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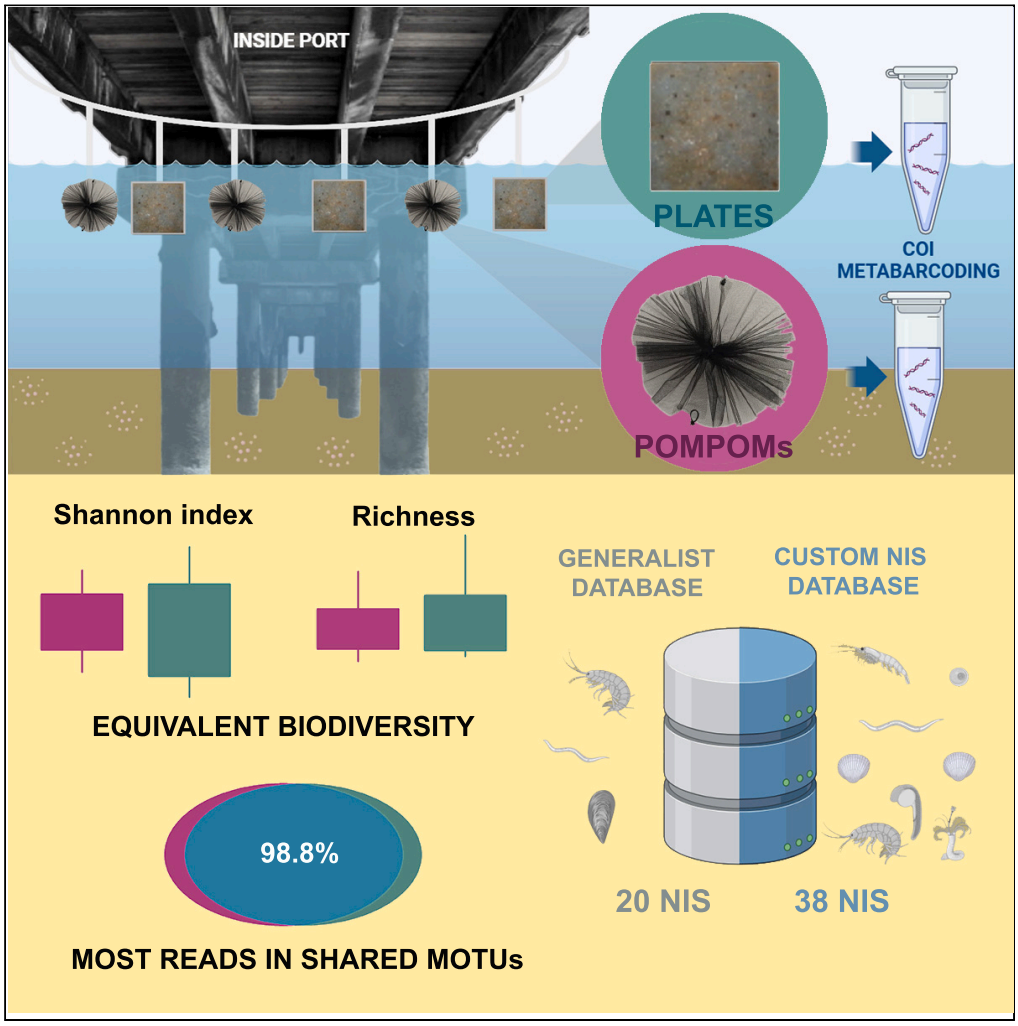

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Highlights

We present a new sampling device for metabarcoding of port organisms (POMPOMs)

POMPOMs capture the biodiversity present as efficiently as settlement plates

A new database with genetic information of Mediterranean NIS is compiled

38 NIS were detected in the studied port, representing ca. 26% of COI reads

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A new sampling device for metabarcoding surveillance of port communities and detection of non-indigenous species

Jesús Zarcero,[1,2,4] Adrià Antich,[1,4] Marc Rius,[1,3] Owen S. Wangensteen,[2] and Xavier Turon[1,5,*]

SUMMARY

Metabarcoding techniques are revolutionizing studies of marine biodiversity. They can be used for monitoring non-indigenous species (NIS) in ports and harbors. However, they are often biased by inconsistent sampling methods and incomplete reference databases. Logistic constraints in ports prompt the development of simple, easy-to-deploy samplers. We tested a new device called polyamide mesh for ports organismal monitoring (POMPOM) with a high surface-to-volume ratio. POMPOMS were deployed inside a fishing and recreational port in the Mediterranean alongside conventional settlement plates. We also compiled a curated database with cytochrome oxidase (COI) sequences of Mediterranean NIS. COI metabarcoding of the communities settled in the POMPOMs captured a similar biodiversity than settlement plates, with shared molecular operational units (MOTUs) representing ca. 99% of reads. 38 NIS were detected in the port accounting for ca. 26% of reads. POMPOMs were easy to deploy and handle and provide an efficient method for NIS surveillance.

INTRODUCTION

The spread of non-indigenous species (NIS) through shipping is a major threat to coastal marine ecosystems.[1–4] In commercial ports, there is a correlation between shipping activity and NIS diversity at the community and population levels.[5] Ports and harbors act as entry gateways and facilitate propagation corridors for NIS.[6–9] Given that these habitats generally exhibit low biodiversity, in part because of NIS outcompeting native communities,[3,10,11] they are considered vulnerable to biological invasions.[3] The movement of NIS and genotypes that would not have naturally encountered each other can have long-term, irreversible consequences on local and global biodiversity,[12–15] together with substantial economic costs.[16]

The expansion of marine urbanization and urban sprawl, which usually includes creation of new ports and marinas or enlargement of existing ones, is quickly altering large stretches of coastlines,[17–21] yet the field of marine urban science remains relatively unexplored.[15,22,23] In particular, there is a need for developing standardized protocols for biodiversity monitoring of port communities to conduct risk analyses of NIS spread.[2] This information, coupled with data on environmental conditions and ship traffic, can facilitate an efficient management of biological invasions.[24] In spite of this growing threat, few countries have in place regular monitoring programs in ports.[25–28]

DNA metabarcoding is a well-established technique for unraveling biodiversity in many ecosystem types, including the marine environment.[29–34] Metabarcoding can be faster and more cost-effective than traditional, morphology-based methods when generating biodiversity inventories to track changes through time and space.[35,36] It also enables the identification of early life-history stages such as larvae and recruits, which are often the stages at which marine NIS are transported.[37,38] Metabarcoding is, therefore, a technique of choice for NIS monitoring and for conducting biosecurity surveillance,[39–42] particularly in communities from ports and harbors.[2] Metabarcoding datasets can be automatically screened for quick flagging of species of concern.[43] Faster and more accurate data collection increase our ability for early NIS detection and ultimately improves biodiversity management.

