

Article

Environmental DNA (eDNA) for the Detection of Marine Vertebrate Diversity in Maltese Waters

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Abstract

Background/Objectives: Environmental DNA (eDNA) is increasingly recognised as a powerful molecular tool for biodiversity monitoring, enabling the detection of species through trace genetic material found in environmental samples. This study investigates the utility of eDNA analysis for identifying vertebrate marine species in the central Mediterranean, with a focus on taxa that serve as ecological indicators to local ecosystems. **Methods:** Seawater samples were collected from nine sites around the Maltese Islands between May and August 2021, at depths ranging from 2 to 5 m. Samples were filtered and DNA was extracted, amplified and sequenced. The resulting sequences were processed through a bioinformatics pipeline, clustered into molecular operational taxonomic units (MOTUs) and assigned taxonomic identities using reference databases. **Results:** This study led to the detection of 70 MOTUs, including ecologically important species such as the loggerhead turtle (*Caretta caretta*), the striped dolphin (*Stenella coeruleoalba*) and the bottlenose dolphin (*Tursiops truncatus*), underscoring the method's effectiveness in the detection of taxa of conservation value. Additionally, we detected a number of overlooked Blenniidae and Gobiidae taxa and deep-water or rarely encountered species such as the ocean sunfish (*Mola mola*), Cornish blackfish (*Schedophilus medusophagus*), Haifa grouper (*Hyporhamphus haifensis*) and Madeira lantern fish (*Ceratoscopelus maderensis*). eDNA of the invasive dusky spinefoot (*Siganus luridus*) and that of the lumpfish (*Cyclopterus lumpus*), a species not previously recorded in Maltese waters, was also detected during this study. The latter's detection highlights the potential of this methodology as an early detection tool for biological invasions. **Conclusions:** These findings support the integration of eDNA surveillance into marine biodiversity monitoring frameworks, particularly within marine protected areas to monitor native indicator taxa and assess the effectiveness of conservation measures, but also in ports and bunkering zones, where the risk of alien species introduction is elevated, with potential subsequent invasive species expansion that impacts native species and habitats.

Keywords: indicator species; alien species; metabarcoding; 12S rDNA; 16S rDNA; Mediterranean Sea



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1. Introduction

The vastness and complexity of marine ecosystems pose significant challenges for assessing and monitoring vertebrate biodiversity. Traditional survey methods such as visual observations, underwater videography and fisheries-dependent and fisheries-independent surveys are often resource intensive and are limited by spatial and temporal constraints [1–3]. These constraints leave knowledge gaps in the understanding of species

distributions, population dynamics and biogeographic patterns [4–7]. Such limitations are critical in biodiversity hotspots like the Mediterranean Sea, which faces growing anthropogenic pressures from climate change, overfishing and habitat degradation [8]. Consequently, there is an urgent need for efficient, non-invasive and scalable methods that deliver accurate, broad-scale biodiversity data that can bridge the gap in data from other sources, such as fishing activities, to improve understanding of species feeding relationships and reconstruct food webs, including species that are not typically considered in other surveying techniques [2,9]. Environmental DNA (eDNA) metabarcoding has demonstrated equal or greater effectiveness compared to traditional methods in capturing the diversity and composition of biological communities [3,5,10].

eDNA analysis has emerged as a transformative tool for biodiversity monitoring in aquatic environments [10,11]. This genetic material, coming from sources such as shed skin cells, mucus, scales, excretions and gametes released by organisms into their surroundings [12], is collected from water samples and analysed, allowing researchers to detect species presence without direct observation or capture [2,3,9]. This non-invasive method enhances the ability to monitor biodiversity with greater sensitivity, accuracy and efficiency [12] and can be used as a valuable tool for monitoring shifts in fish communities in response to natural annual cycles and environmental pressures [4,13].

eDNA offers several advantages over conventional methods, notably enhancing the detection of rare, cryptic, highly mobile or deep-sea species, as well as those that use habitats only transiently, taxa that are often underrepresented in traditional surveys. eDNA is also effective at identifying early life stages, such as planktonic larvae, which are often unrecognisable through traditional taxonomy [14,15]. Moreover, eDNA enables broader spatial coverage and higher sampling frequency, allowing for time series monitoring at a reduced cost [16]. It also minimises habitat disturbance and observer bias and, through metabarcoding, allows for the detection of multiple species from a single sample [2,17,18], while it may be applied to understand population structures of given species [4,6]. Works have also shown that eDNA provides valuable rapid biodiversity assessments in diverse marine habitats [4,13]. It also functions as an early warning system for non-indigenous species [3,19] and a non-intrusive method for monitoring threatened taxa [20,21] and fragile ecosystems [12]. eDNA is increasingly employed to detect non-indigenous species and monitor vulnerable ecosystems, with a growing role in fisheries management including assessments of stock presence, habitat use and distribution, providing essential data for conservation planning and ecosystem-based management [12,22,23].

