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Environmental DNA effectively captures functional diversity of coastal fish communities

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1 **Environmental DNA effectively captures functional diversity of coastal fish communities**

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23 **Abstract**

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

40 **Introduction**

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

50 In marine ecosystems, reef fishes are considered to be well suited for global marine biodiversity studies
51 (Mouillot et al., 2014; Pimm et al., 2014), as they are highly diverse among vertebrates, cover a wide range of

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52 ecological functions, and their taxonomy and biological and ecological traits are relatively well known. Reef fish
53 diversity assessments are traditionally carried out using a range of techniques, the most popular of which include
54 Underwater Visual Census strip transects (UVCt), Baited Underwater Videos (BUV, also known as Baited
55 Remote Underwater Video - BRUV), experimental fishing and catches observations of both Industrial and
56 Small-Scale Fisheries (SSFc) commercial operations (Murphy & Jenkins, 2010). Although none of these
57 methods is specifically designed to capture the whole spectrum of biodiversity, they have been extensively used
58 for evaluating taxonomic and functional diversity patterns (Cappo, De'ath, & Speare, 2007; Micheli et al., 2014;
59 Stuart-Smith et al., 2013). UVCt is an efficient, non-invasive low-cost method. Nevertheless, it can be biased by
60 specific fish behaviours, underestimating the diversity of rare, shy, cryptic and very mobile pelagic species (Pais
61 & Cabral, 2017). BUV is also a widely used non-invasive observational method, less restricted by depth and
62 time. It performs well in recording large and elusive predators, including sharks, but might be less able to detect
63 small-sized or cryptic species (Colton & Swearer, 2010). Extractive fishery-dependent surveys may be limited
64 by differences in catchability of species by fishing gears, as well as habitat characteristics (Erzini et al., 2006). In
65 this context, the environmental DNA metabarcoding (hereafter 'eDNA') approach to marine biodiversity
66 assessments may prove promising (Djurhuus et al., 2020): this non-invasive method allows the detection of
67 marine organisms from species-discriminating amplicons of short DNA fragments 'harvested' from the
68 environment (Bohmann et al., 2014). Although limitations associated with quantification, DNA dispersal and
69 false negative/positive detection (Hansen, Bekkevold, Clausen, & Nielsen, 2018), eDNA is efficient in detecting
70 transient, rare and low abundance aquatic species (Boussarie et al., 2018; Sigsgaard, Carl, Møller, & Thomsen,
71 2015; Thomsen et al., 2012).

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

81 diversity; (ii) infer the contribution of each monitoring technique to the overall taxonomic and functional
82 diversity of the fish assemblages; (iii) evaluate the selectivity of each survey methodology for specific fish
83 functional traits.

84 To achieve these goals and to increase the representativeness of regional reef fish diversity, we concomitantly
85 sampled 22 locations within marine protected areas (MPAs) and unprotected areas in the Mediterranean Sea
86 applying standardized sampling protocols. We investigated fish taxonomic identities at species, genus and family
87 level, and fish functional identities using combinations of morphological, behavioural and trophic traits.
88 Coupling the two approaches, we show that ecological conclusions of field studies might depend from the
89 applied survey methods. We describe an unrecognized feature of eDNA: its lack of selectivity for functional
90 traits and hence its greater ability to capture effectively the spectrum of functional diversity of reef fish in the
91 Mediterranean region.

92 Materials and Methods

93 *Field data collection*

94 We concomitantly conducted field surveys of coastal fish diversity using Underwater Visual Censuses strip
95 transects (UVCt), Baited Underwater Video systems (BUV) and environmental DNA metabarcoding (eDNA)
96 during June and July 2018. We sampled the subtidal rocky zone of 22 locations inside and outside eleven Marine
97 Protected Areas (MPAs) in Italy (Egadi Islands MPA and Trapani coast, Portofino MPA and Camogli-Rapallo
98 coast, Torre Guaceto MPA and the Northern Brindisi coast), Greece (Zákynthos National Marine Park and
99 Zákynthos island), Spain (Es Freus Marine Reserve and Straits of Ibiza and Formentera Islands, Cabo de Palos
100 Marine Reserve and adjacent Murcia coast), France (Bonifacio Natural Reserve and South Corsica, Cap Roux
101 Cantonnement de Pêche and adjacent fished zones, Côte Bleue Marine Park and adjacent fished zones), Croatia
102 (Telašćica Nature Park and Dugi-Otok island) and Slovenia (Strunjan Landscape Park and adjacent coast) (Fig.
103 1- Table S1). Photo-sampling of small-scale fisheries catches (SSFc) using fixed-nets in the same locations was
104 also carried out at landings between May and September 2018. Sampling operations at sea were carried out by
105 two separate teams, one for UVCt and eDNA water sampling and one for the BUV systems. Separation between
106 the two teams was necessary to avoid possible onboard and underwater contamination of the eDNA samples
107 from the BUV baits. In order to reduce the time window between collection, filtration and sample storage, the
108 eDNA water samples were collected at the end of each working day close to UVCt sites.

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109 UVC strip transects were carried out between 8:00 a.m. and 3:00 p.m. inside and outside each MPA by the same
110 three trained diving operators. We randomly selected two sites for each protection level (no-take zone, buffer
111 zone and unprotected area) and performed 12 replicates for each site. Each replicate consisted of a strip transect
112 of 125 m^2 (25x5 m), conducted at 5-12m depth on rocky substrates. Overall, we carried out 760 UVCt transects,
113 with experimental design being adjusted according to the MPA size and characteristics in a few instances (Table
114 S1).

115 BUVs consisted of the deployment of a steel structure equipped with two stereo-cameras and a plastic container
116 containing a standard bait (e.g. ~500 gr of gilt sardines, *Sardinella aurita*) to attract fish. Two operators deployed
117 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
118 of 5-15 m. To avoid the repeated recording of the same individuals, BUVs were deployed at >150 meters apart
119 (Whitmarsh, Fairweather, & Huveneers, 2017). Depending on the spatial extent of the areas, a maximum of six
120 replicates were carried out in each of the two sites randomly chosen within each MPA protection level and
121 surrounding areas. Overall, we deployed 354 BUVs (Table S1). Videos were then analyzed recording all the fish
122 observed in the field of view of the two cameras.

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

129 eDNA samples consisted of four liters of water (two liters from the surface and 2 liters at one meter from the
130 bottom, max depth -20 m) collected from the boat and by scuba diving in three randomly chosen sites inside and
131 three outside each MPA for a total of 66 replicates (Table S1). After collection, the bottles were stored in the
132 dark and in ice. For each site, we placed a bottle filled with deionized water (field blank) among the sample
133 bottles in the transportable refrigerator, and processed identically. Filtration was performed within 3 hours from
134 sampling using a vacuum pump and sterile mixed cellulose esters filters (Merck Millipore; 47 mm diameter;
135 0.45 µm pore size). Potential contamination were controlled sterilizing benches and equipment with 50% bleach.

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136 The filters were stored at -20 C in 2.0 ml airtight cryotubes containing silica beads to dry out and prevent DNA
137 degradation.

138 *eDNA laboratory analyses*

139 We carried out DNA extraction, PCR amplification, library preparation and sequencing in a dedicated eDNA lab
140 with separate rooms for pre-PCR preparations and post-PCR procedures. Benchtops were cleaned with 10%
141 bleach and DNA AWAY^{TM}, pipettes and all the surfaces UV-irradiated daily and before beginning any molecular
142 work. We employed rigorous protocols for contamination control at each step of the process, including field,
143 extraction and PCR blanks. We prepared PCRs in a DNA-free hood and performed all post-PCR work in a room
144 physically separated from pre-PCR work. We extracted the DNA from the filters with the QIAGEN PowerWater
145 DNA Isolation Kit, following the manufacturers' protocol, and assessed its concentration in a Qubit fluorometer
146 (Thermo Fisher Scientific). We amplified a ~167bp fragment of mitochondrial 12S rRNA by PCR using the
147 "Tele02" primer set (Taberlet, Bonin, Coissac, & Zinger, 2018). To facilitate demultiplexing of Illumina
148 sequence reads, samples were 'tagged' using individual, sample-specific primers with attached 8-base oligo-tags
149 differing in at least three bases between samples. Forward and reverse primers carried the same tag within each
150 sample. Sequence diversity, important for Illumina amplicon sequencing, was increased by inserting six fully
151 degenerate positions (Ns) at the beginning of each primer. The PCR mix had a total volume of 20 µl, composed
152 by 10 µl Amplitaq Gold Master Mix (Thermo Fisher Scientific), 0.16 µl BSA, 1 µl of 5 µM forward primer, 1 µl
153 of 5 µM reverse primer, 10 ng of eDNA template and 5.84 µl of molecular biology grade water. The
154 thermocycler profile included an initial denaturing step of 94 ˚C for 10 min, 35 cycles of 94 ˚C 1 min, 54 ˚C
155 1 min and 72 ˚C 1 min and a final extension step of 72 ˚C for 5 minutes. We performed PCR amplifications in
156 triplicate and checked the presence of amplification products by gel electrophoresis (1.5%). We pooled PCR
157 products containing all the samples, 11 field blanks, 10 extraction blanks and PCR blanks into two sample pools.
158 We made two dual-indexed Illumina libraries using the KAPA HyperPrep PCR-free library preparation kit
159 (Roche). We quantified the libraries using the KAPA library quantification kit (Roche) and pooled them in
160 equimolar concentrations along with 10% PhiX (Illumina) serving as a positive sequencing quality control. We
161 sequenced the libraries with a final molarity of 8 pM on an Illumina MiSeq platform using v2, 2 x 150 paired-
162 end chemistry.

