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ORIGINAL ARTICLE

Primer set evaluation and sampling method assessment for the monitoring of fish communities in the North-western part of the Mediterranean Sea through eDNA metabarcoding

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Abstract

Environmental DNA (eDNA) metabarcoding appears to be a promising tool to survey fish communities. However, the effectiveness of this method relies on primer set performance and on a robust sampling strategy. While some studies have evaluated the efficiency of several primers for fish detection, it has not yet been assessed *in situ* for the Mediterranean Sea. In addition, mainly surface waters were sampled and no filter porosity testing was performed. In this pilot study, our aim was to evaluate the ability of six primer sets, targeting 12S rRNA (AcMDB07; MiFish; Tele04) or 16S rRNA (Fish16S; Fish16SFD; Vert16S) loci, to detect fish species in the Mediterranean Sea using a metabarcoding approach. We also assessed the influence of sampling depth and filter pore size (0.45 μm versus 5 μm filters). To achieve this, we developed a novel sampling strategy allowing simultaneous surface and bottom on-site filtration of large water volumes along the same transect. We found that 16S rRNA primer sets enabled more fish taxa to be detected across each taxonomic level. The best combination was Fish16S/Vert16S/AcMDB07, which recovered 95% of the 97 fish species detected in our study. There were highly significant differences in species composition between surface and bottom samples. Filters of 0.45 μm led to the detection of significantly more fish species. Therefore, to maximize fish detection in the studied area, we recommend to filter both surface and bottom waters through 0.45 μm filters and to use a combination of these three primer sets.

KEYWORDS
biodiversity assessment, eDNA metabarcoding, fish monitoring, North-western Mediterranean Sea, PCR primer sets, sampling strategy

1 | INTRODUCTION

Fish account for more than half of the extant vertebrate diversity with over 35,000 species described to date (www.fishbase.org; accessed in June 2023). As part of the food web, marine fish communities support crucial ecological functions (Almany & Webster, 2004; Depczynski et al., 2007). For example, through predation on grazing species such as sea urchins, fish populations help to prevent

cascading effects that would lead to the destruction of algae forests (Tegner, 2000). Fish stocks also contribute to the economy through fishing activities, which serve as a major source of income for small island countries (Charlton et al., 2016; Guillotreau et al., 2012). In addition, more than 4.5 billion people rely on fish as food to meet their protein needs (Béné et al., 2015; Loring et al., 2019). However, human activities, including overfishing, habitat destruction, pollution, introduction of non-indigenous species, and climate change, are having a significant impact on fish communities and the functions they perform (Foo et al., 2021; Graham et al., 2006; Sumaila & Tai, 2020). This highlights the fundamental need for effective conservation of marine ecosystems, which relies on the description and biomonitoring of fish communities (Nicholson & Jennings, 2004).

Efficient monitoring is essential to assess the effects of anthropogenic pressures on fish assemblages and ensure their long-term preservation. Marine fishes have been traditionally monitored using survey methods such as Underwater Visual Census (UVC), video techniques or experimental fishing (e.g., Andradi-Brown et al., 2016; Stobart et al., 2009; Thanopoulou et al., 2018). However, these techniques are time-consuming, require taxonomic expertise, can be destructive (i.e., experimental fishing) and often fail to detect cryptobenthic and rare species (Afzali et al., 2021; Boussarie et al., 2018; Pais & Cabral, 2017).

In recent years, environmental DNA (eDNA) has emerged as a promising tool for surveying fish communities (Pawlowski et al., 2021; Thomsen et al., 2012; West et al., 2020). This non-invasive technique offers a high sensitivity that allows rare, cryptic, and non-indigenous species to be detected (Stat et al., 2019; Thomsen & Willerslev, 2015). The method relies on the analysis of DNA traces released by organisms and extracted from environmental samples (i.e., water and sediment) without capturing target species (Bohmann et al., 2014). At first, the technique was dedicated to single species detection with quantitative Polymerase Chain Reaction (qPCR) (Ficetola et al., 2008). More recently, metabarcoding (i.e., DNA amplification, high throughput sequencing and detection of several taxa at the same time using universal PCR primers), has become an increasingly popular tool to monitor communities including fishes (Sawaya et al., 2019; Shaw et al., 2016).

Since eDNA metabarcoding is a rapidly evolving method, various aspects and emerging applications of this technique require evaluation (Goldberg et al., 2016). One crucial issue is the choice of PCR primer sets (Zhang et al., 2020). The efficiency and accuracy of the metabarcoding technique are primer-dependent, as highlighted by the variability in species detected when different primer sets are used (Bylemans et al., 2018; Shu et al., 2021). The main characteristics of an optimal primer set include: a short barcode size (i.e., <200 bp) to optimize the detection of highly degraded DNA from environmental samples; a comprehensive coverage and specificity for the taxonomic group of interest; a high taxonomic resolving power to enable accurate species identification; and a good completeness and accuracy of reference databases to allow matches with DNA sequences (Clarke et al., 2017; Freeland, 2017). It should also be noted that barcode size is important because it can determine compatibility with short-read sequencing platforms. Given that no single primer set fulfills all these criteria, some researchers have suggested conducting multiprimer surveys to allow the detection of a wider range of fish species (Evans et al., 2017; Kumar et al., 2022; Stauffer et al., 2021). At the time of writing (May 2023), there were over 20 primer sets designed for fish detection. These primer sets target several loci, such as mitochondrial cytochrome B (cytb), cytochrome oxidase subunit I (COI), 12S rRNA and 16S rRNA.

A growing literature aims to evaluate the efficiency of these primer sets for the assessment of fish assemblages (e.g., Bylemans et al., 2018; Shu et al., 2021; Valentini et al., 2016; Zhang et al., 2020). However, these studies have either been conducted *in silico* (e.g., Valentini et al., 2016) or, for those involving *in vitro* metabarcoding analysis, they have primarily focused on freshwater ecosystems (e.g., Bylemans et al., 2018; Shu et al., 2021; Zhang et al., 2020), with the exception of Kumar et al. (2022). Furthermore, primer set efficiency and species detection success depend on community composition and complexity, which vary between regions (Zhang et al., 2020). Therefore, it is crucial to evaluate primer set performance in the survey area (i.e., the North-western part of the Mediterranean Sea in our case) before conducting an eDNA census.

Other aspects of the eDNA metabarcoding method, such as the sampling strategy, still need to be improved to ensure its efficiency and reliability for fish surveys. Currently, water sampling is commonly conducted using bottles with a small volume per sample. However, some studies have highlighted the need for filtration of a larger quantity of water to improve fish detection (Kawakami et al., 2023; Stauffer et al., 2021). Furthermore, water is mainly collected from the surface (e.g., Boulanger et al., 2021; Polanco Fernández et al., 2021) whereas many fish species live close to the substrate in coastal areas (i.e., demersal species) (Fredj & Maurin, 1987). Therefore, further investigation is needed to assess if the standard sampling effort is sufficient to obtain an accurate representation of fish assemblages or if bottom samples (i.e., water samples collected close to the seafloor) with large filtration volumes should also be considered.

Another important aspect of the filtration strategy is the filter itself. Enclosed filters, combined with a pump, allow immediate on-site filtration of large water volumes (Lopes et al., 2017). This method eliminates the need for water storage and filtration occurs in an enclosed environment reducing the risk of DNA degradation and contamination (Spens et al., 2017). In addition, these filter capsules are less prone to clogging than open filters as they possess a larger filtering surface area (Coutant et al., 2021; Peixoto et al., 2021). Enclosed filters are available in a wide range of porosities from 0.2 µm to 20 µm (Bowers et al., 2021). Filters with higher porosity can filter a larger amount of water as they are less sensitive to clogging. However, they may be less effective in capturing small DNA molecules (Coutant et al., 2021), potentially impacting species detection ability (Li et al., 2018). Therefore, the influence of filter porosity should be tested before conducting eDNA surveys to select the optimal porosity depending on the amount of water filtered and the turbidity of the sampling zone. In view of the above ideas,

setting up a pilot study becomes essential before implementing an eDNA survey.

Here, we present the results of a pilot metabarcoding study conducted in the North-western part of the Mediterranean Sea. Our objective was to determine the best strategy to monitor fish communities in this area using an eDNA approach. We evaluated the ability of six different primer sets to detect fish species. These primer sets targeted the 12S rRNA and 16S rRNA loci and were selected from a literature search. We also developed and assessed the performance of a novel sampling strategy combining simultaneous surface and bottom sampling along the same transect using enclosed filters connected to pumps. Finally, we tested the impact of filter porosity by using two different mesh sizes. Our goals were: (1) to select the optimal primer set combination for our study area; (2) to assess the benefits of combining surface and bottom live sampling and (3) to determine the best filter porosity in order to (4) develop an effective eDNA metabarcoding strategy to survey fish communities in the North-western part of the Mediterranean Sea. The findings from this pilot study will serve as a reference for future fish censuses in the region.

