Environmental DNA effectively captures functional diversity of coastal fish communities

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Abstract

Robust assessments of taxonomic and functional diversity are essential components of research programs aimed at understanding current biodiversity patterns and forecasting trajectories of ecological changes. Yet, evaluating marine biodiversity along its dimensions is challenging and dependent on the power and accuracy of the available data collection methods. Here we combine three traditional survey methodologies [Underwater Visual Census strip transects (UVCt), Baited Underwater Videos (BUVs) and Small Scale Fishery catches (SSFc)], and one novel molecular technique [eDNA metabarcoding (eDNA) – 12S rRNA and Cytochrome Oxidase Subunit 1 (COI)] to investigate their efficiency and complementarity in assessing fish diversity. We analysed 1,716 multi-method replicates at a basin scale to measure taxonomic and functional diversity of Mediterranean fish assemblages. Taxonomic identities were investigated at species, genus and family level. Functional identities were assessed using combinations of morphological, behavioral and trophic traits. We show that: i) SSFc provided the higher taxonomic diversity estimates followed by eDNA, and then UVCt and BUV; ii) eDNA was the only method able to gather the whole spectrum of considered functional traits, showing the most functionally diversified and least redundant fish assemblages; iii) the eDNA effectiveness in describing functional structure reflected its lack of selectivity toward any considered functional trait. Our findings suggest that the reach of environmental DNA analysis stretches

beyond taxon detection efficiency and provides new insights about the potential of metabarcoding in ecological studies.

Introduction

Under extreme biodiversity loss, our ability to quantify the magnitude and to forecast the direction of ecological change is imperative to foster sound conservation strategies, maintaining functional ecosystems, and ensuring nature's contributions to people (Díaz et al., 2018; Halpern et al., 2019; Mace, Norris, & Fitter, 2012). At the most fundamental level, such knowledge is dependent upon the reliability of investigations on taxonomic and functional diversity of ecological systems (Loreau, Naeem, & Inchausti, 2002; Micheli & Halpern, 2005). Yet, obtaining comprehensive estimates of biodiversity patterns is challenging. Practically, the breadth of such assessments are contingent to the availability of funds, time and the sampling techniques used (Moore & McCarthy, 2016), and no existing method for taxonomic and functional biodiversity estimation is unbiased (MacNeil et al., 2008).

In marine ecosystems, reef fishes are considered to be well suited for global marine biodiversity studies (Mouillot et al., 2014; Pimm et al., 2014), as they are highly diverse among vertebrates, cover a wide range of ecological functions, and their taxonomy and biological and ecological traits are relatively well known. Reef fish diversity assessments are

traditionally carried out using a range of techniques, the most popular of which include Underwater Visual Census strip transects (UVCt), Baited Underwater Videos (BUV, also known as Baited Remote Underwater Video - BRUV), experimental fishing and catches observations of both Industrial and Small-Scale Fisheries (SSFc) commercial operations (Murphy & Jenkins, 2010) Although none of these methods is specifically designed to capture the whole spectrum of biodiversity, they have been extensively used for evaluating taxonomic and functional diversity patterns (Cappo, De'ath, & Speare, 2007; Micheli et al., 2014; Stuart-Smith et al., 2013). UVCt is an efficient, non-invasive low-cost method. Nevertheless, it can be biased by specific fish behaviours, underestimating the diversity of rare, shy, cryptic and very mobile pelagic species (Pais & Cabral, 2017). BUV is also a widely used non-invasive observational method, less restricted by depth and time. It performs well in recording large and elusive predators, including sharks, but might be less able to detect small-sized or cryptic species (Colton & Swearer, 2010). Extractive fishery-dependent surveys may be limited by differences in catchability of species by fishing gears, as well as habitat characteristics (Erzini et al., 2006). In this context, the environmental DNA metabarcoding (hereafter 'eDNA') approach to marine biodiversity assessments may prove promising (Djurhuus et al., 2020): this non-invasive method allows the detection of marine organisms from species-discriminating amplicons of short DNA fragments 'harvested' from the environment (Bohmann et al., 2014). Although limitations associated with quantification, DNA dispersal and false negative/positive detection

(Hansen, Bekkevold, Clausen, & Nielsen, 2018), eDNA is efficient in detecting transient, rare and low abundance aquatic species (Boussarie et al., 2018; Sigsgaard, Carl, Møller, & Thomsen, 2015; Thomsen et al., 2012).

Although most biodiversity surveys typically emphasise measures of taxonomic diversity, the range of functions that organisms perform in a given community is considered a more responsive descriptor than species diversity to understand how ecosystems respond to natural and anthropogenic perturbations (D'agata et al., 2014). Yet, we know nothing about the ability of available survey methods in capturing functional diversity. Here we present findings of a large-scale field study – spanning the Central and Western Mediterranean Sea – aimed at investigating the proficiency of direct observational techniques, a fishery-dependent method, and a molecular approach in quantifying taxonomic and functional diversity of coastal fish. Our aims were to: (i) assess the relative performance and complementarity of traditional UVCt, BUV and SSFc (fixed-net) and eDNA metabarcoding (12S rDNA and Cytochrome Oxidase Subunit 1 markers) survey techniques in detecting reef fish diversity; (ii) infer the contribution of each monitoring technique to the overall taxonomic and functional diversity of the fish assemblages; (iii) evaluate the selectivity of each survey methodology for specific fish functional traits.

To achieve these goals and to increase the representativeness of regional reef fish diversity, we concomitantly sampled 22 locations within marine

protected areas (MPAs) and unprotected areas in the Mediterranean Sea applying standardized sampling protocols. We investigated fish taxonomic identities at species, genus and family level, and fish functional identities using combinations of morphological, behavioural and trophic traits. Coupling the two approaches, we show that ecological conclusions of field studies might depend from the applied survey methods. We describe an unrecognized feature of eDNA: its lack of selectivity for functional traits and hence its greater ability to capture effectively the spectrum of functional diversity of reef fish in the Mediterranean region.

Materials and Methods

Field data collection

We concomitantly conducted field surveys of coastal fish diversity using Underwater Visual Censuses strip transects (UVCt), Baited Underwater Video systems (BUV) and environmental DNA metabarcoding (eDNA) during June and July 2018. We sampled the subtidal rocky zone of 22 locations inside and outside eleven Marine Protected Areas (MPAs) in Italy (Egadi Islands MPA and Trapani coast, Portofino MPA and Camogli-Rapallo coast, Torre Guaceto MPA and the Northern Brindisi coast), Greece (Zákynthos National Marine Park and Zákynthos island), Spain (Es Freus Marine Reserve and Straits of Ibiza and Formentera Islands, Cabo de Palos Marine Reserve and adjacent Murcia coast), France (Bonifacio Natural Reserve and South Corsica, Cap Roux Cantonnement de Pêche and adjacent fished zones, Côte Bleue Marine Park and adjacent fished zones),

Croatia (Telašćica Nature Park and Dugi-Otok island) and Slovenia (Strunjan Landscape Park and adjacent coast) (Fig. 1- Table S1). Photo-sampling of small-scale fisheries catches (SSFc) using fixed-nets in the same locations was also carried out at landings between May and September 2018. Sampling operations at sea were carried out by two separate teams, one for UVCt and eDNA water sampling and one for the BUV systems. Separation between the two teams was necessary to avoid possible onboard and underwater contamination of the eDNA samples from the BUV baits. In order to reduce the time window between collection, filtration and sample storage, the eDNA water samples were collected at the end of each working day. eDNA samples were collected within 500 meters from the sites where UVC and/or BUV sampling was carried out.

