

1Environmental DNA effectively captures functional diversity of

2coastal fish communities

3Giorgio Aglieri^{a,b*}, Charles Baillie^c, Stefano Mariani^d, Carlo Cattano^{a,b,e},
4Antonio Calò^{a,f}, Gabriele Turco^{a,b}, Davide Spatafora^{a,b}, Antonio Di Franco^{e,f},
5Manfredi Di Lorenzo^h, Paolo Guidetti^{b,f,g}, Marco Milazzo^{a,b*}.

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7a - Department of Earth and Marine Sciences (DiSTeM), University of
8Palermo, via Archirafi 20-22, 90123, Palermo, Italy

9b - Consorzio Nazionale Interuniversitario per le Scienze del Mare
10(CoNISMa), Piazzale Flaminio 9, 00196 Roma, Italy

11c - School of Environment & Life Sciences, University of Salford, The
12Crescent, Salford, M5 4WT

13d - School of Biological & Environmental Sciences, Liverpool John Moores
14University, Byrom Street, Liverpool L3 3AF, UK

15e - Department of Integrative Marine Ecology, Sicily, Stazione Zoologica
16Anton Dohrn, Lungomare Cristoforo Colombo (complesso Roosevelt),
1790149, Palermo, Italy

18f - Université Côte d'Azur, CNRS, UMR 7035 ECOSEAS, Parc Valrose 28,
19Avenue Valrose, 06108 Nice, France

20h - Institute for Biological Resources and Marine Biotechnologies, National
21Research Council (IRBIM-CNR), Via L. Vaccara, Mazara del Vallo 61-91026,
22Italy

23g - Department of Integrative Marine Ecology, Stazione Zoologica Anton
24Dohrn , Villa Comunale, 80121, Naples, Italy

25* Corresponding authors: Giorgio Aglieri, Department of Earth and Marine
26Sciences (DiSTeM), University of Palermo, via Archirafi 20-22, 90123,
27Palermo, Italy. Tel. +39.091.2386286; giorgioaglieri@hotmail.com

28Marco Milazzo, Department of Earth and Marine Sciences (DiSTeM),
29University of Palermo, via Archirafi 20-22, 90123, Palermo, Italy. Tel.
30+39.091.2386286; marco.milazzo@unipa.it

31**Abstract**

32Robust assessments of taxonomic and functional diversity are essential
33components of research programs aimed at understanding current
34biodiversity patterns and forecasting trajectories of ecological changes.
35Yet, evaluating marine biodiversity along its dimensions is challenging and
36dependent on the power and accuracy of the available data collection
37methods. Here we combine three traditional survey methodologies
38[Underwater Visual Census strip transects (UVCT), Baited Underwater
39Videos (BUVs) and Small Scale Fishery catches (SSFc)], and one novel
40molecular technique [eDNA metabarcoding (eDNA) – 12S rRNA and
41Cytochrome Oxidase Subunit 1 (COI)] to investigate their efficiency and
42complementarity in assessing fish diversity. We analysed 1,716 multi-
43method replicates at a basin scale to measure taxonomic and functional
44diversity of Mediterranean fish assemblages. Taxonomic identities were
45investigated at species, genus and family level. Functional identities were
46assessed using combinations of morphological, behavioral and trophic
47traits. We show that: i) SSFc provided the higher taxonomic diversity
48estimates followed by eDNA, and then UVCT and BUV; ii) eDNA was the
49only method able to gather the whole spectrum of considered functional
50traits, showing the most functionally diversified and least redundant fish
51assemblages; iii) the eDNA effectiveness in describing functional structure
52reflected its lack of selectivity toward any considered functional trait. Our
53findings suggest that the reach of environmental DNA analysis stretches

54beyond taxon detection efficiency and provides new insights about the
55potential of metabarcoding in ecological studies.

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58**Introduction**

59Under extreme biodiversity loss, our ability to quantify the magnitude and
60to forecast the direction of ecological change is imperative to foster sound
61conservation strategies, maintaining functional ecosystems, and ensuring
62nature's contributions to people (Díaz et al., 2018; Halpern et al., 2019;
63Mace, Norris, & Fitter, 2012). At the most fundamental level, such
64knowledge is dependent upon the reliability of investigations on taxonomic
65and functional diversity of ecological systems (Loreau, Naeem, &
66Inchausti, 2002; Micheli & Halpern, 2005). Yet, obtaining comprehensive
67estimates of biodiversity patterns is challenging. Practically, the breadth of
68such assessments are contingent to the availability of funds, time and the
69sampling techniques used (Moore & McCarthy, 2016), and no existing
70method for taxonomic and functional biodiversity estimation is unbiased
71(MacNeil et al., 2008).

72In marine ecosystems, reef fishes are considered to be well suited for
73global marine biodiversity studies (Mouillot et al., 2014; Pimm et al.,
742014), as they are highly diverse among vertebrates, cover a wide range
75of ecological functions, and their taxonomy and biological and ecological
76traits 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

101(Hansen, Bekkevold, Clausen, & Nielsen, 2018), eDNA is efficient in
102detecting transient, rare and low abundance aquatic species (Boussarie et
103al., 2018; Sigsgaard, Carl, Møller, & Thomsen, 2015; Thomsen et al.,
1042012).