2 | MATERIALS AND METHODS

2.1 | Primer selection and in vitro qPCR

Several studies (e.g., Bylemans et al., 2018; Collins et al., 2019; Kumar et al., 2022; Shu et al., 2021; Zhang et al., 2020) have conducted *in silico* and *in vitro* analyses to evaluate the ability of many metabarcoding primer sets (up to 22 primers for Zhang et al., 2020) to detect fish species. Based on our literature search, we selected six primer sets that were the best performing *in silico* and *in vitro* for the studied areas. Because their *in silico* evaluation was already well documented in the literature, our focus in this paper was solely on an *in vitro* assessment of these primer sets within the North-western part of the Mediterranean Sea. Of the six primer sets selected, three were designed to target the 16S rRNA locus (Fish16S, Fish16SFD and Vert16S), and three targeted the 12S rRNA locus (Tele04, MiFish and AcMDB07) (see Table 1 for references and information on the primer sets used). Each set was designed for Actinopterygian fish detection except for Vert16S, which targets a wider range of species since it is vertebrate specific. We selected this primer set for our pilot study because we were interested in detecting Chondrichthyan species as well.

Since the Tele04 primer set has not been yet published, we performed real-time quantitative Polymerase Chain Reaction (qPCR) on fish DNA extracts to ensure that this primer set was able to amplify fish DNA. For this purpose, we collected fresh tissue samples from 32 Mediterranean fish species (Table S1) by performing caudal fin clip. Fish tissue was stored in 95% ethanol before extraction. DNA was extracted using the following protocol: fins were blotted using absorbent paper to remove excess ethanol, left under a hood overnight to allow complete ethanol evaporation and subsequently cut into small

TABLE 1 References and information on the six primer sets evaluated in the study.

	AcMDB07	MiFish	Tele04	Fish16S	Fish16SFD	Vert16S
Reference	Bylemans et al. (2018)	Miya et al. (2015)	Taberlet et al., not yet published	Shaw et al. (2016)	DiBattista et al. (2017)	Vences et al. (2016)
Target gene	12S rRNA	12S rRNA	12S rRNA	16S rRNA	16S rRNA	16S rRNA
Forward primer	GCCTATATACCGCCGTCG	GTCGGTAAAACTCGTGCCAGC	GTGCCAGCCACCGCGGTT	CGAGAAGACCCTWTGG AGCTTIAG	GACCCTATGGAGCTT TAGAC	AGACGAGAAGACCCYdT GGAGCTT
Reverse primer	GTACACTTACCATGTTA CGACTT	CATAGTGGGGTATCTAATCCC AGTTTG	GTGGGGTATCTAATCCC AGTTTG	GGTCGCCCCAACCRAAG	CGCTGTTATCCCTADR GTAACT	GATCCAACATCGAGGT CGTAA
Annealing T°C	55	61	63	55	54	65
Amplicon length (without primers)	255–644 bp	133–595 bp	123–297 bp	12–94 bp	23–260 bp	84–304 bp
Illumina MiSeq Run	2×250 bp	2×150 bp	2×150 bp	2×150 bp	2×250 bp	2×250 bp

pieces for extraction using the DNeasy Blood and Tissue kit (Qiagen) following the instructions provided by the manufacturer. DNA was quantified using a spectrophotometer (DS 11 FX, Denovix). qPCR reactions were prepared for each primer pair by placing in each well, 5 μL of extracted DNA (2 ng/μL) and 10 μL of a mix containing: 7.5 μL of Power Sybr Green PCR Master Mix (Applied Biosystems), 1 μL of forward primer (10 μM), 1 μL of reverse primer (10 μM), and 0.5 μL of PCR clean water. Samples were amplified using a StepOnePlus thermal cycler (Applied Biosystems). Thermal cycling consisted in an initial holding stage of 95°C for 20 s followed by 40 PCR cycles of 3 s at 95°C and 30 s at 60°C. Finally, the melt curve stage was carried out at 95°C for 15 sec and 60°C for 1 min. The qPCR results were visualized with the StepOne Software v2.3 (Applied Biosystems) and on a 2% agarose gel imaged by a gel imager (Universal Hood II Gel Doc System, Biorad; software: Quantity One, v4.6.5).

2.2 | Metabarcoding analysis: Water sampling in Cap Roux MPA and at the Monaco Oceanographic Museum

This study was conducted over a two-day period (03/05/2022–04/05/2022) in the Marine Protected Area (MPA) of Cap Roux, located in the North-western part of the Mediterranean Sea, between Cannes and Saint-Raphael, France (Figure 1). Established in 2003, it covers an area of 450 ha spanning from the shoreline to the 100 m isobath. All types of fishing are prohibited but permanent surveillance is lacking. The habitats within the MPA consist of typical ecosystems of the Mediterranean Sea such as *Posidonia oceanica* meadows, rocky shores, and coralligenous reefs. We chose this study area due to its high species richness, making it an ideal sampling location to test our metabarcoding strategy.

For this study, we developed a novel sampling strategy that allowed the simultaneous filtration of surface and bottom water. Two transects of approximately 1.3 km in length were designed that crossed several habitats within the MPA, to allow a broad range of species to be detected (Figure 1). Two samples were collected simultaneously along each of these transects. The first sample consisted of 30 L of surface water filtered one meter below the surface with a diaphragm pump (Argaly; flow: 1.0 L/min) attached to a boat (Figure 2). The second sample consisted of 30 L of bottom water filtered one meter above the substrate with a custom-made waterproof pump (flow: 1.0 L/min) fixed on a Diver Propulsion Vehicle (DPV) driven by a scuba diver (Figure 2). Both pumps were started at the same time. A second diver carried a buoy, which enabled the boat to closely follow the divers and adapt its speed to their pace (Figure 2), to ensure the surface and bottom pumps simultaneously pumped water along the same transect. The scuba divers navigated effectively underwater by identifying the capes and determining the appropriate time to spend in each habitat. Each transect was duplicated, resulting in a total of eight samples (Table S2). Both pumps (i.e., surface and bottom) were connected to a filtration capsule (eDNA water filter, Waterra; 600 cm^2; Polyethersulfone), allowing immediate filtration of large water volumes (Figure 2). Two pore sizes, 0.45 μm and 5 μm, were tested. Since field sampling occurred during the plankton bloom period, we were expecting potential filter clogging. Thus, we wanted to try water filtration through a large mesh size (i.e., 5 μm) to assess if this porosity would perform better than conventional 0.45 μm filters in our area.

After the filtration step, 50 mL of Longmire buffer solution (Longmire et al., 1997) was directly injected into each capsule, which was then shaken by hand. This solution enables effective long-term conservation of eDNA samples (Renshaw et al., 2015; Wegleitner et al., 2015). eDNA capsules were always handled with gloves to avoid contamination. Upon returning to the laboratory, the capsules were vigorously agitated again for 1 minute. The 50 mL extract was finally stored at room temperature in the dark until DNA extraction.

In addition, we collected two water samples in the aquarium of the Monaco Oceanographic Museum (MOM) (Table S2). These samples were used to evaluate the effectiveness of our metabarcoding strategy by comparing the number of detected species with the known list of fish species in the aquarium tanks. Moreover, since fish composition is different between the aquarium and field samples (i.e., some species absent from the field samples might be found in the aquarium), it gave us more information on the taxonomic coverage of each primer set. For each capsule, 30 L of water was filtered from the surface across three different tanks containing only Mediterranean fish species (Table S3). One sample was collected with a 0.45 μm pore size capsule and the other with a 5 μm capsule. Following the filtration step, the capsules were treated in the same manner as the Cap Roux field samples.

2.3 | Metabarcoding analysis: Extraction, PCR, and sequencing

All the metabarcoding laboratory steps were performed by Argaly (Sainte-Hélène-du-Lac, France), using the following protocol: DNA extraction from the 10 samples was carried out in a laboratory dedicated to handling eDNA water samples following the NucleoSpin Soil kit protocol (Macherey Nagel) with the following modifications: the 50 mL falcon tubes were centrifuged for 1 h at 12,000*g*. The pellets were then resuspended in ATL buffer and proteinase K, and placed for 2 h at 56°C to lyse cells and cell debris. The extraction procedure was continued according to the manufacturer's protocol and the resulting DNA extracts were eluted in a final volume of 100 μL of elution buffer.

