOTAG v1.0.1, a lowest common ancestor algorithm, both from the OBITOOLS package (Boyer et al., 2016). Reference databases were built with the Genbank nucleotide database (Sayers et al., 2022; release 249) restricted on mitochondrion sequences for teleo (supplemented with a custom regional reference database) and metazoa markers, and with the SILVA ribosomal RNA database (Quast et al., 2013; SSU Ref NR99 release 138.1) for euka2 and bact2.

Post-processing steps filtered out errors generated by index-hopping (MacConaill et al., 2018) with thresholds empirically calculated per sequencing batch thanks to experimental blanks, and errors generating by tag-jump (Schnell et al., 2015) with a 0.001% abundance cut-off per run for a given MOTU. To avoid spurious sequences originating from PCR errors, MOTUs with a read count inferior to 10, and MOTUs present in only one PCR replicate (only applicable for teleo as PCR replicates were not differentiated with specific tags for the other markers) were dismissed. Sequencing contaminations were managed by removing amplicons sequenced in experimental blanks, in addition to sequences not assigned to target taxa (*Actinopterygii* and *Chondrichthyes* classes for teleo; *Metazoa* kingdom for metazoa). The post-clustering algorithm LULU (Frøslev et al., 2017) curated similar MOTUs by merging erroneous 'daughters' MOTUs to their valid 'parents' by evaluating co-occurrence patterns among samples. A pairwise sequence dissimilarity matchlist, required for this algorithm, was produced with MEGABLAST (Zhang et al., 2000), to record the most similar sequences following default parameters (84% percentage identity and 80% query coverage). At this step, co-occurrence patterns of all MOTUs from top to bottom, ordered by abundance, are compared by pairs (with the most abundant MOTU as the putative 'parent' and the least abundant one as the putative 'daughter'). If the ratio between the number of samples where both the putative 'parent' and 'daughter' occur is above 95% and if the read count of this latter is smaller in every sample where they co-occur, the 'daughter' MOTU is considered as an error from its valid 'parent', and they are aggregated.

A final table of taxonomically assigned MOTU was thus obtained after checking they belong to marine taxa with the WoRMS database through the 'worrms' R package v0.4.2 (Chamberlain & Vanhoorne, 2020). To exclusively compare the four markers without co-occurring taxa, 8 MOTUs amplified with the metazoa marker that were assigned to fish and also detected with the teleo marker were eliminated from the metazoa dataset. After that, we considered there was no taxonomic overlap between our markers.

2.4 | Statistical analyses

For the four markers, MOTU richness in each sample was considered as a measure of α-diversity. Since marine reserves were not

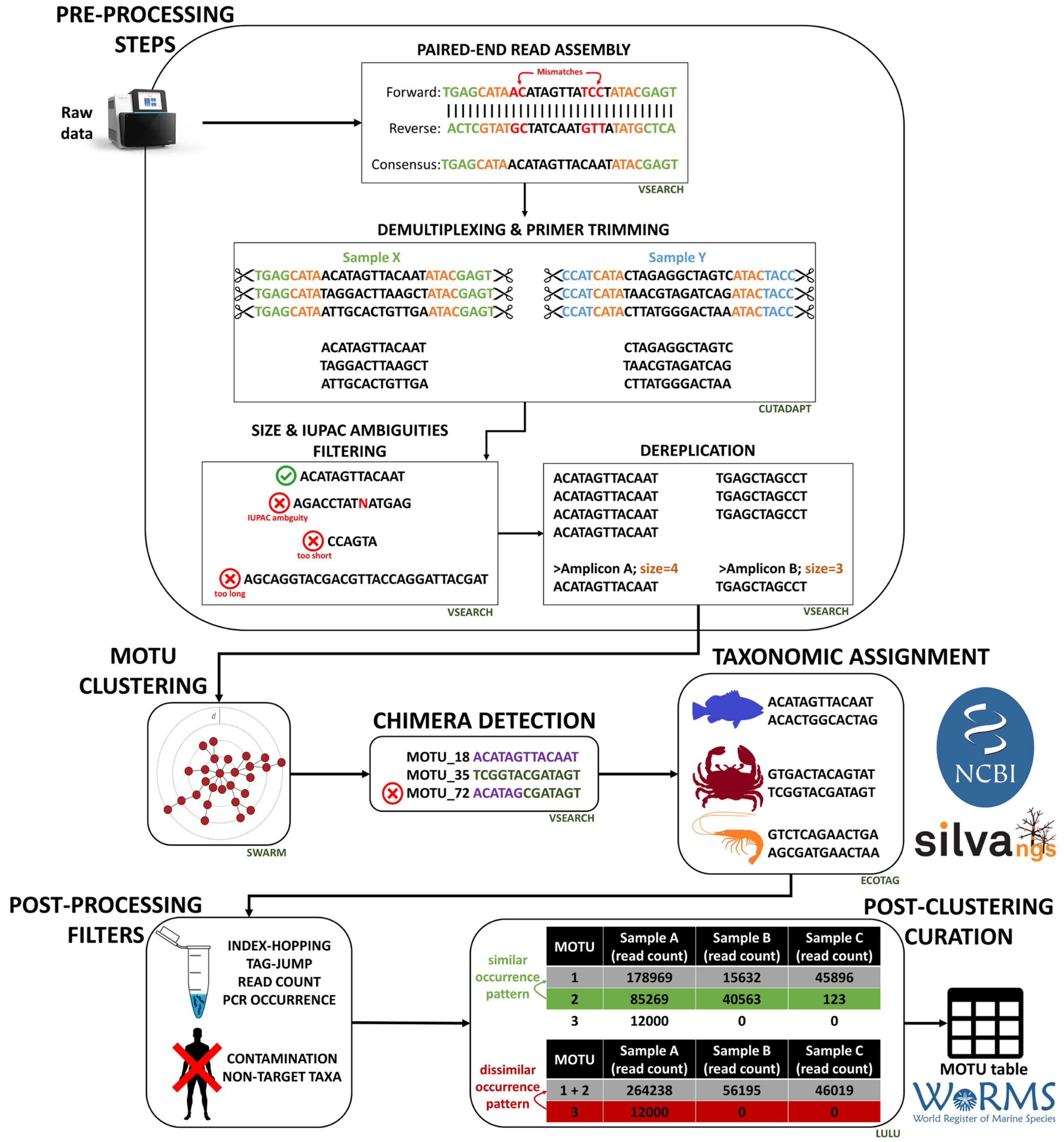

FIGURE 2 Graphical summary of the bioinformatic pipeline. Specific programs used at each step are specified in dark green. The drawing at the MOTU clustering step comes from Mahé et al. (2015).