105Although most biodiversity surveys typically emphasise measures of
106taxonomic diversity, the range of functions that organisms perform in a
107given community is considered a more responsive descriptor than species
108diversity to understand how ecosystems respond to natural and
109anthropogenic perturbations (D'agata et al., 2014). Yet, we know nothing
110about the ability of available survey methods in capturing functional
111diversity. Here we present findings of a large-scale field study – spanning
112the Central and Western Mediterranean Sea – aimed at investigating the
113proficiency of direct observational techniques, a fishery-dependent
114method, and a molecular approach in quantifying taxonomic and
115functional diversity of coastal fish. Our aims were to: (i) assess the relative
116performance and complementarity of traditional UVCT, BUV and SSF_c
117(fixed-net) and eDNA metabarcoding (12S rDNA and Cytochrome Oxidase
118Subunit 1 markers) survey techniques in detecting reef fish diversity; (ii)
119infer the contribution of each monitoring technique to the overall
120taxonomic and functional diversity of the fish assemblages; (iii) evaluate
121the selectivity of each survey methodology for specific fish functional
122traits.

123To achieve these goals and to increase the representativeness of regional
124reef 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),

148Croatia (Telašćica Nature Park and Dugi-Otok island) and Slovenia
149(Strunjan Landscape Park and adjacent coast) (Fig. 1- Table S1). Photo-
150sampling of small-scale fisheries catches (SSFc) using fixed-nets in the
151same locations was also carried out at landings between May and
152September 2018. Sampling operations at sea were carried out by two
153separate teams, one for UVCt and eDNA water sampling and one for the
154BUV systems. Separation between the two teams was necessary to avoid
155possible onboard and underwater contamination of the eDNA samples
156from the BUV baits. In order to reduce the time window between
157collection, filtration and sample storage, the eDNA water samples were
158collected at the end of each working day. eDNA samples were collected
159within 500 meters from the sites where UVC and/or BUV sampling was
160carried out.

161UVC strip transects were carried out between 8:00 a.m. and 3:00 p.m.
162inside and outside each MPA by the same three trained diving operators.
163We haphazardly selected two sites for each protection level (no-take zone,
164buffer zone and unprotected area) and carried out between 8 and 13
165replicated transects per site [on average 11.54 ± 0.41 (mean \pm s.e.)]
166depending on the spatial extent of each site and on the availability of
167suitable habitat (rocky, from 5 to 15 meters). Each replicate consisted of a
168strip transect of 125 m² (25x5 m), conducted at 5-12m depth on rocky
169substrates. Overall, we carried out 760 UVCt replicates (Table S1).

170BUVs consisted of the deployment of a steel structure equipped with two
171stereo-cameras and a plastic container containing a standard bait (e.g.

172~500 gr of gilt sardines, *Sardinella aurita*) to attract fish. Two operators
173deployed the BUVs on rocky bottoms from the boat for 60 minutes
174between 8:00 a.m. and 3:00 p.m. within a depth range of 5-15 m. To avoid
175the repeated recording of the same individuals, BUVs were deployed at
176>150 meters apart (Whitmarsh, Fairweather, & Huveneers, 2017).
177Depending on the spatial extent of the areas, a minimum of three and a
178maximum of six replicates [on average 5.36 ± 0.11 (mean \pm s.e.)] were
179carried out in each of the two sites haphazardly chosen within each MPA
180protection level and surrounding areas. Overall, we deployed 354 BUVs
181(Table S1). Videos were then analyzed recording all the fish observed in
182the field of view of the two cameras.

183SSFc were recorded taking pictures of each catch, placing the fish on a flat
184white plastic surface along with a ruler as length reference. Each picture
185was associated to a unique identifier of the fishing catch and associated to
186the geo-coordinates of the haul. The number of replicates varied among
187locations, ranging from 17 replicates in Telašćica Nature Park and Dugi-
188Otok island to 82 replicates in Egadi Islands MPA and Trapani coast. No
189SSFc data was collected in Strunjan Landscape Park and adjacent coast.
190Overall, 536 SSF landings were recorded (Table S1).

191eDNA samples consisted of four liters of water (two liters from the surface
192and 2 liters at one meter from the bottom, max depth -20 m) collected
193from the boat and by scuba diving in three haphazardly chosen sites inside
194and three outside each MPA for a total of 66 replicates (Table S1). After
195collection, 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 μ l, composed by 10 μ l Amplitaq Gold Master Mix (Thermo Fisher Scientific), 0.16 μ l BSA, 1 μ l of 5 μ M forward primer, 1 μ l of 5 μ M reverse primer, 10 ng of eDNA template and 5.84 μ l 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,

244since the highly degenerated primers used are known to primarily amplify
245micro-eukaryotes and invertebrates and we expected a low yield for fish
246(Collins et al., 2019). We included a subset of replicates for all the
247considered locations and all the negative controls into another
248independent sequencing project. We selected the samples with the
249highest amount of extracted DNA. Amplification was performed using the
250Leray-XT primer set (Wangensteen, Palacín, Guardiola, & Turon, 2018) and
251the PCR profile suggested by the authors. High-depth sequencing was
252carried out in a HiSeq4000 to maximise recovery of rare vertebrate reads.

253*Mediterranean fish species DNA barcoding*

254We collected small fin clip pieces of 25 Mediterranean fish species in local
255fish markets and preserved them in 96% ethanol at -20 C. We extracted
256the DNA using the DNeasy Blood & Tissue Kit (QIAGEN) following the
257manufacturers' protocol. We amplified the "tele02" 12S rRNA fragment
258applying the same conditions reported for eDNA metabarcoding, purified
259the PCR products with the QIAquick PCR Purification Kit (QIAGEN) and
260outsourced the Sanger sequencing to Macrogen Europe (ABI 3730XLs).
261Sequences were edited with BioEdit v.7.2 (Alzohairy, 2011).