Subsequently, DNA from each sample was amplified in 12 replicates for each primer set. Each PCR replicate was uniquely identified by a combination of two eight-base tags appended to the PCR primer

FIGURE 1 Map showing the eDNA transects within the Cap Roux MPA (Mediterranean Sea, Saint-Raphael, France). The lines indicate the four transects for water filtration, each ~1.3 km in length. For each transect, two water samples were filtered, one from the surface, and one above the substrate (The map was generated using Qgis software v3.14.12-Madeira; Background: Donia Expert, Medtrix; eDNA transects: Navionics v19.0.2).

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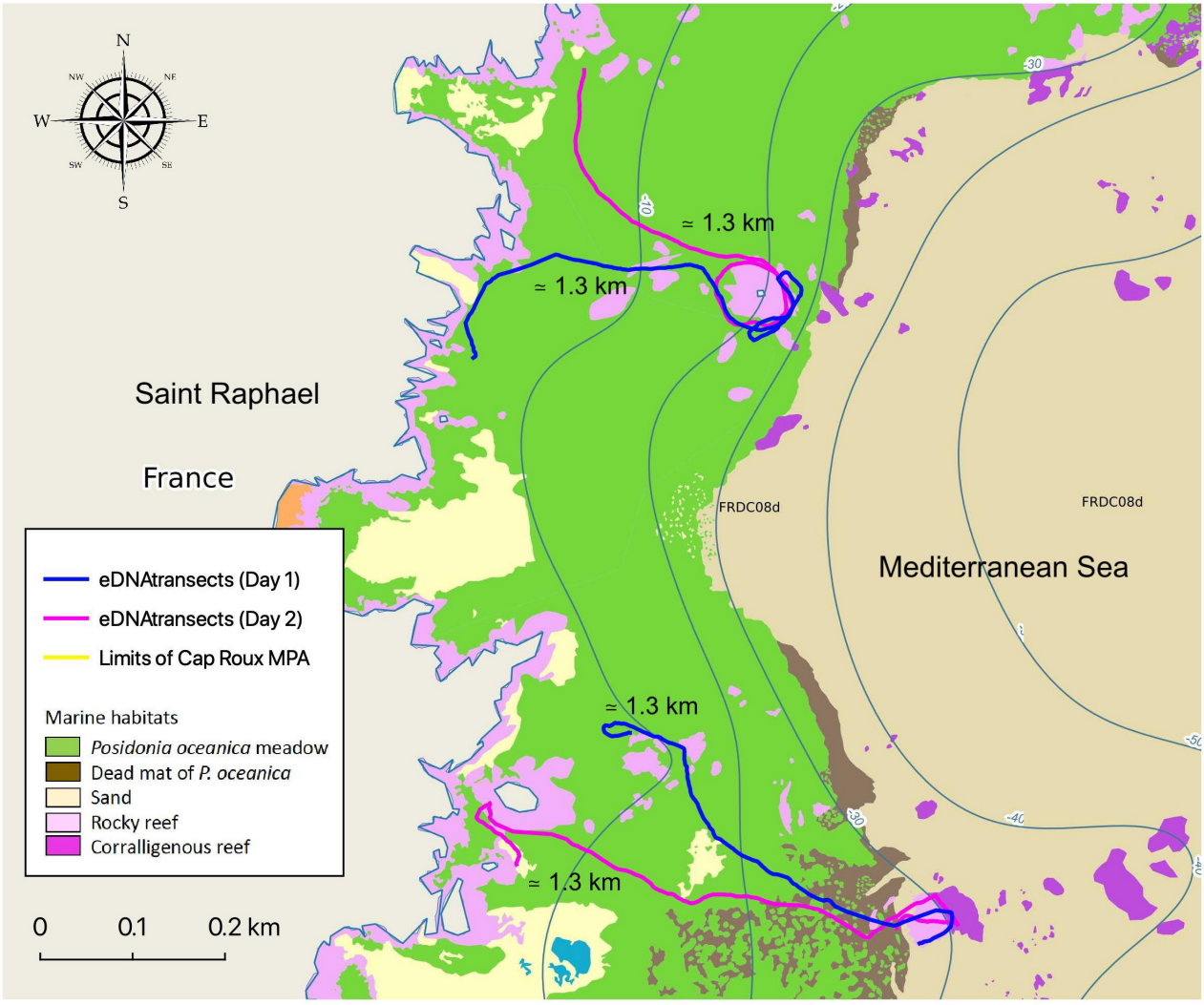

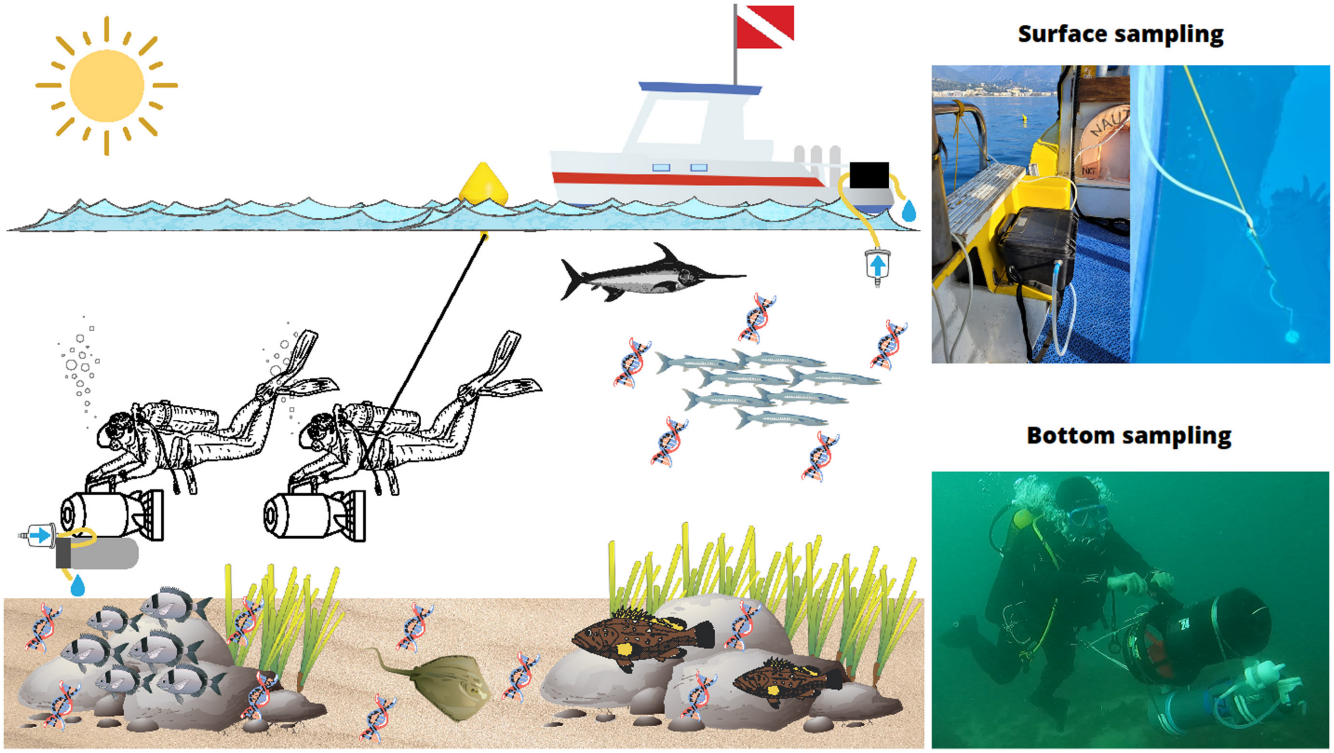

FIGURE 2 Schematic representation of the sampling method. Surface sampling is conducted from a boat with a pump allowing live water filtration through an enclosed capsule. Bottom sampling is performed simultaneously on the same transect, with an underwater pump connected to an enclosed capsule and fixed on a DPV driven by a diver. A second diver is towing a buoy to reveal the divers' position to the boat and allow surface sampling on the same transect (Image' sources: Canva.com, IAN symbols).

at the 5′ end. These tags were used during bioinformatics analysis to assign sequences to the corresponding replicate. Following amplification, all samples were purified with the MinElute purification kit (Qiagen). Library constructions and sequencing were then performed by Fasteris (Geneva, Switzerland). The libraries were prepared according to the Metafast protocol (analysis), designed to minimize sequencing artifacts. The libraries were then sequenced in several Illumina MiSeq runs with paired-end reads of 2×150 bp or 2×250 bp depending on the amplicon's length.

Various quality controls were conducted at each step of the protocol to identify potential contamination, ensuring an accurate interpretation of the results. For each PCR replicate, the following controls were performed: a negative extraction control, a negative PCR control, a positive control, and eight bioinformatic controls. The positive control corresponded to a DNA sample from fish stomach contents diluted to 1/10th previously sequenced by Argaly. The success of the amplifications and purifications was confirmed on a 2% agarose gel (E-Gel Power Snap, Invitrogen).