Despite its promise, marine eDNA research faces several challenges [18]. One focus is improving quantitative interpretations that correlate eDNA signal strength with species abundance or biomass [23,24]. Understanding the effects of environmental variables on eDNA transport, degradation and persistence is also critical for reliable spatial and temporal inference [25]. The continued expansion of genetic reference libraries [26,27] and the standardisation of sampling, extraction and analytical protocols remain essential for enhancing accuracy and comparability [20,28,29].

This study aims to evaluate eDNA metabarcoding for assessing marine vertebrate diversity in Maltese coastal and offshore waters using mtDNA markers, as they typically last longer than nuclear markers [30]. Through targeted water sampling across representative habitats, we contribute to enhanced biodiversity monitoring and refined molecular methodologies for conservation planning. The findings will help advance eDNA as a robust tool for marine vertebrate assessment and long-term ecosystem monitoring.

2. Materials and Methods

Study Area and sample locations:

This study focuses on water samples collected from Maltese waters. The Maltese archipelago, located in the central Mediterranean, supports a high level of marine biodiversity, making it a critical area for marine conservation. Malta has established a 25 nautical mile Fisheries Management Zone (FMZ), covering approximately 11,500 km², over 35% of which is designated as marine Natura 2000 sites under the EU Habitats and Birds Directives, aimed at preserving ecologically important habitats and species [31]. Habitats include *Posidonia oceanica* meadows, coralligenous assemblages and deep-water environments that support a range of protected species, including highly migratory indicator taxa such as cetaceans, sea turtles and elasmobranchs [27,31]. Moreover, Malta's strategic position between the eastern and western Mediterranean basins subjects it to intense maritime traffic, increasing the likelihood of non-indigenous species introduction and range expansion [32–34].

Sampling was conducted between May and August 2021, aiming to capture eDNA diversity during the peak reproductive and gamete release season for most of the fish species in the Mediterranean [35]. During such periods eDNA concentrations are expected to be highest and biodiversity signals most detectable [30,36]. Nine locations, comprising seven open-water sites within Natura 2000 sites and two harbour sites, all situated within a maximum distance of 18 km from the coastline (Figure 1; Table 1), were selected. Each site was sampled once during the study period.