UVC strip transects were carried out between 8:00 a.m. and 3:00 p.m. inside and outside each MPA by the same three trained diving operators. We haphazardly selected two sites for each protection level (no-take zone, buffer zone and unprotected area) and carried out between 8 and 13 replicated transects per site [on average 11.54±0.41 (mean±s.e.)] depending on the spatial extent of each site and on the availability of suitable habitat (rocky, from 5 to 15 meters). Each replicate consisted of a strip transect of 125 m2 (25x5 m), conducted at 5-12m depth on rocky substrates. Overall, we carried out 760 UVCt replicates (Table S1).

BUVs consisted of the deployment of a steel structure equipped with two stereo-cameras and a plastic container containing a standard bait (e.g.

~500 gr of gilt sardines, *Sardinella aurita*) to attract fish. Two operators deployed the BUVs on rocky bottoms from the boat for 60 minutes between 8:00 a.m. and 3:00 p.m. within a depth range of 5-15 m. To avoid the repeated recording of the same individuals, BUVs were deployed at >150 meters apart (Whitmarsh, Fairweather, & Huveneers, 2017). Depending on the spatial extent of the areas, a minimum of three and a maximum of six replicates [on average 5.36±0.11 (mean±s.e.)] were carried out in each of the two sites haphazardly chosen within each MPA protection level and surrounding areas. Overall, we deployed 354 BUVs (Table S1). Videos were then analyzed recording all the fish observed in the field of view of the two cameras.

SSFc were recorded taking pictures of each catch, placing the fish on a flat white plastic surface along with a ruler as length reference. Each picture was associated to a unique identifier of the fishing catch and associated to the geo-coordinates of the haul. The number of replicates varied among locations, ranging from 17 replicates in Telašćica Nature Park and Dugi-Otok island to 82 replicates in Egadi Islands MPA and Trapani coast. No SSFc data was collected in Strunjan Landscape Park and adjacent coast. Overall, 536 SSF landings were recorded (Table S1).

eDNA samples consisted of four liters of water (two liters from the surface and 2 liters at one meter from the bottom, max depth -20 m) collected from the boat and by scuba diving in three haphazardly chosen sites inside and three outside each MPA for a total of 66 replicates (Table S1). After collection, the bottles were stored in the dark and in ice. For each site, we

placed a bottle filled with deionized water (field blank) among the sample bottles in the transportable refrigerator, and processed identically. Filtration was performed within 3 hours from sampling using a vacuum pump and sterile mixed cellulose esters filters (Merck Millipore; 47 mm diameter; 0.45 µm pore size). Potential contamination were controlled sterilizing benches and equipment with 50% bleach. The filters were stored at -20 C in 2.0 ml airtight cryotubes containing silica beads to dry out and prevent DNA degradation.

eDNA laboratory analyses

We carried out DNA extraction, PCR amplification, library preparation and sequencing in a dedicated eDNA lab with separate rooms for pre-PCR preparations and post-PCR procedures. Benchtops were cleaned with 10% bleach and DNA AWAY™, pipettes and all the surfaces UV-irradiated daily and before beginning any molecular work. We employed rigorous protocols for contamination control at each step of the process, including field, extraction and PCR blanks. We prepared PCRs in a DNA-free hood and performed all post-PCR work in a room physically separated from pre-PCR work. We extracted the DNA from the filters with the QIAGEN PowerWater DNA Isolation Kit, following the manufacturers' protocol, and assessed its concentration in a Qubit fluorometer (Thermo Fisher Scientific). We amplified a ~167bp fragment of mitochondrial 12S rRNA by PCR using the "Tele02" primer set (Miya et al., 2015; Taberlet, Bonin, Coissac, & Zinger, 2018). To facilitate demultiplexing of Illumina sequence reads, samples were 'tagged' using individual, sample-specific primers

with attached 8-base oligo-tags differing in at least three bases between samples. Forward and reverse primers carried the same tag within each sample. Sequence diversity, important for Illumina amplicon sequencing, was increased by inserting six fully degenerate positions (Ns) at the beginning of each primer. The PCR mix had a total volume of 20 µl, composed by 10 µl Amplitaq Gold Master Mix (Thermo Fisher Scientific), 0.16 µl BSA, 1 µl of 5 µM forward primer, 1 µl of 5 µM reverse primer, 10 ng of eDNA template and 5.84 µl of molecular biology grade water. The thermocycler profile included an initial denaturing step of 94 ˚C for 10 min, 35 cycles of 94 ˚C 1 min, 54 ˚C 1 min and 72 ˚C 1 min and a final extension step of 72 ˚C for 5 minutes. We performed PCR amplifications in triplicate and checked the presence of amplification products by gel electrophoresis (1.5%). We pooled PCR products containing all the samples, 11 field blanks, 10 extraction blanks and PCR blanks into two sample pools. We made two dual-indexed Illumina libraries using the KAPA HyperPrep PCR-free library preparation kit (Roche). We quantified the libraries using the KAPA library quantification kit (Roche) and pooled them in equimolar concentrations along with 10% PhiX (Illumina) serving as a positive sequencing quality control. We sequenced the libraries with a final molarity of 8 pM on an Illumina MiSeq platform using v2, 2 x 150 paired-end chemistry.

We selected 67 out of 132 samples (Table S2) for further exploration using a 313 bp Cytochrome Oxidase subunit 1 (COI) marker using the same protocol used for 12S rRNA. We applied the selection to contain the costs,

since the highly degenerated primers used are known to primarily amplify micro-eukaryotes and invertebrates and we expected a low yield for fish (Collins et al., 2019). We included a subset of replicates for all the considered locations and all the negative controls into another independent sequencing project. We selected the samples with the highest amount of extracted DNA. Amplification was performed using the Leray-XT primer set (Wangensteen, Palacín, Guardiola, & Turon, 2018) and the PCR profile suggested by the authors. High-depth sequencing was carried out in a HiSeq4000 to maximise recovery of rare vertebrate reads.

Mediterranean fish species DNA barcoding

We collected small fin clip pieces of 25 Mediterranean fish species in local fish markets and preserved them in 96% ethanol at -20 C. We extracted the DNA using the DNeasy Blood & Tissue Kit (QIAGEN) following the manufacturers' protocol. We amplified the "tele02" 12S rRNA fragment applying the same conditions reported for eDNA metabarcoding, purified the PCR products with the QIAquick PCR Purification Kit (QIAGEN) and outsourced the Sanger sequencing to Macrogen Europe (ABI 3730XLs). Sequences were edited with BioEdit v.7.2 (Alzohairy, 2011).