sampled during autumn, only seaport samples collected during the summer campaign were retained when comparing the effect of habitat (see below). Species accumulation curves and their corresponding asymptotes were estimated with the 'iNEXT' R package v3.0.0 (Chao et al., 2014; Hsieh et al., 2016), from sample-based data with the Hill number $q = 0$ (species richness). The effect of habitat (seaport versus reserve) was tested on MOTU and threatened species richness through a linear mixed-effects model (LMM) built with the 'fitme' function from the 'spaMM' R package v4.1.20 (Rousset & Ferdy, 2014). Geographic coordinates of the transects starting point were used as random factors to take into account both spatial autocorrelation and replication of samples—as the same coordinates are affected to each replicate—and the restricted maximum likelihood method was used after checking the significance of the model with a likelihood-ratio test (LRT). Seasonal effect inside seaports (autumn versus summer) was also investigated the same way.

Dissimilarities between samples, so pairwise β-diversity, were estimated with the Jaccard distance applied to the MOTU presence/absence matrices with the 'vegan' R package v2.5.7 (Oksanen et al., 2020). When comparing dissimilarity at higher taxonomic levels (from families to phyla), communities obtained with the four markers were combined, replicates from the same site pooled together, and Bray–Curtis distances calculated from the number of MOTU belonging to each family/order/class/phylum. A principal coordinate analysis (PCoA) was first implemented to visualize community dissimilarities between sites. The difference in community composition between habitats (seaport versus reserve) and seasons (autumn versus summer in seaports only) was assessed by conducting a distance-based redundancy analysis (dbRDA) spatially conditioned by the geographic coordinates of the sites. The significance of these effects on community dissimilarity was evaluated with ANOVA-like permutation tests ('anova.cca' function with 10,000 permutations). Pairwise β-diversity between seaports and reserves were compared at different taxonomic levels (from MOTUs to phyla) and the difference between them was tested with a Kruskal–Wallis' test and post hoc Holm–Bonferroni-corrected Dunn's tests.

When MOTU were assigned to a species, we kept sequences that successfully aligned with a 100% identity to detect non-indigenous and threatened species. We used the latest revised Mediterranean alien species list provided in Zenetos et al. (2022) to identify NIS. Species classified as threatened on the IUCN Mediterranean Red List of Threatened Species were retrieved with the 'rredlist' R package v0.7.1 (Gearty & Chamberlain, 2022). The global IUCN Red List of Threatened Species was used when some were classified as not evaluated (NE), not applicable (NA) or data deficient (DD) on the Mediterranean list.

Statistical analyses were conducted independently on each marker using R v4.2.2 (R Core Team, 2022).

3 | RESULTS

3.1 | Biodiversity overview

We found 17,995,476 sequencing reads after bioinformatic steps, clustered into 1583 non-redundant MOTUs distributed in 180 teleo MOTUs (11,977,244 reads), 264 metazoa MOTUs (4,472,783 reads), 870 euka2 MOTUs (1,111,903 reads) and 269 bact2 MOTUs (433,546 reads). The asymptotes of the MOTU accumulation curves provided an estimation of γ-diversity for the four markers (Figure S1). The proportion of biodiversity we obtained compared with this γ-diversity was unbalanced across markers, some accurately assessed the overall MOTU richness while more sampling effort would have been needed to reach a similar level for others. Indeed, the asymptotic value was almost reached with teleo (Figure S1a; asymptote: 193 MOTUs; 95% confidence interval: 185–214) and bact2 (Figure S1d; 291 MOTUs; 95% CI: 280–315) markers. On the contrary, a hundred of MOTUs were missing with the euka2 marker to reach the asymptote (Figure S1c; 999 MOTUs; 95% CI: 960–1055), and the biodiversity captured with the metazoa marker was far from its highest estimated value (Figure S1b; 430 MOTUs; 95% CI: 367–530). MOTUs cover 58 taxonomic classes (Figure 3) with uneven assignment resolution across markers: 67.68% of MOTUs were assigned to species with teleo, 34.47% with metazoa, 3.45% with euka2 and only 1.11% with bact2. The velvet swimming crab *Necora puber* was the only NIS detected in our samples (with the metazoa marker), in the seaport of Saintes-Maries-de-la-Mer at both seasons. This species is common in the Western Mediterranean for more than 40 years (Holthuis, 1987) and is classified as 'cryptogenic' in Zenetos et al. (2022). It means that its introduction path is unknown, or it comes from a natural range expansion from the Atlantic Ocean. Five threatened species (IUCN statuses detailed in Table S5) were detected in seaports and reserves (*Mola mola*, *Epinephelus marginatus*, *Pomatoschistus microps*, *Merluccius merluccius* and *Sciaena umbra*), all with the teleo marker. Reserves and seaports with the richest and poorest number of unique MOTUs varied across markers (Figure 4). After combining all MOTUs across markers, Porquerolles (in autumn) and Agde (in summer) were the richest and poorest seaports with, respectively, 560 and 148 MOTUs in total, whereas Cap Roux and Banyuls were the richest and poorest reserves with, respectively, 496 and 345 MOTUs. Differences between summer and autumn communities within a single seaport were also noticed (Figure S2). Indeed, the proportion of MOTUs exclusive to one of the two seasons ranged from 55.27% (La Ciotat) to 73.57% (Vendres).

3.2 | Differences in MOTU richness or α-diversity

Except for metazoans, MOTU richness was lower in seaports than in reserves (Figure 5), but the difference between the two habitats was only significant for prokaryotes (LRT-$\chi^2 = 15.63$, p-value <0.001, LMM Pseudo-$R^2 = 59.67\%$). Prokaryotes were also the only clade affected by the sampling season in seaports (LRT-$\chi^2 = 14.58$, p-value <0.001, LMM Pseudo-$R^2 = 45.52\%$), with an increased MOTU richness in autumn compared with summer (Figure S3). For teleo, we were able to assign the majority of MOTUs to species since the reference database was quasi-exhaustive for fish in the Mediterranean Sea. Although fish richness was not influenced by habitat, that of threatened fish species was significantly higher in reserves than seaports (Figure 6; LRT-$\chi^2 = 5.469$, p-value $= 0.019$, LMM Pseudo-$R^2 = 23.90\%$).

