bioRxiv preprint doi: https://doi.org/10.1101/2022.05.10.491303; this version posted May 11, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 **Comparing DNA metabarcoding with morphology in the assessment of macrozoobenthos**
2 **in Portuguese transitional waters in the scope of the Water Framework Directive**
3 **monitoring**
4

5 Sofia Duarte[1,2*], Pedro E. Vieira[1,2], Barbara R. Leite[1,2], Marcos A.L. Teixeira[1,2], João M. Neto[3],
6 Filipe O. Costa[1,2]
7 *corresponding author: sduarte@bio.uminho.pt
8
9
10 [1]Centre of Molecular and Environmental Biology (CBMA), University of Minho, Campus de
11 Gualtar, 4710-057, Braga, Portugal
12 [2]Institute of Science and Innovation for Bio-Sustainability (IB-S), University of Minho, Campus
13 de Gualtar, 4710-057, Braga, Portugal
14 [3]MARE – Marine and Environmental Sciences Centre, Faculty of Sciences and Technology,
15 Department of Life Sciences, University of Coimbra, 3000-456 Coimbra, Portugal
16
17

bioRxiv preprint doi: https://doi.org/10.1101/2022.05.10.491303; this version posted May 11, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

18 **Abstract**

19 Despite the growing use and potential of DNA metabarcoding to improve and expedite
20 macrozoobenthos monitoring, its employment in Water Framework Directive (WFD) monitoring
21 of transitional ecosystems still remains largely unexplored and pending proof-of-concept studies.
22 In the current study, we addressed this research gap by building upon the biomonitoring network
23 program of the Portuguese Environmental Agency (APA) to benchmark metabarcoding against
24 the morphology-based approach for characterizing macrozoobenthic communities. We assessed
25 the ecological condition of 20 sites from four major transitional ecosystems in the west coast of
26 Portugal, namely Minho, Lima, Vouga and Mondego estuaries. A total of 154 marine invertebrate
27 species were detected with both methodologies, distributed by 11 phyla. In the majority of the
28 sites, metabarcoding returned a higher number of species and phyla than the morphology-based
29 approach (up to 2.5 times higher). In parallel, the proportion of species detected concurrently by
30 both methods was low (35 species, 23%). The use of a multi-locus strategy increased recovered
31 diversity through metabarcoding, since 37 species were detected exclusively with COI and 46
32 with 18S. For about 61% of the species recovered through morphology, metabarcoding failed
33 detection, among which 20% was due to the lack of reference sequences in genetic databases. For
34 the remaining, we did not find any plausible reason for only 10%, which could be due either to
35 inefficient DNA extraction or PCR failure. Although morphological and metabarcoding-derived
36 biotic indices did not match completely, similar responses to the environmental gradient were
37 obtained in morphology and metabarcoding based-datasets. We anticipate that metabarcoding can
38 increase the throughput and quality of the assessments, allowing faster assessments with greater
39 spatial-temporal density and robust identifications of all specimens in a sample including larval
40 stages, juveniles, and cryptic lineages, as well as smaller taxonomic groups that cannot be
41 identified to species level using the traditional approach.

42

43 **Keywords:** Estuarine Ecosystems; Water Framework Directive; Benthic Invertebrates;
44 Morphology-based assessments; DNA metabarcoding

45
46
47
48
49
50
51
52
53
54

bioRxiv preprint doi: https://doi.org/10.1101/2022.05.10.491303; this version posted May 11, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

55 **1. Introduction**

56 Coastal and transitional waters are simultaneously among the most important and most threatened
57 ecosystems in the world. They provide important services to humankind, which are at risk due to
58 impactful and diverse anthropogenic pressures (Solan 2004). In the European Union, several
59 legislative measures have been developed aiming to protect and improve the quality of all surface
60 and marine waterbodies, such as the Water Framework Directive (WFD, Directive 2000/60/EC)
61 (European commission 2000) and the Marine Strategy Framework Directive (MSFD, Directive
62 2008/56/EC) (European commission 2008). WFD requires member states to assess the ecological
63 status of aquatic ecosystems at regular intervals, by sampling or surveying Biological Quality
64 Elements (BQE) following national or EU-wide standard methods. Benthic invertebrates are one
65 of the key BQE employed in WFD, given that they provide invaluable ecosystem services (e.g.,
66 support – larval supply, habitat supply; provision – shellfish, genetic resources; regulation –water
67 cleaning and sediments stabilization; cultural – aesthetic), and integrate environmental conditions
68 and changes in a very effective way, which allows the monitoring of long-term responses and
69 site-specific impacts (Salas et al. 2004, Teixeira et al. 2008a,b, Neto et al. 2010, Borja et al. 2011).
70 WFD determines that the macrozoobenthos must be assessed in terms of taxonomic composition,
71 diversity, abundance, disturbance-sensitive and pollution-indicator taxa (European Commission
72 2011) and several biotic indices have been developed to assess the condition of coastal and
73 transitional waters, with these communities (e.g., Borja et al. 2000, Rosenberg et al. 2004,
74 Teixeira et al. 2009). Some of the most widely used include species richness, Shannon-Wiener,
75 Margalef, AZTI Marine Biotic Index – AMBI, among others, that are assessed through
76 morphology-based identification of specimens (Borja et al. 2000, 2011, Salas et al. 2004, Teixeira
77 et al. 2008b). Resulting measurements are then compared against values expected at "reference
78 conditions", and water bodies concomitantly assigned to an ecological status (Vinagre et al.
79 2015).

80 Although well established and harmonized, bioassessment methodologies of the WFD and MSFD
81 are still intensively debated. Main problems include high monitoring costs and some level of
82 variability and subjectivity that raise unsureness about the reliability of the results. Also, the long
83 time required to complete the species identification process has resulted in the low throughput
84 processing of biomonitoring samples and this is no longer compatible with the need to rapidly
85 reach conclusions about the ecological status of the water bodies (Leese et al. 2018). In addition,
86 despite the imperative need of monitoring and assessment, economic constraints are forcing some
87 countries to reduce the budgets dedicated to biomonitoring (Hering et al. 2018). Due to the above-
88 mentioned reasons, benthic invertebrates monitoring has been conducted most of the times in 1
89 or 2 events per 6-year management cycle (Hering et al. 2010). This scenario is far from ideal,
90 since benthic invertebrate communities can be susceptible to several natural events that may occur
91 periodically, such as floods and droughts, and that may mask the effects of anthropogenic

bioRxiv preprint doi: https://doi.org/10.1101/2022.05.10.491303; this version posted May 11, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