Bioinformatic analyses

We processed the sequence reads using the OBITools v.1.01 12 metabarcoding software suite (Boyer et al., 2016). Libraries were demultiplexed with 'bcl2fastq v. 2.20' (Illumina), before assessing read quality using FastQC v.0.11.7 (http://www.bioinformatics.babraham.ac.uk/

projects/fastqc/). Reads were trimmed to a length so each base was, on average, above a score of Q30 using ‘obicut’. Paired-end reads were aligned using ‘illuminapairedend’, retaining alignments with a quality score >40. Sample demultiplexing was performed with ‘ngsfilter’ for each library. Samples from 12S libraries were concatenated and the sequences length filtered (140-180 bp) using ‘obigrep’ to select only fragment lengths known to amplify with our primers. The COI library was filtered between 303bp and 323 bp. Reads containing ambiguous bases were also removed. Remaining reads were de-replicated using “obiuniq” and chimeras were removed with the ‘uchime-denovo’ algorithm (Edgar, Haas, Clemente, Quince, & Knight, 2011) implemented in vsearch v.1.9 (Rognes, Flouri, Nichols, Quince, & Mahé, 2016). Sequence clustering was performed using ‘swarm’ v.2.0 (Mahé, Rognes, Quince, Vargas, & Dunthorn, 2014) with a d-value of 2 for 12S, and 13 for COI. As suggested by the authors, the choice of “d” was made after testing the outcomes of different values. Identification and exclusion of potential contamination was achieved by including field blanks, eDNA extraction blanks and PCR blanks. We removed the reads present in the negative controls from the respective samples. All singletons were discarded.

Taxonomy assignment

We performed the taxonomic assignment of 12S sequences representing each Molecular Taxonomic Unit (MOTU) using ’ecotag’ against a curated database (https://github.com/boopsboops/reference-libraries. Since several Mediterranean fish species were missing in public databases, we

complemented our custom 12S database with ‘Teleo02’ sequences of 25 common coastal species (Table S3). The taxonomic assignment for the COI marker was performed against the db_COI_MBPK database (Bakker et al., 2019) (http://github.com/metabarpark/Reference-databases). Each Molecular taxonomic unit (MOTU) was assigned to a single species when this was the only Mediterranean species with a sequence similarity >97%, a cut-off value that optimizes the recovery of species composition of the studied environment avoiding erroneous taxonomic assignments (Miya et al., 2015). For the COI marker, the similarity threshold used was conservatively increased to 99%, since identifications below 98% of identity with COI markers could be error prone (Clare, Barber, Sweeney, Hebert, & Fenton, 2011). Ambiguous automatic assignments were manually checked through a BLAST search against the NCBI (https://www.ncbi.nlm.nih.gov/) and MitoFish (http://mitofish.aori.u-tokyo.ac.jp/) databases for 12S, and NCBI and BOLD (http://v3.boldsystems.org/index.php/IDS_OpenIdEngine) for COI. If appropriate, we applied an assignment correction accordingly to the up to date knowledge of the species distribution in the Mediterranean Sea provided by FishBase (https://www.fishbase.se/trophiceco/FishEcoList.php?ve_code=13), following these criteria:

1. In case of multiple possible assignment (i.e. more than one Mediterranean species with the same sequence similarity) -> the MOTU was registered at the lowest possible taxonomic rank.

2. In case of a MOTU assigned to a non-Mediterranean taxon despite closely related Mediterranean taxa showed a sequence similarity within the 97-99% threshold -> the assignment was corrected including the Mediterranean taxa at the lowest possible taxonomic rank.
3. In case of a taxonomic assignment at family or genus level when a single Mediterranean species had an equivalent % of similarity to a non-Mediterranean species belonging to the same genus or family -> the assignment was corrected excluding the non-Mediterranean taxon.
4. In case of a record assigned to a non-Mediterranean taxon because of a lack of reference sequences for Mediterranean congeneric species -> the assignment was corrected only in presence of a single Mediterranean congeneric species

After the taxonomic assignment revision, the MOTUs assigned to the same taxa were condensed together.

Statistical analyses

We built datasets containing taxa presence/absence data for each location using the outcomes of each sampling technique. A “unique trait combinations” (UTCs) dataset was also built, representing each taxon as a string of traits for each considered functional category. We considered seven categories: a) maximum length; b) depth range; c) cryptic/nocturnal

behaviour; d) mobility; e) habitat type; f) aggregation behaviour; g) trophic habit (Table S4).

We performed all the statistical analyses in R V. 3.5.2 (R; http://www.R-project.org). Taxa and UTCs accumulation curves were generated using the 'specaccum' function implemented in the package 'vegan' v.2.5-5 (Oksanen et al., 2019), applying the 'random' method and 1000 permutations. Intersections among the datasets generated by the four different sampling methods were represented using 'UpSetR' package v. 1.4.0 (Conway, Lex, & Gehlenborg, 2017). Non-metric multidimensional scaling (nMDS) of similarities (Jaccard) among fish assemblages (taxa and UTCs) was performed with the 'metaMDS' function implemented in 'vegan'. We took specific precautions to avoid bias due to the uneven taxonomic resolution of the different methods. Indeed, some taxa identified at the genus or family level could be potentially redundant whenever other members of the same genus or family were present in the dataset. For this reason, we rearranged the taxa dataset condensing all the possibly redundant identifications among different sampling methods into higher taxonomic ranks (Table S5).

The relative contribution of each sampling technique to the global diversity estimate was analyzed using four measures of diversity: Average Taxonomic Distinctiveness (AvTD) (Clarke & Warwick, 1998), Average Functional Distinctiveness (AvFD) (Somerfield, Clarke, Warwick, & Dulvy, 2008), Rao's quadratic entropy (RaoQ) (Botta-Dukát, 2005) (SI Appendix) and Functional redundancy (FR) (Mouillot et al., 2014). All indices utilize or

can accommodate presence/absence data to explore the diversity of a community (or assemblage). AvTD takes into account the taxonomic distance among the units composing a sample (species). AvFD considers the functional divergence among the same items. Both the indices are able to compare the local diversity (taxonomic or functional) of observed fish assemblages to the expected total diversity extrapolated from the overall list of species known to be present in the considered environment. We built an aggregated fish assemblage list collating all the taxa recorded by each method. This was then used along with sample specific lists to calculate AvTD and AvFD, and to verify potential methods-specific divergences from the expectancies using the ‘taxa2dist’ and ‘taxondive’ functions implemented in ‘vegan’. Taxonomic distinctness estimates were generated using, for each sampling method, the highest possible taxonomic resolution. We excluded the records at family level to avoid the risk of producing spurious variations in the taxonomic breadth of the samples. For similar reasons, we kept the records at taxonomic level higher than species for the AvFD analyses only if appropriate functional categories were applicable for all the considered traits. RaoQ estimates were generated from the same datasets used for the calculation of AvFD, employing the function ‘rao.diversity’ implemented in the package ‘SYNCSA’ v. 1.3.4 (Debastiani & Pillar, 2012). FR estimates were calculated dividing the number of taxa by the number of UTCs observed in each sample. Analysis of variance (ANOVA) was used to test for differences in AvTD, AvFD, RaoQ and FR estimates among the four survey methods using

the ‘aov’ function in R. Normality of distribution and homogeneity of variance were tested using the ‘shapiro.test’ and ‘leveneTest’ functions implemented in the ‘dplyr’ and ‘car’ packages (Fox & Weisberg, 2018). A logarithmic transformation was applied to the AvTD values to ensure normality of distribution. Significant differences among factors were followed by pairwise comparison t-tests. Functional structure variation among fish assemblages was summarized using principal component analysis (PCA) based on the relative proportion of each considered functional trait in the samples provided by each survey technique. The latter was calculated dividing the number of trait occurrences in each sample by the total number of taxa registered in each locality, to account for the intrinsic environmental variability among MPAs. The PCA was performed using the ‘rda’ function in ‘vegan’.