Port ecosystems have different compartments (water, hard artificial substrates, sediment) that provide different windows on the biodiversity present.[44–48] Substrate selection is thus of crucial importance when designing metabarcoding surveys, and the need of analyzing multiple substrates has been proposed.[45,47,48] However, this is not always logistically feasible or cost-effective, and operational compromises should be sought. Environmental DNA (eDNA) metabarcoding of filtered water has often been used in these environments[49,50] and has proven effective in detecting particular species with specific primers.[51–54] However, it falls short of providing a complete picture of the epibenthic

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eukaryotic community when using generalist primers, as shown in studies with paired water and bulk samples both at sea and in freshwater.[55–58] Even at distances of a few cm from the benthos the biodiversity recovered is only a fraction of the one present in the benthic community.[58] Therefore, organismal sampling using bulk-sample methods targeting the so-called community DNA[30,59–61] is necessary in port surveys.[45] Among these methods, settlement plates have been traditionally used to monitor ports either genetically or morphologically,[8,62–64] and they are also a commonly used method outside of them. For instance, they are at the core of the autonomous reef monitoring Structures (ARMS) system.[65] However, their deployment and handling can be difficult, requiring fastening frames and structures with screws underwater.

Here we introduce a novel organismal sampler, called polyamide mesh for ports organismal monitoring (hereafter POMPOM), consisting of a polyamide mesh of ca. 1 mm mesh size, folded to provide a high surface for settlement of organisms and to capture organic particles in the water. POMPOMs are logistically easy to deploy and process. They are little affected by currents and wave exposure, and can be easily placed in both sheltered and exposed habitats. This sampler can also capture both settlers and planktonic stages as well as particulate organic matter, thus providing a comprehensive picture of the present biodiversity with a single sampling method. We used COI metabarcoding to compare the biodiversity information gleaned from POMPOMs with that from the commonly used PVC settlement plates in a fishing and leisure port (Blanes, NW Mediterranean) over a year. The samples obtained were processed using an established bioinformatic pipeline.[34,59] In addition, as DNA-based studies of NIS are crucially dependent on complete DNA barcode reference libraries,[66] we compiled a curated up-to-date NIS database for the Mediterranean Sea. We then compared the results obtained using this new database with those obtained using a generalist reference database.

RESULTS

α-diversity and taxonomic composition

After pairing, demultiplexing, quality and length filtering, and chimera removal we obtained 44,615,630 reads from 10,383,408 COI unique sequences. The denoising procedure resulted in 806,328 exact sequence variants (ESVs), which were grouped into 15,664 molecular operational taxonomic units (MOTUs). Finally, after all the refining steps (see STAR Methods section), our database consisted of 2,955 MOTUs and 24,490,456 reads (Table S1). The different groups of samples will be hereafter denoted as PM (POMPOMs) and PL (plates) for simplicity. Temperature recordings showed a clear seasonal pattern (Figure S2) and showed a clear seasonal pattern. It is also noteworthy that temperatures were higher in spring 2020 than in 2019, reaching 16°C in April 2020.

The final table had 408,174 $\pm$ 63,508 (mean $\pm$ SE) reads per sample. The number of MOTUs per sample was 223.43 $\pm$ 15.94. Rarefaction curves (Figure S1) showed that a plateau in number of MOTUs was achieved in the samples, indicating an adequate sequencing depth. In turn, MOTU accumulation curves (Figure S1) did not reach a plateau, with more MOTUs being added as more samples are combined. However, this is exclusively due to very rare MOTUs, if we consider only those MOTUs representing the 99% of total reads, then as asymptote is reached both in PM and PL samples (Figure S1).

The median values of Shannon diversity after rarefaction to 27,992 reads (corresponding to those found in the sample with less reads) were 2.72 and 2.41 for PM and PL samples, respectively (Figure 1). The median values of MOTU richness for these same samples were 162.5 and 190.0, respectively (Figure 1). Diversity was thus slightly higher in POMPOMs than in plates and the reverse was true for richness values, but the differences were not significant (Mann-Whitney tests, $p = 0.277$ and 0.416, respectively).

Barplots with the taxonomic composition were prepared separately for each group and sampling period in terms of super-group and metazoan phyla composition. Groups accounting for less than 5% of MOTUs were grouped under "Others". The "Unidentified" category corresponded to eukaryotic MOTUs that could not be assigned to a super-group or to metazoan MOTUs that did not get a phylum-level assignment. In terms of proportion of MOTUs (Figure 2), metazoans were by far the dominant group for all collectors, followed by Stramenopiles and Rhodophyta, and the composition of PM and PL samples was similar. When considering read, instead of MOTU, proportions, the dominance of metazoans was still more marked, and some differences between PM and PL were apparent (f.i., a higher abundance of reads of Stramenopiles and Rhodophyta in PL in spring).

In terms of metazoan composition (Figure 3), Annelida, Arthropoda, and Cnidaria were the dominant phyla, followed by Chordata (mostly ascidians) and Bryozoa. In terms of relative number of reads, the same groups were dominant, and a pattern of higher abundance of annelids in the warmer months (from July to October) was apparent in both sample types. The proportion of MOTUs unidentified at the super-group level was 25.24% in PM, and 26.55% in PL (representing 4.11%, and 2.04% of the reads, respectively). Likewise, the metazoan MOTUs that could not be assigned at phylum-level represented 45.12% and 51.44% of the metazoans in PM, and PL (representing 12.12% and 10.39% of the reads, respectively).

As for the overlap between POMPOMs and plates, 1,068 MOTUs (ca. 36%) were shared, with 778 MOTUs exclusive of PM and 1,109 of PL (Figure 4). The non-shared MOTUs had very low abundances in reads, as those exclusive of PM and PL represented 0.26% and 0.92% of the total reads, respectively, while the shared MOTUs corresponded to 98.82% of the total reads (Figure 4). Thus, the non-shared component comprised rare, low abundance MOTUs. If we exclude them by considering only the MOTUs making up the 99% of the total reads, then 83% of the MOTUs were shared, while using the MOTUs adding up to 95% of the total reads, we found that 100% were shared. In addition, there is a highly significant correlation between the mean read abundances of the shared MOTUs in the two types of samples (Pearson's $r = 0.766$, $p < 0.001$) (Figure S3). The taxonomic composition of the MOTUs exclusive of one or another collector is presented in Figure S4 as proportion of MOTUs and reads of the different super-groups and metazoan phyla. The proportions of the different groups are similar across collector types, albeit plates tended to have a higher proportion (in MOTUs and reads) of Stramenopiles and of the Unidentified category in metazoans.