262*Bioinformatic analyses*

263We processed the sequence reads using the OBITools v.1.01 12
264metabarcoding software suite (Boyer et al., 2016). Libraries were
265demultiplexed with 'bcl2fastq v. 2.20' (Illumina), before assessing read
266quality using FastQC v.0.11.7 (<http://www.bioinformatics.babraham.ac.uk/>)

267projects/fastqc/). Reads were trimmed to a length so each base was, on
268average, above a score of Q30 using 'obicut'. Paired-end reads were
269aligned using 'illumina pairedend', retaining alignments with a quality
270score >40. Sample demultiplexing was performed with 'ngsfilter' for each
271library. Samples from 12S libraries were concatenated and the sequences
272length filtered (140-180 bp) using 'obigrep' to select only fragment lengths
273known to amplify with our primers. The COI library was filtered between
274303bp and 323 bp. Reads containing ambiguous bases were also removed.
275Remaining reads were de-replicated using "obiuniq" and chimeras were
276removed with the 'uchime-denovo' algorithm (Edgar, Haas, Clemente,
277Quince, & Knight, 2011) implemented in vsearch v.1.9 (Rognes, Flouri,
278Nichols, Quince, & Mahé, 2016). Sequence clustering was performed using
279'swarm' v.2.0 (Mahé, Rognes, Quince, Vargas, & Dunthorn, 2014) with a d-
280value of 2 for 12S, and 13 for COI. As suggested by the authors, the choice
281of "d" was made after testing the outcomes of different values.
282Identification and exclusion of potential contamination was achieved by
283including field blanks, eDNA extraction blanks and PCR blanks. We
284removed the reads present in the negative controls from the respective
285samples. All singletons were discarded.

286*Taxonomy assignment*

287We performed the taxonomic assignment of 12S sequences representing
288each Molecular Taxonomic Unit (MOTU) using 'ecotag' against a curated
289database (<https://github.com/boopsboops/reference-libraries>). Since
290several Mediterranean fish species were missing in public databases, we

291 complemented our custom 12S database with 'Teleo02' sequences of 25
292 common coastal species (Table S3). The taxonomic assignment for the COI
293 marker was performed against the db_COI_MBPK database (Bakker et al.,
294 2019) (<http://github.com/metabarpark/Reference-databases>). Each
295 Molecular taxonomic unit (MOTU) was assigned to a single species when
296 this was the only Mediterranean species with a sequence similarity >97%,
297 a cut-off value that optimizes the recovery of species composition of the
298 studied environment avoiding erroneous taxonomic assignments (Miya et
299 al., 2015). For the COI marker, the similarity threshold used was
300 conservatively increased to 99%, since identifications below 98% of
301 identity with COI markers could be error prone (Clare, Barber, Sweeney,
302 Hebert, & Fenton, 2011). Ambiguous automatic assignments were
303 manually checked through a BLAST search against the NCBI
304 (<https://www.ncbi.nlm.nih.gov/>) and MitoFish ([http://mitofish.aori.u-](http://mitofish.aori.u-tokyo.ac.jp/)
305 [tokyo.ac.jp/](http://mitofish.aori.u-tokyo.ac.jp/)) databases for 12S, and NCBI and BOLD
306 (http://v3.boldsystems.org/index.php/IDS_OpenIdEngine) for COI. If
307 appropriate, we applied an assignment correction accordingly to the up to
308 date knowledge of the species distribution in the Mediterranean Sea
309 provided by FishBase ([https://www.fishbase.se/trophiceco/FishEcoList.php?](https://www.fishbase.se/trophiceco/FishEcoList.php?ve_code=13)
310 [ve_code=13](https://www.fishbase.se/trophiceco/FishEcoList.php?ve_code=13)), following these criteria:

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

314 **2.** In case of a MOTU assigned to a non-Mediterranean taxon despite
315 closely related Mediterranean taxa showed a sequence similarity
316 within the 97-99% threshold -> the assignment was corrected
317 including the Mediterranean taxa at the lowest possible taxonomic
318 rank.

319 **3.** In case of a taxonomic assignment at family or genus level when a
320 single Mediterranean species had an equivalent % of similarity to a
321 non-Mediterranean species belonging to the same genus or family -
322 > the assignment was corrected excluding the non-Mediterranean
323 taxon.

324 **4.** In case of a record assigned to a non-Mediterranean taxon because
325 of a lack of reference sequences for Mediterranean congeneric
326 species -> the assignment was corrected only in presence of a
327 single Mediterranean congeneric species

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

330 *Statistical analyses*

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

336behaviour; d) mobility; e) habitat type; f) aggregation behaviour; g) trophic
337habit (Table S4).

338We performed all the statistical analyses in R V. 3.5.2 (R; [http://www.R-](http://www.R-project.org)
339project.org). Taxa and UTCs accumulation curves were generated using
340the 'specaccum' function implemented in the package 'vegan' v.2.5-5
341(Oksanen et al., 2019), applying the 'random' method and 1000
342permutations. Intersections among the datasets generated by the four
343different sampling methods were represented using 'UpSetR' package v.
3441.4.0 (Conway, Lex, & Gehlenborg, 2017). Non-metric multidimensional
345scaling (nMDS) of similarities (Jaccard) among fish assemblages (taxa and
346UTCs) was performed with the 'metaMDS' function implemented in
347'vegan'. We took specific precautions to avoid bias due to the uneven
348taxonomic resolution of the different methods. Indeed, some taxa
349identified at the genus or family level could be potentially redundant
350whenever other members of the same genus or family were present in the
351dataset. For this reason, we rearranged the taxa dataset condensing all
352the possibly redundant identifications among different sampling methods
353into higher taxonomic ranks (Table S5).