3.3 | Differences in MOTU composition or β-diversity

dbRDAs on total MOTU composition revealed marked β-diversity patterns with a clear distinction between seaport and reserve habitats for all taxa (p-values ≤0.001, Table S6). Indeed, this dissimilarity was shared between markers and clades, with seaports and reserves being well discriminated along the constrained dbRDA axis, and even for those located nearby (Figure 5). Except for metazoans,

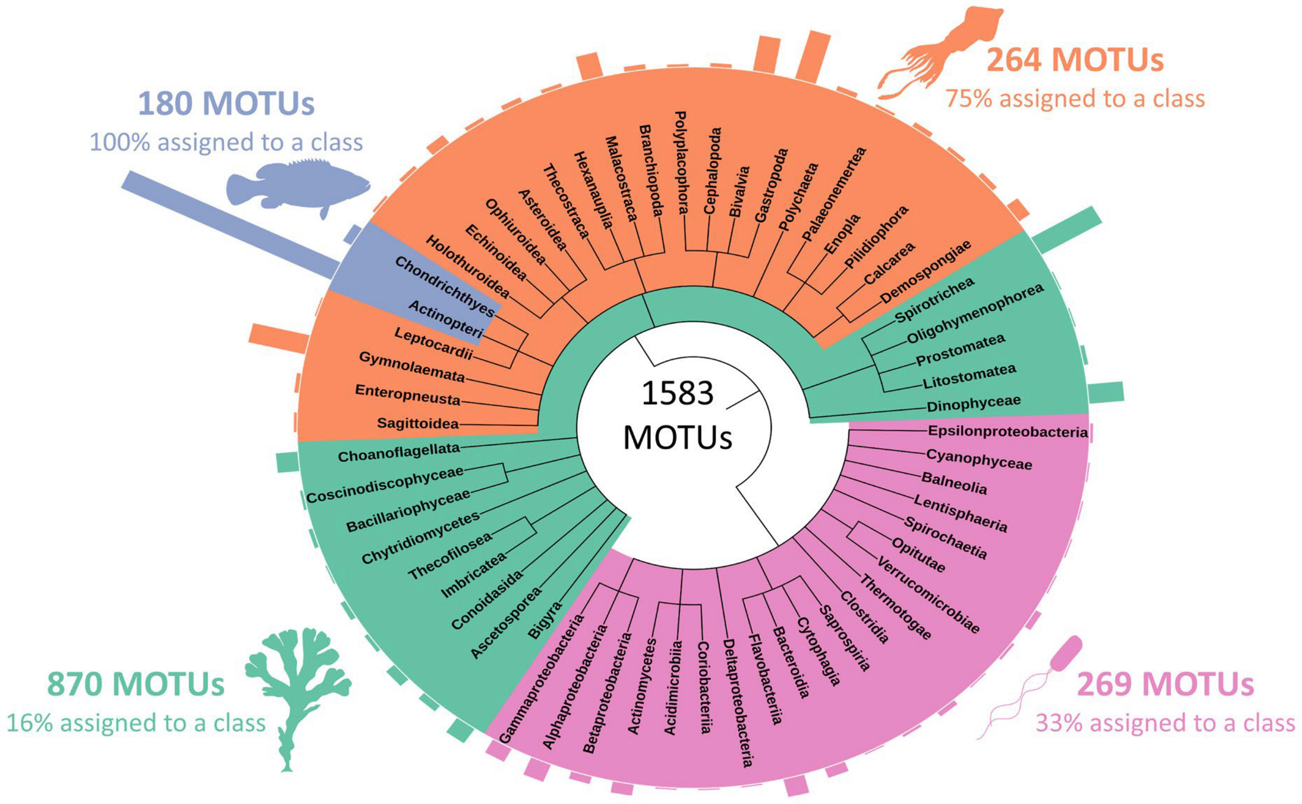

FIGURE 3 Phylogenetic tree of the global community composition at the class level. Bar charts represent the numbers of MOTUs amplified with teleo (blue), metazoa (orange), euka2 (green) and bact2 (pink) markers within each class. Among the 1583 total MOTUs, 970 were not assigned to a class.

differences between autumn and summer communities in seaports were also significant, but with a lower discrimination than between habitats (Table S7). Once again, MOTU communities were distinct along the constrained dbRDA axis (Figure S3), revealing that seaports host different fish, eukaryote and prokaryote taxa depending on the season. These results were confirmed by the PCoAs, showing a clear distinction between seaport and reserve communities along the first axis (Figure S4a), but this signal was blurred when comparing communities between seasons within seaports (Figure S4b).

3.4 | Dissimilarity between communities across taxonomic levels

The marked β-diversity patterns discriminating seaports and reserves that are shared for the four markers were also detected when combining the four communities (Figure 7). More surprisingly, this discrimination was also found significant when scaling up across the Tree of Life at the family level and up to the order level (see Table S8) but is then lost at the class level, so is strongly conserved across evolutionary lineages. This result is consistent with the drop in mean pairwise dissimilarity between seaports and reserves that decreased from 0.42 to 0.30 when moving from the order to the class (Figure 8). Even if dissimilarities between autumn and summer communities in seaports were previously observed for fish, eukaryotes and prokaryotes, it was not significant anymore when combining all MOTUs and for higher taxonomic levels (Figure S5, Table S9). Species scores of the dbRDAs indicate that some taxa are associated with seaports only and others to reserves (Figure 7). Seaports are mainly characterized by three orders of molluscs—two bivalves (Mytilida and Lucinida) and one gastropod (Littorinimorpha)—and one order of bony fish that includes all the gobies and their relatives (Gobiiformes). When considering MOTUs assigned at the species level, three are mainly present in seaports, one from the Lucinida order (*Loripes lacteus*) and two gobies (*Gobius niger* and *Gobius cruentatus*). On the contrary, other orders were marginally detected in seaports while being more prevalent in reserves like Labriformes and Blenniformes for teleosts, and also Enterobacterales and Burkholderiales bacteria that are completely absent from seaports in the assigned sequences of our dataset.

4 | DISCUSSION

We present here one of the first ToL-metabarcoding surveys performed in marine environments and the very first one conducted in seaports. We found that MOTU richness was similar between seaports and reserves for most taxa but significantly higher in reserves for prokaryotes and endangered fish species. We also found more prokaryotes within seaports in autumn compared with summer.