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163 We selected 67 out of 132 samples (Table S2) for further exploration using a 313 bp Cytochrome Oxidase
164 subunit 1 (COI) marker using the same protocol used for 12S rRNA. We applied the selection to contain the
165 costs, since the highly degenerated primers used are known to primarily amplify micro-eukaryotes and
166 invertebrates and we expected a low yield for fish (Collins et al., 2019). We included a subset of replicates for all
167 the considered locations and all the negative controls into another independent sequencing project. We selected
168 the samples with the highest amount of extracted DNA. Amplification was performed using the Leray-XT
169 primer set (Wangensteen, Palacín, Guardiola, & Turon, 2018) and the PCR profile suggested by the authors.
170 High-depth sequencing was carried out in a HiSeq4000 to maximise recovery of rare vertebrate reads.

171 *Mediterranean fish species DNA barcoding*

172 We collected small fin clip pieces of 25 Mediterranean fish species in local fish markets and preserved them in
173 96% ethanol at -20 C. We extracted the DNA using the DNeasy Blood & Tissue Kit (QIAGEN) following the
174 manufacturers' protocol. We amplified the "tele02" 12S rRNA fragment applying the same conditions reported
175 for eDNA metabarcoding, purified the PCR products with the QIAquick PCR Purification Kit (QIAGEN) and
176 performed the Sanger sequencing. Sequences were edited with BioEdit v.7.2 (Alzohairy, 2011).

177 *Bioinformatic analyses*

178 We processed the sequence reads using the OBITools v.1.01 12 metabarcoding software suite (Boyer et al.,
179 2016). Libraries were demultiplexed with 'bcl2fastq v. 2.20' (Illumina), before assessing read quality using
180 FastQC v.0.11.7 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Reads were trimmed to a length
181 so each base was, on average, above a score of Q30 using 'obicut'. Paired-end reads were aligned using
182 'illuminapairedend', retaining alignments with a quality score >40. Sample demultiplexing was performed with
183 'ngsfilter' for each library. Samples from 12S libraries were concatenated and the sequences length filtered (140-
184 180 bp) using 'obigrep' to select only fragment lengths known to amplify with our primers. The COI library was
185 filtered between 303bp and 323 bp. Reads containing ambiguous bases were also removed. Remaining reads
186 were de-replicated using "obiuniq" and chimeras were removed with the 'uchime-denovo' algorithm (Edgar,
187 Haas, Clemente, Quince, & Knight, 2011) implemented in vsearch v.1.9 (Rognes, Flouri, Nichols, Quince, &
188 Mahé, 2016). Sequence clustering was performed using 'swarm' v.2.0 (Mahé, Rognes, Quince, Vargas, &
189 Dunthorn, 2014) with a d-value of 2 for 12S, and 13 for COI. As suggested by the authors, the choice of "d" was
190 made after testing the outcomes of different values. Identification and exclusion of potential contamination was

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191 achieved by including field blanks, eDNA extraction blanks and PCR blanks. We removed the reads present in
192 the negative controls from the respective samples. All singletons were discarded.

193 *Taxonomy assignment*

194 We performed the taxonomic assignment of 12S sequences representing each Molecular Taxonomic Unit
195 (MOTU) using 'ecotag' against a curated database (https://github.com/boopsboops/reference-libraries. Since
196 several Mediterranean fish species were missing in public databases, we complemented our custom 12S database
197 with 'Teleo02' sequences of 25 common coastal species (Table S3). The taxonomic assignment for the COI
198 marker was performed against the db_COI_MBPK database (Bakker et al., 2019)
199 (http://github.com/metabarpark/Reference-databases). Each Molecular taxonomic unit (MOTU) was assigned to
200 a single species when this was the only Mediterranean species with a sequence similarity >97%. For the COI
201 marker the similarity threshold used was 99%. Ambiguous automatic assignments were manually checked
202 through a BLAST search against the NCBI (https://www.ncbi.nlm.nih.gov/) and MitoFish (http://mitofish.aori.u-
203 tokyo.ac.jp/) databases for 12S, and NCBI and BOLD (http://v3.boldsystems.org/index.php/IDS_OpenIdEngine)
204 for COI. If appropriate, we applied an assignment correction accordingly to the up to date knowledge of the
205 species distribution in the Mediterranean Sea provided by FishBase
206 (https://www.fishbase.se/trophiceco/FishEcoList.php?ve_code=13), following these criteria:

207 1. In case of multiple possible assignment (i.e. more than one Mediterranean species with the same
208 sequence similarity) -> the MOTU was registered at the lowest possible taxonomic rank.
209 2. In case of a MOTU assigned to a non-Mediterranean taxon despite closely related Mediterranean taxa
210 showed a sequence similarity within the 97-99% threshold -> the assignment was corrected including
211 the Mediterranean taxa at the lowest possible taxonomic rank.
212 3. In case of a taxonomic assignment at family or genus level when a single Mediterranean species had an
213 equivalent % of similarity to a non-Mediterranean species belonging to the same genus or family -> the
214 assignment was corrected excluding the non-Mediterranean taxon.
215 4. In case of a record assigned to a non-Mediterranean taxon because of a lack of reference sequences for
216 Mediterranean congeneric species -> the assignment was corrected only in presence of a single
217 Mediterranean congeneric species

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

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219 *Statistical analyses*

220 We built datasets containing taxa presence/absence data for each location using the outcomes of each sampling
221 technique. A "unique trait combinations" (UTCs) dataset was also built, representing each taxon as a string of
222 traits for each considered functional category. We considered seven categories were: a) maximum length; b)
223 depth range; c) cryptic/nocturnal behaviour; d) mobility; e) habitat type; f) aggregation behaviour; g) trophic
224 habit (Table S4).

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

235 The relative contribution of each sampling technique to the global diversity estimate was analyzed using four
236 measures of diversity: Average Taxonomic Distinctiveness (AvTD) (Clarke & Warwick, 1998), Average
237 Functional Distinctiveness (AvFD) (Somerfield, Clarke, Warwick, & Dulvy, 2008), Rao's quadratic entropy
238 (RaoQ) (Botta-Dukát, 2005) (SI Appendix) and Functional redundancy (FR) (Mouillot et al., 2014). All indices
239 utilize or can accommodate presence/absence data to explore the diversity of a community (or assemblage).
240 AvTD takes into account the taxonomic distance among the units composing a sample (species). AvFD
241 considers the functional divergence among the same items. Both the indices are able to compare the local
242 diversity (taxonomic or functional) of observed fish assemblages to the expected total diversity extrapolated
243 from the overall list of species known to be present in the considered environment. We built an aggregated fish
244 assemblage list collating all the taxa recorded by each method. This was then used along with sample specific
245 lists to calculate AvTD and AvFD, and to verify potential methods-specific divergences from the expectancies
246 using the 'taxa2dist' and 'taxondive' functions implemented in 'vegan'. Taxonomic distinctness estimates were

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247 generated using, for each sampling method, the highest possible taxonomic resolution. We excluded the records
248 at family level to avoid the risk of producing spurious variations in the taxonomic breadth of the samples. For
249 similar reasons, we kept the records at taxonomic level higher than species for the AvFD analyses only if
250 appropriate functional categories were applicable for all the considered traits. RaoQ estimates were generated
251 from the same datasets used for the calculation of AvFD, employing the function 'rao.diversity' implemented in
252 the package 'SYNCSA' v. 1.3.4 (Debastiani & Pillar, 2012). FR estimates were calculated dividing the number
253 of taxa by the number of UTCs observed in each sample. Analysis of variance (ANOVA) was used to test for
254 differences in AvTD, AvFD, RaoQ and FR estimates among the four survey methods using the 'aov' function in
255 R. Normality of distribution and homogeneity of variance were tested using the 'shapiro.test' and 'leveneTest'
256 functions implemented in the 'dplyr' and 'car' packages (Fox & Weisberg, 2018). A logarithmic transformation
257 was applied to the AvTD values to ensure normality of distribution. Significant differences among factors were
258 followed by pairwise comparison t-tests. Functional structure variation among fish assemblages was summarized
259 using principal component analysis (PCA) based on the relative proportion of each considered functional trait in
260 the samples provided by each survey technique. The latter was calculated dividing the number of trait
261 occurrences in each sample by the total number of taxa registered in each locality, to account for the intrinsic
262 environmental variability among MPAs. The PCA was performed using the 'rda' function in 'vegan'.