Results

The Illumina MiSeq run of the pooled 12S amplicon libraries produced 16,197,599 reads. After sample assignment, quality and sequence-length filtering, singletons and chimera removal, we obtained 10,560,688 reads. After conservative removal of MOTUs unassigned or assigned to non-fish taxa, we obtained 1,863,535 reads, among which 1,422,635 unambiguously assigned to Mediterranean fish taxa. The main contaminants detected and their relative proportions are listed in table S6. The average number of reads per samples was higher for the samples taken at -20 m (13,089 reads) than for the surface samples (8,467 reads). After merging the reads from the two depths for each replicate, the

number of reads ranged from 3 to 196,306, with a mean value of 21,541 reads.

The Illumina MiSeq run of the COI amplicon library produced 45,901,040 reads. After bioinformatic filtering, we obtained 24,242,469 reads of the samples included in the present study. As expected, most of the sequence reads belonged to non-fish taxa. In all, 99,071 reads were assigned to fish taxa. The number of COI reads belonging to Mediterranean fish taxa per sample ranged from zero (three samples failed) to 48,329 with a mean value of 1,479 reads.

Overall, we identified 159 fish taxa belonging to 100 genera and 55 families (Table S7), and 91 unique traits combinations (UTCs) at functional level, with unequal contributions, provided by different survey techniques to locally observed taxonomic and functional richness (Fig. 1). eDNA detected 79 different fish taxa (74 at species and 5 at genus level) belonging to 65 genera and 37 families, and 53 UTCs. UVCt, BUV and SSFc identified respectively 69 taxa (66 at species, two at genus and one at family level; 40 genera and 20 families) and 45 UTCs, 66 taxa (57 at species, 6 at genus and 3 at family level; 41 genera and 28 families) and 34 UTCs, and 102 taxa (all at species level; 67 genera and 40 families) and 61 UTCs. The four different methods shared 14.46% of species (23), 22% of genera (22) and 20% of families (Fig. 2, S1, S2). SSFc recorded the highest proportion of exclusive records (25.16%, 40 taxa), followed by eDNA (17.61%; 28 taxa), UVCt (7.55%; 12 taxa) and BUV (2.52%; 4 taxa). Overall, 42.85% of UTCs (39) was not shared between any of the survey

techniques. SSFc showed the highest proportion of exclusive UTCs (18.68%, 17 UTCs), followed by eDNA (14.29%; 13), UVCt (7.69%; 7) and BUV (2.20%; 2) (Fig. S3).

On average, eDNA performed better in detecting a higher number of fish taxa and trait combinations per replicate (10.76 ± 7.15 and 8.73 ± 5.55, respectively; N=66; mean ±SD) than BUV (Taxa: 9.86 ± 3.46; UTCs: 7.13 ± 2.37; N=354), UVCt (Taxa: 9.13 ± 2.88; UTCs: 7.33 ± 1.92; N=760) and SSFc (Taxa: 5.51 ±3.81; UTCs: 5.16 ± 3.38; N=536) as shown by the taxa and UTCs accumulation curves (Fig.3).

Non-metric multidimensional scaling (nMDS) on trait-based (Fig. 4) and taxon-based fish assemblages (Fig. S4) confirmed little overlap among fish assemblages, showing a sharp separation in the 2-D space among three well-defined groups. As expected, UVCt and BUV samples clustered together and were well separated from the more scattered eDNA and SSFc samples groups.

The Average Taxonomic Distinctiveness (AvTD) estimates returned significantly different variances among survey methods, with pairwise comparisons being also highly significant ($p<0.001$), except for the UVCt-BUV comparison (Table S8). AvTD funnel plot shows that the highest proportion of taxonomic breadth of the identified pool of Mediterranean coastal fish was provided by SSFc, with most of the samples matching the expected distribution at 95% C.I., and, to a lesser extent, by eDNA (few samples outside the 95% C.I.) (Fig. 5A). UVCt and BUV samples exhibited a

narrower taxonomic breadth (i.e., close fish taxonomic composition and all samples below the 95% C.I.). Average Functional Distinctiveness (AvFD) estimates were significantly different between each pair of methods (Table S8). eDNA samples were unique in showing estimates of functional distinctness consistent with the expectations (AvFD= 29.53 ± 2.47; Mean ± SD), while almost all the UVCt, BUV and SSFc samples were below the lower boundary of the 95% confidence limit (Fig. 5B). BUV was the survey technique with the lowest average functional distance among the detected fish (AvFD= 20.62 ± 2.15). UVCt produced functionally broader samples than BUV, performing slightly better in detecting functional than taxonomical diversity (AvFD= 23.41 ± 2.38). Despite the larger taxonomic diversity observed, SSFc data returned AvFD estimates below expectations (AvFD= 27.07 ± 2.17) (Fig. 5B). We complemented information on the Mediterranean fish functional dimensions using the Rao's quadratic entropy (RaoQ) index on presence-absence data (Fig. 5C) and the Functional Redundancy (FR) index (Fig. 5D). RaoQ estimates showed similar patterns to AvFD with eDNA exhibiting the highest values (RaoQ= 0.50 ± 0.03; Mean ± SD), which were similar to SSFc (0.48 ± 0.03), but significantly different from UVCt (RaoQ= 0.44 ± 0.03) and BUV (RaoQ= 0.41 ± 0.03) (Table S8). The functional redundancy (FR) of the fish assemblages described by the observational methods was significantly higher than those identified by the molecular and fishing methods, with eDNA samples having the less redundant fish assemblages (Table S8).

The principal component analysis (PCA) used to visualize functional structure variations among fish assemblages shows a clear separation among three distinct clouds represented by eDNA, SSFc and UVCt-BUV fish assemblages (Fig. 6). Most of the variability was explained by differences between the UVCt-BUV and SSFc data clouds, clearly separated along the first PC axis. eDNA data in the functional space were neutral with respect to the first PC axis, suggesting a balanced proportion of traits related to the variability along this dimension. The trait categories best explaining the variability along the first axis were “fish size”, “cryptic/nocturnal behaviour”, “depth range” and “trophic category”, while “aggregative behaviour” and “habitat type” were mostly responsible for variation along the second PC axis.

Discussion

Several observational methods are routinely employed to describe biodiversity worldwide. Data collected are then used to test hypotheses on ecological processes and functioning. Although the power of most of the survey techniques in capturing marine taxon richness is relatively well understood, we know little about their suitability for describing the diverse functional attributes of fish assemblages. Here we investigated the ability of two observational (UVCt and BUV) and one fishery-related (SSFc) survey methods and one molecular approach (eDNA) to describe the taxonomic and functional diversity of coastal fish assemblages. We show that eDNA metabarcoding is more powerful in describing the functional attributes of

fish assemblages than other techniques. We attribute such feature to a substantial lack of selectivity towards any functional trait.

We applied standardized procedures for carrying out traditional visual observation in coastal environment. We also designed the eDNA sampling strategy with the aim of balancing the effort and the potential yield of useful information. Our results showed that levels of diversity saturation were different, with eDNA that could have probably benefited from a larger sample size in the case of taxonomic diversity, and all the techniques well approximating the asymptote for functional diversity accumulation curves.