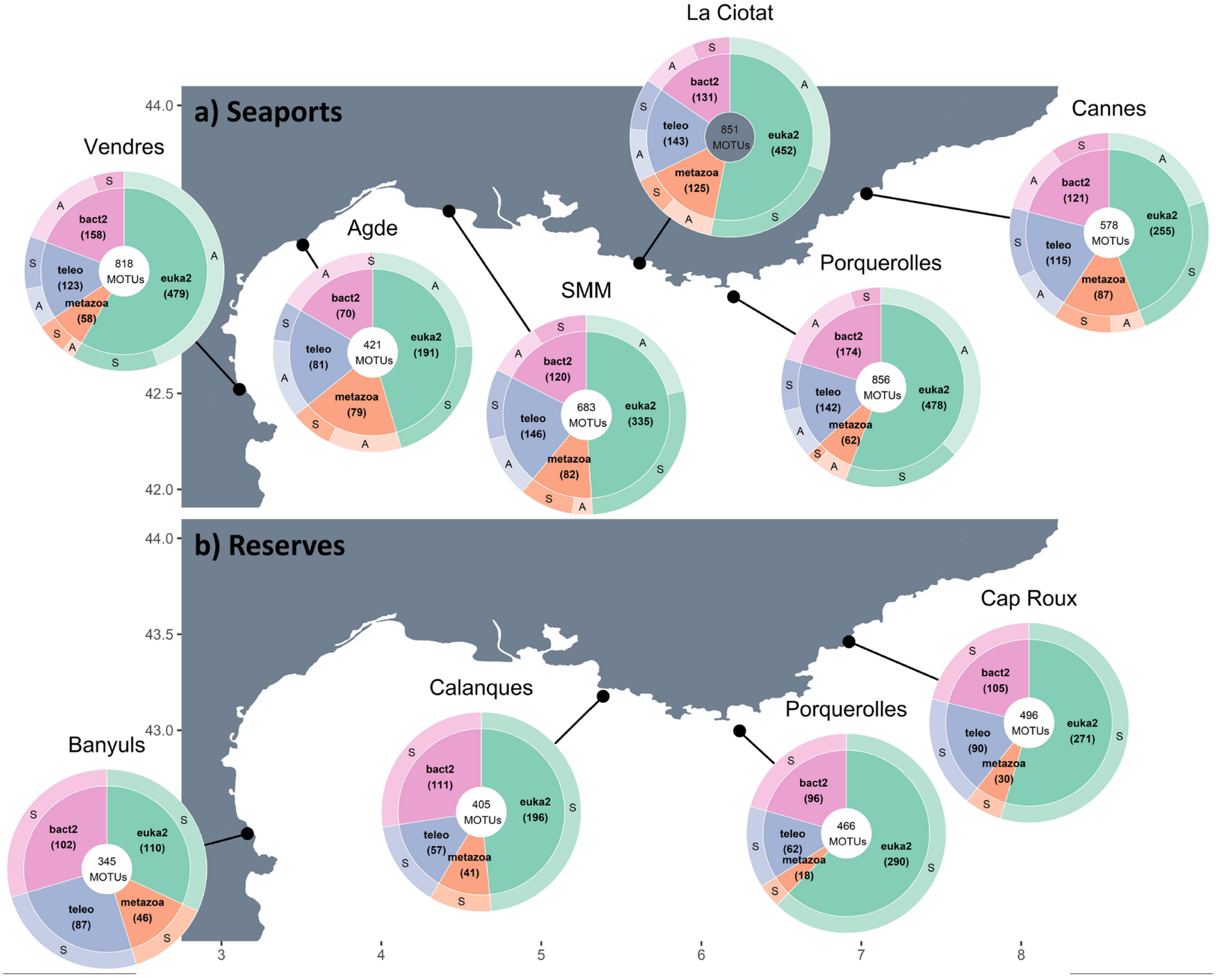

FIGURE 4 MOTU richness in the sampling area (Western Mediterranean Sea). Pie charts represent the numbers of MOTUs obtained at each sampling site in seaports (a) and adjacent 'no-take' marine reserves (b) for the four markers after pooling replicates. For every site, the overall number of MOTUs detected is mentioned in the centre of the pie, and the season of sampling is depicted in the outer circle (A, Autumn; S, Summer). SMM, Saintes-Maries-de-la-Mer.

Communities were different between the two habitats, revealing shared dissimilarity in biodiversity patterns between the four taxonomic groups targeted. A seasonal effect was observed as well within seaports on community composition for fish, eukaryotes and prokaryotes. We identified some taxa as specifically associated with seaports, and others with reserves. Finally, the dissimilar biodiversity patterns found between seaports and reserves were conserved along the Tree of Life, up to the order level.

The lack of difference in MOTU richness between seaports and reserves for all taxonomic groups except prokaryotes and threatened species confirms the results found in Manel et al. (2024) for fish only in a similar study area. Other eDNA-based studies already highlighted the role of no-take marine reserves as refuges for threatened species in comparison to fished areas on the Mediterranean coast (Boulanger et al., 2021; Dalongeville et al., 2022). The significantly higher prokaryote taxonomic diversity in reserves suggests an effect of protective measures on this group. This result is surprising because prokaryotes are not exploited, but it is in line with a previous study comparing microbial communities between one Mediterranean seaport and a nearby MPA from sedimentary ancient DNA (Catania et al., 2017). Prokaryotes were also the only group to show a seasonal difference in MOTU richness, as we found more prokaryote taxa in autumn than in summer within seaports. This is consistent with studies investigating microbial seasonal variations that cycle through the year and peak during the winter in northern marine temperate regions (Gilbert et al., 2012; Ladau et al., 2013).