92 disturbances and alter ecosystems assessments (Neto et al. 2010). Thus, a more frequent
93 biomonitoring would definitively provide a more accurate and comprehensive view of the present
94 and changing status of benthic ecosystems.
95 One of the most promising tools for the simultaneous identification of bulk organism assemblages
96 is DNA metabarcoding, where amplicons of standardized DNA-barcode markers, obtained from
97 bulk communities or environmental samples, are massively-parallel sequenced via high-
98 throughput sequencing (HTS) (Hajibabaei 2012, Cristescu 2014). This approach has a number of
99 potential benefits over the morphology-based method, including the simultaneous processing of
100 a large number of samples, increased sensitivity, accuracy and specificity, as well as greater time
101 and cost effectiveness in biodiversity monitoring (Hajibabaei 2012, Cristescu 2014, Duarte et al.
102 2021).
103 However, despite the demonstrated utility of metabarcoding to reliably generate assessments of
104 aquatic environmental status, in particular based on benthic invertebrates (Aylagas et al. 2014,
105 2018, Cowart et al. 2015, Lobo et al. 2017b, Derycke et al. 2021, Duarte et al. 2021, Van den
106 Bulcke et al. 2021), the adoption of metabarcoding in biomonitoring still face several challenges,
107 in particular in coastal and transitional ecosystems. These ecosystems hold very different features
108 from freshwaters, and are highly diverse, thereby requiring a tailored tool in order to overcome
109 existing technological shortcomings that can prevent the detection of all taxa within a sample
110 (e.g., Leite et al. 2021, Wangensteen et al. 2018a,b). In addition, one of the greatest challenges
111 would be to establish a framework for implementation of metabarcoding into monitoring
112 programs, which is currently lacking. To that end it would be imperative to conduct extensive
113 cross-validation studies under realistic scenarios, involving stakeholders, and benchmarking
114 against morphotaxonomic approaches.
115 In the current study, we evaluated the sensitivity and accuracy of DNA metabarcoding for species
116 detection and identification in the scope of WFD monitoring of macrozoobenthos in coastal and
117 transitional waters in Portugal. To that end we conducted a metabarcoding-morphology
118 comparison on the course of a WFD survey in 20 monitoring sites belonging to four transitional
119 ecosystems – the estuaries of the Rivers Minho, Lima, Vouga and Mondego. This will enable the
120 parallel comparison of morphology and DNA-based data outputs and to test the sensitivity and
121 discriminatory power of metabarcoding on estuarine ecological condition assessment, along
122 selected environmental gradients. To our best knowledge this will be the first attempt in Portugal
123 to address this topic comprehensively (but see Martins et al. 2020, for freshwaters) using
124 metabarcoding, and involving an End-user stakeholder (APA).
125
126
127
128

bioRxiv preprint doi: https://doi.org/10.1101/2022.05.10.491303; this version posted May 11, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

129 ## 2. Material and Methods

130 ### 2.1. Sampling sites and environmental characterization

131 For the current study, 20 sampling sites were selected within 4 estuaries of the west Atlantic coast
132 of Portugal, that are included in the national WFD monitoring program: the estuaries of Rivers
133 Minho (02D/05, 02D/04, 02E/06, 02E/05, 02E/07), Lima (03D/06, 03E/06, 03E/04, 03E/03,
134 03E/28), Vouga (10E/05, 10E/06, 09F/04, 09F/08, 09E/05, 09F/07) and Mondego (13E/08,
135 13E/03, 13D/03, 13E/02) and were sampled in late summer 2019 (Fig. 1, please see more details
136 of sampling sites and dates on Supplementary Material: Table S1).
137 The Minho estuary is a mesotidal stratified estuary, partially mixed (Sousa et al. 2008) and has
138 been classified as a Natura 2000 site, covering a total estuarine area of 23 km^2 and located in the
139 Northwest of the Iberian Peninsula. The Lima estuary, is also a mesotidal stratified estuary,
140 partially mixed, and located in the Northwest of the Iberian Peninsula (Sousa et al. 2006, 2007).
141 It is an important harbour in the region, serving trade navigation and fishing activities. Because
142 of this, it has been subjected to constant dredging of the navigation channel within its first 3 km.
143 Other sources of disturbance include the input of agricultural runoff and urban and industrial
144 sewage (Sousa et al. 2007), which changed the physical nature of the lower part of the estuary
145 and have also been responsible for eutrophication (Sousa et al. 2006, 2007). As a consequence of
146 these activities, three of the selected sites are heavily impacted (03D/06, 03E/06 and 03E/04),
147 while the other two remain relatively pristine (sites 03E/03 and 03E/28).
148 The Vouga estuary is a shallow coastal lagoon and a homogeneous mesotidal estuary, with
149 irregular river discharges, spanning about 75 km^2 along the central west coast of Iberian
150 Peninsula, where it merges with the freshwater flow of the Vouga River's catchment area. It has
151 a complex geometry, forming 4 main channels with several branches, exposed to the impact of
152 diverse industrial, shipping, aquaculture and other regional activities (Rodrigues et al. 2011). It is
153 a LTER site (Long Term Ecosystem Research; http://www.lter-europe.net/). Most of the selected
154 sites are pristine (10E/05, 10E/06, 09F/08, 09F/07), but two of them are heavily modified (09E/04,
155 09E/05). The Mondego estuary is a relatively small warm-temperate homogenous mesotidal
156 system, with irregular river discharges, located in the central west coast of Iberia, formed by two
157 arms; the North and the South arm, which are highly affected by eutrophication (Teixeira et al.
158 2009). Most of the sites are heavily modified (13E/08, 13E/03, 13E/02), and only one site can be
159 considered pristine (13D/03).
160

bioRxiv preprint doi: https://doi.org/10.1101/2022.05.10.491303; this version posted May 11, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