263 **Results**

264 The Illumina MiSeq run of the pooled 12S amplicon libraries produced 16,197,599 reads. After sample
265 assignment, quality and sequence-length filtering, singletons and chimera removal, we obtained 10,560,688
266 reads belonging to the samples used in the present study. After conservative removal of MOTUs unassigned or
267 assigned to non-fish taxa, we obtained 1,863,535 reads, among which 1,422,635 unambiguously assigned to
268 Mediterranean fish taxa (Dataset 1). The average number of 12S reads included was higher for the samples taken
269 at -20 m (13,089 reads) than for the surface samples (8,467 reads). After merging the reads from the two depths
270 for each replicate, the number of reads ranged from 3 to 196,306, with a mean value of 21,541 reads.

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

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276 Overall, we identified 159 fish taxa belonging to 100 genera and 55 families (Table S6), and 91 unique traits
277 combinations (UTCs) at functional level, with unequal contributions, provided by different survey techniques to
278 locally observed taxonomic and functional richness (Fig. 1). eDNA detected 79 different fish taxa (74 at species
279 and 5 at genus level) belonging to 65 genera and 37 families, and 53 UTCs (see SI Appendix section IV. and
280 Dataset 1 for details). UVCt, BUV and SSFc identified respectively 69 taxa (66 at species, two at genus and one
281 at family level; 40 genera and 20 families) and 45 UTCs, 66 taxa (57 at species, 6 at genus and 3 at family level;
282 41 genera and 28 families) and 34 UTCs, and 102 taxa (all at species level; 67 genera and 40 families) and 61
283 UTCs, (see Dataset 2 for details). The four different methods shared 14.46% of species (23), 22% of genera (22)
284 and 20% of families (Fig. 2, S1, S2). SSFc recorded the highest proportion of exclusive records (25.16%, 40
285 taxa), followed by eDNA (17.61%; 28 taxa), UVCt (7.55%; 12 taxa) and BUV (2.52%; 4 taxa). Overall, 42.85%
286 of UTCs (39) was not shared between any of the survey techniques. SSFc showed the highest proportion of
287 exclusive UTCs (18.68%, 17 UTCs), followed by eDNA (14.29%; 13), UVCt (7.69%; 7) and BUV (2.20%; 2)
288 (Fig. S3).

289 On average, eDNA performed better in detecting a higher number of fish taxa and trait combinations per
290 replicate (10.76 ± 7.15 and 8.73 ± 5.55, respectively; N=66; mean ±SD) than BUV (Taxa: 9.86 ± 3.46; UTCs:
291 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:
292 5.16 ± 3.38; N=536) as shown by the taxa and UTCs accumulation curves (Fig.3).

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

297 The Average Taxonomic Distinctiveness (AvTD) estimates returned significantly different variances among
298 survey methods, with pairwise comparisons being also highly significant ($p<0.001$), except for the UVCt-BUV
299 comparison (Table S7). AvTD funnel plot shows that the highest proportion of taxonomic breadth of the
300 identified pool of Mediterranean coastal fish was provided by SSFc, with most of the samples matching the
301 expected distribution at 95% C.I., and, to a lesser extent, by eDNA (few samples outside the 95% C.I.) (Fig. 5A).
302 UVCt and BUV samples exhibited a narrower taxonomic breadth (i.e., close fish taxonomic composition and all
303 samples below the 95% C.I.). Average Functional Distinctiveness (AvFD) estimates were significantly different

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304 between each pair of methods (Table S7). eDNA samples were unique in showing estimates of functional
305 distinctness consistent with the expectations (AvFD= 29.53 ± 2.47; Mean ± SD), while almost all the UVCt,
306 BUV and SSFc samples were below the lower boundary of the 95% confidence limit (Fig. 5B). BUV was the
307 survey technique with the lowest average functional distance among the detected fish (AvFD= 20.62 ± 2.15).
308 UVCt produced functionally broader samples than BUV, performing slightly better in detecting functional than
309 taxonomical diversity (AvFD= 23.41 ± 2.38). Despite the larger taxonomic diversity observed, SSFc data
310 returned AvFD estimates below expectations (AvFD= 27.07 ± 2.17) (Fig. 5B). We complemented information
311 on the Mediterranean fish functional dimensions using the Rao's quadratic entropy (RaoQ) index on presence-
312 absence data (Fig. 5C) and the Functional Redundancy (FR) index (Fig. 5D). RaoQ estimates showed similar
313 patterns to AvFD with eDNA exhibiting the highest values (RaoQ= 0.50 ± 0.03; Mean ± SD), which were
314 similar to SSFc (0.48 ± 0.03), but significantly different from UVCt (RaoQ= 0.44 ± 0.03) and BUV (RaoQ= 0.41
315 ± 0.03) (Table S7). The functional redundancy (FR) of the fish assemblages described by the observational
316 methods was significantly higher than those identified by the molecular and fishing methods, with eDNA
317 samples having the less redundant fish assemblages (Table S7).

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

326

327 **Discussion**

328 Several observational methods are routinely employed to describe biodiversity worldwide. Data collected are
329 then used to test hypotheses on ecological processes and functioning. Although the power of most of the survey
330 techniques in capturing marine taxon richness is relatively well understood, we know little about their suitability
331 for describing the diverse functional attributes of fish assemblages. Here we investigated the ability of two

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332 observational (UVCt and BUV) and one fishery-related (SSFc) survey methods and one molecular approach
333 (eDNA) to describe the taxonomic and functional diversity of coastal fish assemblages. We show that eDNA
334 metabarcoding is more powerful in describing the functional attributes of fish assemblages than other techniques.
335 We attribute such feature to a substantial lack of selectivity towards any functional trait.

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

341 The public reference sequences databases do lack for many Mediterranean fish species. We partially contributed
342 to fill these knowledge gaps by adding new 12S rRNA barcodes data. This point remained perhaps the main
343 source of bias leading to incomplete taxa detection by eDNA: approximately 40% of the 12S rRNA fish MOTUs
344 have been discarded due to low sequence similarity with the records available in the databases. Despite these
345 limitations, eDNA showed the ability to identify more taxa per sample than the other techniques, adding on
346 previous evidences (Boussarie et al., 2018; Yamamoto et al., 2017), even if replicates of different methods are
347 not formally comparable and need to be considered only as qualitative proxy of sampling effort. eDNA and SSFc
348 also showed the highest variability of number of records per replicate compared to UVCt and BUV. This likely
349 relates to the probability of detecting a species with the observational methods and the relative abundance of the
350 recognized taxa in the environment. Indeed, UVCt and BUV mostly rely on the detection of abundant and
351 homogeneously distributed fish species, while rare or cryptic fish may have a lower probability to be detected
352 (Colton & Swearer, 2010; Pais & Cabral, 2017). On the other hand, eDNA and SSFc exhibited a more
353 pronounced ability to 'capture' rare and cryptic species and their susceptibility to variations of environmental
354 conditions (Evans et al., 2017; Frid & Belmaker, 2019) can explain the variability of their outcomes.

355 In addition to the substantial differences in taxon detection power among the considered methods, we found also
356 a pronounced compartmentalization of the information provided by each of them, supporting previous findings
357 suggesting that different methods capture different subsets of biodiversity (Kelly et al., 2017). Indeed, excluding
358 the pair UVC-BUV, the four methods turned out to be more complementary than convergent, each one
359 contributing with exclusive findings to the overall fish diversity assessment. The observational techniques were

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360 mainly able to capture the most common Mediterranean reef fish families (Fig. 2), containing several congeneric
361 species, such as sea breams (Sparidae), wrasses (Labridae) and combers/groupers (Serranidae) (Guidetti, 2000).
362 This result likely pertains to their limited spatio-temporal scales compared with SSFc and eDNA, which in turn
363 are able to capture the presence of additional taxa in longer times and from a wider area. Indeed, both methods
364 can capture nocturnal species, since SSF fixed-nets often operate overnight and eDNA can detect genetic signal
365 of organisms that have been in the area several hours before water collection, depending on the environmental
366 conditions driving the environmental DNA decay rate and sinking/resuspension processes (Barnes & Turner,
367 2016; Collins et al., 2018). Local oceanography is also accountable for the eDNA broader spatial context, since
368 water movement may favour the transfer of environmental DNA from adjacent areas and habitats, even if
369 different studies have proved good spatial resolution (Jeunen et al., 2019; Port et al., 2016; Yamamoto et al.,
370 2017). This is a particularly important aspect for evaluating fish assemblage diversity in very patchy
371 environments as Mediterranean coastal areas. Indeed, if eDNA has the advantage of simultaneously providing
372 information on different habitats closely distributed in a certain area, the higher spatial fidelity of the
373 observational methods might allow to assign species and traits more punctually to particular habitats.