The dissimilarity in community composition between seaports and reserves found in our study was shared between taxonomic groups. To our knowledge, only Holman et al. (2021) have detected shared community dissimilarity patterns across a wide range of taxonomic groups from eDNA survey before. Their study extended the macroecological pattern shaping the three major South African marine coasts for metazoans to protists and bacteria. Our study shows that this phenomenon can be witnessed at small spatial scales. For instance, the seaport and the reserve of Porquerolles are far from <4 km apart (see Figure 1) but are very different in terms of communities. This finding supports the primary role of habitat to drive community composition compared with the geographic

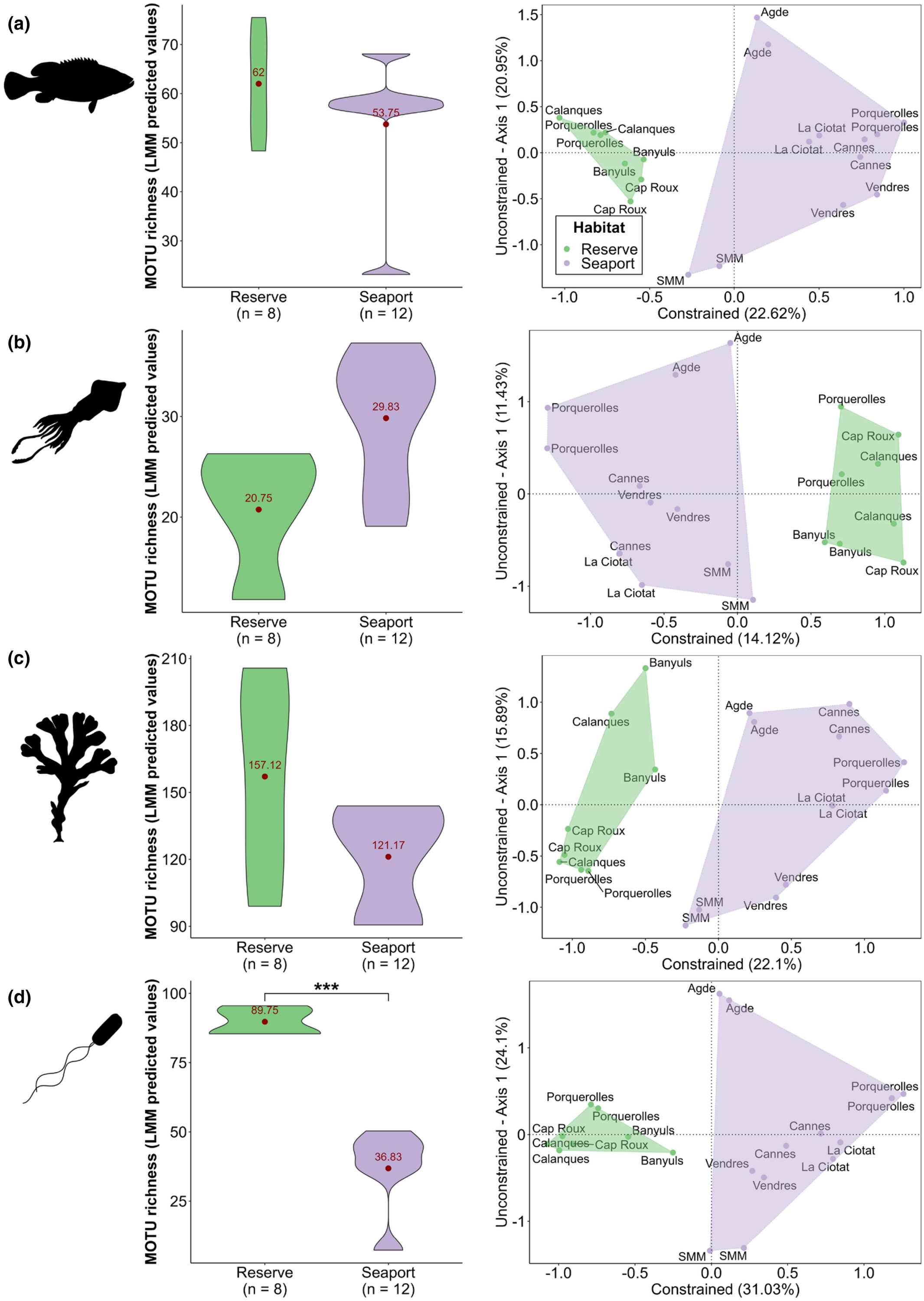

FIGURE 5 Comparison of seaport and reserve communities. Violin plots (left) represent linear mixed-effects model predicted values of MOTU richness for fish (a), animals (b), eukaryotes (c) and prokaryotes (d). Red dots indicate mean values. Asterisks (***) indicate a significance level at 0.001. Site scores plots (right) represent the two first dbRDA axes conditioned by spatial coordinates when testing the effect of habitat on Jaccard distances between samples (significant for all markers; Table S6). SMM, Saintes-Maries-de-la-Mer.

distance. Discrepancies in species assemblages between natural and urban marine habitats were also detected in previous studies, but they were restricted to one or few taxonomic groups (Bulleri et al., 2005; Bulleri & Chapman, 2004; Pennino et al., 2024; Piazzi & Ceccherelli, 2020; Scherner et al., 2013). In the Mediterranean Sea, this have been observed in a variety of natural environments with traditional biomonitoring methods like in seamounts benthic communities (de la Torriente et al., 2020), corals and gorgonians