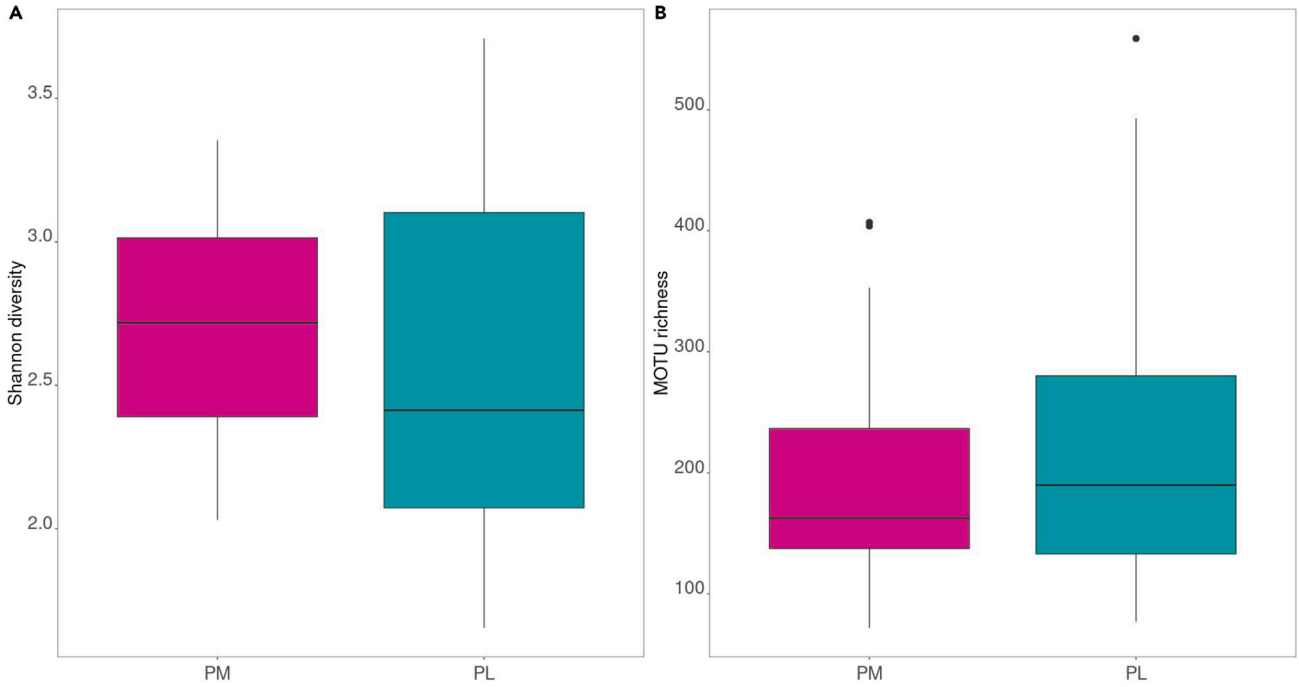

Figure 1. Shannon diversity and molecular operational taxonomic unit (MOTU) richness

Box-plots of the values of Shannon diversity (A) and MOTU richness (B) of the different types of samples. PM: polyamide mesh for port organismal monitoring (POMPOMs), PL: settlement plates. Horizontal lines are medians, boxes encompass the first and third quartiles, whiskers indicate 10th and 90th percentiles, and outliers are indicated as dot symbols.

β-diversity and indicator species

The nmMDS configuration (Figure 5) showed no spatial separation between POMPOMs and plates, and the samples of the same months tended to appear close to each other in the configuration (except in December 19 and April 20). The three replicate samples of each month appear close together for both sampler types. A seasonal pattern was also apparent, with samples from the warmest months concentrated at the positive extreme of the first axis. However, the annual cycle did not close properly, with April 2020 behaving differently to April 2019. This is likely attributable to higher temperatures detected in 2020 (Figure S2). The PERMANOVA results indicated a significant effect of both main factors (collector type and month) and the interaction (Table S2). There was also a different degree of heterogeneity among levels of both factors, as indicated by significant permdisp tests. The dispersion was significantly higher in the PL samples, and in the spring months (April 2019 and 2020). In the presence of a significant interaction term, we performed pairwise comparisons between collector types within levels of month, and only the comparison corresponding to December 2019 was significant after correction. In addition, the Mantel test comparing dissimilarity matrices obtained with the PM and PL samples revealed a highly significant correlation ($r = 0.694$, $p < 0.001$).

The indicator species analysis showed low numbers of indicator MOTUs (37 in PM, 118 in PL) representing a low percentage of the total reads in these collectors (7.03% and 4.83%, respectively). A MOTU identified as *Bugula stolonifera* and an unidentified Ctenostomatida were the two most abundant indicator MOTUs for PM, while in PL samples the two most abundant were the calanoid copepod *Acartia discaudata* and an unidentified MOTU.

NIS database and detection

The NIS database generated for the Mediterranean is presented in Table S4. Future updates of the table will be publicly available in GitHub (github.com/jesuszarcero/NISdb). Using this database, 38 MOTUs were assigned to an NIS with similarities above 95% (all but five with ≥99% similarity) (Table S5). These MOTUs accounted for 25.82% of the total number of reads obtained. When comparing the new assignment of these 38 MOTUs with the one obtained with the general database (Table S5), we found that in 24 of them the assignment was coincident (same species), but of these, in 6 the general database returned a best hit below 95% identity. In 9 MOTUs the assignment obtained with the general database was coherent with the one from the NIS database, but was made at a higher taxonomic level. Finally, in five cases the assignments were conflicting (different genus assignment in the same superfamily in one case, different genus in the same family in three instances, and different species in the same genus in one case, Table S5). The overall similarity with the best match of these MOTUs increased from 95.27% to 99.59% when using the NIS database. It should be noted that, with the taxonomic assignment obtained with the general database, only 20 MOTUs were assigned to species included in the NIS table with a similarity of 0.95 or more, that is, almost half (18) of the NIS identified would have gone undetected.