The public reference sequences databases do lack for many Mediterranean fish species. We partially contributed to fill these knowledge gaps by adding new 12S rRNA barcodes data. This point remained perhaps the main source of bias leading to incomplete taxa detection by eDNA: approximately 40% of the 12S rRNA fish MOTUs have been discarded due to low sequence similarity with the records available in the databases. Despite these limitations, eDNA showed the ability to identify more taxa per sample than the other techniques, adding on previous evidences (Boussarie et al., 2018; Yamamoto et al., 2017), even if replicates of different methods are not formally comparable and need to be considered only as qualitative proxy of sampling effort. eDNA and SSFc also showed the highest variability of number of records per replicate compared to UVCt and BUV. This likely relates to the probability of detecting a species with the observational methods and the relative abundance of the recognized taxa in the environment. Indeed, UVCt and

BUV mostly rely on the detection of abundant and homogeneously distributed fish species, while rare or cryptic fish may have a lower probability to be detected (Colton & Swearer, 2010; Pais & Cabral, 2017). On the other hand, eDNA and SSFc exhibited a more pronounced ability to ‘capture’ rare and cryptic species and their susceptibility to variations of environmental conditions (Evans et al., 2017; Frid & Belmaker, 2019) can explain the variability of their outcomes.

In addition to the substantial differences in taxon detection power among the considered methods, we found also a pronounced compartmentalization of the information provided by each of them, supporting previous findings suggesting that different methods capture different subsets of biodiversity (Kelly et al., 2017; Stat et al., 2019). Indeed, excluding the pair UVC-BUV, the four methods turned out to be more complementary than convergent, each one contributing with exclusive findings to the overall fish diversity assessment. The observational techniques were mainly able to capture the most common Mediterranean reef fish families (Fig. 2), containing several congeneric species, such as sea breams (Sparidae), wrasses (Labridae) and combers/groupers (Serranidae) (Guidetti, 2000). This result likely pertains to their limited spatio-temporal scales compared with SSFc and eDNA, which in turn are able to capture the presence of additional taxa in longer times and from a wider area. Indeed, both methods can capture nocturnal species, since SSF fixed-nets often operate overnight and eDNA can detect genetic signal of organisms that have been in the area several hours

before water collection, depending on the environmental conditions driving the environmental DNA decay rate and sinking/resuspension processes (Barnes & Turner, 2016; Collins et al., 2018). Local oceanography is also accountable for the eDNA broader spatial context, since water movement may favour the transfer of environmental DNA from adjacent areas and habitats, even if different studies have proved good spatial resolution (Jeunen et al., 2019; Port et al., 2016; Yamamoto et al., 2017). This is a particularly important aspect for evaluating fish assemblage diversity in very patchy environments as Mediterranean coastal areas. Indeed, if eDNA has the advantage of simultaneously providing information on different habitats closely distributed in a certain area, the higher spatial fidelity of the observational methods might allow to assign species and traits more punctually to particular habitats.

As natural communities are composed of taxa with disparate levels of relatedness and ecological functions (Cardoso, Rigal, Borges, & Carvalho, 2014), a robust estimate of their complexity depends on the comprehensive collection of as many variants as possible. In this respect, we found for the first time the greater efficiency of eDNA in detecting fish functional diversity compared to the other methods (Fig.3 and Fig. 5), a competence only partially linked to its proficiency in detecting single species. Indeed, SSFc was more efficient in collecting taxonomic variants than eDNA and subsequently observational methods. By contrast, eDNA samples showed the highest functional diversity (RaoQ) values, the lowest levels of functional redundancy (FR) and were unique in showing estimates

of fish functional distinctness consistent with expectations (Fig. 5). We attribute these results to the link between taxonomic and functional diversity, strictly dependent on the redundancy of functional entities inside a given community (Micheli & Halpern, 2005), so that fractions of taxonomic diversity captured by different sampling systems are not necessarily reflected in functional diversity. At present, the non-quantitative nature of eDNA (Lamb et al., 2019) prevents a fully implementation of functional diversity indices as RaoQ, that should include the relative abundances of fish. However, even if this reduces the accuracy of the estimates, it does not nullify its informative content (Kim, Blomberg, & Pandolfi, 2018), which tends to be positively correlated to species richness when the functional redundancy inside the assemblage is lower (Granger et al., 2015). In addition to this, our findings suggest that the application of different survey methods may lead to different ecological conclusions when assessing ecological redundancies within fish assemblages. The lower functional redundancy we found using eDNA may have important implications for reliable predictions of the effects of biodiversity loss on the functioning of Mediterranean marine ecosystems, and the consequent management and conservation interventions. By contrast, the higher levels of functional redundancies detected by the observational methods would rather suggest that ecosystem functions might be robust to changes in diversity, likely not reflecting the real situation of the overexploited, and highly disturbed Mediterranean marine ecosystems (Coll et al., 2010).

Potential method-specific functional selectivity might be a major factor in shaping the distribution of functional traits within fish assemblages. As such, we suggest that the selectivity of traditional survey tools, and the consequent imbalanced proportion of traits in the estimated fish assemblages, might be accountable for their low functional representation. The active selection of certain traits by specific fishing gears, fish behavioural characteristics, or the use of a bait might intrinsically generate functional redundancies within fish assemblages, consequently reducing their functional diversity levels even when taxonomic diversity is high. UVCt and BUV selectivity for shallow-dwelling and for mobile bentho-pelagic species, as well as the tendency of SSFc to collect preferentially benthic fish with a broad depth range and large predators, arguably represent intrinsic features of these traditional methodologies such as operational time (day vs night), depth, and employed gears. UVCt and BUV resulted less suitable for detecting “cryptic” species, corroborating previous evidence that a substrate-blending coloration and an inconspicuous behaviour could be accountable for the low proportion of benthic fish, while colourful and curious bentho-pelagic species are more easily detected (Willis, 2001). Similarly, highly mobile pelagic species were underrepresented in UVCt, arguably due to fish behavioural response to divers, other than the limited width of the strip transects (Prato, Thiriet, Di Franco, & Francour, 2017; Watson, Carlos, & Samoilys, 1995). As for SSFc, since the probability to catch fish of different size ranges varies with the mesh size (Hubert, Pope, & Dettmers, 2012), the only way to reduce the

fish size bias would be the simultaneous use of gears with a wide range of net mesh sizes. Yet, adopting such a strategy would increase efforts and environmental impact of the surveys. Collecting fish data from fishers may represent a low-cost option for diversity assessments, as small-scale fishery commercial operations are usually carried out regardless of scientific purposes.

As opposed to visual and capture-based methods, eDNA does not imply any kind of selection other than the presence of genetic material in the sampled medium, as suggested by the neutrality of eDNA data with respect to the first PC axis. Technical, bio-molecular and biochemical factors also play a role in determining accuracy and completeness of metabarcoding biodiversity estimates (Zinger et al., 2019). In addition to this, abundant species should be more represented inside an ideal eDNA sample (Takahara, Minamoto, Yamanaka, Doi, & Kawabata, 2012). This relationship still needs to be fully validated in order to confer a quantitative power to eDNA (Lamb et al., 2019), but a certain proportionality between eDNA abundance and detection probability is recognized (Lacoursière-Roussel, Rosabal, & Bernatchez, 2016). In this regard, our study indirectly supports such assertions, as half of the traits discriminating between eDNA and traditional methods were related to fish schooling behaviours. It is reasonable to expect that schooling species, releasing high amounts of DNA, may be more easily detected.