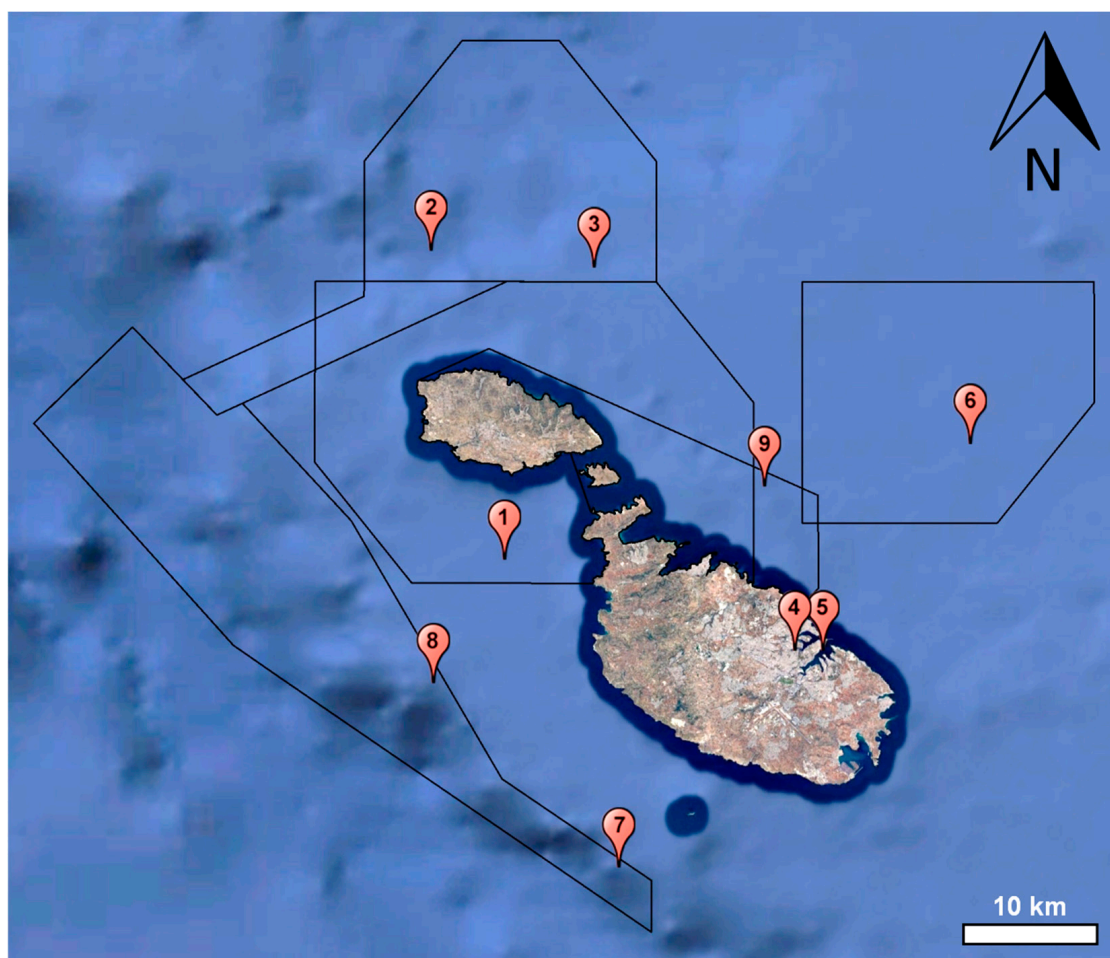


Figure 1. Map showing the nine sampling locations and the Natura 2000 sites associated with the open-water sampling sites [37,38].

Table 1. Details of locations sampled during this study.

Sample	Sampling Month	GPS	Description	Natura 2000 Site Code [37]
S1	May 2021	35.956; 14.256	6 km off-shore	MT0000112
S2	May 2021	36.161; 14.195	9 km off-shore	MT0000115
S3	June 2021	36.150; 14.329	10 km off-shore	MT0000115
S4	June 2021	35.896; 14.494	Marsamxett Harbour	–
S5	June 2021	35.896; 14.517	Grand Harbour	–
S6	June 2021	36.033; 14.638	18 km off-shore	MT0000107
S7	July 2021	35.752; 14.350	10 km off-shore	MT0000113
S8	July 2021	35.874; 14.198	12 km off-shore	MT0000113
S9	August 2021	36.005; 14.469	6 km off-shore	MT0000105

Sampling and molecular processing:

Three 5 L seawater samples were collected from a depth of 2 to 5 m at each sampling site. Samples were immediately cooled to 4 °C upon collection and transported to the laboratory. Each 5 L sample was independently filtered cold within 24 h of collection using 0.45 µm nitrocellulose filters. During each filtration event, a 5 L sample of deionised water was processed as a negative control. This control was prepared by placing deionised water in containers identical to those used for sampling and was processed in the same way as the other samples. Immediately after filtration, the membranes were folded, placed in 15 mL centrifuge tubes and stored at –80 °C until DNA extraction.

eDNA was extracted from two filters at each sampling site and from the respective negative control, while keeping the third filter as a backup. DNA extractions were carried out within one month of collection using the QIAamp Fast DNA Stool Mini Kit (Qiagen, MD, USA), following the manufacturer’s protocol. The initial step was modified to enable DNA extraction from nitrocellulose filters using 1.6 mL of buffer, which was added to each 15 mL centrifuge tube and briefly vortexed to release DNA from the filters. DNA was eluted in 200 µL of elution buffer and stored at –20 °C until use in PCR.

All eDNA extracts together with their respective negative controls were processed as described in this section. The first amplification was carried out using MarVer1 primers targeting approximately 202 bp of the 12S rDNA gene and MarVer3 primers targeting approximately 245 bp of the 16S rDNA gene [20]. PCR reactions were performed in a 12.5 µL reaction volume containing 3 µL of extract, 0.125 µL of each primer (0.5 µM each), 2.5 µL of 5X HOT FIREPol® Blend Master Mix (Solis BioDyne, Tartu, Estonia) (containing 2.5 mM Mg²⁺) and 6.25 µL of sterile dH₂O.

The temperature profile used consisted of an initial denaturation of 15 min at 95 °C, followed by 38 cycles of 30 s at 95 °C, 30 s at annealing temperatures 54/55/56 °C (10/10/18 cycles) for MarVer1 and 54/55/56/57 °C (8/10/10/10 cycles) for MarVer3, and a final extension step for 5 min at 72 °C.