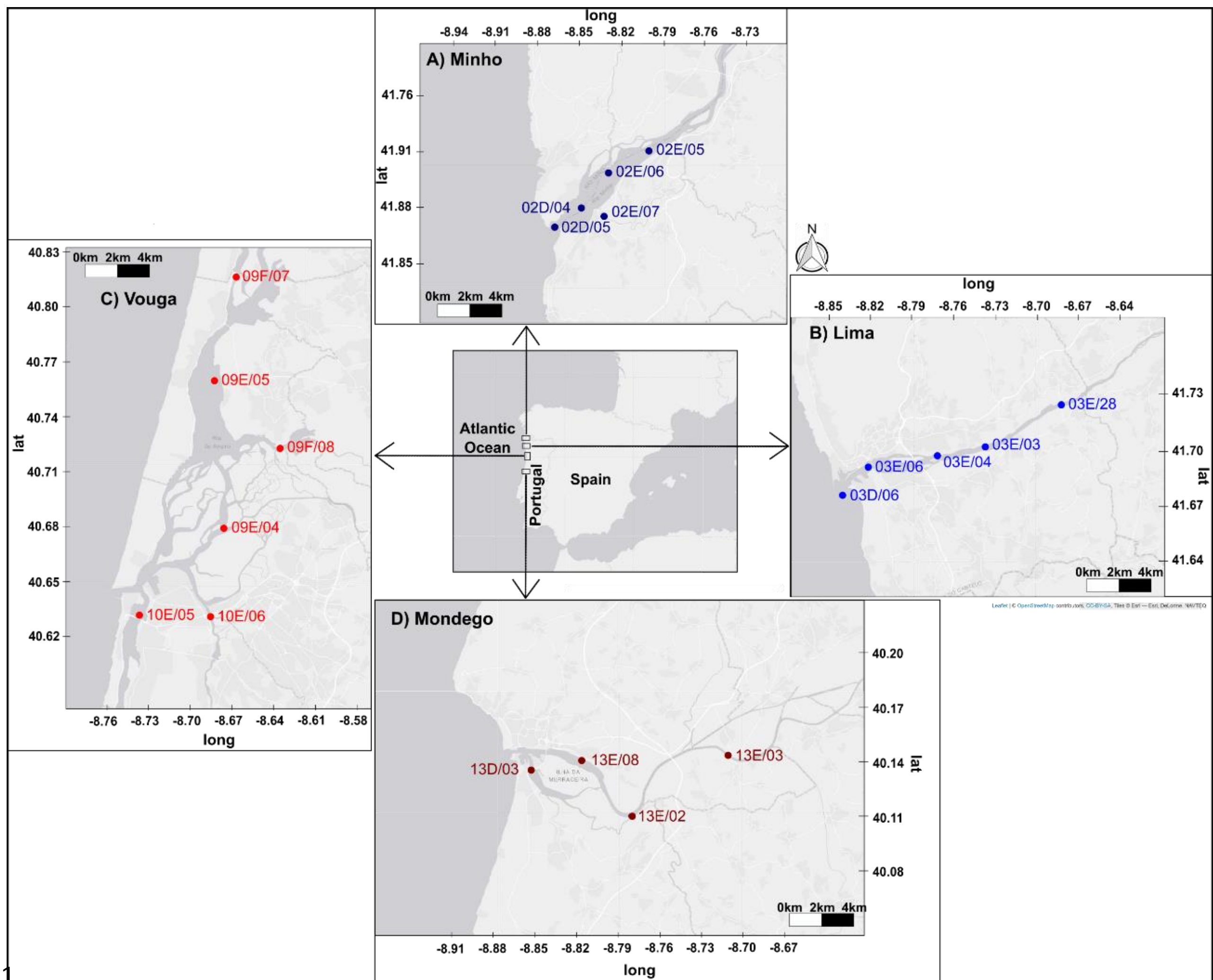


Figure 1. Location of the 20 sites sampled for macrozoobenthos in the scope of the WFD monitoring program, and belonging to four transitional ecosystems: the estuaries of the Rivers Minho, Lima, Vouga and Mondego. See Supplementary Material: Table S1, for more details of the sampling sites.

In situ water temperature, dissolved oxygen, conductivity, pH and salinity values were registered at high tide conditions, using a YSI professional plus/HANNA HI98194 Ph/EC/DO multiparameter probe. A Niskin/Van Dorn (Horizontal) bottle was used to collect water at the bottom of each sampling site (except for 03E/28, 09F/07 and 13E/03 sites, where water was collected at surface), for total suspended solids (TSS), particulate organic matter (POM), nutrients (NO_3^--N and NH_4^+-N) and Silicon (Si) analyses. In the laboratory, the concentrations of NO_3^--N, NH_4^+-N and Si were measured using a Skalar San++ Autoanalyser, following adapted and optimised methodologies: NO_3^--N (Houba et al. 1987, Kroon et al. 1993) and NH_4^+-N (Krom 1980). Total suspended solids (TSS) were determined by filtering water aliquots, until pre-clogging, through GF/F (0.7 µm pore size, 47 mm diameter, Whatman), previously stuffed and weighed. The filters were then washed with distilled water and dried for 24 hours in a 60 ºC oven,

bioRxiv preprint doi: https://doi.org/10.1101/2022.05.10.491303; this version posted May 11, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

178 and then weighed to the nearest ± 0.00001 g, after attaining room temperature in a desiccated
179 environment. TSS were assessed, as the difference between dried filters and initial filters weight.
180 For determination of POM, the filters were burned at 450 ºC, for 4 h, and then weighed to the
181 nearest ± 0.00001 g, after attaining room temperature in a desiccated environment. POM was
182 assessed as the difference between dried and burned filters weights. The transparency of water
183 samples was assessed with Secchi disks.
184

185 **2.2. Morphology-based sample processing and taxonomic identifications**

186 Subtidal soft-bottom macrozoobenthic assemblages were sampled with a van Veen grab
187 (sampling area 0.1 m^2), in the 20 sampling sites of the four transitional ecosystems in late summer
188 of 2019 (for details on sampling dates please see Supplementary Material: Table S1). Three
189 sediment samples were collected from each sampling site, sieved through 1 mm mesh size, and
190 the invertebrate specimens manually sorted from the sediment in the laboratory. Two samples (R1
191 and R2) were preserved in 4% buffered formalin solution at room temperature and one sample
192 (R3) was preserved in absolute ethanol and placed at 4 ºC until further analysis. Due to logistical
193 reasons only R3 was used for metabarcoding (out of 3 replicates per sampling site). Benthic
194 invertebrates preserved in formalin were counted and identified at the stereomicroscope to the
195 lowest possible taxonomic level, with the assistance of taxonomic identification keys and
196 monographs (e.g., Lincoln 1979, Hayward et al. 1996, Campbell and Nicholls 2008, Hayward
197 and Ryland 2017). Ethanol-preserved bulk invertebrate samples were first subjected to non-
198 destructive DNA extraction, as described below, and subsequently used in morphology-based
199 identification to the lowest possible taxonomic level, as described above.
200

201 **2.3. DNA extraction, preparation of amplicon libraries and high-throughput sequencing**
202 **(HTS)**