In summary, we show that the choice of the survey method can influence the ecological conclusions of biodiversity studies and that eDNA is well set

to capture most of the functional fish diversity of coastal marine environments. Beside the advantages in terms of sampling ease and emancipation from taxonomic expertise, this new generation monitoring tool appears now geared to boost the collection of complex information from marine environments, including their functional dimension. We recognize the generalization of our approach still needs to be tested in other environmental and geographical settings, or using different biological descriptors. We are also aware that the full eDNA potential in providing accurate taxonomic and functional diversity estimates can be reached only by lending a quantitative power to the technique. Until that time, association of eDNA with some of the traditional quantitative methods is advisable.

This study also provides a thorough outlook on the pitfall we might encounter trying to obtain robust marine diversity estimates upon which our understanding of the functioning of marine ecological systems greatly depend (Mouillot et al., 2014; Stuart-Smith et al., 2013). As eDNA analysis continues to develop beyond its original descriptive nature (Djurhuus et al., 2020), these new, diverse pathways of investigations promise to significantly enhance our ability to understand, interpret and ultimately sustainably manage the ocean and its resources.

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Data Accessibility

Mediterranean Fish DNA barcodes (Tele02 fragment): NCBI accession numbers (from MT903884 to MT903923) are provided in detail in supporting information, Table S3.

12S and COI raw data and Presence-Absence datasets are available at the public repository Driad: https://doi.org/10.5061/dryad.5qfttdz30

Author Contributions

G.A, M.M. S.M. designed research; G.A, G.T., C.C., D.S., A.D.F., A.C., M.D.L, P.G. performed field work; G.A, C.B. performed laboratory work; G.T., C.C. performed video analyses; G.A, C.B., A.C. analyzed data; G.A. and M.M. wrote the paper with input from all co-authors.

Figures captions

Figure 1. Map of study areas in the Central and the Western Mediterranean Sea (black dots). Each name identifies an MPA and its flanking unprotected location. Lollipop charts on the right side display the number of taxa (upper chart) and unique traits combinations (UTCs – upside down lower chart). Overall number of taxa and UTCs are displayed in the smaller chart on the upper left side of the map. BF: Bonifacio; CP: Cabo de Palos; CR: Cap Roux; CB: Côte Bleue; EG: Egadi Islands; EF: Es Freus; PF: Portofino; ST: Strunjan; TS: Telašćica; TG: Torre Guaceto; ZK: Zákynthos.

Figure 2. UpSet plot displaying the fish families detected by each of the four applied techniques. Horizontal bars, coupled with lines and dots on the left panel, show the exclusive families for each method and the intersection among them. The set size histograms show the overall yield of each sampling method. Fish images are modified free of right pictures. Sources: *NOAA photo library, Wikipedia, Rawpixel public domain, Fionasplace.net, Freepng.es, CleanPNG.com, Freepik.com, Wpclipart.com, Pngimag.com, NicePNG.com*

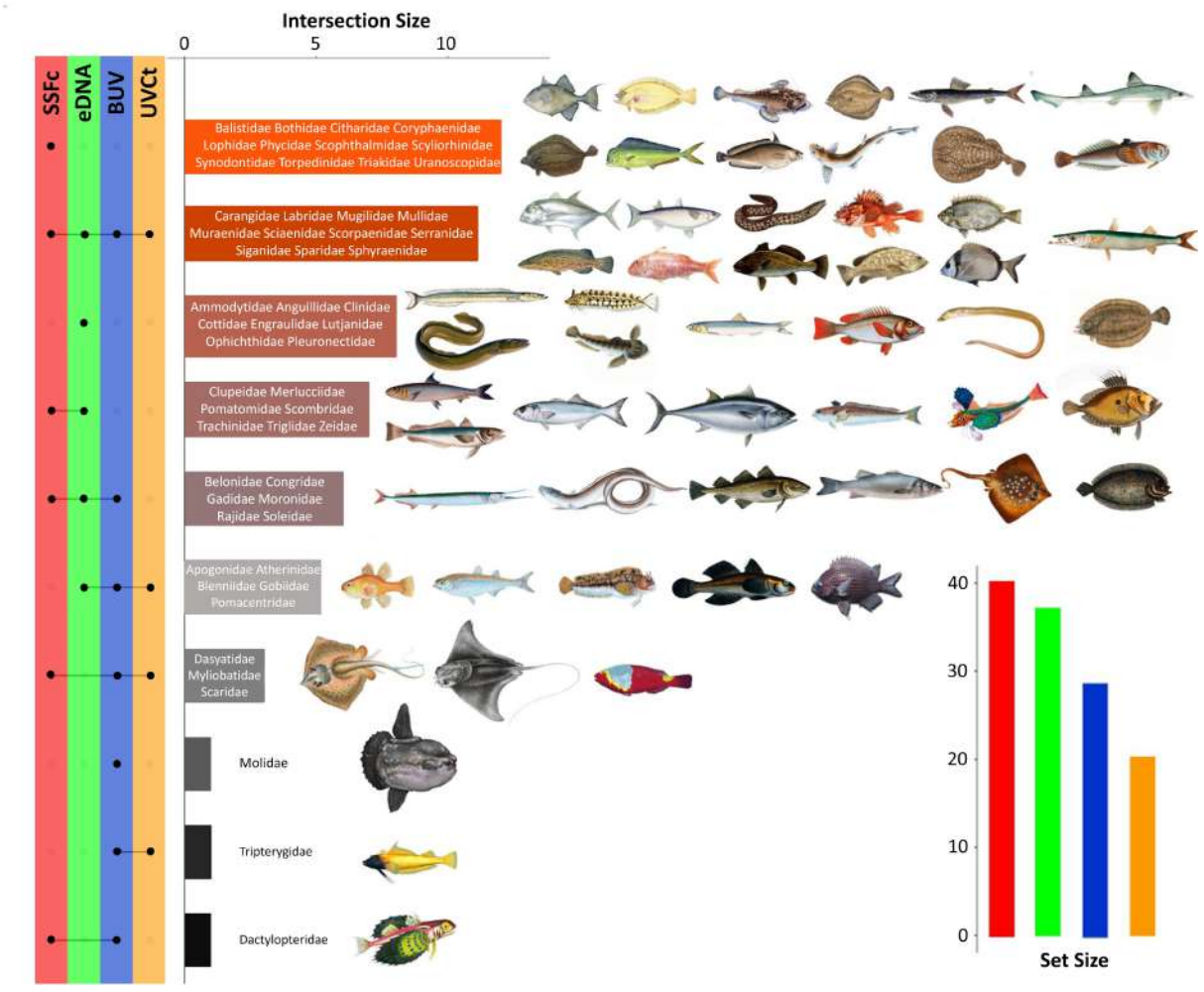


Figure 3. Taxa and Unique traits combinations accumulation curves. The zoomed plots in the insets help visualizing the more rapid accumulation of taxa and trait combinations achieved through eDNA analyses. Multi-method accumulation curves have been plotted together for graphical reasons.