A second PCR reaction was carried out using the respective MarVer1 and MarVer3 primers with a unique 5-bp MID tag, adapted from Shokralla et al. [39], and an Illumina overhang adaptor on both the forward and reverse primers. This second PCR was used to attach the MID tag and Illumina overhang adaptors to the amplicons generated in the first PCR. The second PCR reaction was performed in a 12.5 µL volume containing the same final concentrations of reagents as the first PCR, except that instead of genomic DNA, 1 µL of PCR product from the first reaction was used as the template. The thermal cycling profile was the same as for the first PCR but with fewer cycles (17 cycles) and a longer elongation time of 2 min per cycle.

The second PCR procedure was carried out three times to produce three replicates from each filter analysed. For each replicate, the only variable changed was the MID tag combination. Therefore, each sample and replicate had a unique tag during the second PCR. Using a different MID tag for each replicate helps to minimise potential tag bias and also allows for the identification and exclusion of tag jumps in downstream analyses [40]. A total of 2 µL of each second PCR product was visualised alongside a 100 bp DNA ladder (Solis BioDyne, Tartu, Estonia) on a 1.5% agarose gel stained with ethidium bromide to verify amplification success.

Amplicons from the second PCR were then cleaned using the GF-1 PCR Clean-up Kit (Vivantis, Selangor, Malaysia) following the manufacturer's instructions. The nucleic acid concentration of each PCR product was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, MA, USA). PCR products were then pooled in equimolar ratios. The template size of the PCR-enriched fragments was verified using a DNA 1000 chip on a 2100 Bioanalyzer (Agilent Technologies, CA, USA). Sequencing was performed on a MiSeq platform using 250 bp paired-end chemistry with an overall sequencing depth of 3.5 Gb.

Bioinformatics and data analyses:

The paired-end raw data were obtained and stripped of adaptor sequences, and raw reads from each library were initially processed separately using the AMPtk pipeline v1.4.2 [41]). The paired-end raw reads from each library were first demultiplexed using the forward and reverse MID tags, without allowing mismatches in the barcodes. Demultiplexed reads were then renamed to reflect the MID tag combinations. The MID tags, together with the MarVer1 and MarVer3 primer sequences, were subsequently trimmed and the paired reads were then merged using USEARCH v9.2.64 [42]. From the merged reads, only sequences matching the expected dual MID tag combinations were retained for further processing using the 'select' command. Reads identified for each sample were then denoised using UNOISE3 [43] with USEARCH v10.0.240 [42]. The UNOISE3 command implemented in the AMPtk pipeline first filtered reads based on quality by setting a maximum expected error of 1.0, then dereplicated reads were denoised with UNOISE3 to remove all putative chimeric and erroneous sequences. Denoised sequences were then clustered into MOTUs at 98% similarity [21] using the UCLUST algorithm [42]. Misaligned and MOTUs shorter than 85% of the expected amplicon length were also removed to produce the final MOTU list for taxonomic assignment. The resulting MOTUs were then filtered using the AMPtk 'filter' against the respective negative controls. Any MOTUs detected in the controls were removed from the final dataset per site. MOTUs that appeared in only one PCR per sampling site per locus were also omitted. Only those occurring in at least two PCRs from the same site and locus were retained for taxonomic assignment. For these retained MOTUs, read counts from multiple PCRs were pooled when presented in the results section.

MOTUs were taxonomically assigned at the species level using records from the NCBI GenBank database [44], with BLASTn searches [45,46] performed against taxonomic group information available as of January 2024. Taxonomic groups with fewer than 20 reads were also removed from the list. Scientific names were checked and any unaccepted names were updated to their accepted taxon name using the WoRMS database [47]. Trophic levels and habitat types of the fish species detected were obtained from www.fishBase.org (accessed on 30 August 2025) [48].

Pairwise Bray–Curtis dissimilarities between samples were calculated and visualised using non-metric multidimensional scaling (nMDS) plots in PRIMER 7 [35]. Two separate analyses were conducted. The first analysis compared read count data between sampling sites; however, actual count values may be biased. Therefore, a second analyses assessed

3. Results

Table 2. List of MOTUs detected per marker at each sampling location. Values represent the read count for each MOTU. Trophic levels, in brackets, for fish species were obtained from www.fishbase.se (accessed on 30 August 2025) [48].

[illegible]

Table 2. Cont.