203 Up to 30 g of ethanol-preserved invertebrate samples were used to extract DNA by means of a
204 non-destructive procedure, using a silica-based method, adapted from Ivanova et al. (2006), and
205 as described by Steinke et al. (2022). Briefly, samples were placed in autoclaved flasks,
206 previously washed with 10% bleach and ultra-pure water, to which an adequate volume of a lysis
207 buffer (100 mM NaCl, 50 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0 and 0.5% SDS) (depending
208 on the sample wet weight) was added. Samples were then digested overnight in an orbital
209 incubator (Infors) at 140 rpm and 56 ºC. Two aliquots of 1 mL, collected from each lysate, were
210 used in two independent DNA extractions, which were pooled together before PCR amplification.
211 Lysates were then centrifuged and supernatants mixed with a binding mix (6M GuSCN, 20mM
212 EDTA pH 8.0, 10mM Tris-HCl pH 6.4 and 4% Triton X-100) and purified through silica columns
213 and 3 washing steps, with two ethanol-based solutions. DNA was finally eluted from the columns
214 by using autoclaved deionized water. Negative controls were processed along the DNA extraction

bioRxiv preprint doi: https://doi.org/10.1101/2022.05.10.491303; this version posted May 11, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

215 procedure for checking for contaminations of the solutions used for DNA extractions and labware
216 materials used. These negative controls were used as template in subsequent PCR amplification
217 reactions.
218 Amplicon libraries and high-throughput sequencing (HTS) were carried out at Genoinseq
219 (Biocant, Portugal). The primer pair mlCOIintF (5'-
220 GGWACWGGWTGAACWGTWTAYCCYCC -3') (Leray et al. 2013) and LoboR1 (5'-
221 TAAACYTCWGGRTGWCCRAARAAYCA -3') (Lobo et al. 2013) was used to amplify an
222 internal region of 313 bp of the mitochondrial cytochrome c oxidase I (COI) gene and the primer
223 pair TAReuk454FWD1 (5'- CCAGCASCYGCGGTAATTCC -3') and TAReukREV3 (5'-
224 ACTTTCGTTCTTGATYRA -3') (Stoeck et al. 2010, Lejzerowicz et al. 2015) was used to
225 amplify ~400 bp of the V4 hypervariable region of the 18S rRNA gene (18S). The two primer
226 pairs were selected based on previous studies on marine invertebrates of the region
227 (macrozoobenthos and meiofauna) as the ones that captured the most diverse taxa among four
228 tested primers pairs for COI (Fais et al. 2020, Leite et al. 2021) and three tested primers for 18S
229 (Fais et al. 2020). PCR reactions were performed for each sample using KAPA HiFi HotStart
230 PCR kit according to manufacturer instructions, 0.3 µM of each primer and 50 ng of template
231 DNA, in a total volume of 25 µL. For the mlCOIintF/LoboR1 primer pair, the PCR conditions
232 involved a 3 min denaturation at 95 ºC, followed by 35 cycles of 98 ºC for 20 s, 60 ºC for 30 s
233 and 72 ºC for 30 s and a final extension at 72 ºC for 5 min. For the
234 TAReuk454FWD1/TAReukREV3 primer pair, the PCR conditions involved a 3 min denaturation
235 at 95 ºC, followed by 10 cycles of 98 ºC for 20 s, 57 ºC for 30 s and 72 ºC for 30 s and 25 cycles
236 of 98 ºC for 20 s, 47 ºC for 30 s and 72 ºC for 30s, and a final extension at 72 ºC for 5 min.
237 Second limited-cycle PCR reactions added indexes and sequencing adapters to both ends of the
238 amplified target regions according to manufacturer recommendations (Illumina 2013). PCR
239 products were then one-step purified and normalized using SequalPrep 96-well plate kit
240 (ThermoFisher Scientific, Waltham, USA) (Comeau et al. 2017), pooled and pair-end sequenced
241 in the Illumina MiSeq® sequencer with the V3 chemistry, according to manufacturer instructions
242 (Illumina, San Diego, CA, USA) at Genoinseq (Biocant, Portugal).
243

244 **2.4. Bioinformatics pipelines**

245 Raw reads, extracted from Illumina MiSeq® System in fastq format, were quality-filtered with
246 PRINSEQ version 0.20.4 (Schmieder and Edwards 2011). This entailed the removal of
247 sequencing adapters and of short reads (<100 bp and <150 bp, for COI and 18S, respectively).
248 Bases with an average quality lower than Q25, in a window of 5 bases were also trimmed. The
249 filtered forward and reverse reads provided by the sequencing facility were merged by
250 overlapping paired-end reads in mothur (make.contigs function, default alignment) (version

bioRxiv preprint doi: https://doi.org/10.1101/2022.05.10.491303; this version posted May 11, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

251 1.39.5), where primers sequences were also removed (trim.seqs function, default) (Schloss et al.
252 2009, Kozich et al. 2013).
253 The usable reads were then processed in two public databases pipelines (Leite et al. 2021): COI
254 reads were submitted to mBrave – Multiplex Barcode Research and Visualization Environment
255 (www.mbrave.net; Ratnasingham 2019), which is linked with BOLD (Ratnasingham and Hebert
256 2007) and 18S reads were analysed in SILVAngs database (https://ngs.arb-silva.de/silvangs/;
257 Quast et al. 2013).
258 In mBrave, COI reads were uploaded using the sample batch function and only the trimming by
259 length was applied (maximum of 313 bp). Low quality reads were then removed if the average
260 quality value (QV) was less than 20 or sequences shorter than 150 bp. Reads fulfilling the previous
261 criteria were further de-replicated and clustered in Operational Taxonomic Units (OTUs) using a
262 distance threshold of 3%. The resultant OTUs were then taxonomically assigned at species level
263 using a 97% similarity threshold against BOLD database that includes several publicly available
264 reference libraries for marine invertebrates of the Northeast Atlantic (e.g., Hollatz et al. 2017,
265 Leite et al. 2020, Vieira et al. 2020).
266 In SILVAngs, each 18S read was aligned using the SILVA Incremental Aligner (SINA v1.2.10
267 for ARB SVN (revision 21008)) (Pruesse et al. 2012) against the SILVA SSU rRNA SEED and
268 quality controlled (Quast et al. 2013). Reads shorter than 150 aligned nucleotides and reads with
269 more than 1% ambiguities, or 2% homopolymers, respectively, were excluded from further
270 processing. Putative contaminations and artefacts, reads with a low alignment quality (80
271 alignment identity, 40 alignment score reported by SINA), were identified and excluded from
272 downstream analysis. After these initial steps of quality control, identical reads were identified
273 (dereplication), the unique reads were clustered (OTUs) on a per sample basis, and the reference
274 read of each OTU was then taxonomically assigned. VSEARCH (version 2.14.2;
275 https://github.com/torognes/vsearch) (Rognes et al. 2016) was used for dereplication and
276 clustering, applying identity criteria of 1.00 and 0.99, respectively. The taxonomic assignment
277 was performed using BLASTn (2.2.30+; http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Camacho et al.
278 2009) with standard settings and the non-redundant version of the SILVA SSU Ref dataset
279 (release 138.1; http://www.arb-silva.de). The taxonomic classification of each OTU reference
280 read was mapped onto all reads that were assigned to the respective OTU. Reads without any or
281 weak classifications, where the function "(% sequence identity + % alignment coverage)/2" did
282 not exceed the value of 70, remained unclassified and were assigned to "No Taxonomic Match".
283 In the end, only OTUs taxonomically identified with a similarity threshold of 99% were kept for
284 further analysis.
285 For both markers, only reads with match at species level were used for further analysis, and
286 taxonomic assignments with less than 9 sequences were discarded (Fais et al. 2020, Leite et al.

bioRxiv preprint doi: https://doi.org/10.1101/2022.05.10.491303; this version posted May 11, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

2021). Any read that matched to non-metazoan and metazoan non-invertebrate groups were also excluded.