354The relative contribution of each sampling technique to the global
355diversity estimate was analyzed using four measures of diversity: Average
356Taxonomic Distinctiveness (AvTD) (Clarke & Warwick, 1998), Average
357Functional Distinctiveness (AvFD) (Somerfield, Clarke, Warwick, & Dulvy,
3582008), Rao's quadratic entropy (RaoQ) (Botta-Dukát, 2005) (SI Appendix)
359and 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

408number of reads ranged from 3 to 196,306, with a mean value of 21,541
409reads.

410The Illumina MiSeq run of the COI amplicon library produced 45,901,040
411reads. After bioinformatic filtering, we obtained 24,242,469 reads of the
412samples included in the present study. As expected, most of the sequence
413reads belonged to non-fish taxa. In all, 99,071 reads were assigned to fish
414taxa. The number of COI reads belonging to Mediterranean fish taxa per
415sample ranged from zero (three samples failed) to 48,329 with a mean
416value of 1,479 reads.

417Overall, we identified 159 fish taxa belonging to 100 genera and 55
418families (Table S7), and 91 unique traits combinations (UTCs) at functional
419level, with unequal contributions, provided by different survey techniques
420to locally observed taxonomic and functional richness (Fig. 1). eDNA
421detected 79 different fish taxa (74 at species and 5 at genus level)
422belonging to 65 genera and 37 families, and 53 UTCs. UVCT, BUV and SSFc
423identified respectively 69 taxa (66 at species, two at genus and one at
424family level; 40 genera and 20 families) and 45 UTCs, 66 taxa (57 at
425species, 6 at genus and 3 at family level; 41 genera and 28 families) and
42634 UTCs, and 102 taxa (all at species level; 67 genera and 40 families) and
42761 UTCs. The four different methods shared 14.46% of species (23), 22%
428of genera (22) and 20% of families (Fig. 2, S1, S2). SSFc recorded the
429highest proportion of exclusive records (25.16%, 40 taxa), followed by
430eDNA (17.61%; 28 taxa), UVCT (7.55%; 12 taxa) and BUV (2.52%; 4 taxa).
431Overall, 42.85% of UTCs (39) was not shared between any of the survey

432 techniques. SSFc showed the highest proportion of exclusive UTCs
433 (18.68%, 17 UTCs), followed by eDNA (14.29%; 13), UVct (7.69%; 7) and
434 BUV (2.20%; 2) (Fig. S3).

435 On average, eDNA performed better in detecting a higher number of fish
436 taxa and trait combinations per replicate (10.76 ± 7.15 and 8.73 ± 5.55 ,
437 respectively; $N=66$; mean \pm SD) than BUV (Taxa: 9.86 ± 3.46 ; UTCs: 7.13
438 ± 2.37 ; $N=354$), UVct (Taxa: 9.13 ± 2.88 ; UTCs: 7.33 ± 1.92 ; $N=760$) and
439 SSFc (Taxa: 5.51 ± 3.81 ; UTCs: 5.16 ± 3.38 ; $N=536$) as shown by the taxa
440 and UTCs accumulation curves (Fig.3).

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

447 The Average Taxonomic Distinctiveness (AvTD) estimates returned
448 significantly different variances among survey methods, with pairwise
449 comparisons being also highly significant ($p < 0.001$), except for the UVct-
450 BUV comparison (Table S8). AvTD funnel plot shows that the highest
451 proportion of taxonomic breadth of the identified pool of Mediterranean
452 coastal fish was provided by SSFc, with most of the samples matching the
453 expected distribution at 95% C.I., and, to a lesser extent, by eDNA (few
454 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 ($\text{AvFD} = 29.53 \pm 2.47$; Mean \pm 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 ($\text{AvFD} = 20.62 \pm 2.15$). UVCT produced functionally broader samples than BUV, performing slightly better in detecting functional than taxonomical diversity ($\text{AvFD} = 23.41 \pm 2.38$). Despite the larger taxonomic diversity observed, SSFc data returned AvFD estimates below expectations ($\text{AvFD} = 27.07 \pm 2.17$) (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 ($\text{RaoQ} = 0.50 \pm 0.03$; Mean \pm SD), which were similar to SSFc (0.48 ± 0.03), but significantly different from UVCT ($\text{RaoQ} = 0.44 \pm 0.03$) and BUV ($\text{RaoQ} = 0.41 \pm 0.03$) (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

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

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

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

525BUV mostly rely on the detection of abundant and homogeneously
526distributed fish species, while rare or cryptic fish may have a lower
527probability to be detected (Colton & Swearer, 2010; Pais & Cabral, 2017).
528On the other hand, eDNA and SSFc exhibited a more pronounced ability to
529‘capture’ rare and cryptic species and their susceptibility to variations of
530environmental conditions (Evans et al., 2017; Frid & Belmaker, 2019) can
531explain the variability of their outcomes.