2.4 | Metabarcoding analysis: Bioinformatics

Argaly conducted the bioinformatic steps, using the following procedure: the raw sequence data for each primer were analyzed using the suite of OBITools programs (https://pythonhosted.org/OBITools/welcome.html; version 2; Boyer et al., 2016) and the SumaClust clustering tool (Mercier et al., 2013), which are specifically designed for analyzing metabarcoding data. More specifically, the paired sequences were first assembled ("illuminapairedend" command), then only the sequences with an alignment score ≥40 (i.e., corresponding to an overlap of at least 10 bases) were assigned to the corresponding amplification replicate, thanks to the tags inserted in the 5′ of the primers ("ngsfilter" command). The resulting dataset was dereplicated ("obiuniq" command), then filtered ("obigrep" command) to remove low-quality sequences (i.e., containing at least one N), sequences whose length did not belong to the length range observed in silico for the target group, and singletons (i.e., sequences observed only once in the dataset). SumaClust was then used to group sequences sharing 97% identity into clusters. The abundances of sequences belonging to each cluster were summed for each PCR replicate. The cluster head, representing the most abundant sequence in the cluster, was chosen as the representative sequence, and clusters appearing less than 10 times in a sample were deleted. A taxonomic assignment of the cluster heads was then performed with the "ecotag" command, to obtain a list of MOTUs (Molecular Operational Taxonomic Units). The reference sequences used for this taxonomic assignment were obtained by performing an *in silico* PCR on the public sequence database GenBank (v.249) with the ecoPCR program (Ficetola et al., 2010). This *in silico* PCR was conducted using the PCR primers associated with each marker allowing a maximum of three mismatches per primer and retaining only sequences assigned at least at the family level.

The R package "metabaR" (Zinger et al., 2021) was then used to remove artifactual sequences from the resulting dataset that are present in low abundance in the metabarcoding data, but which may influence the ecological conclusions that can be drawn from them (Calderón-Sanou et al., 2020). This included removing (1) MOTUs with sequence similarity to any sequence in the reference database below 0.95, as they are potential chimeras; (2) MOTUs whose frequency over the entire dataset is maximum in at least one negative control ("max" method of the "contaslayer" function), because they are potential contaminants; and (3) MOTUs with a relative frequency <0.03% within a PCR replicate ("tagjumpslayer" function), because they are potentially artifacts generated during sequencing library construction (i.e., "tag jumps"; Schnell et al., 2015). PCR replicates with a sequencing coverage <1000 sequences were also removed and then the remaining PCR replicates were aggregated by sample using the "aggregate_pcrs" function. Finally, MOTUs observed less than 10 times in a sample were recoded as absent in that sample.

After receiving the results from Argaly, manual verification and modification of the taxonomic assignations were performed. Non-fish taxa and freshwater fish MOTUs were deleted. Then, marine fish MOTUs were reviewed by blasting the sequences on Genbank and were modified if needed following these criteria:

- Based on biogeographic data, when a sequence was assigned to a non-Mediterranean species, we changed the assignment to the next lowest possible taxonomic rank known to occur in the Mediterranean Sea. If there was only one species of this particular genus or family occurring in the Mediterranean Sea, we changed the assignment to this species.
- Based on biogeographic data, when a sequence was assigned to a taxonomic rank higher than the species level and there was only one species with this genus or family occurring in the Mediterranean Sea, we changed the assignment to this species.

2.5 | Statistical analysis of metabarcoding data: Primer set efficiency

All statistical analyses were performed in R v4.2.2 (http://www.R-project.org; R Core Team, 2022). The fish MOTU datasets for each primer set resulting from the metabarcoding process were analyzed qualitatively (i.e., presence/absence matrix). As we were not interested in intraspecific diversity, MOTUs assigned to the same taxa were grouped for the evaluation of primer set performance. For a given primer set, samples with an insufficient number of PCR replicates were removed from the analyses.

Initially, taxonomic coverage of the six primer sets was assessed by calculating the number of fish taxa identified for each set and for each combination of two and three primer sets. This analysis included samples from Cap Roux MPA and from the MOM aquarium. In addition, we analyzed the MOM aquarium samples separately to determine the proportion of fish species recovered from a known list of species for each primer set.

After this step, we decided to remove samples collected through the 5 μm pore size filters from the dataset for subsequent primer set performance analyses. These samples yielded a very limited number of fish taxa, thereby making comparison and evaluation of the primer sets difficult. We evaluated differences in the mean number of fish species detected per Cap Roux sample between marker genes through a Welch's *t*-test ("t.test" function in R) and between primer sets through an analysis of variance (ANOVA) ("aov" function in R) combined with the Scheffe post hoc test ("ScheffeTest" function of "DescTools" package; v0.99.49). Finally, to assess the influence of primer set choice on the fish species composition detected, we performed a permutational multivariate analysis of variance (PERMANOVA). This analysis was conducted using the Jaccard similarity index on the presence/absence community matrix, combining each field sample from Cap Roux MPA with each primer set ("adonis2" function of the "vegan" package; v2.6.4; Dixon, 2003). Dissimilarity values were ordinated using a nonmetric multidimensional scaling (nMDS), with the function "metaMDS" of the "vegan" package, to visualize the discrimination between samples according to the primer set.

2.6 | Statistical analysis of metabarcoding data: Influence of the sampling strategy

First, we evaluated the influence of the sampling strategy by assessing the effect of filter porosity on the fish detection ability. We compared species detection in Cap Roux MPA across filter pore sizes using a Venn diagram (function "venn.diagram" of "VennDiagram" package; v1.7.3; Chen & Boutros, 2011). The impact of filter porosity on the mean number of fish species detected per Cap Roux sample was assessed through a Wilcoxon rank sum test (function "wilcox.test" in R). We considered each combination of primer set – field sample for this test.

Then, to compare the surface and bottom sampling methods, intersections among the datasets from Cap Roux MPA and at the two sampling depths were highlighted through a Venn diagram and an UpSet plot at the family level (function "upset" of "UpSetR" package; v1.4.0; Conway et al., 2017). Samples collected using the 5 μm filters were removed for the subsequent analyses because of the limited number of fish species detected with these capsules. Differences in the mean number of fish species detected per Cap Roux sample across sampling depth was investigated through the student *t*-test (function "t.test"). Each combination of primer set – field sample was considered for this test. A PERMANOVA was also performed to assess whether the depth of sampling had an impact on the fish composition detected. Dissimilarity values were ordinated on the same nMDS plot as for the primer set evaluation.

Finally, we evaluated our sampling effort by computing species rarefaction curves for different taxonomic levels according to the number of field samples collected in Cap Roux MPA (function "ggiNEXT" of "iNEXT" package; v3.0.0; Hsieh et al., 2016). To conduct this analysis, we used a merged dataset combining the data of

the best performing primer sets selected from the previous analyses (i.e., if a species was detected in a given sample with at least one of the primer sets selected, we put 1, if not, 0).

3 | RESULTS

3.1 | Primer set efficiency

Tele04 was first tested by qPCR on DNA extracted from 32 Mediterranean fish species. This primer set was able to amplify the DNA of each of the 32 fish species, qualifying it for further analyses.

For the metabarcoding analysis, a total of 19,789,780 paired-end reads were obtained from the 2×150 bp (Fish16S, MiFish, Tele04) and the 2×250 bp (AcMDB07, Fish16SFD, Vert16S) Illumina MiSeq runs. After applying the filtration steps, 9,444,499 reads were retained, accounting for 47.72% of the raw reads with an average read count of 1,570,749 per library, ranging from 775,877 for AcMDB07 to 2,075,560 for MiFish. The MOTU clustering process resulted in 444 MOTUs but 42 were assigned to non-marine or non-fish taxa and so were removed. The number of fish MOTUs varied a lot across primer sets, from 40 MOTUs for MiFish up to 125 MOTUs for Fish16S, and 97.79% of these MOTUs were resolved at species level (Figure 3).

The six primer sets also showed a good specificity for fish, with more than 99.3% of the sequences associated with Actinopteri and Chondrichthyes classes (after the removal of MOTUs with a sequence similarity lower than 0.95 with reference sequences) (Figure S1). Using the six primer sets, we successfully detected 97 fish species encompassing 67 genera, 38 families, 22 orders, and two classes (Table S4). These two classes consisted of Actinopteri with 93 species and Chondrichthyes with four species (including three species of rays, *Torpedo marmorata*, *Pteroplatytrygon violacea*, *Dasyatis tortonesei* and one species of shark, *Scyliorhinus canicula*). As expected, the Vert16S primer set led to the detection of Chondrichthyes species since it is vertebrate specific, but this was also the case for the AcMDB07 and Fish16SFD primer sets. Among the 97 fish species detected, 86 were detected in Cap Roux samples and 40 in the MOM aquarium samples with 29 species shared between the two sampling areas (details on the list of fish species detected for each sample are shown in Table S5).