374 As natural communities are composed of taxa with disparate levels of relatedness and ecological functions
375 (Cardoso, Rigal, Borges, & Carvalho, 2014), a robust estimate of their complexity depends on the
376 comprehensive collection of as many variants as possible. In this respect, we found for the first time the greater
377 efficiency of eDNA in detecting fish functional diversity compared to the other methods (Fig.3 and Fig. 5), a
378 competence only partially linked to its proficiency in detecting single species. Indeed, SSFc was more efficient
379 in collecting taxonomic variants than eDNA and subsequently observational methods. By contrast, eDNA
380 samples showed the highest functional diversity (RaoQ) values, the lowest levels of functional redundancy (FR)
381 and were unique in showing estimates of fish functional distinctness consistent with expectations (Fig. 5). We
382 attribute this results to the link between taxonomic and functional diversity, strictly dependent on the redundancy
383 of functional entities inside a given community (Micheli & Halpern, 2005), so that fractions of taxonomic
384 diversity captured by different sampling systems are not necessarily reflected in functional diversity. At present,
385 the non-quantitative nature of eDNA (Lamb et al., 2019) prevents a fully implementation of functional diversity
386 indices as RaoQ, that should include the relative abundances of fish. However, even if this reduces the accuracy
387 of the estimates, it does not nullify its informative content (Kim, Blomberg, & Pandolfi, 2018), which tends to be
388 positively correlated to species richness when the functional redundancy inside the assemblage is lower (Granger

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389 et al., 2015). In addition to this, our findings suggest that the application of different survey methods may lead to
390 different ecological conclusions when assessing ecological redundancies within fish assemblages. The lower
391 functional redundancy we found using eDNA may have important implications for reliable predictions of the
392 effects of biodiversity loss on the functioning of Mediterranean marine ecosystems, and the consequent
393 management and conservation interventions. By contrast, the higher levels of functional redundancies detected
394 by the observational methods would rather suggest that ecosystem functions might be robust to changes in
395 diversity, likely not reflecting the real situation of the overexploited, and highly disturbed Mediterranean marine
396 ecosystems (Coll et al., 2010).

397 Potential method-specific functional selectivity might be a major factor in shaping the distribution of functional
398 traits within fish assemblages. As such, we suggest that the selectivity of traditional survey tools, and the
399 consequent imbalanced proportion of traits in the estimated fish assemblages, might be accountable for their low
400 functional representation. The active selection of certain traits by specific fishing gears, fish behavioural
401 characteristics, or the use of a bait might intrinsically generate functional redundancies within fish assemblages,
402 consequently reducing their functional diversity levels even when taxonomic diversity is high. UVCt and BUV
403 selectivity for shallow-dwelling and for mobile bentho-pelagic species, as well as the tendency of SSFc to collect
404 preferentially benthic fish with a broad depth range and large predators, arguably represent intrinsic features of
405 these traditional methodologies such as operational time (day vs night), depth, and employed gears. UVCt and
406 BUV resulted less suitable for detecting "cryptic" species, corroborating previous evidence that a substrate-
407 blending coloration and an inconspicuous behaviour could be accountable for the low proportion of benthic fish,
408 while colourful and curious bentho-pelagic species are more easily detected (Willis, 2001). Similarly, highly
409 mobile pelagic species were underrepresented in UVCt, arguably due to fish behavioural response to divers,
410 other than the limited width of the strip transects (Watson, Carlos, & Samoilys, 1995). As for SSFc, since the
411 probability to catch fish of different size ranges varies with the mesh size (Hubert, Pope, & Dettmers, 2012), the
412 only way to reduce the fish size bias would be the simultaneous use of gears with a wide range of net mesh sizes.
413 Yet, adopting such a strategy would increase efforts and environmental impact of the surveys. Collecting fish
414 data from fishers may represent a low-cost option for diversity assessments, as small-scale fishery commercial
415 operations are usually carried out regardless of scientific purposes.

416 As opposed to visual and capture-based methods, eDNA does not imply any kind of selection other than the
417 presence of genetic material in the sampled medium, as suggested by the neutrality of eDNA data with respect to

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418 the first PC axis. Technical, bio-molecular and biochemical factors also play a role in determining accuracy and
419 completeness of metabarcoding biodiversity estimates (Zinger et al., 2019). In addition to this, abundant species
420 should be more represented inside an ideal eDNA sample (Takahara, Minamoto, Yamanaka, Doi, & Kawabata,
421 2012). This relationship still needs to be fully validated in order to confer a quantitative power to eDNA (Lamb
422 et al., 2019), but a certain proportionality between eDNA abundance and detection probability is recognized
423 (Lacoursière-Roussel, Rosabal, & Bernatchez, 2016). In this regard, our study indirectly supports such
424 assertions, as half of the traits discriminating between eDNA and traditional methods were related to fish
425 schooling behaviours. It is reasonable to expect that schooling species, releasing high amounts of DNA, may be
426 more easily detected.

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427 In summary, we show that the choice of the survey method can influence the ecological conclusions of
428 biodiversity studies and that eDNA is well set to capture most of the functional fish diversity of coastal marine
429 environments. Beside the advantages in terms of sampling ease and emancipation from taxonomic expertise, this
430 new generation monitoring tool appears now geared to boost the collection of complex information from marine
431 environments, including their functional dimension. We recognize the generalization of our approach still needs
432 to be tested in other environmental and geographical settings, or using different biological descriptors. We are
433 also aware that the full eDNA potential in providing accurate taxonomic and functional diversity estimates can
434 be reached only by lending a quantitative power to the technique. Until that time, association of eDNA with
435 some of the traditional quantitative methods is advisable.

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

442

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451

452 **References**

453 Alzohairy, T. H. (2011). *BioEdit: An important software for molecular biology*. 2.

454 Bakker, J., Wangensteen, O. S., Baillie, C., Buddo, D., Chapman, D. D., Gallagher, A. J., … Mariani, S. (2019).
455 Biodiversity assessment of tropical shelf eukaryotic communities via pelagic eDNA metabarcoding.
456 *Ecology and Evolution*, *9*(24), 14341–14355. doi: 10.1002/ece3.5871

457 Barnes, M. A., & Turner, C. R. (2016). The ecology of environmental DNA and implications for conservation
458 genetics. *Conservation Genetics*, *17*(1), 1–17. doi: 10.1007/s10592-015-0775-4

459 Bohmann, K., Evans, A., Gilbert, M. T. P., Carvalho, G. R., Creer, S., Knapp, M., … de Bruyn, M. (2014).
460 Environmental DNA for wildlife biology and biodiversity monitoring. *Trends in Ecology & Evolution*,
461 *29*(6), 358–367. doi: 10.1016/j.tree.2014.04.003

462 Botta-Dukát, Z. (2005). Rao's quadratic entropy as a measure of functional diversity based on multiple traits.
463 *Journal of Vegetation Science*, *16*(5), 533–540. doi: 10.1111/j.1654-1103.2005.tb02393.x

464 Boussarie, G., Bakker, J., Wangensteen, O. S., Mariani, S., Bonnin, L., Juhel, J.-B., … Mouillot, D. (2018).
465 Environmental DNA illuminates the dark diversity of sharks. *Science Advances*, *4*(5), eaap9661. doi:
466 10.1126/sciadv.aap9661

467 Boyer, F., Mercier, C., Bonin, A., Bras, Y. L., Taberlet, P., & Coissac, E. (2016). obitools: a unix-inspired
468 software package for DNA metabarcoding. *Molecular Ecology Resources*, *16*(1), 176–182. doi:
469 10.1111/1755-0998.12428

470 Cappo, M., De'ath, G., & Speare, P. (2007). Inter-reef vertebrate communities of the Great Barrier Reef Marine
471 Park determined by baited remote underwater video stations. *Marine Ecology Progress Series*, *350*,
472 209–221. doi: 10.3354/meps07189

473 Cardoso, P., Rigal, F., Borges, P. A. V., & Carvalho, J. C. (2014). A new frontier in biodiversity inventory: a
474 proposal for estimators of phylogenetic and functional diversity. *Methods in Ecology and Evolution*,
475 *5*(5), 452–461. doi: 10.1111/2041-210X.12173

476 Clarke, K. R., & Warwick, R. M. (1998). A taxonomic distinctness index and its statistical properties. *Journal of*
477 *Applied Ecology*, *35*(4), 523–531. doi: 10.1046/j.1365-2664.1998.3540523.x

478 Coll, M., Piroddi, C., Steenbeek, J., Kaschner, K., Lasram, F. B. R., Aguzzi, J., … Voultsiadou, E. (2010). The
479 Biodiversity of the Mediterranean Sea: Estimates, Patterns, and Threats. *PLOS ONE*, *5*(8), e11842. doi:
480 10.1371/journal.pone.0011842

481 Collins, R. A., Bakker, J., Wangensteen, O. S., Soto, A. Z., Corrigan, L., Sims, D. W., … Mariani, S. (2019).
482 Non-specific amplification compromises environmental DNA metabarcoding with COI. *Methods in*
483 *Ecology and Evolution*, *10*(11), 1985–2001. doi: 10.1111/2041-210X.13276

484 Collins, R. A., Wangensteen, O. S., O'Gorman, E. J., Mariani, S., Sims, D. W., & Genner, M. J. (2018).
485 Persistence of environmental DNA in marine systems. *Communications Biology*, *1*(1), 1–11. doi:
486 10.1038/s42003-018-0192-6

For Review Only

487 Colton, M. A., & Swearer, S. E. (2010). A comparison of two survey methods: differences between underwater
488 visual census and baited remote underwater video. *Marine Ecology Progress Series*, *400*, 19–36. doi:
489 10.3354/meps08377

490 Conway, J. R., Lex, A., & Gehlenborg, N. (2017). UpSetR: an R package for the visualization of intersecting
491 sets and their properties. *Bioinformatics*, *33*(18), 2938–2940.