532In addition to the substantial differences in taxon detection power among
533the considered methods, we found also a pronounced
534compartmentalization of the information provided by each of them,
535supporting previous findings suggesting that different methods capture
536different subsets of biodiversity (Kelly et al., 2017; Stat et al., 2019).
537Indeed, excluding the pair UVC-BUV, the four methods turned out to be
538more complementary than convergent, each one contributing with
539exclusive findings to the overall fish diversity assessment. The
540observational techniques were mainly able to capture the most common
541Mediterranean reef fish families (Fig. 2), containing several congeneric
542species, such as sea breams (Sparidae), wrasses (Labridae) and
543combers/groupers (Serranidae) (Guidetti, 2000). This result likely pertains
544to their limited spatio-temporal scales compared with SSFc and eDNA,
545which in turn are able to capture the presence of additional taxa in longer
546times and from a wider area. Indeed, both methods can capture nocturnal
547species, since SSF fixed-nets often operate overnight and eDNA can detect
548genetic signal of organisms that have been in the area several hours

549before water collection, depending on the environmental conditions
550driving the environmental DNA decay rate and sinking/resuspension
551processes (Barnes & Turner, 2016; Collins et al., 2018). Local
552oceanography is also accountable for the eDNA broader spatial context,
553since water movement may favour the transfer of environmental DNA
554from adjacent areas and habitats, even if different studies have proved
555good spatial resolution (Jeunen et al., 2019; Port et al., 2016; Yamamoto et
556al., 2017). This is a particularly important aspect for evaluating fish
557assemblage diversity in very patchy environments as Mediterranean
558coastal areas. Indeed, if eDNA has the advantage of simultaneously
559providing information on different habitats closely distributed in a certain
560area, the higher spatial fidelity of the observational methods might allow
561to assign species and traits more punctually to particular habitats.

562As natural communities are composed of taxa with disparate levels of
563relatedness and ecological functions (Cardoso, Rigal, Borges, & Carvalho,
5642014), a robust estimate of their complexity depends on the
565comprehensive collection of as many variants as possible. In this respect,
566we found for the first time the greater efficiency of eDNA in detecting fish
567functional diversity compared to the other methods (Fig.3 and Fig. 5), a
568competence only partially linked to its proficiency in detecting single
569species. Indeed, SSFc was more efficient in collecting taxonomic variants
570than eDNA and subsequently observational methods. By contrast, eDNA
571samples showed the highest functional diversity (RaoQ) values, the lowest
572levels of functional redundancy (FR) and were unique in showing estimates

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

621fish size bias would be the simultaneous use of gears with a wide range of
622net mesh sizes. Yet, adopting such a strategy would increase efforts and
623environmental impact of the surveys. Collecting fish data from fishers may
624represent a low-cost option for diversity assessments, as small-scale
625fishery commercial operations are usually carried out regardless of
626scientific purposes.

627As opposed to visual and capture-based methods, eDNA does not imply
628any kind of selection other than the presence of genetic material in the
629sampled medium, as suggested by the neutrality of eDNA data with
630respect to the first PC axis. Technical, bio-molecular and biochemical
631factors also play a role in determining accuracy and completeness of
632metabarcoding biodiversity estimates (Zinger et al., 2019). In addition to
633this, abundant species should be more represented inside an ideal eDNA
634sample (Takahara, Minamoto, Yamanaka, Doi, & Kawabata, 2012). This
635relationship still needs to be fully validated in order to confer a
636quantitative power to eDNA (Lamb et al., 2019), but a certain
637proportionality between eDNA abundance and detection probability is
638recognized (Lacoursière-Roussel, Rosabal, & Bernatchez, 2016). In this
639regard, our study indirectly supports such assertions, as half of the traits
640discriminating between eDNA and traditional methods were related to fish
641schooling behaviours. It is reasonable to expect that schooling species,
642releasing high amounts of DNA, may be more easily detected.

643In summary, we show that the choice of the survey method can influence
644the ecological conclusions of biodiversity studies and that eDNA is well set

645to capture most of the functional fish diversity of coastal marine
646environments. Beside the advantages in terms of sampling ease and
647emancipation from taxonomic expertise, this new generation monitoring
648tool appears now geared to boost the collection of complex information
649from marine environments, including their functional dimension. We
650recognize the generalization of our approach still needs to be tested in
651other environmental and geographical settings, or using different
652biological descriptors. We are also aware that the full eDNA potential in
653providing accurate taxonomic and functional diversity estimates can be
654reached only by lending a quantitative power to the technique. Until that
655time, association of eDNA with some of the traditional quantitative
656methods is advisable.

657This study also provides a thorough outlook on the pitfall we might
658encounter trying to obtain robust marine diversity estimates upon which
659our understanding of the functioning of marine ecological systems greatly
660depend (Mouillot et al., 2014; Stuart-Smith et al., 2013). As eDNA analysis
661continues to develop beyond its original descriptive nature (Djurhuus et
662al., 2020), these new, diverse pathways of investigations promise to
663significantly enhance our ability to understand, interpret and ultimately
664sustainably manage the ocean and its resources.

665

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925 **Data Accessibility**

926 Mediterranean Fish DNA barcodes (Tele02 fragment): NCBI accession
 927 numbers (from MT903884 to MT903923) are provided in detail in
 928 supporting information, Table S3.
 929 12S and COI raw data and Presence-Absence datasets are available at the
 930 public repository Driad: <https://doi.org/10.5061/dryad.5qfttdz30>

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933**Author Contributions**

934G.A, M.M. S.M. designed research; G.A, G.T., C.C., D.S., A.D.F., A.C., M.D.L,
935P.G. performed field work; G.A, C.B. performed laboratory work; G.T., C.C.
936performed video analyses; G.A, C.B., A.C. analyzed data; G.A. and M.M.
937wrote the paper with input from all co-authors.