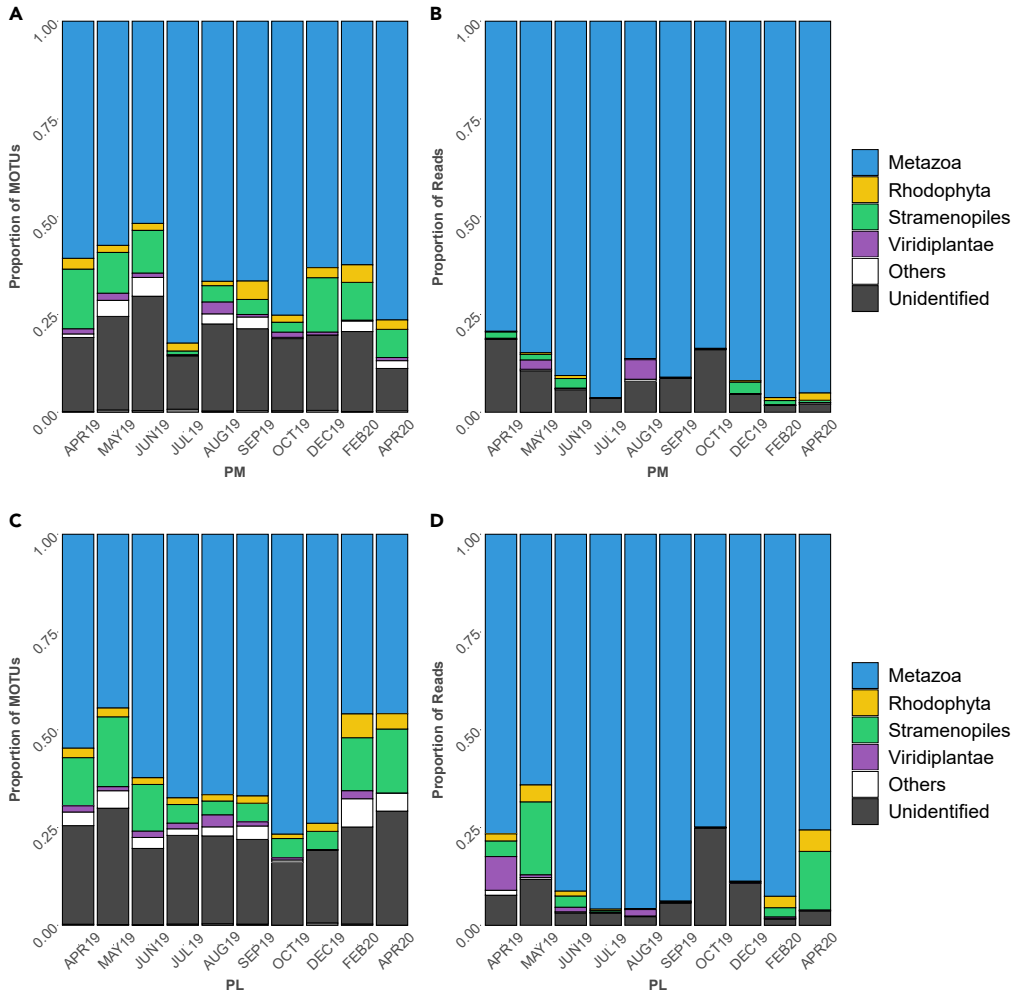

Figure 2. Taxonomic composition in super-groups

Barplots of the relative proportion of molecular operational taxonomic units (MOTUs) (A and C) and reads (B and D) of the different super-groups considered at each sampling time. Category "Others" pools the groups representing less than 5% of the MOTUs. Category "Unidentified" refers to eukaryote MOTUs that could not be assigned to any super-group.

The abundance of MOTUs assigned to NIS in the different sample types are also presented in Table S5. Overall, the three most abundant NIS in percent reads were *Jassa slattery* (8.01%), the colonial ascidian *Diplosoma listerianum* (5.78%), and the cirripede *Amphibalanus amphitrite* (3.11%). When comparing the information provided by POMPOMs and plates, both had a similar percentage of NIS reads (23.76% in PM, 26.28% in PL). 28 NIS were detected by both types of collector and the percent of NIS reads in the shared NIS was 99.98%, so those NIS exclusive of one or another collector were very rare MOTUs. There was a significant correlation between the relative number of reads detected in POMPOMs and plates for shared NIS (Pearson's $r = 0.816$, $p < 0.001$, Figure S5). Heatmaps representing the abundance of the different NIS over the study period are presented in Figure 6, showing similar temporal patterns for both sampler types. Accordingly, the overall relative frequencies of each MOTU at each sampling month were significantly correlated between sampler types (Pearson's $r = 0.474$, $p < 0.001$).

DISCUSSION

Our results showed that POMPOMs and settlement plates captured a similar picture of the biodiversity present inside a middle-sized port over a yearly cycle, in terms of diversity, MOTU richness, and taxa abundances. They also perform almost equally in NIS detection success. Settlement plates represent the most commonly used sampling method in ports[67,68] and link with a well-established tradition in NIS studies on fouling communities.[64] The results therefore validate POMPOMs as an efficient sampling method.

Rarefaction curves indicated that our sequencing depth was adequate to capture the diversity in each sample, while sample accumulation curves, as usual in studies with generalist primers,[34,58,60,69] pointed to an increasing number of MOTUs as more samples are combined. However, this is due to the addition of very rare MOTUs, as those representing 99% of the reads are well captured with just a few samples. More