492 D'agata, S., Mouillot, D., Kulbicki, M., Andréfouët, S., Bellwood, D. R., Cinner, J. E., … Vigliola, L. (2014).
493 Human-Mediated Loss of Phylogenetic and Functional Diversity in Coral Reef Fishes. *Current Biology*,
494 *24*(5), 555–560. doi: 10.1016/j.cub.2014.01.049

495 Debastiani, V. J., & Pillar, V. D. (2012). SYNCSA—R tool for analysis of metacommunities based on functional
496 traits and phylogeny of the community components. *Bioinformatics*, *28*(15), 2067–2068. doi:
497 10.1093/bioinformatics/bts325

498 Díaz, S., Pascual, U., Stenseke, M., Martín-López, B., Watson, R. T., Molnár, Z., … Shirayama, Y. (2018).
499 Assessing nature's contributions to people. *Science*, *359*(6373), 270–272. doi: 10.1126/science.aap8826

500 Djurhuus, A., Closek, C. J., Kelly, R. P., Pitz, K. J., Michisaki, R. P., Starks, H. A., … Breitbart, M. (2020).
501 Environmental DNA reveals seasonal shifts and potential interactions in a marine community. *Nature*
502 *Communications*, *11*(1), 1–9. doi: 10.1038/s41467-019-14105-1

503 Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., & Knight, R. (2011). UCHIME improves sensitivity and
504 speed of chimera detection. *Bioinformatics*, *27*(16), 2194–2200. doi: 10.1093/bioinformatics/btr381

505 Erzini, K., Gonçalves, J. M. S., Bentes, L., Moutopoulos, D. K., Casal, J. A. H., Soriguer, M. C., … Stergiou, K.
506 I. (2006). Size selectivity of trammel nets in southern European small-scale fisheries. *Fisheries*
507 *Research*, *79*(1), 183–201. doi: 10.1016/j.fishres.2006.03.004

508 Evans, N. T., Li, Y., Renshaw, M. A., Olds, B. P., Deiner, K., Turner, C. R., … Pfrender, M. E. (2017). Fish
509 community assessment with eDNA metabarcoding: effects of sampling design and bioinformatic
510 filtering. *Canadian Journal of Fisheries and Aquatic Sciences*, *74*(9), 1362–1374. doi: 10.1139/cjfas-
511 2016-0306

512 Fox, J., & Weisberg, S. (2018). *An R companion to applied regression*. Sage publications.

513 Frid, O., & Belmaker, J. (2019). Catch dynamics of set net fisheries in Israel. *Fisheries Research*, *213*, 1–11. doi:
514 10.1016/j.fishres.2018.12.021

515 Granger, V., Bez, N., Fromentin, J.-M., Meynard, C., Jadaud, A., & Mérigot, B. (2015). Mapping diversity
516 indices: not a trivial issue. *Methods in Ecology and Evolution*, *6*(6), 688–696. doi: 10.1111/2041-
517 210X.12357

518 Guidetti, P. (2000). Differences Among Fish Assemblages Associated with Nearshore Posidonia oceanica
519 Seagrass Beds, Rocky–algal Reefs and Unvegetated Sand Habitats in the Adriatic Sea. *Estuarine,*
520 *Coastal and Shelf Science*, *50*(4), 515–529. doi: 10.1006/ecss.1999.0584

521 Halpern, B. S., Frazier, M., Afflerbach, J., Lowndes, J. S., Micheli, F., O'Hara, C., … Selkoe, K. A. (2019).
522 Recent pace of change in human impact on the world's ocean. *Scientific Reports*, *9*(1), 1–8. doi:
523 10.1038/s41598-019-47201-9

524 Hansen, B. K., Bekkevold, D., Clausen, L. W., & Nielsen, E. E. (2018). The sceptical optimist: challenges and
525 perspectives for the application of environmental DNA in marine fisheries. *Fish and Fisheries*, *19*(5),
526 751–768. doi: 10.1111/faf.12286

527 Hubert, W. A., Pope, K. L., & Dettmers, J. M. (2012). *Passive Capture Techniques*. 45.

528 Jeunen, G.-J., Knapp, M., Spencer, H. G., Lamare, M. D., Taylor, H. R., Stat, M., … Gemmell, N. J. (2019).
529 Environmental DNA (eDNA) metabarcoding reveals strong discrimination among diverse marine
530 habitats connected by water movement. *Molecular Ecology Resources*, *19*(2), 426–438. doi:
531 10.1111/1755-0998.12982

532 Kelly, R. P., Closek, C. J., O'Donnell, J. L., Kralj, J. E., Shelton, A. O., & Samhouri, J. F. (2017). Genetic and
533 Manual Survey Methods Yield Different and Complementary Views of an Ecosystem. *Frontiers in*
534 *Marine Science*, *3*. doi: 10.3389/fmars.2016.00283

For Review Only

535 Kim, S. W., Blomberg, S. P., & Pandolfi, J. M. (2018). Transcending data gaps: a framework to reduce
536 inferential errors in ecological analyses. *Ecology Letters*, *21*(8), 1200–1210. doi: 10.1111/ele.13089

537 Lacoursière-Roussel, A., Rosabal, M., & Bernatchez, L. (2016). Estimating fish abundance and biomass from
538 eDNA concentrations: variability among capture methods and environmental conditions. *Molecular*
539 *Ecology Resources*, *16*(6), 1401–1414. doi: 10.1111/1755-0998.12522

540 Lamb, P. D., Hunter, E., Pinnegar, J. K., Creer, S., Davies, R. G., & Taylor, M. I. (2019). How quantitative is
541 metabarcoding: A meta-analytical approach. *Molecular Ecology*, *28*(2), 420–430. doi:
542 10.1111/mec.14920

543 Loreau, M., Naeem, S., & Inchausti, P. (2002). *Biodiversity and Ecosystem Functioning: Synthesis and*
544 *Perspectives*. Oxford University Press.

545 Mace, G. M., Norris, K., & Fitter, A. H. (2012). Biodiversity and ecosystem services: a multilayered
546 relationship. *Trends in Ecology & Evolution*, *27*(1), 19–26. doi: 10.1016/j.tree.2011.08.006

547 MacNeil, M. A., Tyler, E. H. M., Fonnesbeck, C. J., Rushton, S. P., Polunin, N. V. C., & Conroy, M. J. (2008).
548 Accounting for detectability in reef-fish biodiversity estimates. *Marine Ecology Progress Series*, *367*,
549 249–260. doi: 10.3354/meps07580

550 Mahé, F., Rognes, T., Quince, C., Vargas, C. de, & Dunthorn, M. (2014). Swarm: robust and fast clustering
551 method for amplicon-based studies. *PeerJ*, *2*, e593. doi: 10.7717/peerj.593

552 Micheli, F., & Halpern, B. S. (2005). Low functional redundancy in coastal marine assemblages. *Ecology*
553 *Letters*, *8*(4), 391–400. doi: 10.1111/j.1461-0248.2005.00731.x

554 Micheli, F., Mumby, P. J., Brumbaugh, D. R., Broad, K., Dahlgren, C. P., Harborne, A. R., … Sanchirico, J. N.
555 (2014). High vulnerability of ecosystem function and services to diversity loss in Caribbean coral reefs.
556 *Biological Conservation*, *171*, 186–194. doi: 10.1016/j.biocon.2013.12.029

557 Moore, A. L., & McCarthy, M. A. (2016). Optimizing ecological survey effort over space and time. *Methods in*
558 *Ecology and Evolution*, 891–899. doi: 10.1111/2041-210X.12564@10.1111/(ISSN)2041-
559 210X.StatisticalEcology

560 Mouillot, D., Villéger, S., Parravicini, V., Kulbicki, M., Arias-González, J. E., Bender, M., … Bellwood, D. R.
561 (2014). Functional over-redundancy and high functional vulnerability in global fish faunas on tropical
562 reefs. *Proceedings of the National Academy of Sciences*, *111*(38), 13757–13762. doi:
563 10.1073/pnas.1317625111

564 Murphy, H. M., & Jenkins, G. P. (2010). Observational methods used in marine spatial monitoring of fishes and
565 associated habitats: a review. *Marine and Freshwater Research*, *61*(2), 236–252. doi:
566 10.1071/MF09068

567 Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., … Solymos, P. (2019). *vegan:*
568 *Community Ecology Package (R package version 2.5–5); 2019*.