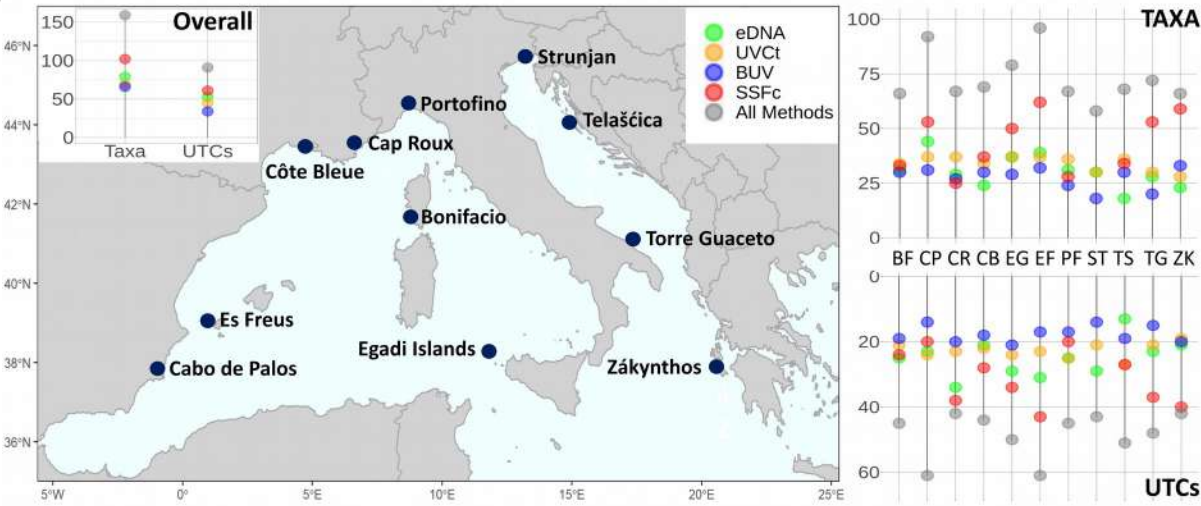
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939**Figures captions**

940**Figure 1.** Map of study areas in the Central and the Western
941Mediterranean Sea (black dots). Each name identifies an MPA and its
942flanking unprotected location. Lollipop charts on the right side display the
943number of taxa (upper chart) and unique traits combinations (UTCs –
944upside down lower chart). Overall number of taxa and UTCs are displayed
945in the smaller chart on the upper left side of the map. BF: Bonifacio; CP:
946Cabo de Palos; CR: Cap Roux; CB: Côte Bleue; EG: Egadi Islands; EF: Es
947Freus; PF: Portofino; ST: Strunjan; TS: Telašćica; TG: Torre Guaceto; ZK:
948Zákynthos.

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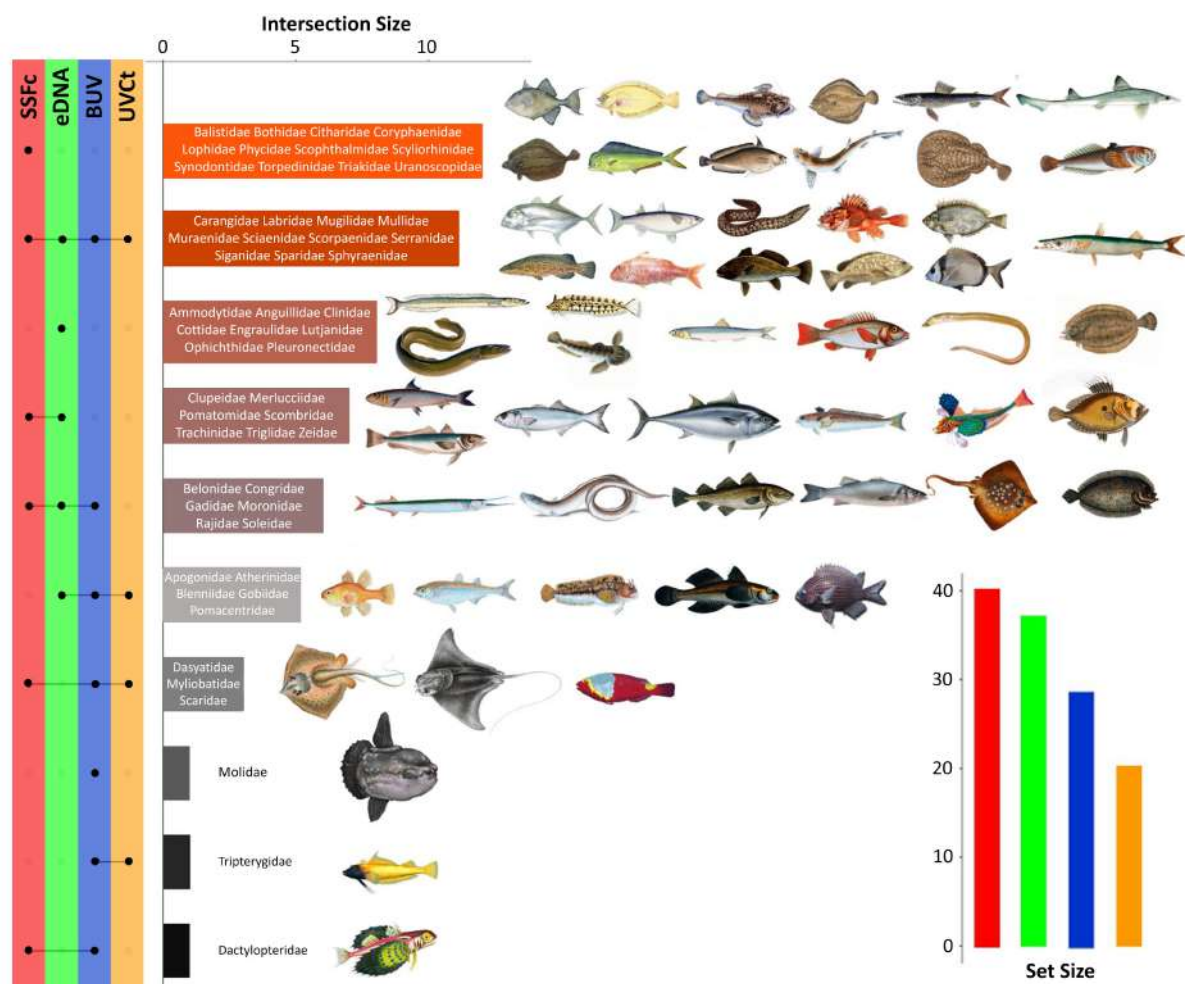
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952**Figure 2.** UpSet plot displaying the fish families detected by each of the
953four applied techniques. Horizontal bars, coupled with lines and dots on
954the left panel, show the exclusive families for each method and the
955intersection among them. The set size histograms show the overall yield of
956each sampling method. Fish images are modified free of right pictures.
957Sources: NOAA photo library, Wikipedia, Rawpixel public domain,
958Fionasplace.net, Freepng.es, CleanPNG.com, Freepik.com, Wpclipart.com,
959Pngimag.com, NicePNG.com
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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.