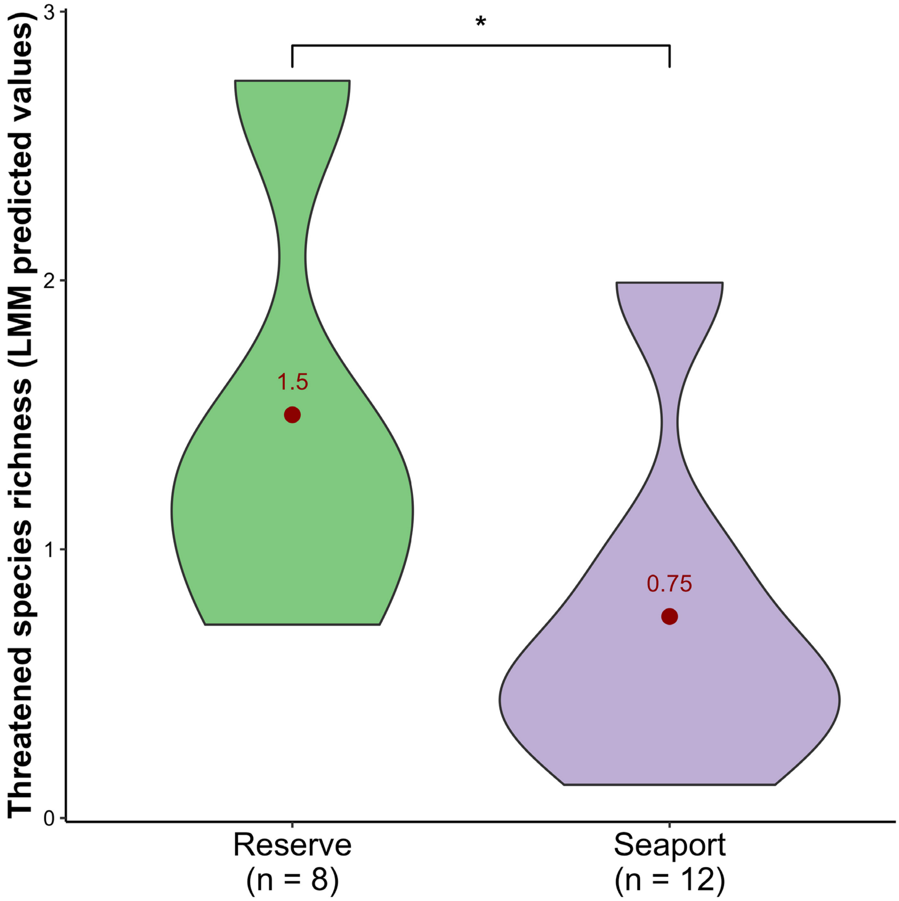

FIGURE 6 Comparison of threatened species richness between seaports and reserves. Violin plots represent linear mixed-effects model predicted values. Red dots indicate mean values. Asterisk (*) indicates a significance level at 0.05.

inhabiting rocky pinnacles (Cau et al., 2015) or fish assemblages associated with *Posidonia oceanica* beds under different protection levels (Appolloni et al., 2023). Metabarcoding also shed light on marked changes in fish communities between Mediterranean fished areas and reserves (Boulanger et al., 2021; Manel et al., 2024), or eukaryotic communities between *Posidonia oceanica* meadows and nearby rocky reefs (Turon et al., 2023). We also observed seasonal variations in community composition within seaports for fish, eukaryotes and prokaryotes. So, as the sampling season is a non-negligible driver of biodiversity, we recommend sampling at different period of the year to provide comprehensive biodiversity reports, or to sample at the same season for biodiversity monitoring in time. This assessment is shared by other ToL-metabarcoding surveys (Berry et al., 2019; Djurhuus et al., 2020).

The dissimilarity in community composition between seaports and reserves was also conserved across evolutionary lineages. This conserved split uncovers that seaports and marine reserves host completely different communities and are thus two highly different ecosystems. Therefore, seaports and reserves depending on the present communities would have different ecological functions. Characterizing seaports and reserves functions based on identified taxonomic groups based on traits databases would give additional knowledge by indirectly assess ecological functioning of the habitats sampled (Aglieri et al., 2021; Condachou et al., 2023; Marques et al., 2021). This approach would require wide taxonomic coverage and resolution, and would allow to point out which functions are altered in urbanized habitats. However, eDNA metabarcoding alone cannot yet provide information on organism life stages and functional traits vary across the life cycle in many marine taxa.

Taxonomic assignment of MOTUs allows to unveil taxa responsible for community differences between seaports and reserves. Fish assemblages in seaports were mostly marked by gobies while Labriformes and blennies were mainly detected in reserves. The metazoan taxa that most contributed to seaport biodiversity were sessile invertebrates like mussels. Indeed, it is known that artificial structures offer new habitat opportunities for sessile organisms (Connell, 2001; Firth et al., 2013) and do not favour vagile ones (Chapman, 2003). Some prokaryote and unicellular eukaryote lineages characterizing seaports and reserve communities were also identified, but this should be carefully interpreted as taxonomic resolution was very poor for these taxonomic groups.

Some Mediterranean lineages reported in Coll et al. (2010) were completely missing from the genetic reference databases we built with in silico PCRs before taxonomic assignment. Particularly, sequences cannot be assigned to plants, tunicates, cnidarians and flatworms in our dataset. This is due to the incompleteness of