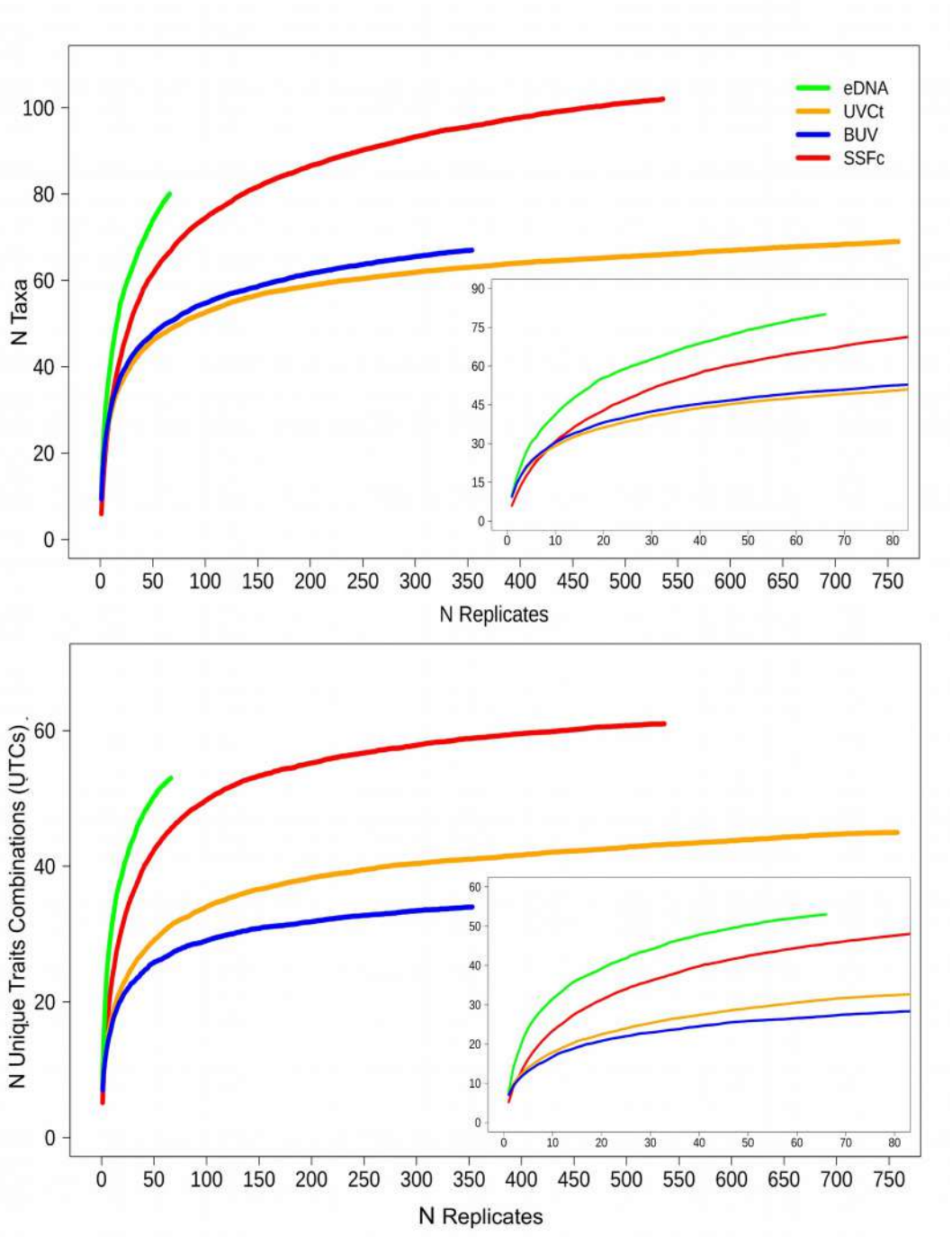


Figure 4. Non-metric multidimensional scaling (nMDS) based on Jaccard similarity index of the composition of fish functional traits recorded in MPAs and their flanking unprotected locations (i.e., overall 22 locations) by eDNA, BUV, UVCt and SSFc. Lines connect each point to the centroid; ellipses represent the SD of point scores.

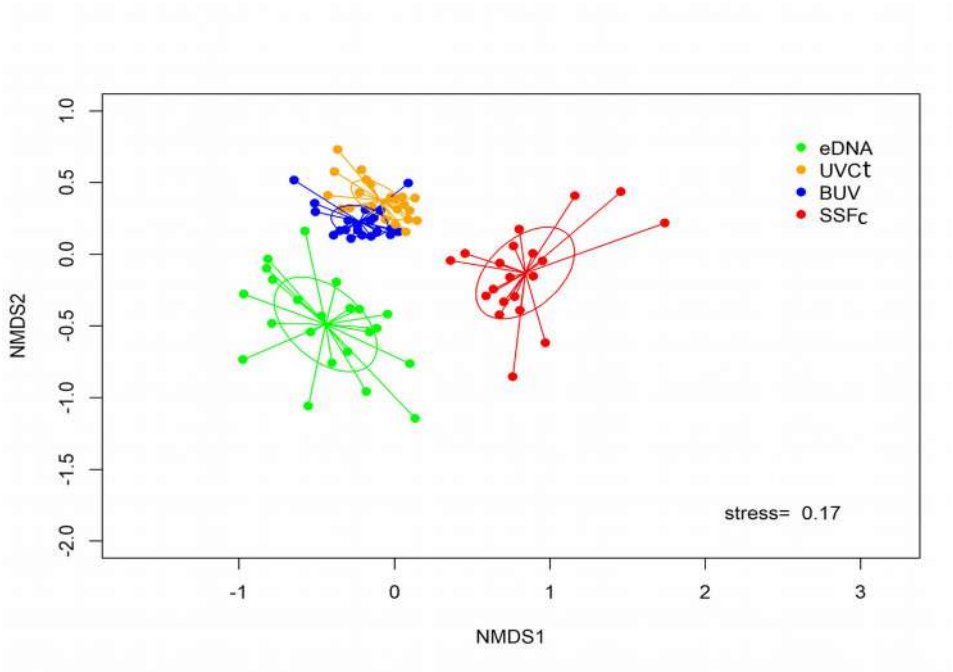


Figure 5. Funnel plots of Average taxonomic distinctness (AvTD) (A.) and Average functional distinctness (AvFD) (B.) of the fish assemblages recorded in eleven Mediterranean MPAs and eleven unprotected zones by eDNA, BUV, UVCt and SSFc. Dotted lines and dashed lines delimit respectively the 95% and 99% confidence interval areas of the expected diversity distributions based on all species detected across methods and locations. Box plots displays overall Rao's quadratic entropy (RaoQ) (C.) and Functional Redundancy (FR) (D.) estimates for each method.