In terms of taxonomic coverage, we detected 75, 65, 60, 44, 39, and 32 fish species using Fish16S, Vert16S, Fish16SFD, AcMDB07, Tele04 and MiFish, respectively (Figure 4). Overall, primer sets targeting the 16S rRNA locus led to the detection of more fish taxa than primer sets targeting the 12S rRNA locus. This was the case for every taxonomic rank (Figure 4). Of the 97 fish species, 25 were detected by all primer sets. Fish16S had the best taxonomic coverage for the 16S rRNA locus whereas AcMDB07 showed the best performance for the 12S rRNA locus (Figure 4). In addition, these two primer sets detected the highest number of unique fish species (i.e., detected species that were not detected by any other primer set) (Table S6). In the case of the MOM aquarium samples, primer sets targeting the 16S rRNA locus again demonstrated better performance, with Vert16S and Fish16SFD recovering up to 70.3% of fish species while the highest value for the 12S rRNA primer sets was 51.4% for Tele04 and AcMDB07 (Figure S2).

Following the removal of the 5 µm pore size filters from the analyses due to their poor fish detection ability, taxonomic coverage was then assessed as the mean number of species detected per field sample (i.e., Cap Roux samples) depending on the targeted locus and the primer set. The number of fish species detected was significantly higher for the primer sets targeting the 16S rRNA locus (28.3 ± 8.33) compared to the 12S rRNA primer sets (15.1 ± 2.91) (Welch's *t*-test, $p < 0.05$) (Figure S3). When comparing primer sets individually, we found that the Fish16S set detected the highest number of species

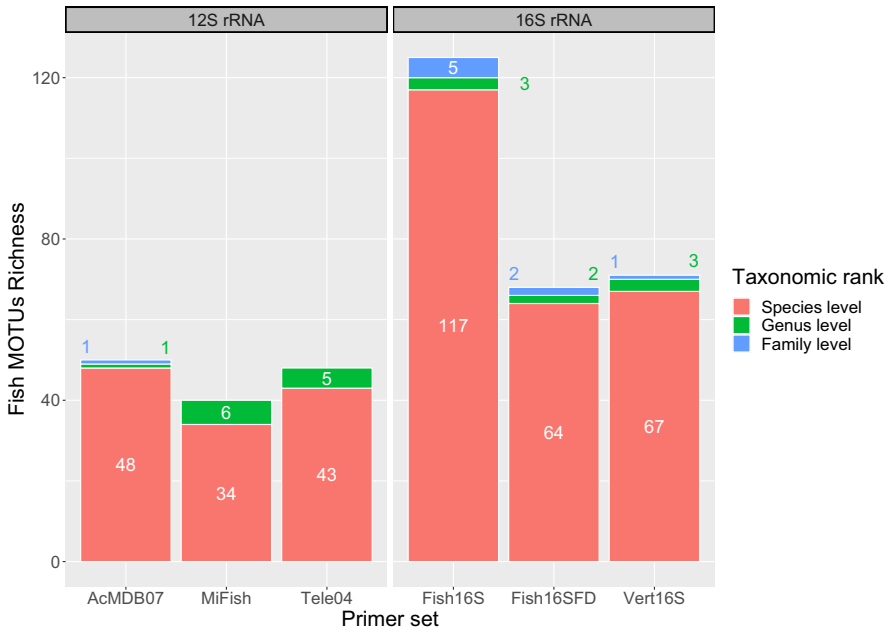

FIGURE 3 Histogram showing the number of fish MOTUs detected using a given primer set, according to the taxonomic rank.

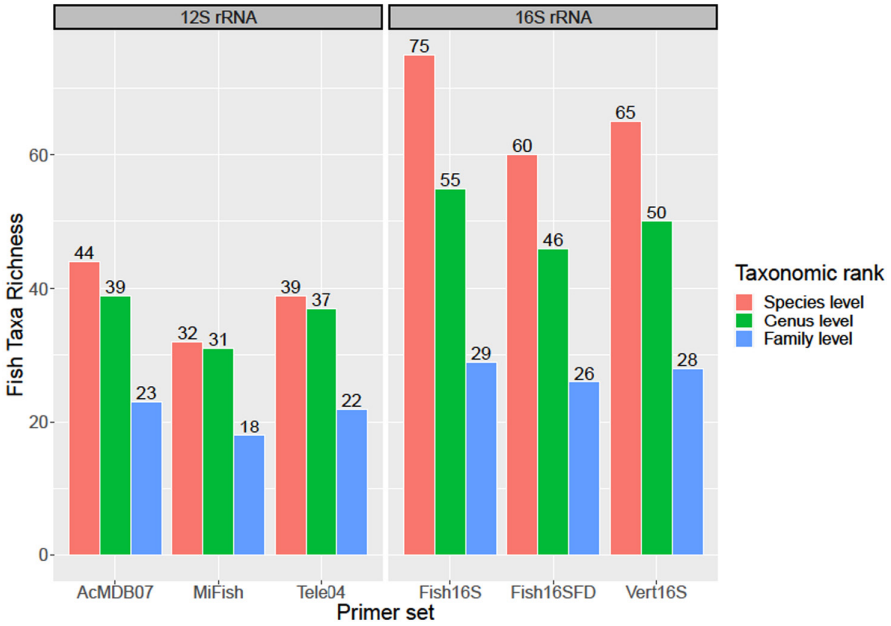

FIGURE 4 Histogram showing the number of fish taxa detected using a given primer set, according to the taxonomic rank.

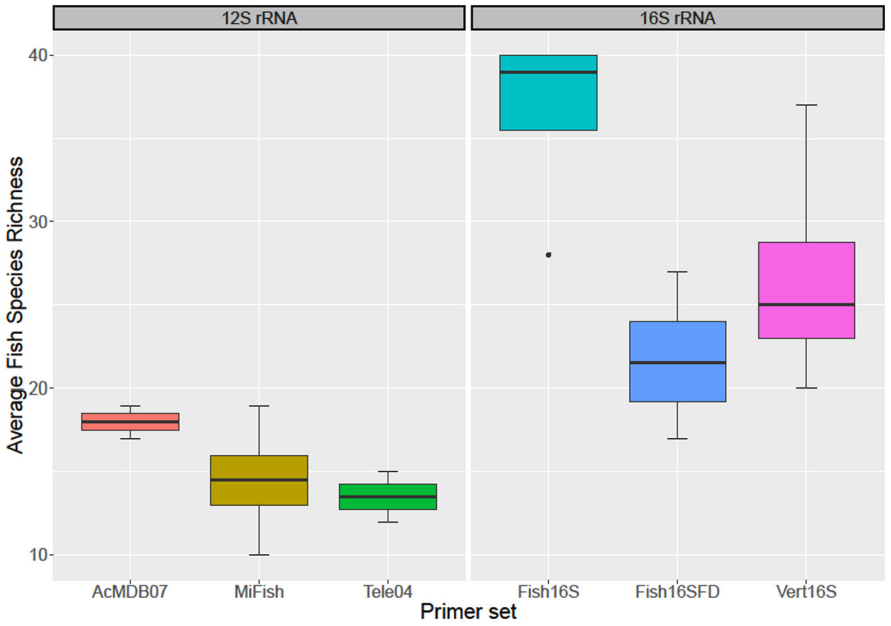

FIGURE 5 Boxplot showing the mean number of fish species detected per water sample using a given primer set. Water samples were collected in Cap Roux MPA using the 0.45 μm pore size filters ($n = 4$ for each primer set except for AcMDB07, $n = 3$).

(36.5 ± 5.74) (Figure 5). The mean number of species detected using this primer set was significantly higher than for primer sets MiFish (14.5 ± 3.70), Tele04 (13.5 ± 1.29), AcMDB07 (18 ± 1), and Fish16SFD (21.8 ± 4.27) (Figure 5, Table S7). Moreover, Vert16S (26.8 ± 6.27) resulted in the detection of significantly more species than Tele04 and MiFish (Figure 5, Table S7).

Since one of our goals was to select the best combination of primer sets, we also evaluated primer combinations. Among pairs of primer sets, Fish16S/AcMDB07 detected the highest number of species (i.e., 87 species). For a combination of three primer sets, Fish16S/Vert16S/AcMDB07 provided the best results with 92 species detected, which accounted for 94.85% of the 97 species detected across the six primer sets.