In order to maximize the results, the taxonomic assignments were made using the full databases (i.e., BOLD for COI and SILVA for 18S), and each species match was reviewed individually to assess the reliability of the taxonomic assignments. Discordances in the taxonomic assignments were carefully inspected and if they were possible to resolve (i.e., synonyms, clear cases of misidentification), the most probable identification was kept.

2.5. Gap-analysis and reasons for the absence of species detection in the metabarcoding dataset

The presence of representative sequences of all the species detected in the present study was assessed in BOLD and SILVA. All the available COI sequences matching the detected species names were mined from BOLD using BAGS (Fontes et al. 2021). All the Animalia records were mined directly from SILVA (version 138.1) to assess which species have representative sequences in this database. A species was considered represented if at least one sequence was available. The reasons for the no-detection of the species in the metabarcoding dataset and that were exclusively identified through morphology were further investigated. Failed detection by one marker or by both may simply have occurred because that particular species was absent in the respective reference databases. However, if a species was present in both reference databases, but was only detected by one marker, or not detected at all through metabarcoding, this would be an indication of inefficient DNA extraction or PCR amplification failure with the primers used for targeting each marker. Other possible reasons pointed out included: 1) species generating less reads than the minimum threshold set in the bioinformatic pipeline (<9 reads); 2) species detection in replicate 1 (R1) and/or 2 (R2), but not in replicate 3 (R3) and 3) inefficient DNA extraction or PCR failure.

2.6. Statistical analyses

A principal component analysis (PCA) was used to ordinate sampling sites according to physical and chemical parameters, after data standardization.

Only OTUs with matches to marine invertebrate species (i.e., <3% and <1% ID distance from matching sequences, for COI and 18S, respectively) and with a minimum of 9 reads, were used in further analyses. The number of reads of different OTUs were summed up if they were assigned to the same species, for each marker separately. The validity of the species names for the morphology and the metabarcoding datasets were verified in the World Register of Marine Species (WoRMS) database (WoRMS Editorial Board 2021).

The proportion of species with overlapping or exclusive detections by morphology-based identification and metabarcoding-based identification was determined: 1) for the overall of the

bioRxiv preprint doi: https://doi.org/10.1101/2022.05.10.491303; this version posted May 11, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

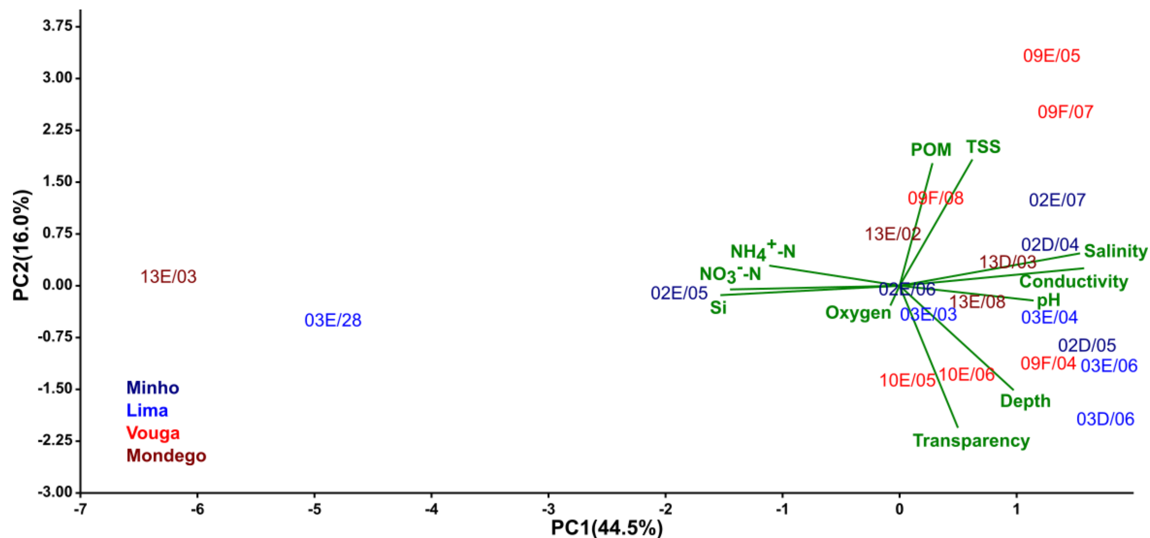
324 species detected, 2) for the species detected within each estuary, and within each estuary 3) for
325 the species detected on each sampling site, using Venn diagrams (http://www.venndiagrams.net/).
326 For each replicate, within each sampling site, the distribution of species among high-rank
327 taxonomic groups (i.e., phyla) was displayed through barplots for the morphology and
328 metabarcoding datasets using GraphPad Prism v6 (GraphPad Software, Inc.).
329 The AZTI Marine Biotic Index (AMBI) was calculated based on the presence/absence of each
330 species identified through metabarcoding for each marker, and for all species detected using both
331 markers and, based on the presence/absence of each species identified through morphology
332 ((p/a)AMBI) (Aylagas et al. 2014, 2018). This version uses only the presence/absence of each
333 species, ignoring the number of individuals or the number of reads. Values were calculated based
334 on pollution tolerances of the species present, where tolerance is expressed categorically as one
335 of five ecological groups: I, sensitive to pressure; II, indifferent; III, tolerant; IV, second order
336 opportunistic, and V, first order opportunistic (http://ambi.azti.es, Borja et al. 2000) by using the
337 formula: (p/a)AMBI = [(0 x % GI) + (1.5 x % GII) + (3 x %GIII) + (4.5 x % GIV) + (6 x % GV)]
338 / 100; where % represent the number of species falling on each ecological group. (p/a)AMBI
339 index values vary from 0 to 7 indicating different ecosystem status according to pollution
340 tolerances of the species present: 0 to 1.2, unpolluted; 1.3 to 3.3, slightly polluted; 3.4 to 5,
341 moderately polluted; 5.1 to 6, heavily polluted and 6.1 to 7, extremely polluted (Borja et al. 2000,
342 Aylagas et al. 2014, 2018).
343 Canonical correspondence analysis (CCA) was used to determine the relationships between
344 environmental variables and marine benthic invertebrate's species detected through morphology
345 and metabarcoding (Ter Braak and Verdonschot 1995). Pearson correlation coefficients were
346 determined to assess the correlations between: i) each environmental variable and PC1 and PC2
347 axes scores; ii) each environmental variable and CC1 and CC2 axes scores; iii) the no. of
348 specimens (morphology) and the no. of reads (metabarcoding), for species detected with both
349 methodologies in R3; iv) (p/a)AMBI based on morphology assessments and metabarcoding-based
350 assessments; v) (p/a)AMBI and PC1 and PC2 axes scores.
351 Correlation, PCA and CCA analyses were conducted using PAleontological STatistics (PAST)
352 version 4.03 for Windows (Hammer et al. 2001).
353