569 Pais, M. P., & Cabral, H. N. (2017). Fish behaviour effects on the accuracy and precision of underwater visual
570 census surveys. A virtual ecologist approach using an individual-based model. *Ecological Modelling*,
571 *346*, 58–69. doi: 10.1016/j.ecolmodel.2016.12.011

572 Pimm, S. L., Jenkins, C. N., Abell, R., Brooks, T. M., Gittleman, J. L., Joppa, L. N., … Sexton, J. O. (2014). The
573 biodiversity of species and their rates of extinction, distribution, and protection. *Science*, *344*(6187),
574 1246752–1246752. doi: 10.1126/science.1246752

575 Port, J. A., O'Donnell, J. L., Romero-Maraccini, O. C., Leary, P. R., Litvin, S. Y., Nickols, K. J., … Kelly, R. P.
576 (2016). Assessing vertebrate biodiversity in a kelp forest ecosystem using environmental DNA.
577 *Molecular Ecology*, *25*(2), 527–541. doi: 10.1111/mec.13481

578 Rognes, T., Flouri, T., Nichols, B., Quince, C., & Mahé, F. (2016). VSEARCH: a versatile open source tool for
579 metagenomics. *PeerJ*, *4*, e2584. doi: 10.7717/peerj.2584

580 Sigsgaard, E. E., Carl, H., Møller, P. R., & Thomsen, P. F. (2015). Monitoring the near-extinct European
581 weather loach in Denmark based on environmental DNA from water samples. *Biological Conservation*,
582 *183*, 46–52. doi: 10.1016/j.biocon.2014.11.023

For Review Only

583 Somerfield, P. J., Clarke, K. R., Warwick, R. M., & Dulvy, N. K. (2008). Average functional distinctness as a
584 measure of the composition of assemblages. *ICES Journal of Marine Science*, *65*(8), 1462–1468. doi:
585 10.1093/icesjms/fsn118

586 Stuart-Smith, R. D., Bates, A. E., Lefcheck, J. S., Duffy, J. E., Baker, S. C., Thomson, R. J., … Edgar, G. J.
587 (2013). Integrating abundance and functional traits reveals new global hotspots of fish diversity.
588 *Nature*, *501*(7468), 539–542. doi: 10.1038/nature12529

589 Taberlet, P., Bonin, A., Coissac, E., & Zinger, L. (2018). *Environmental DNA: For Biodiversity Research and*
590 *Monitoring*. Oxford University Press.

591 Takahara, T., Minamoto, T., Yamanaka, H., Doi, H., & Kawabata, Z. (2012). Estimation of Fish Biomass Using
592 Environmental DNA. *PLoS ONE*, *7*(4). doi: 10.1371/journal.pone.0035868

593 Thomsen, P. F., Kielgast, J., Iversen, L. L., Wiuf, C., Rasmussen, M., Gilbert, M. T. P., … Willerslev, E. (2012).
594 Monitoring endangered freshwater biodiversity using environmental DNA. *Molecular Ecology*, *21*(11),
595 2565–2573. doi: 10.1111/j.1365-294X.2011.05418.x

596 Wangensteen, O. S., Palacín, C., Guardiola, M., & Turon, X. (2018). DNA metabarcoding of littoral hard-bottom
597 communities: high diversity and database gaps revealed by two molecular markers. *PeerJ*, *6*, e4705.
598 doi: 10.7717/peerj.4705

599 Watson, R. A., Carlos, G. M., & Samoilys, M. A. (1995). Bias introduced by the non-random movement of fish
600 in visual transect surveys. *Ecological Modelling*, *77*(2), 205–214. doi: 10.1016/0304-3800(93)E0085-H

601 Whitmarsh, S. K., Fairweather, P. G., & Huveneers, C. (2017). What is Big BRUVver up to? Methods and uses
602 of baited underwater video. *Reviews in Fish Biology and Fisheries*, *27*(1), 53–73. doi: 10.1007/s11160-
603 016-9450-1

604 Willis, T. J. (2001). Visual census methods underestimate density and diversity of cryptic reef fishes. *Journal of*
605 *Fish Biology*, *59*(5), 1408–1411. doi: 10.1111/j.1095-8649.2001.tb00202.x

606 Yamamoto, S., Masuda, R., Sato, Y., Sado, T., Araki, H., Kondoh, M., … Miya, M. (2017). Environmental DNA
607 metabarcoding reveals local fish communities in a species-rich coastal sea. *Scientific Reports*, *7*(1), 1–
608 12. doi: 10.1038/srep40368

609 Zinger, L., Bonin, A., Alsos, I. G., Bálint, M., Bik, H., Boyer, F., … Taberlet, P. (2019). DNA metabarcoding—
610 Need for robust experimental designs to draw sound ecological conclusions. *Molecular Ecology*, *28*(8),
611 1857–1862. doi: 10.1111/mec.15060

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613 **Data Accessibility**

614 The authors declare that the main data supporting the findings of this study are available within the article and its
615 Supplementary information files. Extra data are available from the corresponding author upon request. Datasets
616 will be archived in an appropriate public repository upon acceptance.

617 **Author Contributions**

618 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;
619 G.A, C.B. performed laboratory work; G.T., C.C. performed video analyses; G.A, C.B., A.C. analyzed data;
620 G.A. and M.M. wrote the paper with input from all co-authors.

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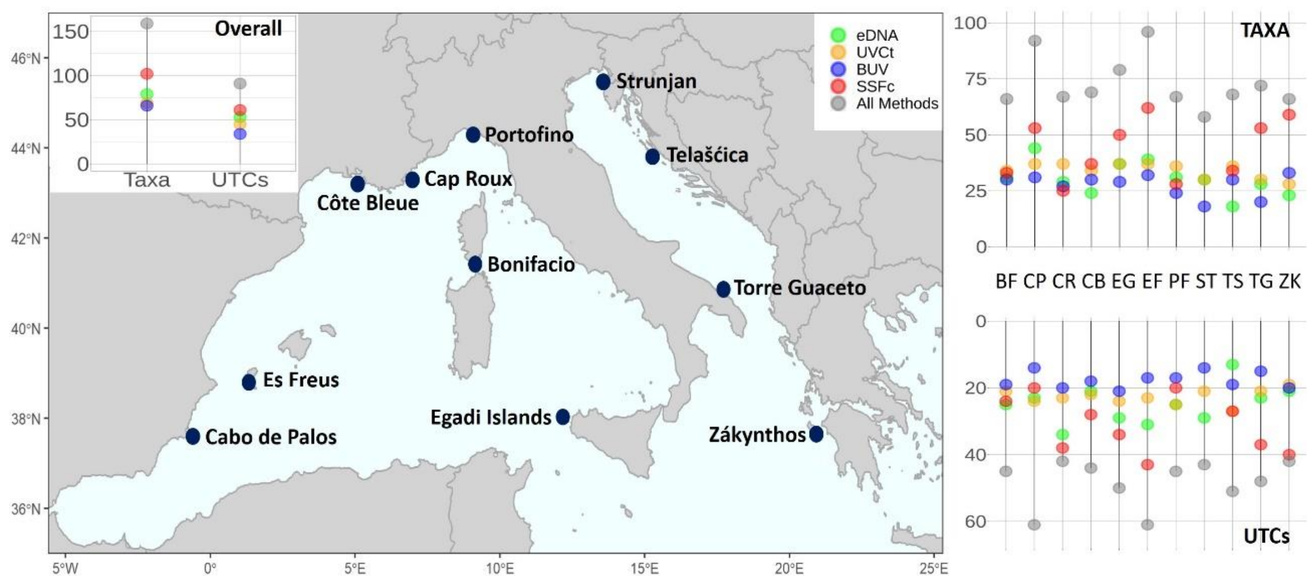
621 **Tables and Figures**