Family	12S									16S								
Species (Trophic Level)	S1	S2	S3	S4	S5	S6	S7	S8	S9	S1	S2	S3	S4	S5	S6	S7	S8	S9
Mullidae																		
<i>Mullus barbatus</i> (3.1)										2375			1449	33			1431	
<i>Mullus surmuletus</i> (3.5)				436									21	1161				
Muraenidae																		
<i>Muraena helenae</i> (4.2)					1270									251				
Myctophidae																		
<i>Ceratoscopelus maderensis</i> (3.3)	219																	
Pomacentridae																		
<i>Chromis chromis</i> (3.8)				26	79					2218			16,381	17,119				
Scaridae																		
<i>Sparisoma cretense</i> (2.9)													75					
Scombridae																		
<i>Auxis rochei</i> (4.4)							699	2621										229
<i>Euthynnus alletteratus</i> (4.5)					574													
<i>Thunnus thynnus</i> (4.5)					831			866	1241									
<i>Thunnus</i> sp.													39	200				
Scorpaenidae																		
<i>Scorpaena notata</i> (3.7)													365	282				
Serranidae																		
<i>Serranus scriba</i> (3.8)				2535	1273	641							315	29				
Siganidae																		
<i>Siganus luridus</i> (2.0)				36	698													
Sparidae																		
<i>Boops boops</i> (2.8)	493			2784	931					1551			262	1001				
<i>Diplodus annularis</i> (3.6)					2180								521	47				
<i>Diplodus puntazzo</i> (3.2)														66				
<i>Diplodus vulgaris</i> (3.5)				1378	1421								2705	998				
<i>Lithognathus mormyrus</i> (3.5)														110				
<i>Oblada melanura</i> (3.4)	29	560	1087	16,672	3339	822						228	5825	42	1278			
<i>Pagellus bogaraveo</i> (4.2)				56	48								841	268				
<i>Sarpa salpa</i> (2.0)	70			5681	261				52				2415	20				
<i>Sparus aurata</i> (3.7)	52			13,744	2343				895	117			31,657	761				
<i>Spicara smaris</i> (3.0)														316				
Tripterygiidae																		
<i>Tripterygion tripteronotum</i> (3.4)													1033	1181				
Xiphiidae																		
<i>Xiphius gladius</i> (4.5)								197					230					
Total reads	4569	757	2321	65,278	45,806	4307	1132	5399	3367	14,623	508	2348	103,552	55,660	4403	479	2185	695
MOTUs	8	2	3	18	23	6	2	5	6	8	1	3	35	38	6	3	3	2
Scale:	10	100	1000	10,000	100,000													

In total, 31 families were detected (24 for 12S and 26 for 16S), including two species of cetaceans, one species of sea turtles, two species of elasmobranchs and a number of bony fish species. The latter accounted for 98.3% of the total reads (Table 2). Overall, the combined data show that 27 MOTUs appear in both 12S and 16S datasets, while the rest were detected in one of the datasets only.

For the 12S dataset (MarVer1), 97.5% of the reads were assigned to bony fish. The remaining reads included *Stenella coeruleoalba* (835 reads), *Caretta caretta* (853 reads), *Raja clavata* (620 reads) and *Torpedo marmorata* (985 reads). In total, 39 MOTUs were detected, of which 35 corresponded to bony fish distributed across 21 families. The most represented family was Sparidae, with seven MOTUs across 54,898 reads (41.3%), predominantly originating from samples collected in harbour areas. 12S data from harbour samples also revealed the presence of the non-indigenous lumpfish, *Cyclopterus lumpus*, representing a previously unrecorded species for the Maltese Islands [49] and the invasive dusky spinefoot, *Siganus luridus*, an invasive alien species in the region [33]. Open-water samples yielded indicator species such as *Stenella coeruleoalba* and *Caretta caretta*, together with benthic taxa including *Raja clavata* and *Torpedo marmorata*, as well as deep-water species like *Ceratoscopelus maderensis* and *Hyporthodus haifensis*, along with commercially important pelagic taxa such as *Thunnus thynnus* and *Auxis rochei* and the coastal pelagic species *Engraulis encrasicolus* (Table 2).

For the 16S dataset (MarVer3), 98.8% of the reads were attributed to bony fish species. The remaining reads were assigned to *Tursiops truncatus* (32 reads), *Caretta caretta* (189 reads) and *Raja clavata* (2067 reads). In total, the 16S data led to the identification of 58 MOTUs, including 55 MOTUs corresponding to bony fish across 23 different families. The most represented families were Sparidae (10 MOTUs; 51,029 reads) and Gobiidae (12 MOTUs;

7885 reads). Similar to the 12S results, most detections originated from harbour samples. Samples from open waters revealed the presence of pelagic species such as the rarely recorded *Schedophilus medusophagus*, *Mola mola* and the deep-water grouper *Hyporthodus haifensis* (Table 2) [50–52].