354 **3. Results**

355 **3.1. Environmental characterization of the sampling sites within each estuarine system**

356 The full environmental characterization of each sampling site within each estuary is detailed in
357 Supplementary Material: Table S1 and summarized in the PCA diagram displayed in Fig. 2.

bioRxiv preprint doi: https://doi.org/10.1101/2022.05.10.491303; this version posted May 11, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.



358

359 **Figure 2.** Principal components analysis (PCA) of physical and chemical stream water parameters
360 of the 20 sampling sites along the 4 estuaries.

361

362 PCA ordination of the 20 sites according to water variables showed that axes 1 and 2 explained
363 60.5% of the total variance (Fig. 2). The first axis was significantly correlated with salinity,
364 conductivity and pH, and nitrates, ammonia and Si concentrations which changed in opposite
365 directions (more details of the coefficients of correlation and p values on Supplementary Material:
366 Table S2). The second axis was more correlated with TSS and POM, and transparency and depth,
367 which also changed in opposite directions (Supplementary Material: Table S2). There was a clear
368 separation of the oligohaline sites 13E/03 (Mondego) and 03E/28 (Lima) from the remaining sites,
369 which according to the available salinity values were classified as poly-mesohaline (Minho -
370 02E/06, 02E/05; Lima - 03E/03; Mondego – 13E/02) and euhaline (Minho - 02D/05, 02D/04,
371 02E/07; Lima - 03D/06, 03E/06, 03E/04; Vouga - 09F/08, 09E/05, 09F/07; Mondego - 13E/08).

372

373 **3.2. Initial metabarcoding dataset processing**

374 The number of initial raw reads was 1111837 and 770841 for COI and 18S, respectively (Table
375 1). Subsequent filtering steps (short length reads removal, de-multiplexing, primers removal, de-
376 replication and chimera's removal) reduced the number of sequences to 718120 and 448299 for
377 COI and 18S, respectively (Table 1). From these, 663364 and 448291 were taxonomically
378 classified for COI and 18S (Supplementary Material: Table S3), respectively, and from these
379 55.4% and 38.8%, of the initial reads, matched with marine invertebrate taxa, while 52.5% and
380 37.3% had species level assignments with sequence numbers superior to 8, for COI and 18S,
381 respectively (Table 1). The % of COI reads that matched marine invertebrate species varied
382 between 2.4% and 85.1%, for 13D/03 (Mondego) and 02E/06 (Minho), respectively, while the %
383 of 18S reads that matched marine invertebrate species varied between 0 and 64.2%, for 02E/06
384 (Minho) and 03D/06 (Lima), respectively (Supplementary Material: Table S3).

bioRxiv preprint doi: https://doi.org/10.1101/2022.05.10.491303; this version posted May 11, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

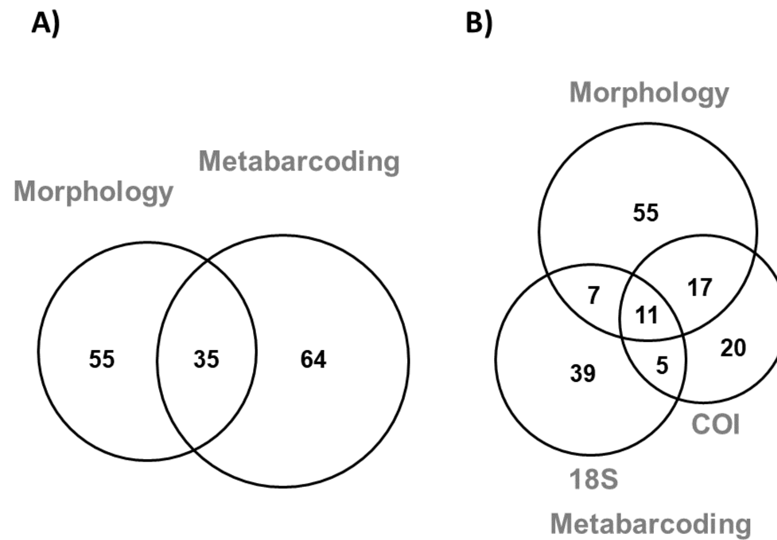
385 **Table 1.** Total number of sequences generated in Illumina MiSeq high-throughput sequencing
386 (raw reads) and %, in comparison with initial raw reads, retained after all the processing steps of
387 the bioinformatics pipeline (demultiplexing, primers removal and quality filter) (usable reads),
388 and assigned to marine invertebrate taxa and species, for each marker (COI and 18S). *, reads
389 submitted to taxonomic assignment; **, reads with taxonomic classification at species level
390 (>97% for COI and >99% for 18S) and sequence number higher than 8.