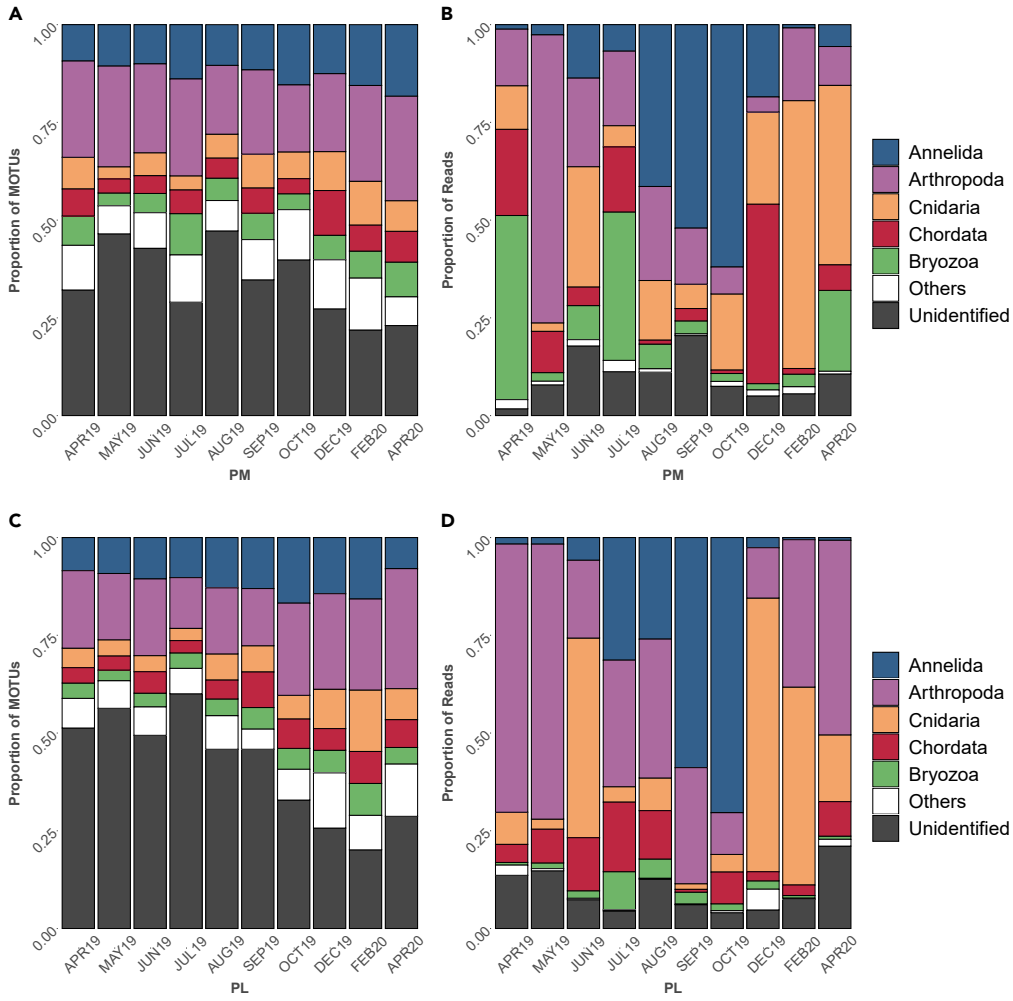

Figure 3. Taxonomic composition in metazoan phyla

Barplots of the relative proportion of molecular operational taxonomic units (MOTUs) (A and C) and reads (B and D) of the different metazoan phyla at each sampling time. Category "Others" pools the phyla representing less than 5% of the MOTUs. Category "Unidentified" refer to metazoan MOTUs that could not be assigned to any phylum.

replicates, rather than more sequencing depth, can be considered in future studies for a more complete characterization of the rare biodiversity present.

The patterns of MOTU overlap confirmed this trend, with shared MOTUs between POMPOMs and plates representing the overwhelming majority of the reads obtained (ca. 99%). Thus, the non-shared MOTUs are most likely small, rare organisms belonging to the so-called cryptobiome,[70] which remain underrepresented in the existing databases.[71] This can also explain the high percentage of unassigned non-shared MOTUs. Accordingly, indicator species analyses revealed that the species associated with one or another collector type were few and represented a small percentage of the reads (<8%). Reduced-dimension analyses (nmMDS) also highlighted wide overlap between sample types inside the port. The POMPOM and plate centroids of most of the months (with only a couple of exceptions) appear close in the final configuration. The Mantel test indicated a highly significant correlation of the two sets of samples. The PERMANOVA results, however, revealed significant differences between POMPOMs and plates, but they were mostly due to a higher heterogeneity of the composition of the plate samples, with only one pairwise comparison between collector types within months being significant. Our monthly sampling also revealed some temporal trends, with a differentiation of the warmer months (July-October) at one extreme of the first axis, while spring samples were concentrated at the other extreme, probably reflecting recruitment patterns in the area, with many invertebrates reproducing in spring. However, anomalous high temperatures in spring 2020 led to a different behavior of April samples with respect to 2019. Consequently, clear seasonal trends could not be discerned with just one year of sampling.

The new collector type presented is suitable for sampling inside ports as tested here. Notwithstanding, it can be used in other habitats, as it is easy to deploy and recover, and due to its flexibility, is able to withstand currents and tides, thus reducing the risk of losing samples due to physical factors and simplifying the logistics of the experiments.[8,67,72] They can also be used in traditional morpho-taxonomic approaches.

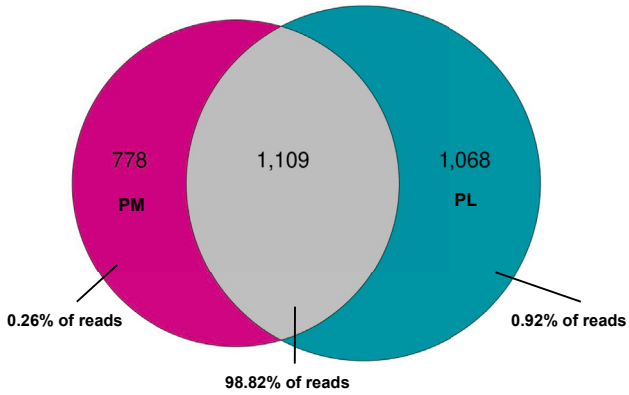

Figure 4. Molecular operational taxonomic unit (MOTU) overlap

Venn diagrams showing the overlap in MOTU composition between POMPOMs (polyamide mesh for port organismal monitoring) and plates. We also provide the percent of the total reads that the different compartments represent.

POMPOMs are cheap, simple, and practical, enabling the standardized monitoring of multiple marine environments without the need of complex sampling structures that need to be bolted to the bottom. This is particularly handy in studies of ports and harbors, where diving and/or deploying structures can be logistically unfeasible. Albeit there is no single fit-for-all method, if one type of sampling device is to be chosen, we contend that POMPOMs will provide an accurate assessment of the biodiversity present. As one potential drawback of POMPOMs, their flexibility would be an inconvenient for the development of mature sessile communities, and so for long immersion times (e.g., one year) plates would be preferable. For short interval monitoring (one or two months in our case), however, POMPOMs are a sensible choice.