From the fish taxa detected and resolved to species level, 10 (17.5%) were at trophic level 2. The majority, 36 taxa (63.2%), were at trophic level 3, while 11 species (19.3%) were at trophic level 4 and were therefore considered top predators (Table 2; Figure 2). The latter included species from Carangidae, Epinephelinae, Scombridae and Xiphiidae, representing species that are both ecologically and commercially important. The majority of the fish species detected occur in benthic habitats, including demersal (40.3%), benthopelagic (12.3%) and reef-associated (19.3%) species, which together accounted for 71.9%. The rest were more pelagic in nature, including bathypelagic (1.8%), pelagic-neritic (15.8%) and pelagic-oceanic (10.5%) species (Figure 2).

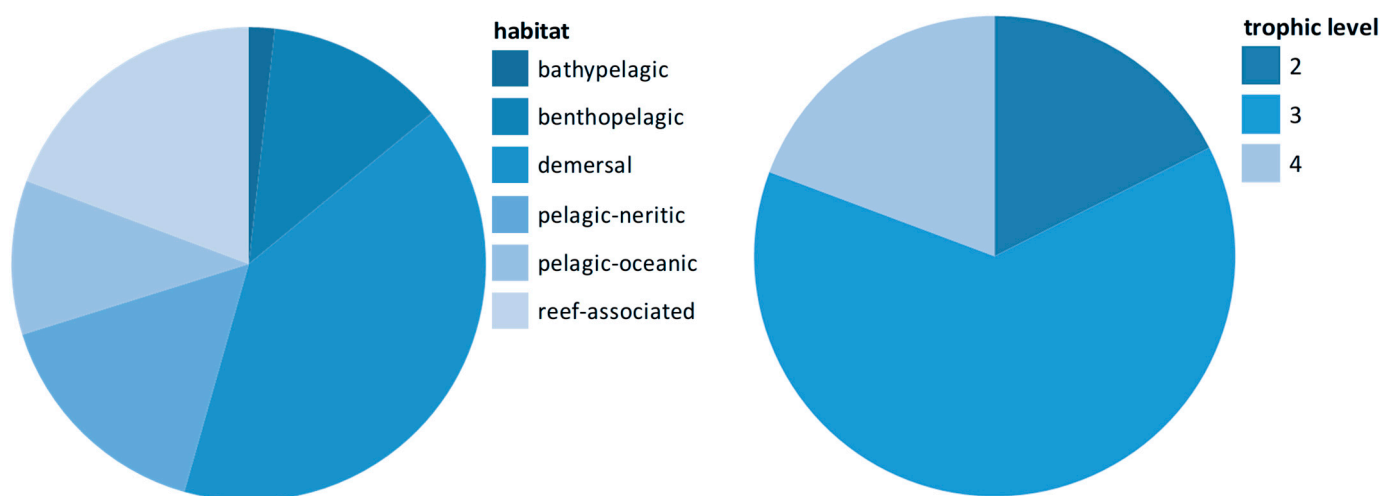


Figure 2. A summary of the proportions of fish species per habitat type (left) and trophic level (right) for the MOTUs identified down to species level. Habitat types and trophic levels were assigned based on www.fishbase.se (accessed on 30 August 2025) [48].

A comparison of eDNA assemblages at each sampling site, using pooled data from both markers, was visualised with three-dimensional nMDS plots (Figure 3). When counts were used as the variable, there was clear separation between all sampling sites except the harbour samples (S4 and S5). However, given the caveats associated with count data, the comparison was repeated using presence/absence. In this case, S4 and S5 again clustered closely, while S7 grouped with S8, and S2 with S3. These similarities may reflect the spatial and temporal proximity of the sampling.