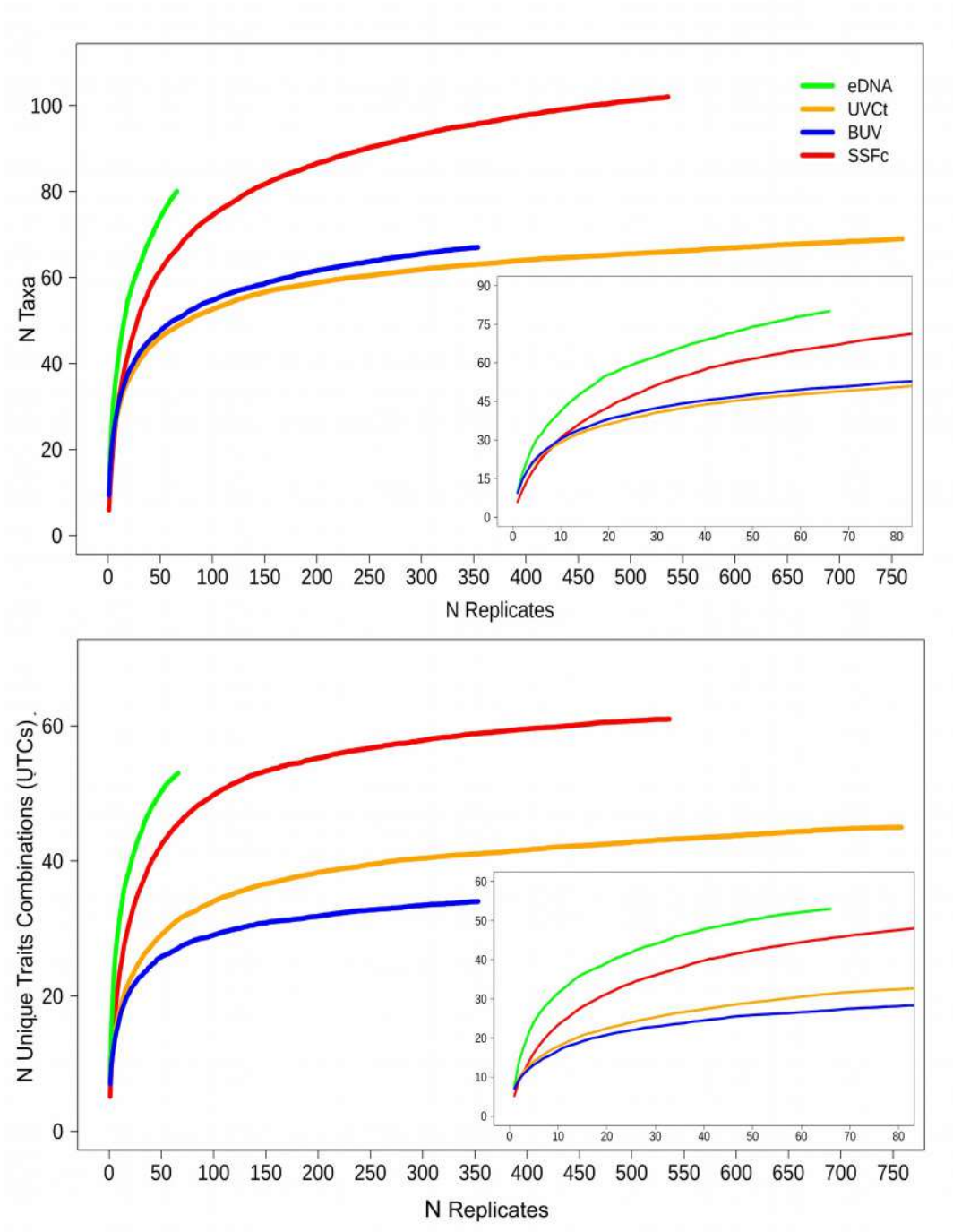


Figure 4. Non-metric multidimensional scaling (nMDS) based on Jaccard similarity index of the composition of fish functional traits recorded in MPAs and their flanking unprotected locations (i.e., overall 22 locations) by eDNA, BUV, UVCT and SSFc. Lines connect each point to the centroid; ellipses represent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