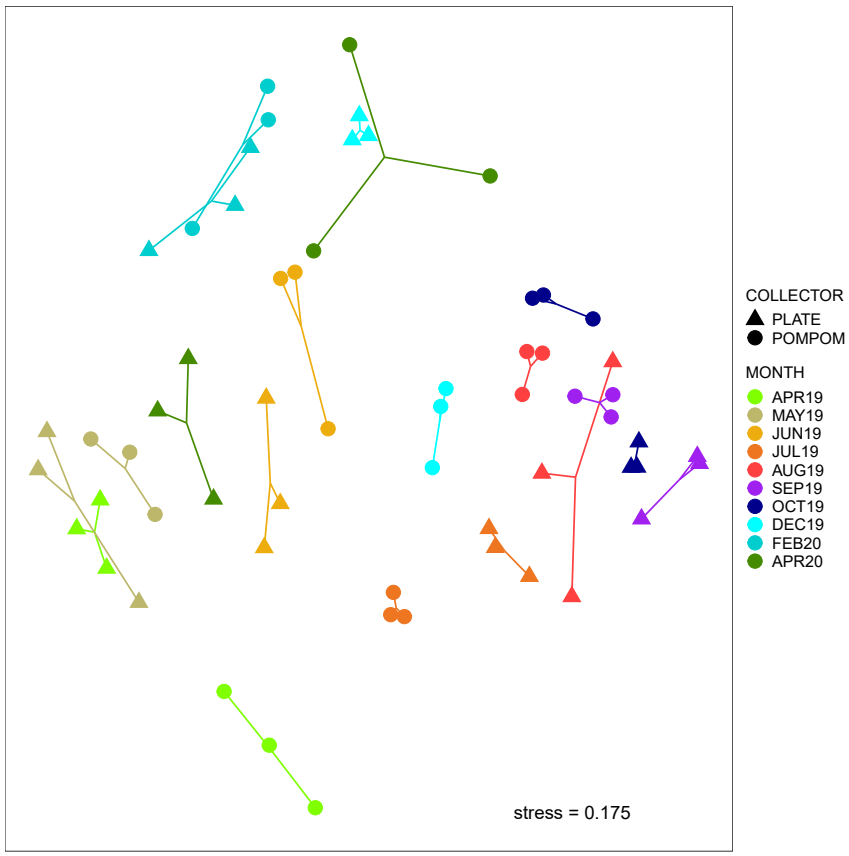

Figure 5. Non-metric multidimensional scaling (nmMDS) ordinations

nmMDS configuration of POMPOM (polyamide mesh for port organismal monitoring) and plate samples inside the port. Lines join samples from the same date and collector type. Stress of the final configuration is indicated.

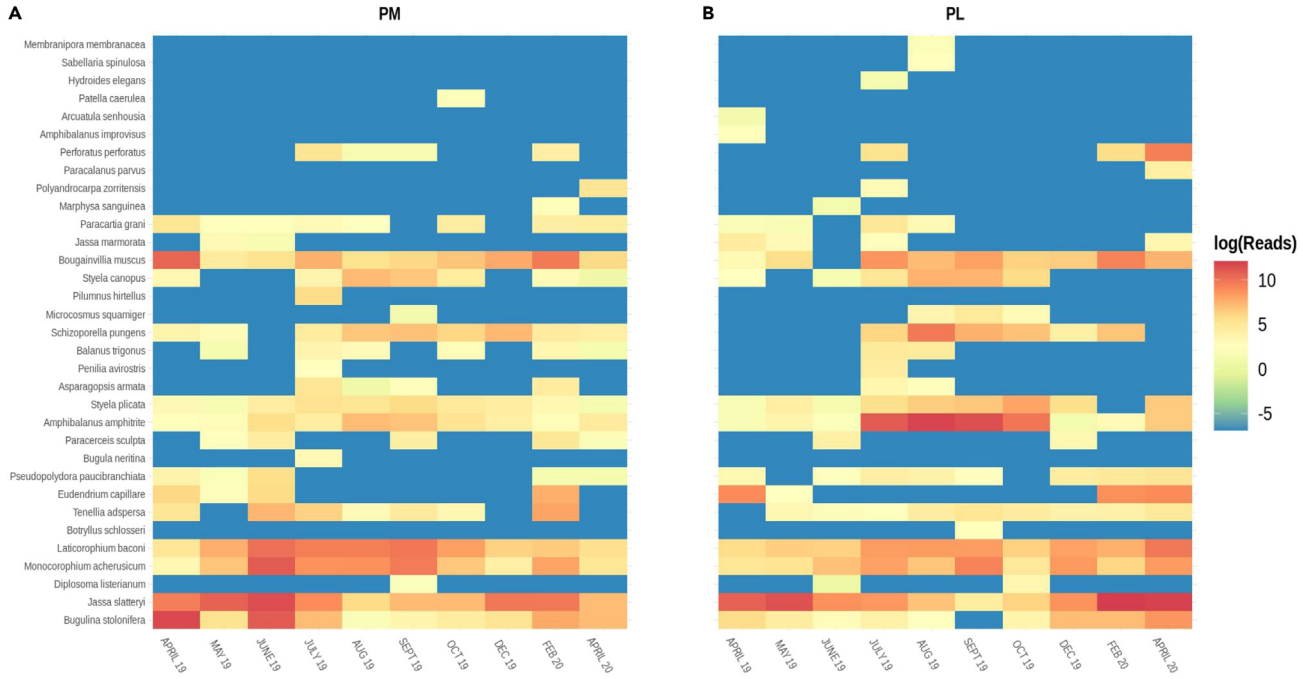

Figure 6. Heatmap of non-indigenous species (NIS) abundance

Heatmaps showing read abundances (log-transformed) of the identified NIS in the two types of collector at each date: POMPOMs (A) and plates (B). Blue cells indicate 0 reads. Note that we have pooled molecular operational taxonomic units (MOTUs) assigned to the same nominal species, so the heatmaps show results for 33 species-level taxa.

We also emphasize how the monitoring of NIS in these artificial environments is highly dependent on accurate and curated reference databases.[66] Our newly created database yielded almost twice as many NIS detections than a widely used generic database. Nevertheless, many reported Mediterranean NIS still lack genetic information, so more DNA sequencing efforts guided by adequate taxonomic expertise are needed. Unlike other survey methods, metabarcoding allows re-examining existing MOTU datasets as more complete reference databases become available.

The combination of a generalist and a custom database is a way forward to mitigate the problem of database completeness.[73] While general databases can be generated manually or with the help of specialized software[74,75]), they inherit errors and misidentifications from the source databases. Furthermore, in most cases they are not continuously updated. A custom, reduced database such as the NIS dataset in our case can be pruned of errors and curated manually. Importantly, it can also be maintained up-to-date as primary literature is being produced and critically screened. The updated versions of the database will become automatically publicly available. We favor this dual database approach to assess biodiversity of specific groups of organisms, such as NIS.

In summary, we have shown here the usefulness of POMPOMs as a standardized method for sampling in ports, easy to deploy and process. It allows monitoring over time or space of fouling communities and, combined with suitable databases, constitutes a useful tool for NIS detection and surveillance in these introduction hotspots. It has the potential to be used also in other types of marine ecosystems.

Limitations of the study

One likely limitation of our new sampling device is attributable to its flexibility (as compared with rigid systems such as plates). While this flexibility allows POMPOMs to withstand currents and impacts, it can interfere with the growth of organisms, particularly calcified invertebrates. Thus, we do not expect representative mature communities to be able to develop on POMPOMs over time, and our sampler is more convenient for short-term monitoring with a time frame of one-two months between units replacement. In addition, our study would have benefitted from comparison with other compartments of the port biota; for instance, with eDNA metabarcoding of water samples, and with sediment samples close to the point of deployment of POMPOMS. This will be a direction for future research, but for this study we chose as a counterpart for comparison settlement plates, as they were a standard method in morphological and metabarcoding studies, and they were *a priori* expected to yield results similar to our collectors. Another limitation of our study is that detection of seasonal patterns would have required a longer study period. Although the analysis of these patterns was not our primary objective, the fact that we monitored over one year (with a two-month overlap) compromised the assessment of temporal trends. Continuing the port monitoring over longer time frames is an ongoing line of research. Finally, we should also note that the newly built NIS database generated should be subject to continuous updates and curation based on a close screening of new relevant literature as it appears, otherwise it can become quickly outdated.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Sampling and sample processing
- METHOD DETAILS
  - DNA extraction and sequencing
  - NIS database compilation
  - Bioinformatic analyses
- QUANTIFICATION AND STATISTICAL ANALYSIS
  - Community analyses

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.108588.