Concerning the Beta diversity, the nMDS plot based on presence/absence data of Cap Roux MPA displayed a clear separation among samples according to the marker gene used (12S rRNA vs 16S rRNA) (Figure 6). This pattern was statistically supported by a PERMANOVA highlighting a significant difference in species composition not only across genes but also across primer sets (gene: $p < 0.001$; primer set: $p < 0.001$). *Sarpa salpa* was the species contributing the most to these differences as it was not detected in any samples amplified by 12S rRNA primer sets whereas it was detected in all the 16S rRNA primer set samples.

3.2 | Influence of the sampling strategy

In our investigation of the sampling strategy with regards to filter porosity, we detected significantly more fish species per Cap Roux sample with the 0.45 μm filters (22 ± 9.18) than the 5 μm

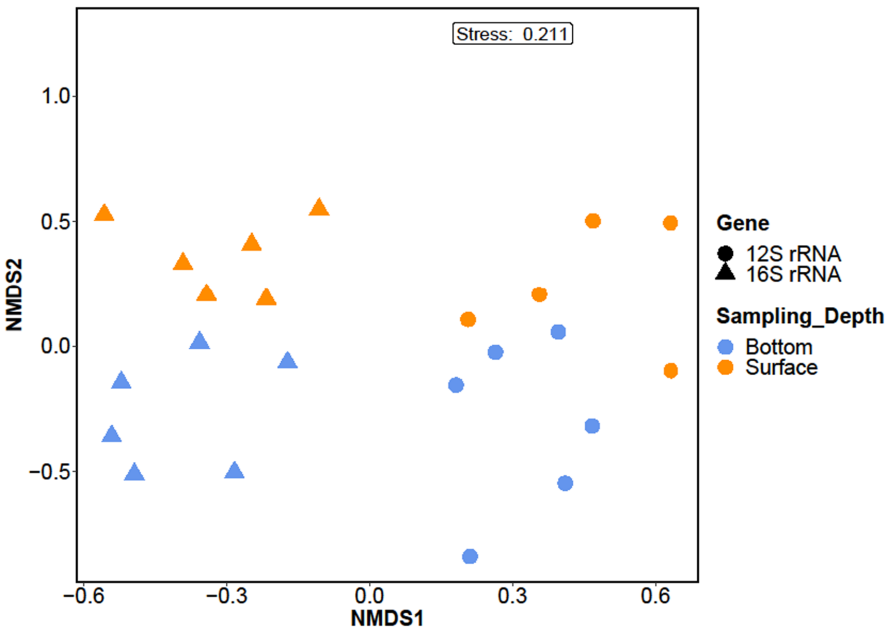

FIGURE 6 Nonmetric multidimensional scaling (nMDS) based on the Jaccard similarity index on the composition of the fish community (presence–absence matrix) for Cap Roux water samples filtered through the 0.45 µm capsule. Each dot corresponds to the combination of a given field sample with a given primer set ($n = 23$). Colors represent the sampling depth (blue: bottom; orange: surface). Shapes represent the marker gene (diamond: 12S rRNA; triangle: 16S rRNA).

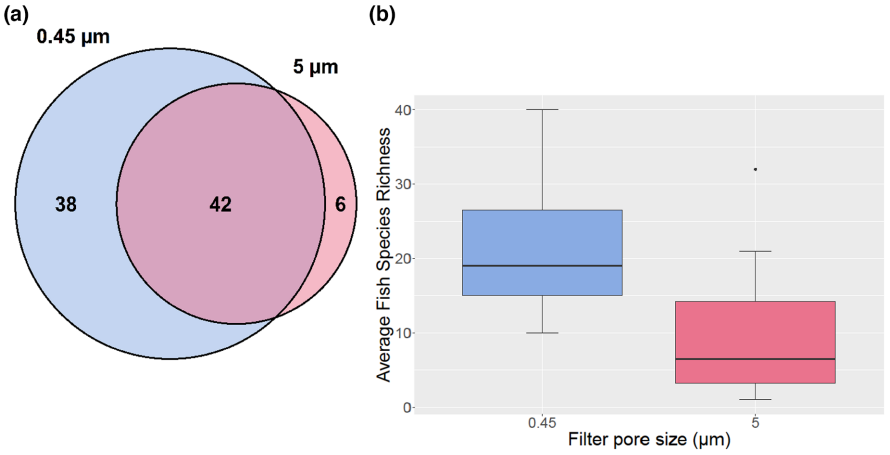

FIGURE 7 (a) Venn diagram showing the number of fish species detected in Cap Roux MPA for each filter pore size. (b) Boxplot of the mean number of fish species detected per Cap Roux water sample for each porosity. Each combination of field sample (Cap Roux MPA, 0.45 µm and 5 µm filters) – primer set, was considered ($n = 23$ for 0.45 µm filters; $n = 18$ for 5 µm filters).

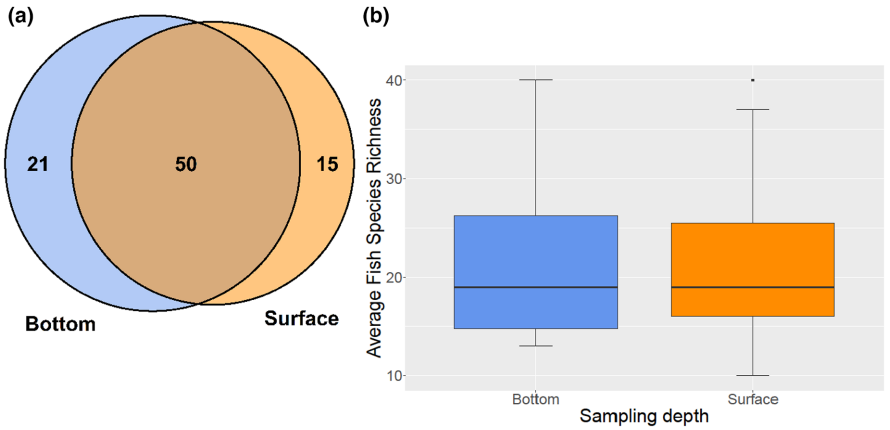

FIGURE 8 (a) Venn diagram showing the number of fish species detected in Cap Roux MPA for each sampling depth. (b) Boxplot of the mean number of fish species detected per Cap Roux water sample for each sampling depth. Each combination of field sample (Cap Roux MPA, 0.45 µm filter) – primer set, was considered ($n = 11$ for surface samples; $n = 12$ for bottom samples).

filters (9.33 ± 8.30) (Wilcoxon rank sum test, $p < 0.001$) (Figure 7b). Furthermore, out of the 86 species detected in Cap Roux samples, 93.02% were detected using the 0.45 µm capsules (44.19% with the 0.45 µm filter only and 6.98% with the 5 µm filter only) (Figure 7a).

Regarding the sampling depth, we found no significant difference in the number of species detected per Cap Roux sample between surface samples (21.8 ± 9.62) and bottom samples (22.2 ± 9.18) (student t-test, $p = 0.93$) (Figure 8b). Bottom samples added 21 fish