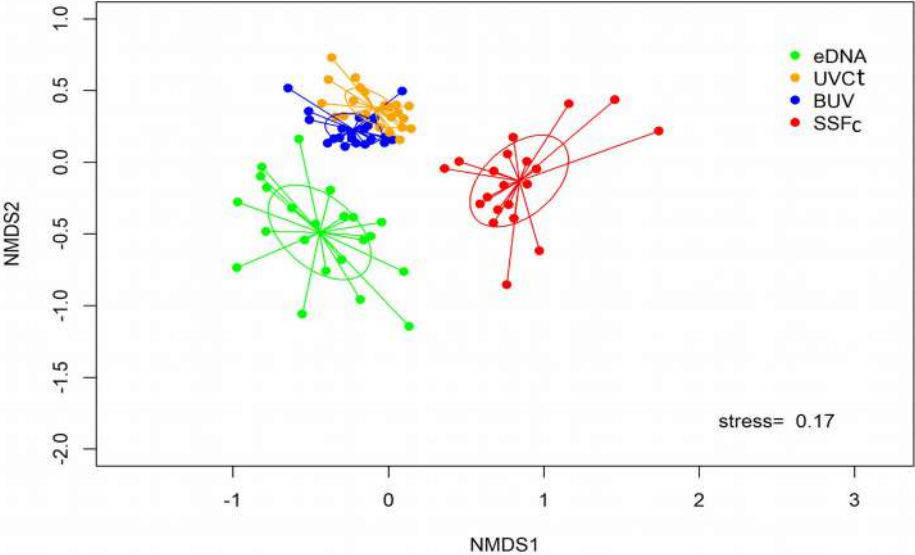


Figure 5. Funnel plots of Average taxonomic distinctness (AvTD) (A.) and Average functional distinctness (AvFD) (B.) of the fish assemblages recorded in eleven Mediterranean MPAs and eleven unprotected zones by eDNA, BUV, UVCT and SSFc. Dotted lines and dashed lines delimit respectively the 95% and 99% 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.

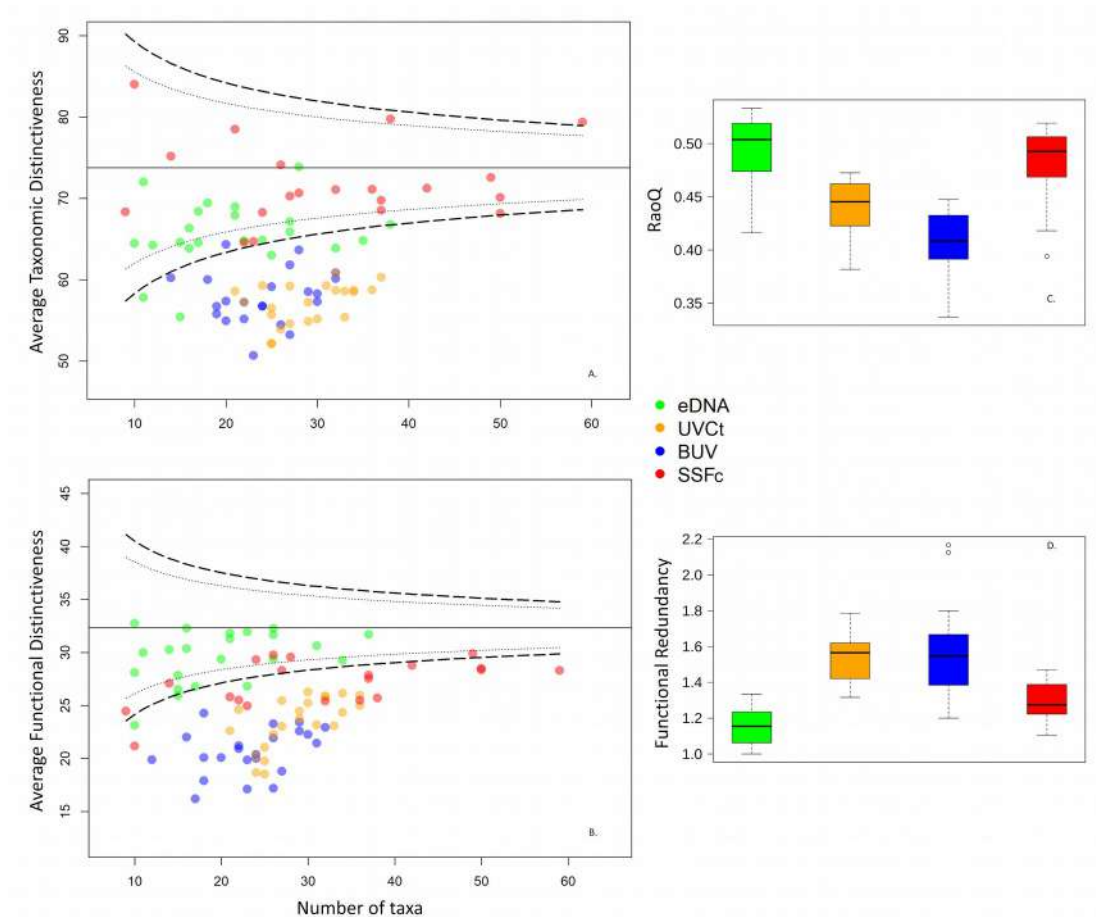


Figure 6. Principal Component Analysis (PCA) of the functional traits proportions of fish assemblages identified by eDNA, UVCT, BUV and SSFc techniques. The first four dimensions of the PCA cumulatively explained 88.24% of the projected inertia in the distribution of fish species traits, 174.82% of which 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*.