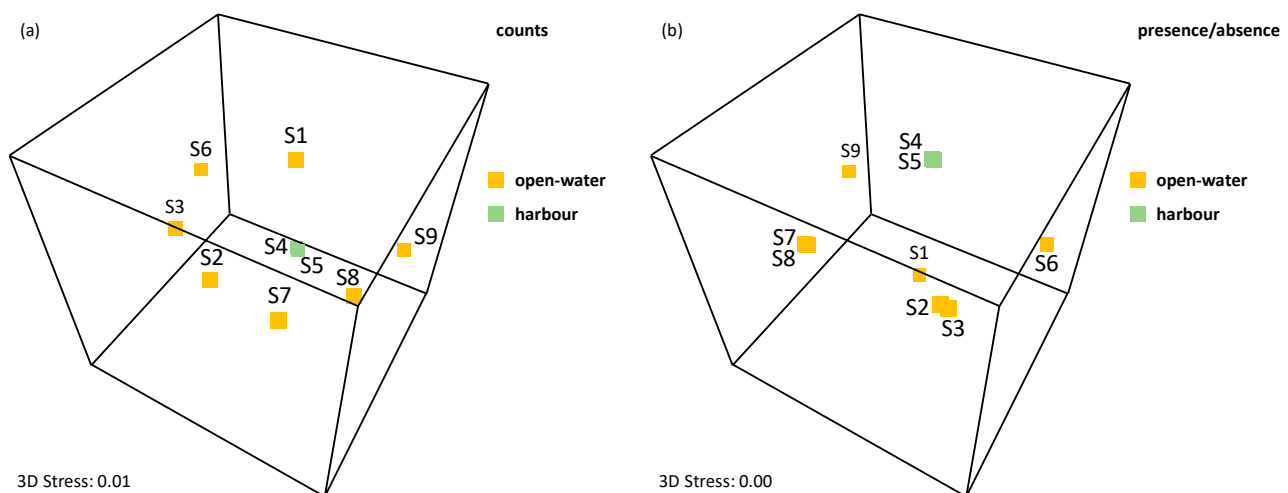


Figure 3. Three-dimensional nMDS ordinations of assemblage comparisons between the various sampling sites: (a) using read counts of MOTUs; (b) using presence/absence of MOTUs.

4. Discussion

Our results highlight the effectiveness of the MarVer1 and MarVer3 primer sets [20] in detecting vertebrate biodiversity through eDNA analysis. The use of a multi-marker approach significantly improved the taxonomic resolution and provided a comprehensive snapshot of the vertebrate communities across varied marine habitats. The complementary nature of the multi-markers enhances species level assignments [21,53–55], particularly in ecologically complex environments, where diverse taxa from different niches coexist in a confined space. All open-water sampling sites were located within marine Natura 2000 sites (Figure 1), adding further conservation relevance to our findings by demonstrating the utility of eDNA monitoring within protected areas [56]. As anticipated, in our study we observed reads from benthic and coastal taxa, reflecting the localised nature of our sampling, together with a few taxa from migratory or deeper water species.

The MarVer primers detected broad taxonomic diversity, with particularly high reads in harbours. In these areas, filtration was noticeably slower, likely due to elevated particulate loads, which may have enhanced eDNA retention. Additionally, harbours are known to exhibit higher eDNA concentrations, resulting from restricted water circulation and limited dispersal [57]. As expected, in these harbour samples we observed reads from benthic and coastal taxa, consistent with other harbour samples from the Mediterranean [57]. In our harbour samples, we also detected eDNA from large migratory pelagic species such as *Euthynnus alletteratus*, *Thunnus thynnus* and *Xiphias gladius*, which are typically associated with open-water habitats. Their presence in harbour environments suggests that these areas may serve as sheltered habitats for the early life stages of these species, including prelarval, larval or juvenile phases.

In the samples from Marsamxett Harbour, we also detected eDNA of the alien Lumpfish, *Cyclopterus lumpus*, a species not previously recorded in Malta. If confirmed through a specimen, this would represent the third record of the species in the Mediterranean Sea and a new record for Malta [49,58,59]. The eDNA, or possibly individuals of this species, likely reached this local harbour via shipping activity [58]. We also detected the presence of the invasive alien *Siganus luridus* in both harbours. This finding is consistent with observations of the species' range expansion and growing population in Maltese waters [33]. Concerns about the expanding range and impacts from the large population size of *Siganus luridus* have increased due to the negative impact of this species' grazing activity on algal biomass [60]. The detection of invasive species promotes the utility of such techniques in monitoring alien taxa. Continued monitoring of non-indigenous species using eDNA tools

may provide an effective early warning system and contribute to marine biosecurity and mitigation efforts [57,61,62].

In open-water samples, where eDNA may persist longer due to slower degradation rates [63], we detected pelagic species, including cetaceans and sea turtles, which are often regarded as indicators of ecosystem health [64,65]. Additionally, we detected deep-sea species such as *Ceratoscopelus maderensis* and *Hyporthodus haifensis*. The presence of these taxa may be attributed to the release of eDNA from pelagic prelarval or larval stages or through vertical transport processes that carry biological material from deeper layers to the surface. The effect of vertical transport appears particularly evident in the detection of species lacking a pelagic life stage, such as *Raja clavata* and *Torpedo marmorata*. The presence of their eDNA in surface waters underscores the role of dynamic oceanographic processes leading to vertical mixing, redistributing eDNA throughout the water column [66,67]. These mechanisms allow for the detection of demersal and deep-water species in surface samples, even when the organisms themselves remain near or on the seabed. Additionally, it cannot be excluded that eDNA from benthic organisms may reach surface waters during fishing activities [68] using gear such as trawls, trammel nets or demersal longlines, especially through the discarding of bycatch. The coastal habitats analysed in this study are frequented by both professional and recreational fishermen, especially during the summer months.