622

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

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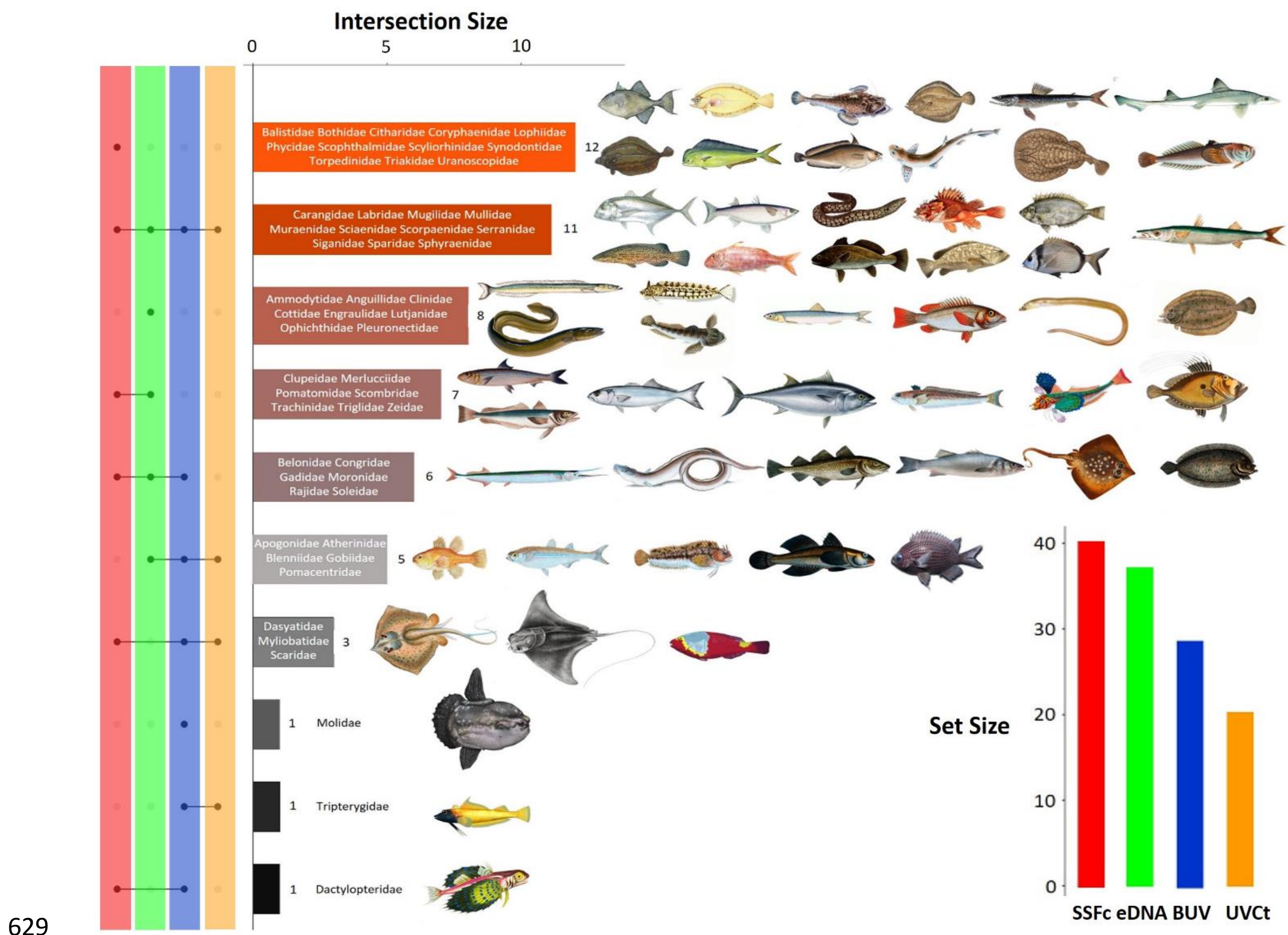

630 **Figure 2.** UpSet plot displaying the fish families detected by each of the four applied techniques. Horizontal bars, coupled
631 with lines and dots on the left panel, show the exclusive families for each method and the intersection among them. The set
632 size histograms show the overall yield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

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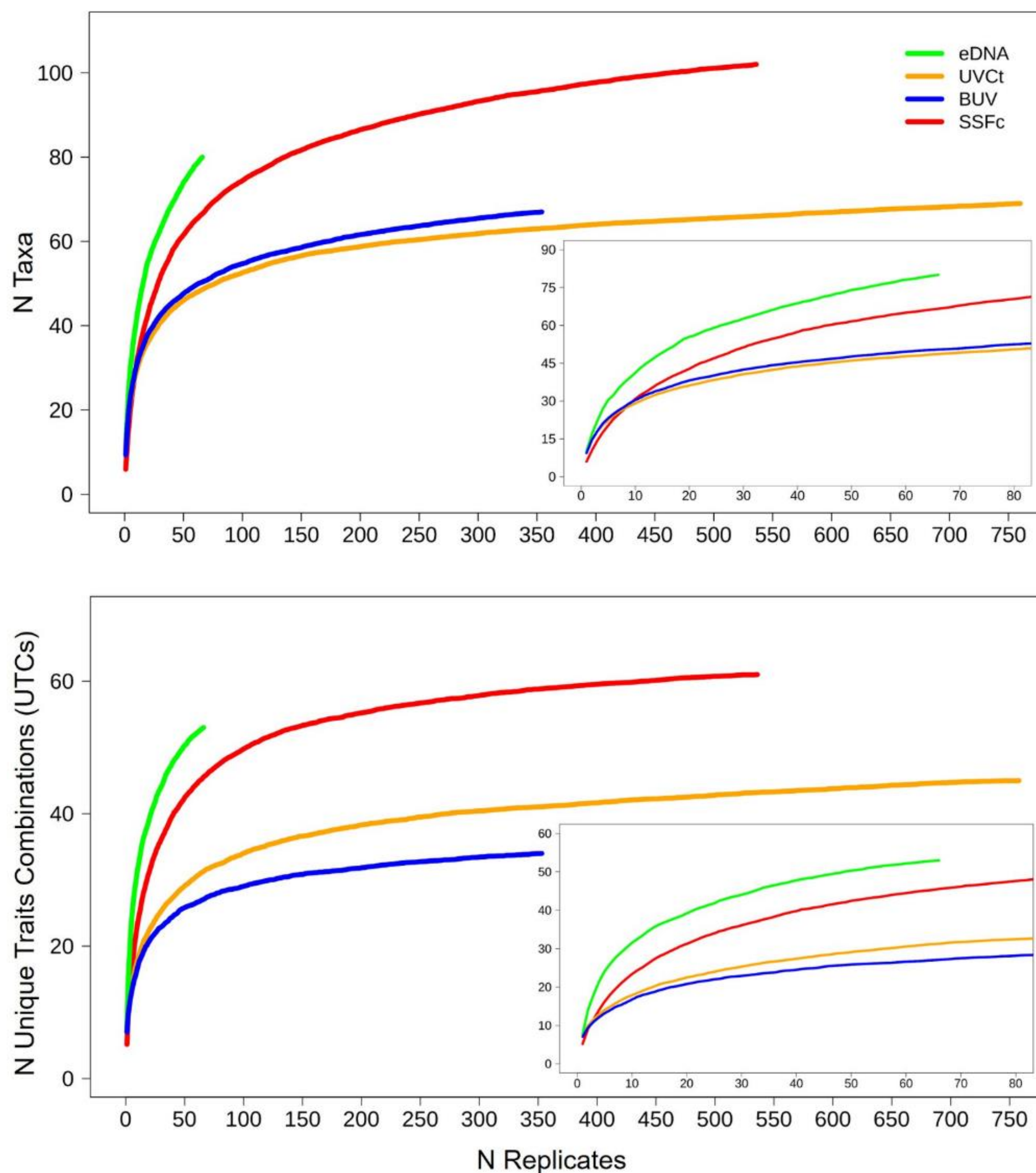
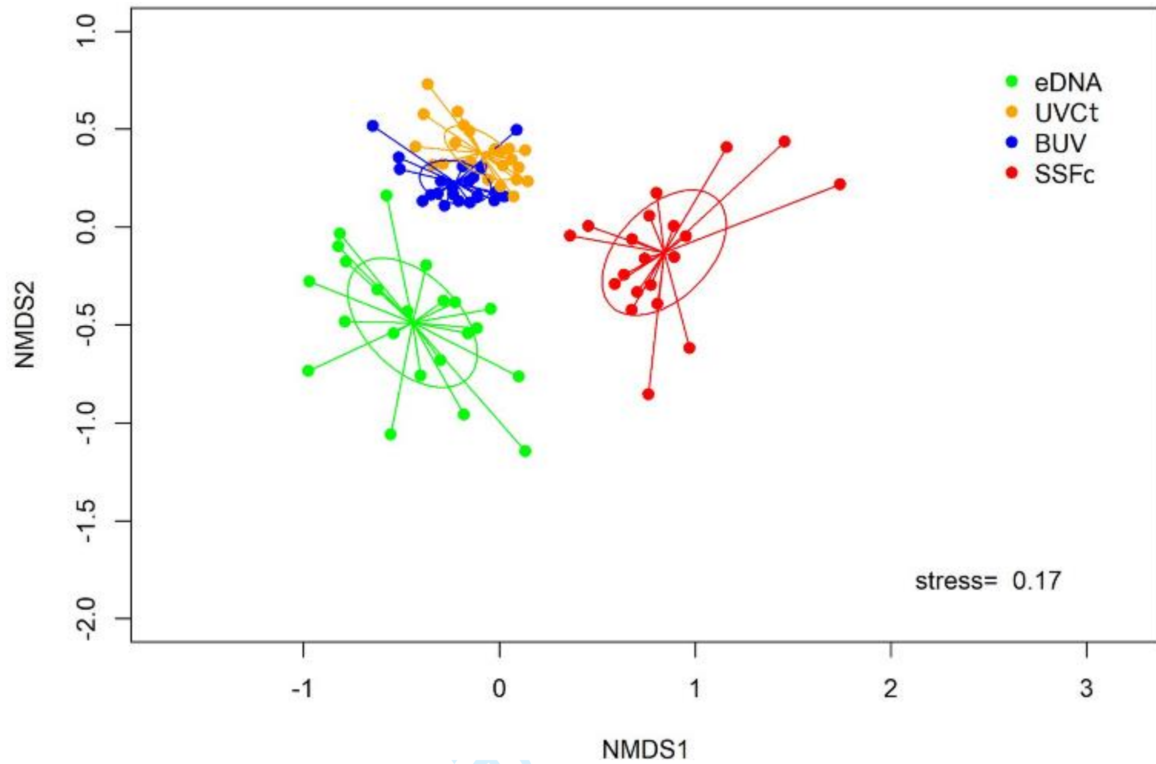