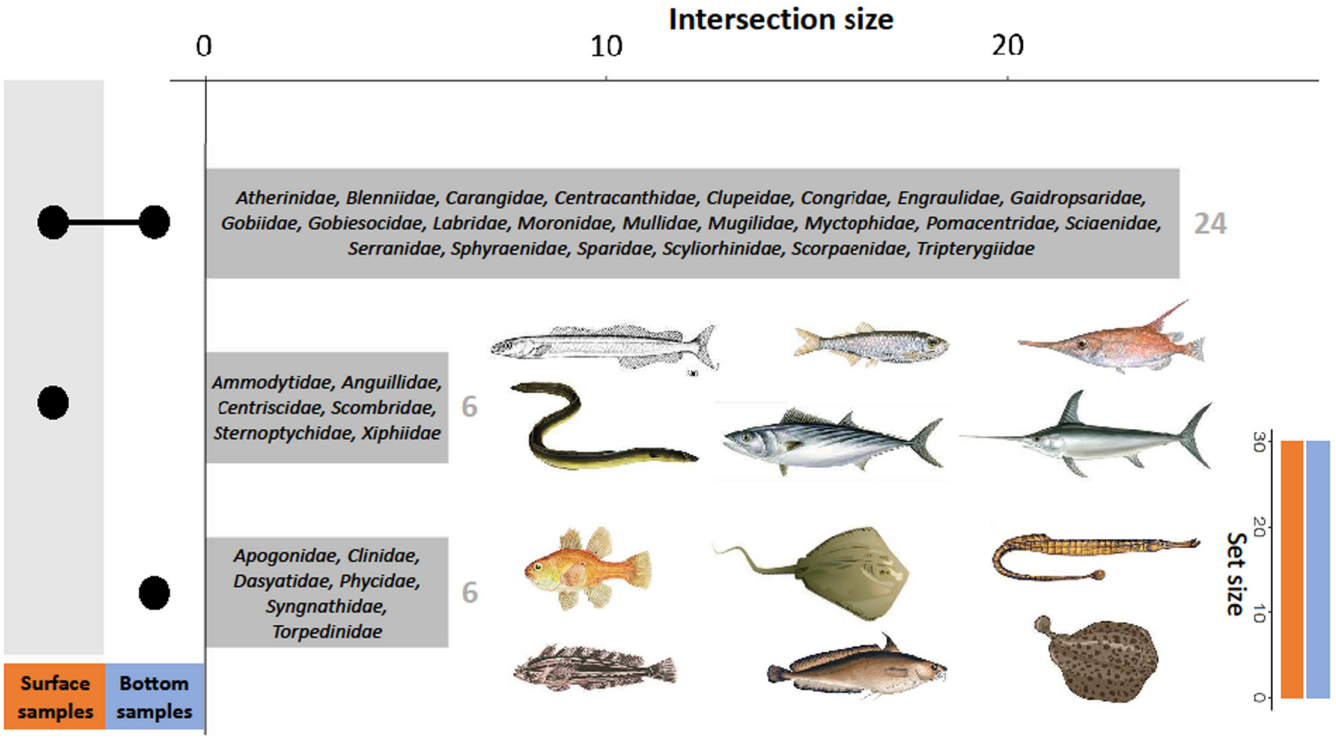

FIGURE 9 UpSet plot displaying the fish families detected at each sampling depth. The Intersection Size histogram shows the families detected exclusively from surface samples, from bottom samples and those detected at both sampling depths. The Set Size histogram represents to the total number of families detected from surface (orange) and from bottom samples (blue) (Image' sources: Canva.com, Dreamstime.com, FAO.org, IAN symbols, Livenisyros.com, Michigan Science Art, Scandinavian Fishing Year Book, Wikimedia, Wikipedia).

species that were not detected by surface samples, including patrimonial species such as the dusky grouper *Epinephelus marginatus*, while surface samples alone returned 15 species. 50 species were detected at both sampling depths (Figure 8a). At the family level, six fish families were exclusively detected through bottom sampling. These families were all typically benthic and included two families of rays (i.e., *Dasyatidae*, *Torpedinidae*). Conversely, six families were solely identified from surface samples including pelagic/highly mobile fish families (i.e., *Scombridae*, *Xiphiidae*) (Figure 9). Furthermore, the nDMS plot (Figure 6), suggests that fish assemblages seem to be structured according to the sampling depth, with a clear separation in the two-dimensional space between surface and bottom samples. The PERMANOVA analysis performed on the same distance matrix supports this result, indicating a significant effect of sampling depth on the composition of the community ($p < 0.001$).

Finally, employing the optimal strategy consisting of a combination of three primer sets (Fish16S/Vert16S/AcMDB07), a sampling effort of four field replicates in Cap Roux MPA and the combination of surface and bottom sampling using 0.45 µm filters, was sufficient to detect most of the fish orders and families (Figure 10). However, this was not the case at genus and species level and additional samples would be required to reach an asymptotic value for these taxonomic ranks. Extrapolation curves indicated that collecting eight samples in Cap Roux MPA might be necessary to reach this asymptomatic value (Figure 10). The confidence interval of the species curve constructed from the dataset using the combination of six primers overlaps considerably with that based on the three best performing primer sets. This highlights that the combination of these three primer sets gave comparable results to that of the six primer combination.

4 | DISCUSSION

Our study presents the first *in vitro* evaluation of fish metabarcoding primers conducted in the Mediterranean Sea to date. This work was crucial before initiating fish monitoring studies in this region, as primer set performance can vary among different studies due to variations in fish communities and complexity in different geographical areas (Kumar et al., 2022; Zhang et al., 2020). With this in mind, we evaluated the effectiveness of six metabarcoding primer sets previously shown to be successful in other assessment studies (e.g., Bylemans et al., 2018; Collins et al., 2019; Kumar et al., 2022; Shu et al., 2021; Zhang et al., 2020), to determine their suitability for the Mediterranean Sea.

Through the analysis of metabarcoding data from samples collected in Cap Roux MPA and MOM aquarium, we observed that primer sets targeting the 16S rRNA locus detected a higher number of fish taxa across all taxonomic levels than primer sets targeting the 12S rRNA locus. This result was unexpected, as 12S rRNA primer sets are typically chosen for fish detection in most studies (Miya, 2022) and have shown better performance in evaluation

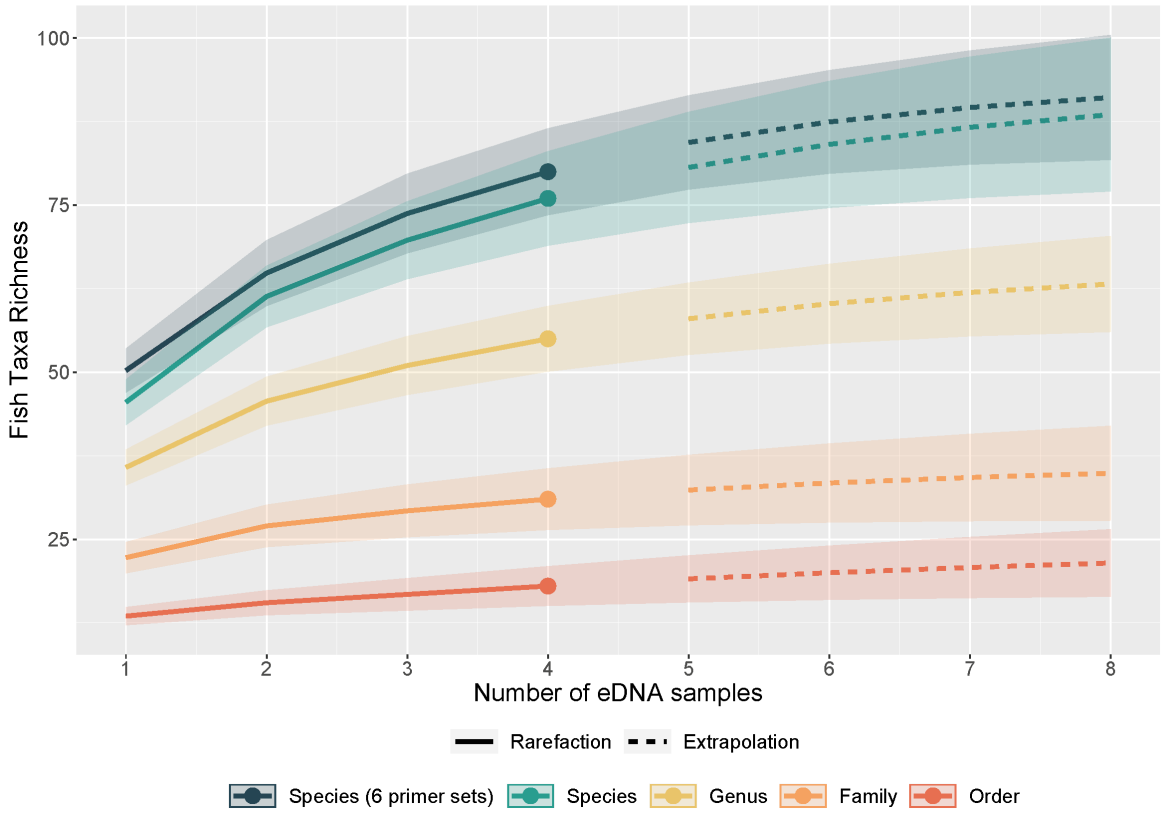

FIGURE 10 Rarefaction curves with confidence intervals (95%), showing the number of fish taxa identified at various taxonomic levels depending on the number of field replicates. The curves for Species (Purple), Genus (Blue), Family (Orange), and Order (Pink) were generated from a dataset combining Fish16S, Vert16S, and AcMDB07 primer sets. The Species curve (Green) was constructed from a dataset combining the six primer sets used in this study. Extrapolation curves are shown in dashed lines for each taxonomic level.

papers. This highlights the need to conduct a pilot study before launching eDNA surveys, to ensure the selection of the most suitable primer sets for a given area. Among the 16S rRNA primer sets, Fish16S led to the identification of the highest number of fish species, both in total and on average per Cap Roux sample. This result may be due to the shortness of amplicons generated by this primer set (~100 bp). Primers amplifying short barcodes (i.e., <200 bp) generally have better amplification success due to the degraded nature of DNA sequences in environmental samples (Zhang et al., 2020).