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AUTHOR CONTRIBUTIONS

Conceptualization: X.T., M.R., O.S.W., and A.A.; Methodology: X.T. and A.A.; Investigation: J.Z., A.A., X.T., and M.R.; Formal Analysis: J.Z., A.A., and X.T.; Resources: X.T., O.S.W., and M.R.; Funding Acquisition: X.T. and M.R.; Writing – Original Draft: J.Z. and X.T.; Writing – Review and Editing: J.Z., A.A., M.R., O.S.W., and X.T.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Organismal community of Blanes	This paper	Table S1
Chemicals, peptides, and recombinant proteins		
DNeasy PowerMax Soil Kit	Qiagen	Cat#12988-10
Leray-XT primers	Wangensteen et al.[33]	https://doi.org/10.1016/j.marpolbul.2017.11.033
MinElute PCR Purification Kit	Qiagen	Cat#11732668001
BIOO NEXTFLEX PCR-Free DNA-Seq Kit	Perkin-Elmer	Cat#5142-02
Deposited data		
Raw COI amplicon sequence data	This paper	PRJNA977116
NIS database	This paper	Table S4, future updates available at https://github.com/jesuszarcero/NISdb
DUFA reference database	Unpublished.	https://github.com/uit-metabarcoding/DUFA
Software and algorithms		
MJOLNIR3 pipeline	Unpublished.	https://github.com/adriantich/MJOLNIR3
OBITools3	Boyer et al.[76]	https://doi.org/10.1111/1755-0998.12428
VSEARCH v 2.7.1	Rognes et al.[77]	https://bioweb.pasteur.fr/packages/pack@vsearch@2.7.1
DnoisE	Antich et al.[78]	https://doi.org/10.7717/peerj.12758
SWARM v2.1.7	Mahé et al.[79]	https://github.com/torognes/swarm
'vegan' v. 2.5-6 R package	Oksanen et al.[80]	https://cran.r-hub.io/web/packages/vegan/vegan.pdf
'ggplot2' R package	Wickham[81]	https://doi.org/10.1002/wics.147
Primer v6 statistical package	Anderson[82]	https://doi.org/10.1016/j.jembe.2008.07.006
Other		
Polyamide mesh	MaterialsWorld®	Cat#103820
Data loggers	HOBO®	Cat#UA-001-64
PVC settlement plates	SKU®	Cat#188412

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Xavier Turon (xturon@ceab.csic.es).

Materials availability

This study did not generate new unique reagents or materials.

Data and code availability

- The raw DNA sequences generated are deposited in the NCBI Sequence Read Archive and are publicly available as of the date of publication. Accession numbers are provided in the key resources table.
- Fully reproducible code associated with bioinformatic analyses is deposited at GitHub and is publicly available as of the date of publication. Accession links are provided in the key resources table.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Sampling and sample processing

POMPOMs consist of a 100 x 25 cm strip of polyamide mesh (MaterialsWorld®) with hexagonal openings of ca. 1 mm, folded in a zig-zag pattern and fastened with cable ties, resulting in a circular shape (ca. 25 cm in diameter) reminiscent of the garments that go with the same name (Figure S6). A step-by-step procedure for preparing a POMPOM is presented in Video S1.

Four POMPOMs were deployed at a depth of 50 cm below the surface, hanging from ropes stretched across a dock in the Blanes port (NW Mediterranean, 41°40′33.0"N 2°47′57.2"E) from February 2019 to April 2020. Blanes is a medium-sized port, with 1,486 lineal metres of fishing docks and marinas. Three of the collectors were used for metabarcoding analyses, while the other was kept as backup. We replaced collectors monthly during the warmer months (from April to October) and bimonthly during cold periods (December, February), for a total of 10 time points sampled. The temperature was recorded hourly using a HOBO® data logger next to the collectors.

In addition to the POMPOMs, we deployed 20x20 cm PVC settlement plates inside the port. They were placed in the same dock and depth as the POMPOMs and were replaced at the same time with the same number of replicates. Plates were placed inside plastic zip bags and kept at -20°C.

After collecting the samples, the POMPOMs were unfolded and all biofouling attached to them was removed using sterilized nail brushes, replaced at each replicate, and the material was recovered using a stainless-steel sieve of 64 μm. The plates were scraped clean with sterilized spatulas, and the obtained material was also filtered. The handling time for a POMPOM was ca. 10 minutes, similar to the time needed for scraping and filtering the plates. The resulting material was a homogenate that was preserved in 50 mL sterile Falcon tubes filled with absolute ethanol and stored at -20°C. Blanks consisted of sterile collectors processed in the same way. Negatives were obtained using sand charred in a muffle furnace (400°C for 4 hr) and then spread on sterile POMPOMs and processed following the same methodology as the biological samples.

In total, we obtained 30 samples of POMPOMs and 30 samples of settlement plates inside the port, plus 10 blanks and 9 negatives. The targeted community includes the eukaryotes, organic particles, and DNA traces settling or being passively captured in the different collectors, so sex/gender cannot be reported and do not apply to our study.

METHOD DETAILS

DNA extraction and sequencing

All procedures were performed in a sterilized laminar flow cabinet with activated UV light between samples. We followed the DNA extraction, library preparation, and sequencing protocols outlined in.[59] In short, DNA was extracted from 5-6 g of homogenized material from the samples with the DNeasy PowerMax Soil Kit (Qiagen). We amplified a region of the Cytochrome Oxidase 1 (COI) gene of ca. 313 bp using generalist primers that targeted eukaryotes. Specifically, we used the Leray-XT primer set[33,71] which includes the forward primer jgHCO2198;[83] 5′-TAIACYTCIGGRTGICCRAARAAYCA-3′ and the reverse primer mlCOIintF-XT: 5′-GGWACWRGWTGRACWITITAYCCYCC-3′.[33] Both primers had an 8-base tag added at the 5′ end, and different tags were used for each sample with a minimum difference of 3 bases from each other. The same tag was used for both the forward and reverse primers of each sample (thus making the elimination of inter-sample chimeras straightforward). To increase sequence diversity and facilitate Illumina base calling, a variable number of degenerate bases (N), ranging from two to four, were also added before the tags to the forward and reverse primers. PCR amplification conditions followed.[34] Amplification was then followed by purification and concentration using the MinElute PCR Purification Kit (Qiagen). Amplification success was checked using electrophoresis gels. Two PCR blanks were included by amplifying the PCR mix without any DNA template. Libraries were prepared with the BIOO NEXTFLEX PCR-Free DNA-Seq Kit (Perkin-Elmer) and sequenced in a partial Illumina NovaSeq lane using 2 × 250 bp paired-end sequencing at Novogene Company.