636
637 **Figure 3.** Taxa and Unique traits combinations accumulation curves. The zoomed plots in the insets help visualizing the
638 more rapid accumulation of taxa and trait combinations achieved through eDNA analyses. Multi-method accumulation
639 curves have been plotted together for graphical reason.
640



641

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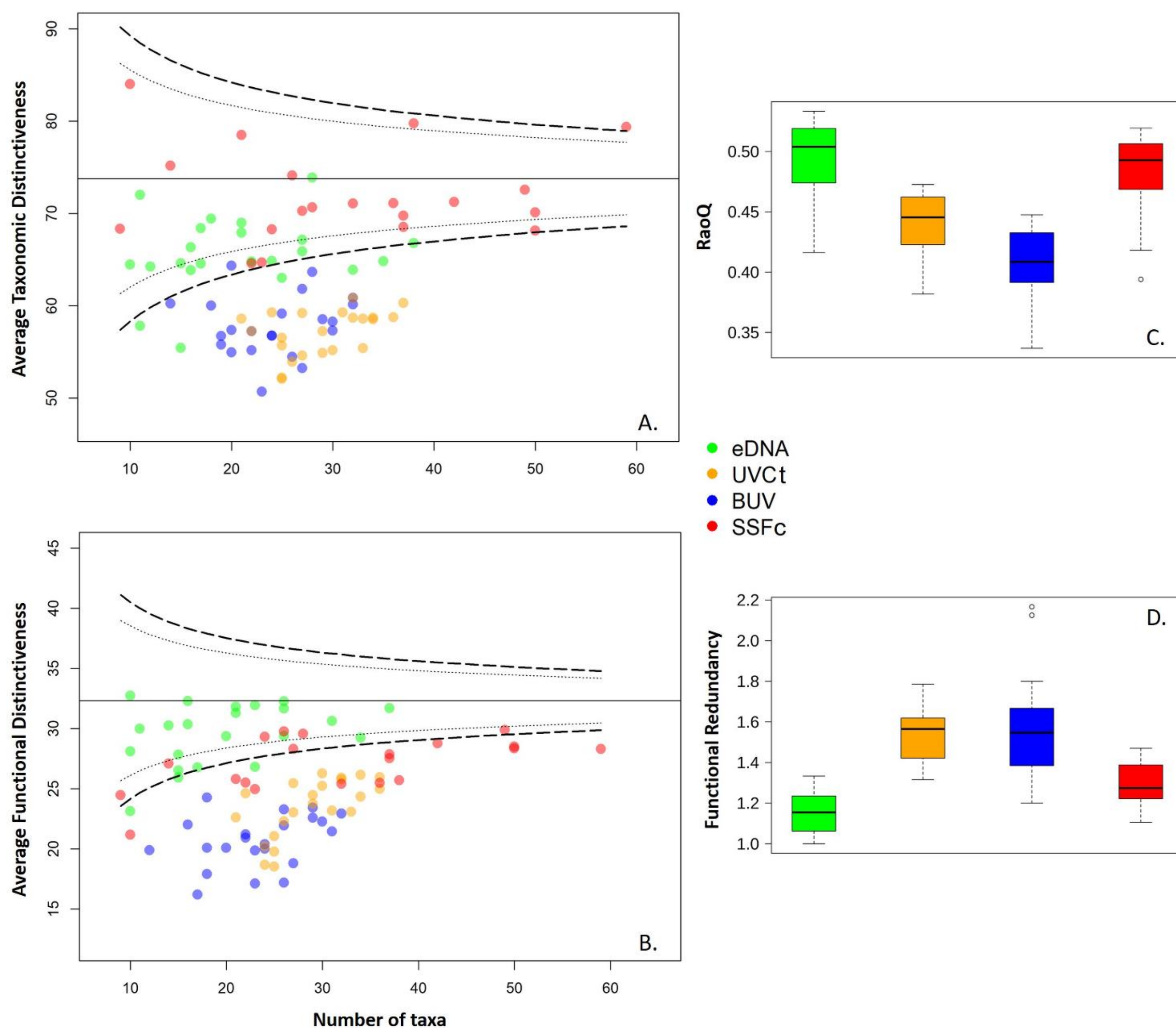

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647 **Figure 5.** Funnel plots of Average taxonomic distinctness (AvTD) (A.) and Average functional distinctness (AvFD) (B.) of the
648 fish assemblages recorded in eleven Mediterranean MPAs and eleven unprotected zones by eDNA, BUV, UVCt and SSF.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

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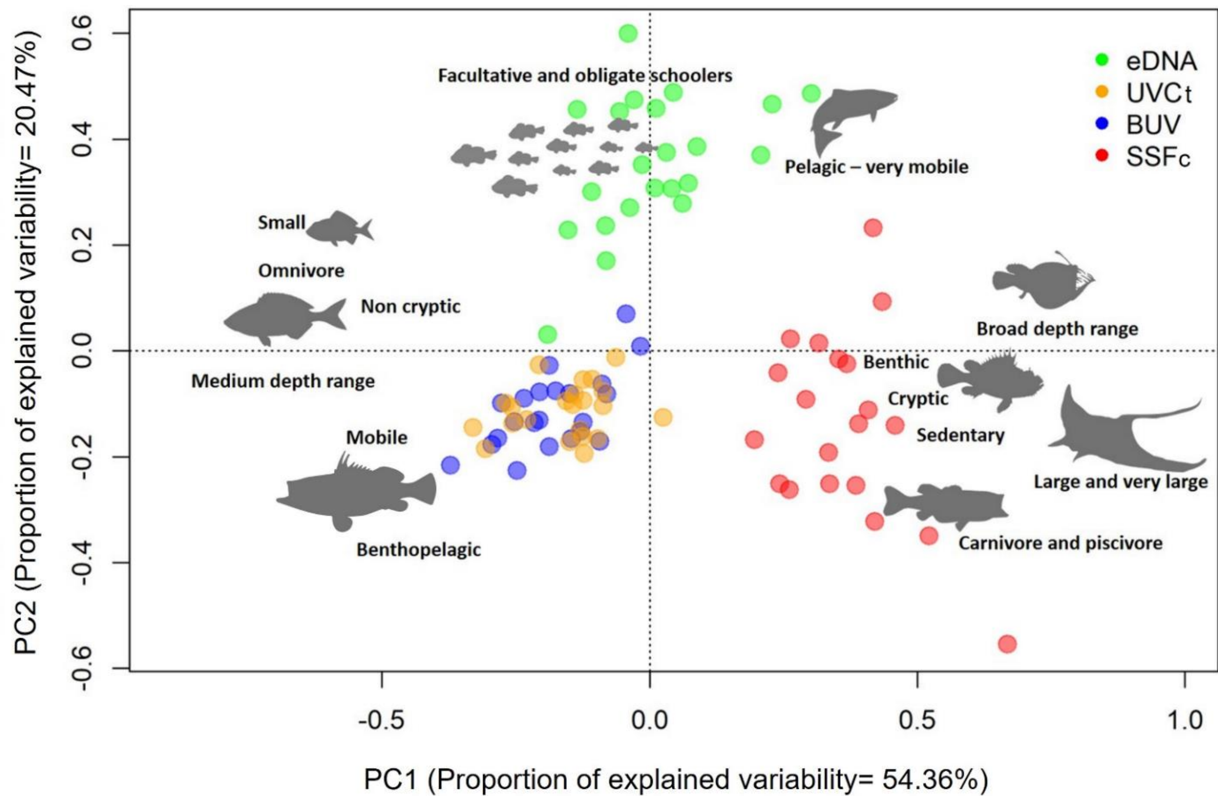

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654 **Figure 6.** Principal Component Analysis (PCA) of the functional traits proportions of fish assemblages
655 identified by eDNA, UVCt, BUV and SSFc techniques. The first four dimensions of the PCA cumulatively
656 explained 88.24% of the projected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

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