Our findings were consistent with those of Valsecchi et al. [21], who using the same primer sets demonstrated that eDNA sampling can effectively capture marine vertebrate diversity, with low detection read counts for marine mammals. Additionally, our study revealed enhanced detection of several taxa from families such as Gobiidae, Blenniidae and Sparidae, which are often underrepresented in traditional surveys. Similar outcomes have been reported in other harbour-based eDNA studies across the Mediterranean, where the most diverse families detected were Sparidae and Gobiidae, respectively [57]. Rarely recorded taxa such as *Mola mola* and *Schedophilus medusophagus* were detected [50,51], the latter of which was confirmed in Maltese waters through a specimen collection in 2023 [50]. This highlights the capacity of the MarVer primers to resolve species diversity in structurally complex environments, such as harbours, seagrass beds and rocky reefs, even for species that are typically overlooked or previously unrecorded in the area.

The detection of cetacean MOTUs in our study was slightly lower than that reported by Valsecchi et al. [21]. This discrepancy likely reflects differences in thresholds, with Valsecchi et al. [21] including MOTUs tables with read counts as low as 10. Additionally, differences in sampling methodology may also explain the variation, as they used underway sampling guided by visual cetacean sightings along extended transects, which allows a higher chance of eDNA collection compared to our fixed-point sampling, independent of visual cues.

Several fish species, such as *Oblada melanura* and *Sparus aurata*, produced relatively high read counts (9.4% and 15.6% of the total reads, respectively). This may be indicative of more frequent eDNA shedding, leading to greater detectability in the water column [23,69], though such quantification must be interpreted with caution as the link between the number of reads and actual species biomass may not always be correlated, due to potential bias within the experimental procedure itself [66,70]. *Oblada melanura* was detected in six of the nine sampling locations during a period that coincides with the time when these fish exhibit their highest gonadosomatic index or shortly thereafter [71,72], when the presence of gametes and larvae may be at its peak, thus increasing the eDNA after spawning events [30,36,73]. The occurrence of *Sparus aurata*, *Dicentrarchus labrax* and *Thunnus thynnus* may also be partially attributed to eDNA shed from coastal fish farms and tuna ranches [74] that are present mostly along the north-eastern coast and offshore Maltese waters. These anthropogenic activities contribute eDNA from a few taxa, which coincidentally have been

recorded with high counts, possibly resulting from elevated eDNA concentrations that could mask or skew natural biodiversity patterns using this procedure [75].

This molecular approach effectively detected both common, overlooked and rare taxa. Notably, the open-water sampling sites overlapped with marine Natura 2000 zones, adding further conservation relevance to this work by providing data on some species present in the areas, while highlighting the utility of eDNA monitoring for conservation purposes [56]. Our results reinforce the value of eDNA as a complementary tool to traditional monitoring with the potential of enhancing biodiversity assessments, supporting early detection of invasive species and contribute to ongoing conservation and management efforts in both harbour and Natura 2000 sites. This is particularly relevant given the growing need for improved monitoring tools, as current monitoring strategies are often insufficient to efficiently meet conservation goals. In this respect, an eDNA metabarcoding based approach can help bridge this gap by enhancing timely species detection [56,61,76].

While our analyses provide insight into community composition across sampling sites, some aspects should be considered for future work. Rare species may have been underestimated due to low eDNA concentrations, potentially biasing diversity estimates. Increasing sampling effort may help improve detection of these rare taxa [13]. Sampling was also restricted to surface waters, which means that any vertical stratification of eDNA may have been missed. Future studies could benefit from depth-stratified sampling to better capture the full spatial distribution of taxa, especially for deep-sea taxa [66,77]. Additionally, incorporating seasonal sampling would provide a more comprehensive understanding of temporal variability [13] in community composition, especially for taxa that are migratory or spawn during months not covered by the current study.

5. Conclusions

This study demonstrates that an eDNA-based approach represents an effective early warning system for the detection of non-indigenous species, providing greater sensitivity and cost efficiency compared to conventional methods, especially for rare species such as the alien *Cyclopterus lumpus* or the invasive *Siganus luridus*, whose eDNA was recorded in this study. Our findings further highlight that molecular tools can facilitate rapid biodiversity assessment and support research on indicator and conservation flagship species, including marine mammals and sea turtles, as well as typically overlooked taxa such as members of the families Blennidae and Gobiidae, which nonetheless contribute to ecosystem health. Future applications may benefit from increased spatio-temporal sampling to better capture patterns in the dynamics of eDNA presence in marine systems.

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