NIS database compilation

We compiled a database using NIS information from the Mediterranean Sea. We completed the existing database on European NIS by Lavrador et al.[66] using available literature reviews[17,84–87] and our own searches. We then searched for COI-5P sequences of the species not present in Lavrador et al.[66] in the Barcode of Life Data System (BOLD, https://www.boldsystems.org/, queried in February 2023). Whenever NIS could not be found in BOLD, we referred to the NCBI database and checked species identification in the taxonomic literature. For each NIS species, the obtained sequences were collapsed into unique haplotypes. To ensure data accuracy, a thorough manual curation was conducted, involving comprehensive BLAST searches and the removal of potentially erroneous sequences whenever the BLAST results indicated a different species or conflicting assignments. In total, out of the 777 listed species, 557 had COI sequences, providing a total of 6,008 validated sequences. Different tags such as taxID and process ID were also included to facilitate data tracking and integration into various databases.

Bioinformatic analyses

The bioinformatic analyses followed the MJOLNIR3 pipeline scheme (https://github.com/adriantich/MJOLNIR3) in the R 4.0.2. environment. MJOLNIR3 is based on the OBITools3[76] software toolkit and also calls other software at the diverse steps, as detailed below. In short, alignpairedend was used to align paired-end reads and keep only those with >40 alignment quality score. Reads were demultiplexed using ngsfilter. Those with mismatched primer tags at any end were discarded. Functions grep and uniq were used to perform a length filter (retaining only those between 310–319 bp) and dereplicate sequences within each sample. The Uchime *de novo* algorithm from VSEARCH v2.7.1 was

used to remove chimeric amplicons. For the mjolnir4_ODIN function from MJOLNIR3 the "DnoisE_SWARM" algorithm was used. Sequences were denoised with the DnoisE program,[78] which is a modification of the Unoise algorithm[88] that incorporates the natural variability in the three codon positions (https://github.com/adriantich/DnoisE). Denoising was performed within samples with an alpha parameter of 4 and an auto-computed entropy correction to generate Exact Sequence Variants (ESVs).[60] ESVs were then clustered into molecular operational taxonomic units (MOTUs) with SWARM v3.1.3 using d = 13[60,89,90]). SWARM is a threshold-free and fast algorithm that connects all reads with distance less than d in a first step, and then breaks down the resulting clusters using a topological criterion based on the internal abundance structures of the clusters.[79] We assigned taxonomically the representative sequences of the MOTUs with a generalist database containing sequences from the EMBL nucleotide database and sequences obtained from the Barcode of Life Database (BOLD). This database is available at GitHub (https://github.com/uit-metabarcoding/DUFA), and the last version (July 2021) comprises 175,045 sequences. This database is widely used in metabarcoding studies of aquatic ecosystems.[34,89,90,91]

Taxonomic assignment was performed using ecotag from Obitools v1.01 against the reference database and then higher rank taxonomy was added with mjolnir5_THOR function. Ecotag searches the best hit in the reference database and selects this and all sequences that are as similar to the best-hit as the query sequence is. The sequence is then assigned to the lowest common ancestor to all database sequences selected using the NCBI taxonomy tree. We performed a second ecotag against the NIS database generated in this work (see above), and selected those MOTUs assigned with a best-match similarity higher than 0.95 as confirmed NIS records. We then compared the assignment of these selected MOTUs with that obtained with the general reference database.

Even after the denoising procedures, undetected errors still remain,[60] including numts, tag switching and PCR errors, among others. Therefore, stringent cleaning is necessary to get rid of as many of them as possible. The final dataset refining consisted of five steps: 1. LULU filtering[92] using mjolnir7_LOKI, which combines similarity and co-occurrence metrics to detect erroneous MOTUs. 2. The deletion of all MOTUs that were not assigned to marine eukaryotes (i.e., MOTUs assigned to non-marine organisms, prokaryotes, or to the root of the Tree of Life). 3. The deletion of any MOTU for which reads in blank or negative controls represented more than 10% of total reads for that MOTU in all samples, as these are suspect to correspond to contaminations. 4. For each sample, we established a dual abundance filtering, setting to zero the reads of (i) MOTUs that represent less than 0.005% of the sample total reads, and (ii) MOTUs with less than 5 reads. 5. The deletion of MOTUs identified as numts following Turon et al. (2020) and Antich et al. (2023): we deleted all MOTUs with codon stops, checking the 12 mitochondrial genetic codes from the Biostrings R package,[93] and those MOTUs assigned to metazoans with changes in five conserved amino acids.

QUANTIFICATION AND STATISTICAL ANALYSIS

Community analyses

Most analyses were done with the 'vegan' v. 2.5-6 R package,[80] and plots were created with the 'ggplot2' R package.[81] Rarefaction curves and species accumulation curves were generated with functions *rarecurve* and *specaccum*, respectively.

MOTU richness and Shannon diversity values were computed for the two types of sampler. Prior to these analyses, samples were rarefied to the minimal number of reads in the samples (function *rarefy*). The resulting metrics were compared across categories with ANOVAs and post-hoc Tukey tests.

We determined the degree of MOTU and read overlap among the two sets of collectors with Venn diagrams. To assess the taxonomic composition of the samples, we grouped the MOTUs into the major eukaryotic super-groups following,[94] and metazoans were further sorted into phyla. Barplots were plotted with the composition (in terms of relative abundance of reads and of MOTUs) of each group.

For β-diversity analyses we used the relative read abundance of each MOTU in each sample without rarefaction, and computed the Bray-Curtis dissimilarity index (BC). These values were used to generate reduced-space representations of the samples in a non-metric multidimensional scaling (nmMDS) configuration using the *metaMDS* function. We also performed permutational analyses of variance (PERMANOVA) on the BC matrix with the PERMANOVA module of the Primer v6 statistical package.[82] Tests of multivariate dispersions (permdisp) were run for significant main factors to determine whether the outcome was a result of different multivariate means or different heterogeneity (spread) of the groups. The p-values obtained in pairwise comparisons were corrected using the Benjamini-Yekutieli False Discovery Rate (FDR) correction.[95] We also performed a Mantel test (function *mantel*) comparing the dissimilarity matrices obtained with pompoms and plates.

Finally, we performed an Indicator Species analysis using the IndVal index[96] with the R package 'indicspecies'.[97] This analysis allowed us to identify the MOTUs significantly associated with the two types of samples.