	Marker	
	COI	18S
Raw reads	1111837 (100%)	770841 (100%)
Usable sequences*	718120 (64.6%)	448299 (58.2%)
Taxonomic match w/ marine invertebrates	615521 (55.4%)	299082 (38.8%)
Species level taxonomic assignment (>8 reads)**	584220 (52.5%)	287397 (37.3%)

391

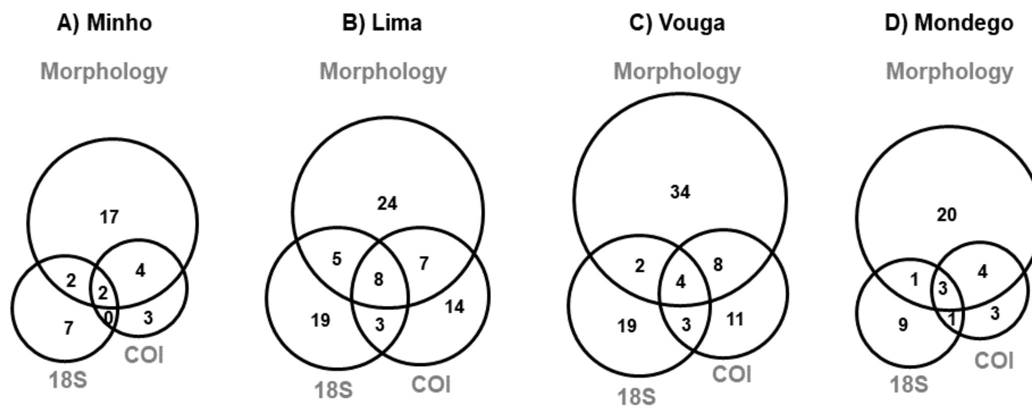
392 **3.3. Morphology and metabarcoding-based benthic invertebrates' taxonomic assignments**
393 A total number of 154 marine invertebrate taxa was identified at species level, by using both
394 methodologies (Fig. 3, Supplementary Material: Table S4). From these, 90 species were identified
395 through morphology, while 99 species through metabarcoding (Fig. 3, Supplementary Material:
396 Table S4). Thirty-five species were identified by using both methodologies (ca. 23%), while 55
397 species were exclusively detected through morphology (ca. 36%) and 64 through metabarcoding
398 (ca. 42%) (Fig. 3A, Supplementary Material: Table S4).
399 Within the metabarcoding dataset, 16 species were detected with both markers (ca. 16%), while
400 37 species were exclusively detected with COI (ca. 37%) and 46 with 18S (ca. 47%) (Fig. 3B,
401 Supplementary Material: Table S4).

bioRxiv preprint doi: https://doi.org/10.1101/2022.05.10.491303; this version posted May 11, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.



402
403 **Figure 3.** Partitioning of the marine invertebrate species detected with both methodologies (A)
404 and detected with both methodologies, but discriminated by genetic marker for the metabarcoding
405 dataset (B). All sampling sites were considered.
406
407 The highest numbers of total marine invertebrate species were found in Lima (80 species) and
408 Vouga estuaries (81 species), while lower numbers were recovered from Minho (35 species) and
409 Mondego estuaries (41 species) (Fig. 4). In general, metabarcoding recovered equal or higher
410 diversity of species than morphology-based identification in the more diverse estuaries (Lima and
411 Vouga), while the opposite was found for the lowest diverse estuaries (Minho and Mondego). The
412 % of species recovered with both methodologies varied between 17% and 25%, for Vouga and
413 Lima estuaries, respectively. A closer look into each sampling site revealed that for about half of
414 the sites, metabarcoding recovered a higher diversity of species than morphology-based
415 identification (Supplementary Material: Fig. S1) and the % of the diversity recovered with both
416 methodologies varied between 0 and 20%, for Minho; 6 to 40%, for Lima; 5 to 36%, for Vouga
417 and 0 to 27%, for Mondego, respectively (Supplementary Material: Fig. S1).
418

bioRxiv preprint doi: https://doi.org/10.1101/2022.05.10.491303; this version posted May 11, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.



419

420 **Figure 4.** Partitioning of the marine invertebrate species detected with both methodologies for
421 Minho (A), Lima (B), Vouga (C) and Mondego (D) estuaries. All sampling sites were considered,
422 within each estuary.