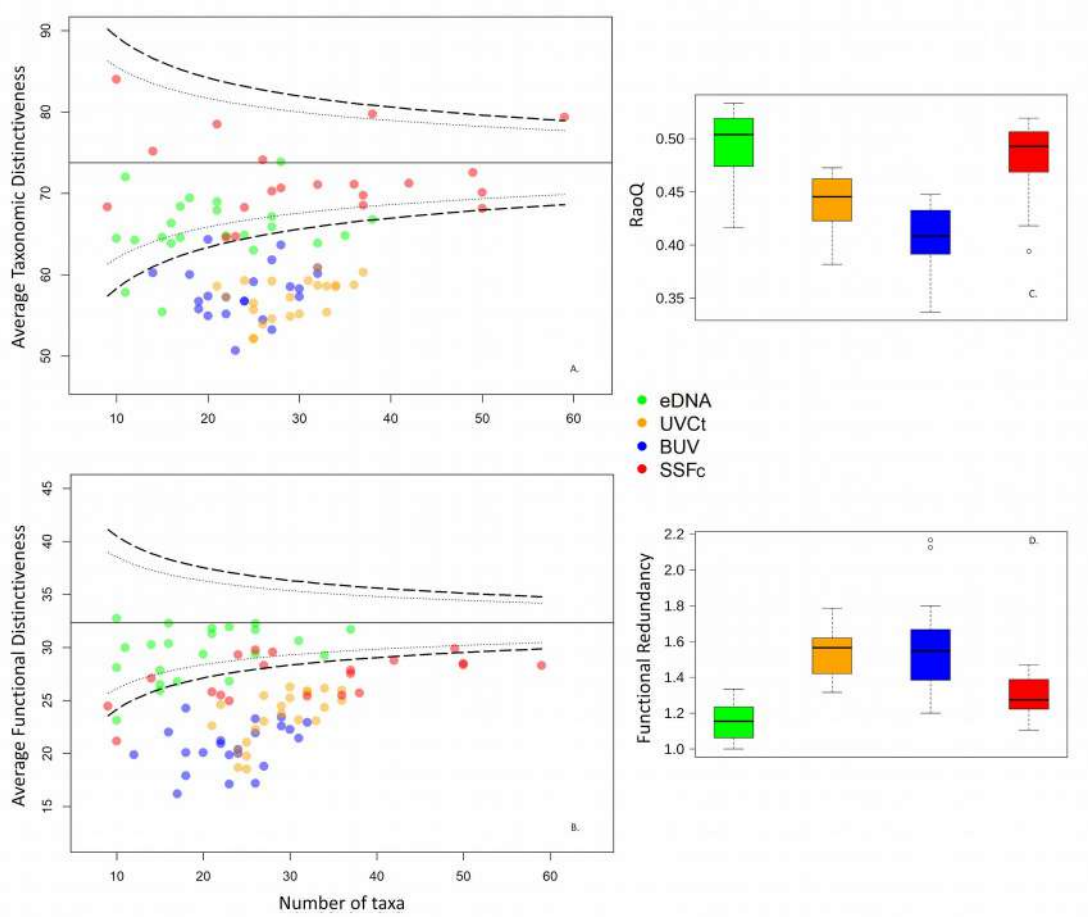
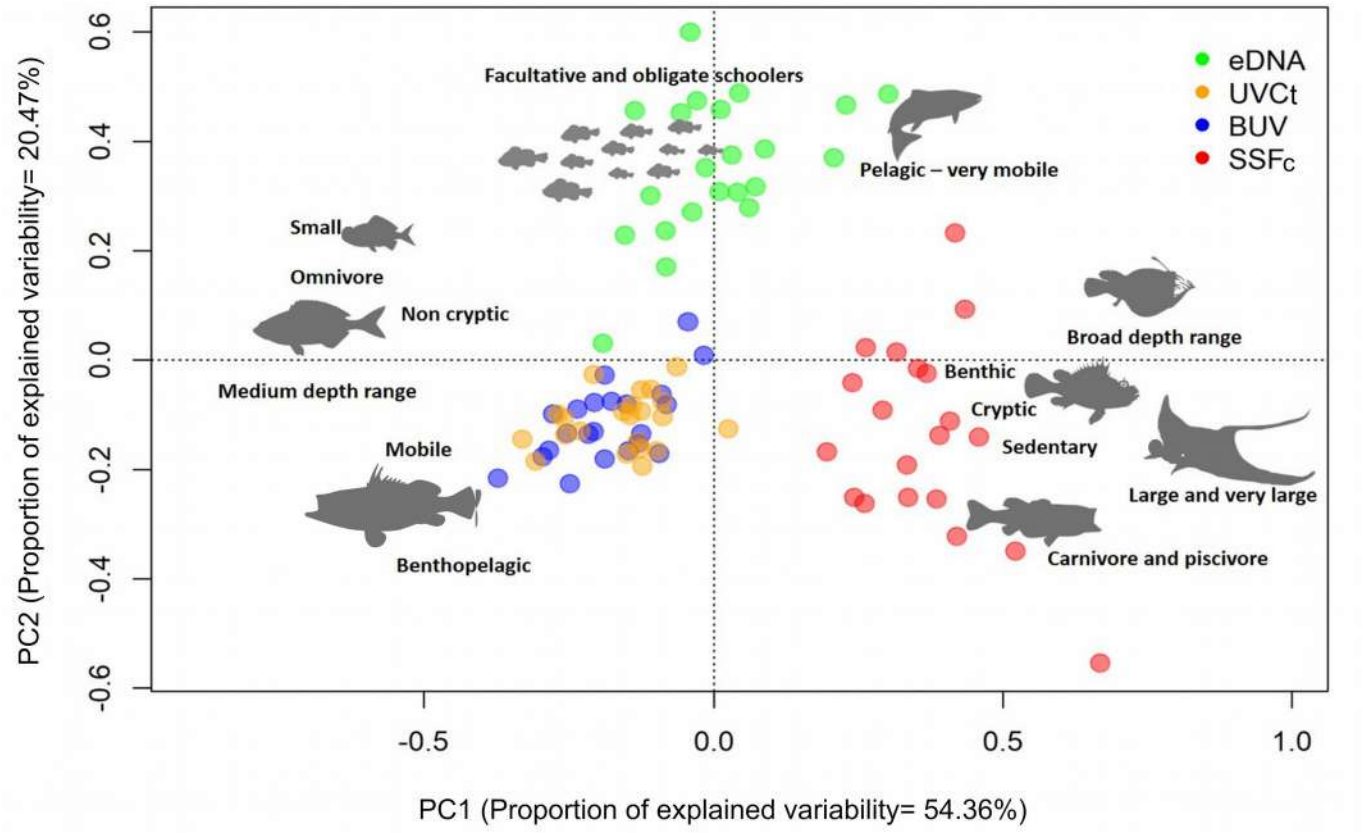


Figure 6. Principal Component Analysis (PCA) of the functional traits proportions of fish assemblages identified by eDNA, UVCt, BUV and SSFc techniques. The first four dimensions of the PCA cumulatively explained 88.24% of the projected inertia in the distribution of fish species traits, 74.82% of which was explained by the first two axes. Each point refers to samples collected in MPAs and their flanking unprotected locations (i.e., a total of 22 locations). Correlations with main fish traits (represented by different fish shapes) are also super-imposed. The original PCA graph is provided in Fig. S5. Fish shapes are modified free of rights images. Sources: *flyclipart.com, cleanpng.com, www.shareicon.net, netclipart.com, publicdomainvectors.org.*


eDNA
UVCt
BUV
SSFc
Facultative and obligate schoolers
Pelagic – very mobile
Small
Omnivore
Non cryptic
Medium depth range
Broad depth range
Benthic
Cryptic
Sedentary
Large and very large
Mobile
Benthopelagic
Carnivore and piscivore
PC2 (Proportion of explained variability= 20.47%)
PC1 (Proportion of explained variability= 54.36%)
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.5
0.0
0.5
1.0