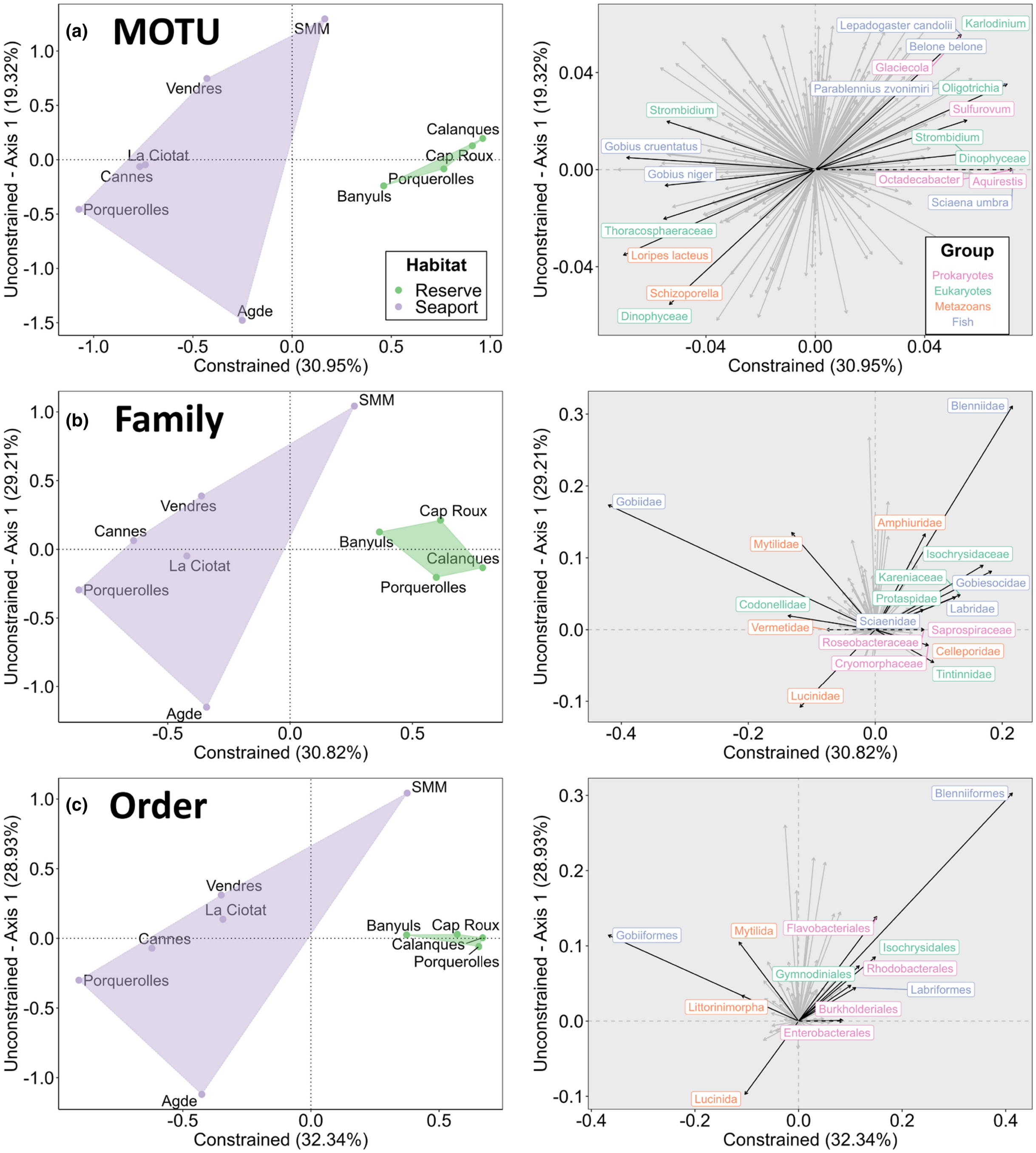

FIGURE 7 Comparison of seaport and reserve communities across taxonomic levels. Site scores (left) and species scores (right) plots represent the two first dbRDA axes conditioned by spatial coordinates testing the effect of habitat on Jaccard/Bray–Curtis distances between sites after pooling replicates and combining all MOTUs (significant from the MOTU level to the order; Table S8). Jaccard dissimilarity index is calculated at the MOTU level (a), whereas Bray–Curtis' dissimilarities based on MOTU abundance are used for family (b) and order (c) levels. Top 10% taxa contributing to the constrained axis are displayed. Among them, only those with an assignment identity higher than 97% are kept at the MOTU level. SMM, Saintes-Maries-de-la-Mer.

Genbank and SILVA databases for the markers chosen that impairs the capacity to assign MOTU to these taxonomic groups. Thus, sequences from these organisms may have been amplified, but if so, they were left as unassigned MOTUs. The uneven taxonomic resolution across the markers we used may explain why we only detected one NIS (*Necora puber*). Among the 150 animal NIS recorded in the French Mediterranean waters (Massé et al., 2023), only 19 are present in our metazoa reference database. With a better taxonomic resolution, we would be able to better assign MOTU to NIS, an important issue here since seaports are recognized as primary steppingstones for NIS introductions and spread (Aglieri et al., 2023; Andrés et al., 2023; Darling et al., 2020; Rey et al., 2020), recreational shipping seaports included (Ferrario et al., 2017; Ulman et al., 2017). To address these limits, it is

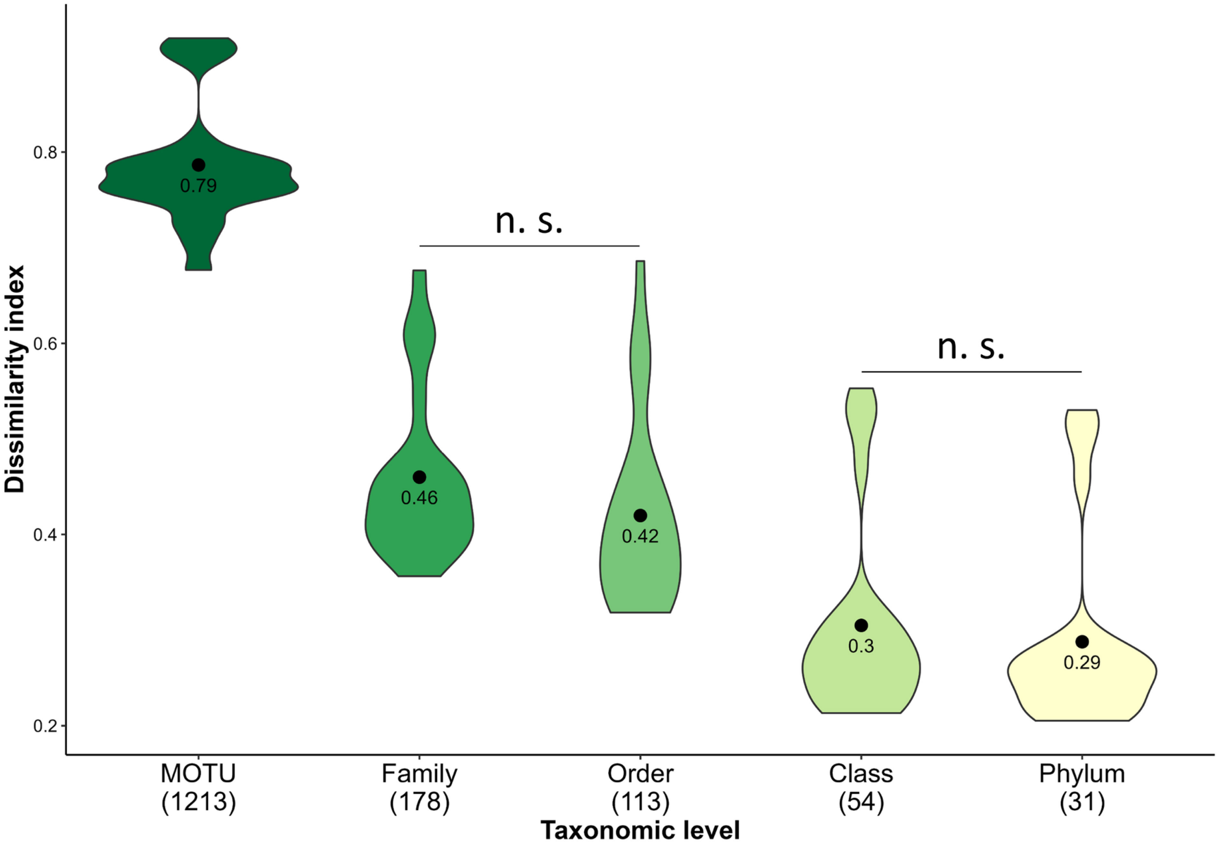

FIGURE 8 Dissimilarity index between seaport and reserve communities across taxonomic levels. Pairwise dissimilarities are computed between sites from seaports and sites from reserves after pooling replicates and combining all MOTUs. Jaccard dissimilarity index is calculated at the MOTU level, whereas Bray–Curtis' dissimilarities based on MOTU abundance are used for higher taxonomic levels. The number of unique MOTUs/families/orders/classes/phyla in the dataset when comparing seaports and reserves are given within parentheses. Black dots indicate mean values and 'ns' couples where differences are not significant (see Table S10 for the Dunn's test output).