However, no primer set successfully identified all the 97 species recovered in this study. This finding supports the idea that complete description of fish communities requires the use of multiple primer sets to maximize species detection probability (Shaw et al., 2016). For example, including primer sets that target Chondrichthyan species should be considered if the goal is to have an overview of the entire fish community (Zhang et al., 2020). Based on our findings, we recommend adding Vert16S or AcMDB07 for effective detection of Chondrichthyan species. Additionally, as suggested by Kumar et al. (2022), using a combination of primer sets targeting different genes enhances species detection. Our results further support this notion, as the best performing combination of two primer sets was Fish16S (16S rRNA locus) and AcMDB07 (12S rRNA locus). We therefore recommend combining 12S and 16S rRNA primer sets for a more comprehensive description of the community composition. This finding may be explained by the complementarity of databases, that is, sequences of species absent from one primer set database may be present in another (Shaw et al., 2016). This is exemplified by the species *S. salpa*, which contributed the most to the differences in species composition across marker genes. The reference sequence for this species was present in the 16s rRNA databases but absent from the 12S rRNA databases. Therefore, more work is needed to complete reference databases with sequences of missing species. Although the completeness of databases is a key parameter in species detection through the metabarcoding approach, we did not assess it in this study. Finally, the most effective combination of three primer sets was Fish16S/Vert16S/AcMDB07, which enabled the recovery of 95% of the species detected across the six primer sets. Increasing the number of primer sets from one (Fish16S) to three (Fish16S/Vert16S/AcMDB07), enabled the detection of 17 additional species, while using six primer sets only led to the identification of a further five species. This finding was also supported by the species rarefaction curves, which showed an overlap between the curves derived from the dataset combining these three primer sets and the dataset combining all six primer sets. Moreover, metabarcoding results confirmed that Fish16S and AcMDB07 exhibited high specificity for fish and did not amplify human DNA. This feature is important as a lack of taxonomic specificity may result in a loss of sequencing depth for non-fish taxa leading to false negatives (Kumar et al., 2022).

The effectiveness of the eDNA metabarcoding approach relies on a robust sampling strategy to optimize species detection (Bessey et al., 2020; Kawakami et al., 2023). Our results showed that filter porosity and sampling depth significantly influenced our ability to identify fish species. We found that filters with 5 μm pore size were significantly less effective in detecting fish species compared to 0.45 μm filters. This finding can be attributed to the fact that small pore sizes generally yield higher amounts of eDNA thereby increasing the probability of species detection (Majaneva et al., 2018). The PES 0.45 μm filters performed well in our study, recovering 93% of the overall species detected. This pore size is widely used in the literature (Wang et al., 2021) and has shown good results in association with PES membranes (Coutant et al., 2021). No clogging issues were encountered for this pore size, even though we collected 30 L per sample, which is more than for most studies (reviewed in Rees et al., 2014 and Shu et al., 2020). This is particularly important given that the filtration of large water volumes enhances the likelihood of collecting eDNA molecules (Bessey et al., 2020). This lack of clogging may be attributed to the use of encapsulated filters, thanks to their large membrane surface (Peixoto et al., 2021). Consequently, we recommend using PES 0.45 μm encapsulated filters connected to pumps for water filtration, as it is likely to maximize species detection. It should be noted that our results are based on the evaluation of two mesh sizes and that encapsulated filters with smaller porosity than 0.45 μm may yield even better results. In addition, the transferability of our results to other areas might be influenced by variations in water turbidity.

This study introduced a novel sampling method that involved simultaneous surface and bottom on-site filtration of sea water along the same transect, enabling a direct comparison between these two sampling depths. This sampling strategy was highly effective leading to the detection of numerous species from both surface and bottom samples. Bottom sampling relied on the development of a custom-made underwater pump to collect seawater one meter above the substrate while traveling from one habitat to another using underwater scooters. The collection of water from multiple habitats for each sample (e.g., *P. oceanica* meadows and rocky substrates), allowed the identification of a diverse range of species inhabiting these different ecosystems. Although we were expecting to detect more species within bottom samples, since water was collected closer to the habitat of demersal fishes, our findings did not reveal any significant difference in the number of species detected per sample between surface and bottom samples. This result corroborates some previous studies (e.g., Andruszkiewicz et al., 2017; Stoeckle et al., 2021) that have also reported no significant difference in the number of species detected at these two depths. Nevertheless, our results on beta diversity showed that the fish composition is significantly different between surface and bottom samples. This finding is consistent with several studies that have showed differences in fish composition between these two sampling depths (e.g., Andruszkiewicz et al., 2017; Jeunen et al., 2020; Sigsgaard et al., 2020; Yamamoto et al., 2017). Notably, six fish families were exclusively detected in bottom samples. These families were typically cryptobenthic suggesting that bottom samples could be more effective in recovering species associated with the seafloor, as well as species living in caves and rocky faults (e.g., families *Apogonidae* and *Phycidae*). At the species level, we detected 21 species uniquely associated with bottom samples, including threatened and patrimonial species such as the dusky grouper *E. marginatus* (IUCN, 2016). Conversely, 15 fish species were detected solely from surface samples, including two families of highly mobile predatory fish (i.e., *Scombridae* and *Xiiphidae*). Our findings suggest that surface sampling might be more efficient to recover these pelagic species. This result aligns with those of Sigsgaard et al. (2020), who found that many species were detected only in surface samples because the DNA released at the surface was not reaching the bottom, potentially due to degradation, horizontal transport, or to the presence of a thermocline limiting vertical transport. We thus recommend combining simultaneous surface and bottom sampling to obtain a more comprehensive understanding of the fish communities. However, as bottom sampling relied on scuba divers, this method might be more challenging for deeper areas (30 m max in our study).

Finally, our results showed that four field samples of 30 L each, taken from Cap Roux MPA were insufficient to recover every species at a local scale, as indicated by the rarefaction curve which did not reach saturation at species level. In tropical regions, known to host many fish species, Stauffer et al. (2021), found that between 23 and 58 replicates of 30 L were required to reach an asymptotic value for local MOTU richness. However, in our case, the extrapolation curve suggested that we might reach saturation by collecting fewer replicates (~ eight). This discrepancy could be due to the fact that our sampling region, located in the North-western part of the Mediterranean Sea, has a lower species richness than the tropical coral reef areas (Stauffer et al., 2021). However, it is more likely that this result can be attributed to the effectiveness of our sampling strategy, which was based on the combination of surface and bottom sampling of 30 L of sea water filtered through 0.45 μm PES enclosed filters, followed by the amplification of extracted DNA using the combination of three primer sets (Fish16S/Vert16S/AcMDB07). Our approach incorporated several recommendations suggested in the literature, including the use of multiple primer sets targeting different marker genes to enable Actinopterygian and Chondrichthyan detection, filtration of a large water volume through small pore size, and sampling water closer to the substrate (Bessey et al., 2020; Jeunen et al., 2020; Stauffer et al., 2021; Zhang et al., 2020). Thanks to this eDNA metabarcoding approach, we successfully detected up to 76 fish species in Cap Roux MPA, using only four replicates. For comparison, Aglieri et al. (2021), recovered fewer species (i.e., 74) while sampling in 11 Mediterranean MPAs, including Cap Roux, with a total of 66 replicates.

In summary, this pilot study allowed us to design an effective strategy for monitoring fish communities in the North-western part of the Mediterranean Sea with eDNA metabarcoding. Primer set efficiency as well as sampling methodology are key parameters that need to be carefully evaluated before launching eDNA surveys.

Reliable surveys are important, especially in MPAs, to monitor fish communities in the context of global changes. This study should therefore be useful for future research and conservation efforts in the field of fish community monitoring. More work is needed in the future to enhance our understanding of the "ecology" of eDNA and improve the reliability of quantitative estimates of fish diversity using this method. Such progress will be crucial for effective MPA monitoring because quantitative indices (e.g., fish density or biomass) are preferred to species richness to highlight a potential reserve effect.

AUTHOR CONTRIBUTIONS

C.S. was awarded the grant and managed the MARE project; S.R., C.S., B.D. and F.P. designed the research; F.P. developed the underwater pump; B.D., G.G., S.R. and F.P. performed fieldwork; C.S., S.R., F.P. and B.D. performed laboratory work; S.R. analyzed the data; G.G. made the maps; S.R. wrote the paper with input from all co-authors.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Presence–absence datasets for each primer set and each sample can be found in the Supporting Information Material as well as samples metadata. AcMDB07, MiFish, Tele04, Fish16S, Fish16SFD, and Vert16S raw sequence reads as well as the bioinformatic pipeline code are available at the public repository: https://doi.org/10.5061/dryad.612jm648m.

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SUPPORTING INFORMATION

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