essential to complete genetic reference databases. Accumulation curves revealed that we did not reach the asymptotic values of MOTU richness for eukaryotes and metazoans (Figure S1). This would have been overcome by increasing the number of field replicates. In addition to complete reference databases, increasing the number of markers is also an alternative to improve the results, but it would increase financial costs. With markers targeting smaller groups inside metazoans and eukaryotes, it is likely that we would have obtained finer results and reached saturation earlier as less species would have been possible to amplify with each marker. For example, in addition to a general metazoan marker, Stat et al. (2017) used markers specific to fish, crustaceans and cephalopods. We only did it for fish here with the teleo marker. Concerning prokaryotes, combining 16S rRNA metabarcoding with shotgun sequencing would certainly provide more sequences (Stat et al., 2017) with better chances to assign them to known taxa as reference databases would not be restricted to one short metabarcode only. We did not explore this option here as the objective of this study was to capture a biodiversity snapshot with a similar methodology for the whole Tree of Life.

The ability of eDNA to outperform conventional surveys is now recognized in many studies and for different taxonomic groups (Eble et al., 2020; Miya, 2022; Pawlowski et al., 2021). It allows a better detection sensitivity, particularly in environments where organisms are challenging to catch or observe, with reduced costs and limited taxonomic expertise required. However, eDNA-based assessments are not free from type I and type II errors, and it is better to use them in conjunction with other methods. Our protocol tried to avoid contamination at each step of DNA handling and applying stringent filters in the bioinformatic pipeline likely reduced false-positive detections. False-negative ones are more difficult to avoid, as it can be highly dependent of abiotic factors and primer bias (Burian et al., 2021; Elbrecht & Leese, 2015). Disparity in eDNA concentration and persistence in seaports and open-sea reserves may also influence eDNA detectability, as seaports are semi-enclosed systems and so less influenced by waves, swell and flows. Comparative studies are needed to better assess whether DNA concentration and persistence are higher or not inside seaports. In addition, we should notice that eDNA cannot yet accurately assess the abundance of organisms in real conditions, even though positive correlations between sequencing read counts and biomass were recently corroborated (Rourke et al., 2022). It is possible that some taxa were detected in both seaports and reserves but actually show significant differences in abundance levels. Consequently, eDNA monitoring should still be completed by traditional surveys to estimate population densities.

There is a growing worldwide trend towards recreational and commercial boating, and the number of seaports is expected to increase to meet this demand (Hanson & Nicholls, 2020; Madon et al., 2023). For this reason, surveying seaport biodiversity is a major but overlooked conservation issue. The Mediterranean Sea, as a biodiversity hotspot for marine wildlife (Coll et al., 2010) and highly anthropized area, is a perfect case study. Here, we compared biodiversity patterns between highly urbanized (seaports) and preserved areas (marine reserves), but seaports and reserves are not necessarily the extremes of anthropogenic degradation gradients. Seaports may represent a shelter against exploitation as fishing is usually forbidden within their boundaries, and their enclosed architecture can provide protection from coastal streams. Seaports have been shown to play the key role of nursery grounds for some fish species when adding habitat complexity (Bouchoucha et al., 2016; Joubert et al., 2023; Selfati et al., 2018). However, artificial structures designed to promote biodiversity may also favour a subset of opportunistic species (Bishop et al., 2017; Dafforn et al., 2015) and NIS establishment (Gauff et al., 2023). On the other hand, an MPA cannot be considered as a perfectly clean environment or reference condition, even no-take reserves (D'agata et al., 2016). In particular, although the Calanques National Park sampled in this study has a high protection status, industrial effluents are known to be discharged directly into its waters (see the map associated with Holon et al., 2015). Yet, each seaport and reserve has its inherent characteristics that may influence the hosted communities. In particular, seaports are commonly built within river mouths, where both marine and brackish water species may co-occur (Arthington et al., 2016). Among the seaports we surveyed, two of them are located in the vicinity of an estuary, Agde being 5km east from the Hérault river mouth, and Saintes-Maries-de-la-Mer 2km east from the Little Rhône river mouth. Urbanization of the French coastline has been under strict control since the 'Coastal law' of 1986, so building new seaports along the French Mediterranean shore is unexpected, but their expansion is still possible. Extending the scope of our present study by associating finer disturbance, health status or preservation and functional indicators to biotic assemblages would reinforce the use of the Tree of Life eDNA metabarcoding in urbanized seascapes. Such indicators would help to monitor and evaluate the efficiency of eco-certifications that are sometimes attributed to seaports (Hossain et al., 2021). Taxon-independent indices have recently been developed to overcome reference database incompleteness (Wilkinson et al., 2024). To go further, future works should explore beyond α- and β-biodiversity patterns to answer whether intraspecific variation is affected or not by coastal urbanization. This was recently implemented in DNA metabarcoding studies by comparing COI haplotype occurrence frequencies (Antich et al., 2023; Thomasdotter et al., 2023).

AUTHOR CONTRIBUTIONS

SM conceived the study. BM, DM, AD and SM performed research. BM analysed data. BM, DM and SM wrote the manuscript. JD, AV, AG and PB helped for the sampling design. MB provided bioinformatic support. All authors gave valuable feedbacks and contributed to the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The raw datasets containing the NGS eDNA sequencing outputs are available on Zenodo (teleo: https://zenodo.org/records/10930835; metazoa: https://zenodo.org/records/10930861; euka2: https://zenodo.org/records/10930847; bact2: https://zenodo.org/records/10930858). The eDNA-seq Metabarcoding OTU-clustering pipeline (version 1.2.0) that was used in this study is deposited in a Software Heritage archive (https://archive.softwareheritage.org/browse/directory/adb93daff2ec7ca6471f0e84a0d7da32d829e1de/). Script of the post-processing steps and statistical analyses are available at https://github.com/bastien-mace/Med_Port_eDNA.

BENEFIT-SHARING STATEMENT

Benefits from this research accrue from the sharing of our data, bioinformatic pipeline and scripts on public databases as described in the 'Data Availability Statement'.

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