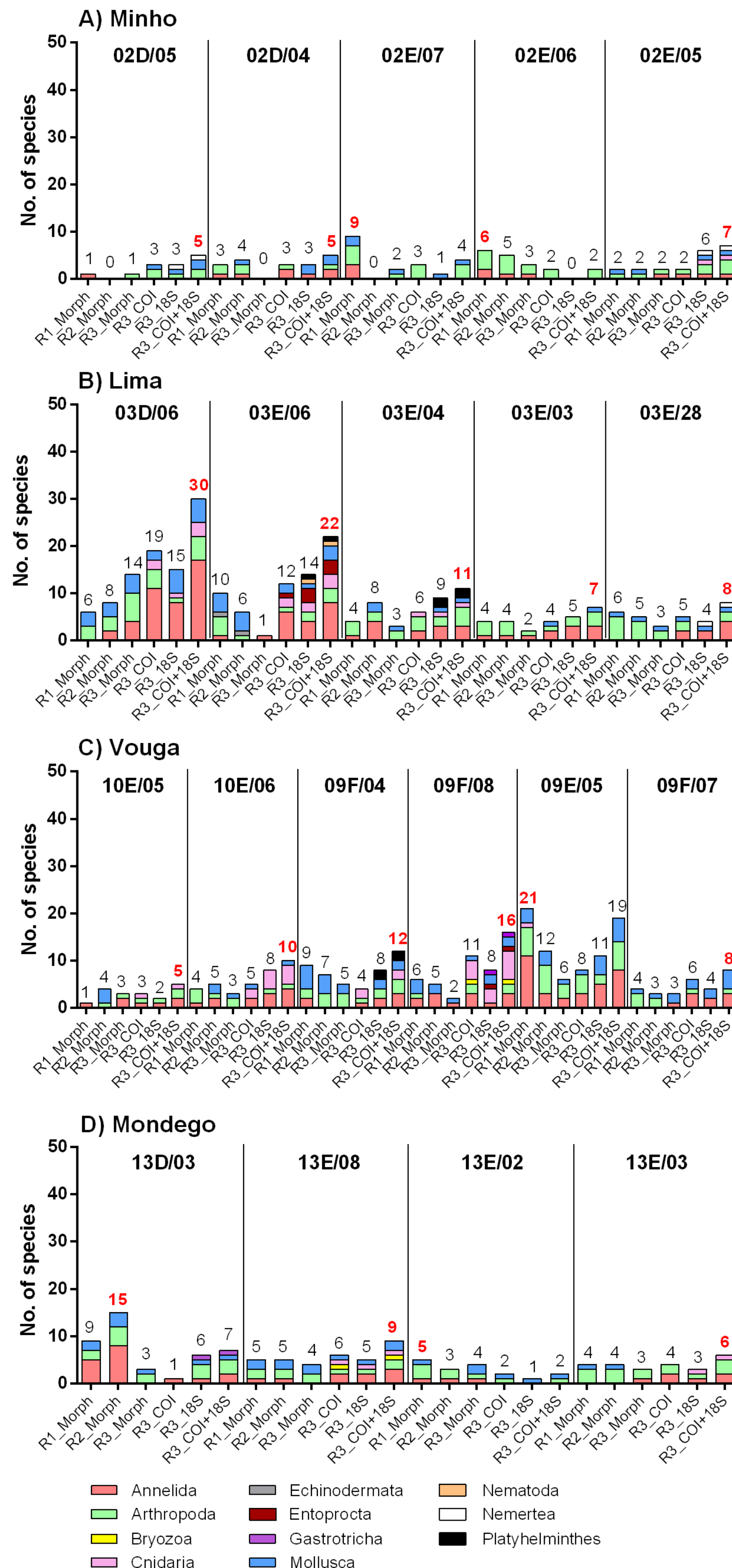
423

424 A closer look within each estuary indicated that maximum numbers of marine invertebrate species
425 were always detected with metabarcoding for all sampling sites in Lima and almost all in Vouga
426 estuaries (except 09E/05) (maximum of 30 in 03D/06 and 21 in 09E/05, for Lima and Vouga,
427 respectively) (Fig. 5). In Minho estuary, while higher numbers of species were retrieved through
428 metabarcoding in 3 out of 5 sampling sites (02D/05, 02D/04, 02E/05), a maximum number of 9
429 species was found through morphology-based identification in 02E/07. In Mondego, a higher
430 number of species was also retrieved through metabarcoding in 2 out 4 sampling sites (13E/08
431 and 13E/03), but the highest diversity retrieved (15 species) was attained in 13D/03, through
432 morphology-based identification.

433 The 154 marine invertebrate taxa identified at species level were distributed by 11 taxonomic
434 groups: Annelida, Arthropoda, Bryozoa, Cnidaria, Echinodermata, Entoprocta, Gastrotricha,
435 Mollusca, Nematoda, Nemertea and Platyhelminthes (more details of the species names and the
436 associated taxonomic groups in Supplementary Material: Table S4). Echinodermata species were
437 exclusively detected through morphology, but not by metabarcoding, while Bryozoa, Entoprocta,
438 Gastrotricha, Nematoda, Nemertea and Platyhelminthes were exclusively detected through
439 metabarcoding, with the 4 later being exclusively detected with the 18S marker (Fig. 5). In
440 general, the most well represented groups included Arthropoda (42.2%), Annelida (33.3%) and
441 Mollusca (22.2%), in the morphology dataset, and Annelida (42.3% and 27.4%), Arthropoda
442 (28.8% and 14.5%), Mollusca (13.5% and 17.7%) and Cnidaria (13.5% and 16.1%, for COI and
443 18S, respectively), in the metabarcoding datasets. Cnidaria, were particularly abundant in Vouga
444 estuary, more specifically in sampling sites 10E/06 and 09F/08.

445

bioRxiv preprint doi: https://doi.org/10.1101/2022.05.10.491303; this version posted May 11, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.



446

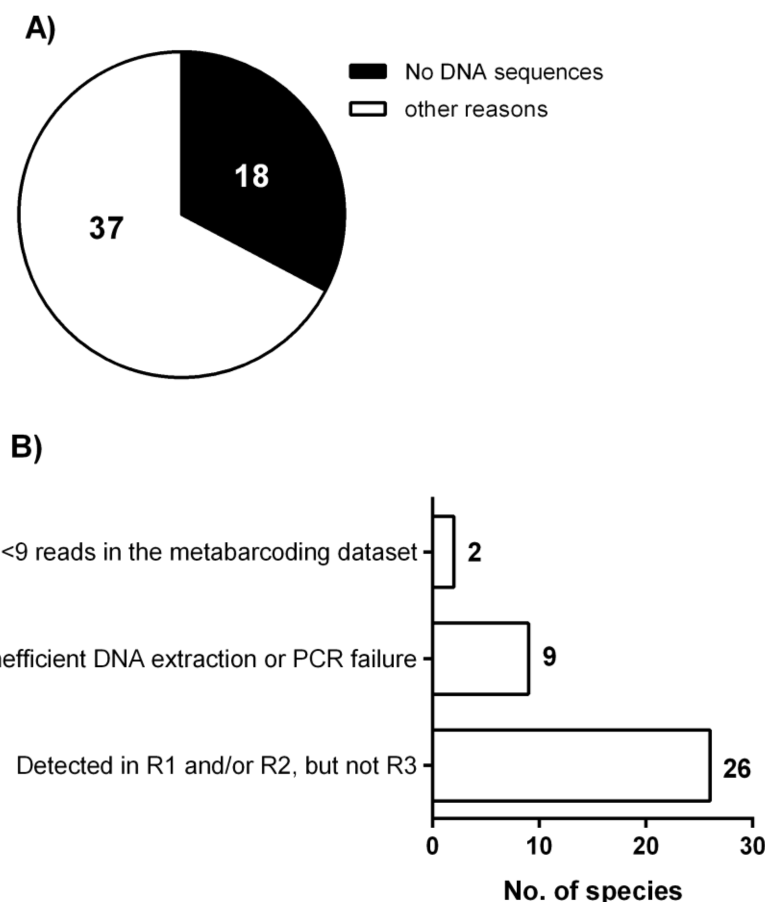
447 **Figure 5.** Taxonomic profiles of marine invertebrate species detected through morphology and

448 metabarcoding (COI, 18S and COI + 18S) on each analysed replicate collected on each sampling

449 site on Minho (A), Lima (B), Vouga (C) and Mondego (D) estuaries. Numbers above bars indicate

bioRxiv preprint doi: https://doi.org/10.1101/2022.05.10.491303; this version posted May 11, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

450 the total number of species detected on each sampled replicate (in red is represented the highest
451 number of species detected on each sampling site).
452
453 Since 55 invertebrate species (out of a total of 90, 61%) were exclusively detected through
454 morphology (Supplementary Material: Table S5), we further investigated the reasons for this.
455 From this list, 18 species do not have sequences belonging to any of the targeted markers on
456 genetic databases (20%) (Fig. 6A, Supplementary Material: Table S5). A closer look revealed that
457 26 species were detected in replicates R1 and/or R2, but not in R3, the replicate analysed through
458 metabarcoding (Fig. 6B). Other reason included 2 species producing less reads than the minimum
459 threshold set in the bioinformatic pipeline (<9 reads). For the remaining 9 species (10%) the
460 absence of detection in the metabarcoding dataset could be due either to inefficient DNA
461 extraction or PCR amplification failure with the primers used for targeting each marker.



462
463 **Figure 6.** Number of marine invertebrate species exclusively detected with the morphology-based
464 approach, represented and not represented with DNA sequences in genetic databases (COI and/or
465 18S) (A) and possible reasons for the no detection through metabarcoding for species that were
466 represented with DNA sequences in genetic databases (B).
467

bioRxiv preprint doi: https://doi.org/10.1101/2022.05.10.491303; this version posted May 11, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

468 **3.4. Morphology and metabarcoding-derived biotic indices**

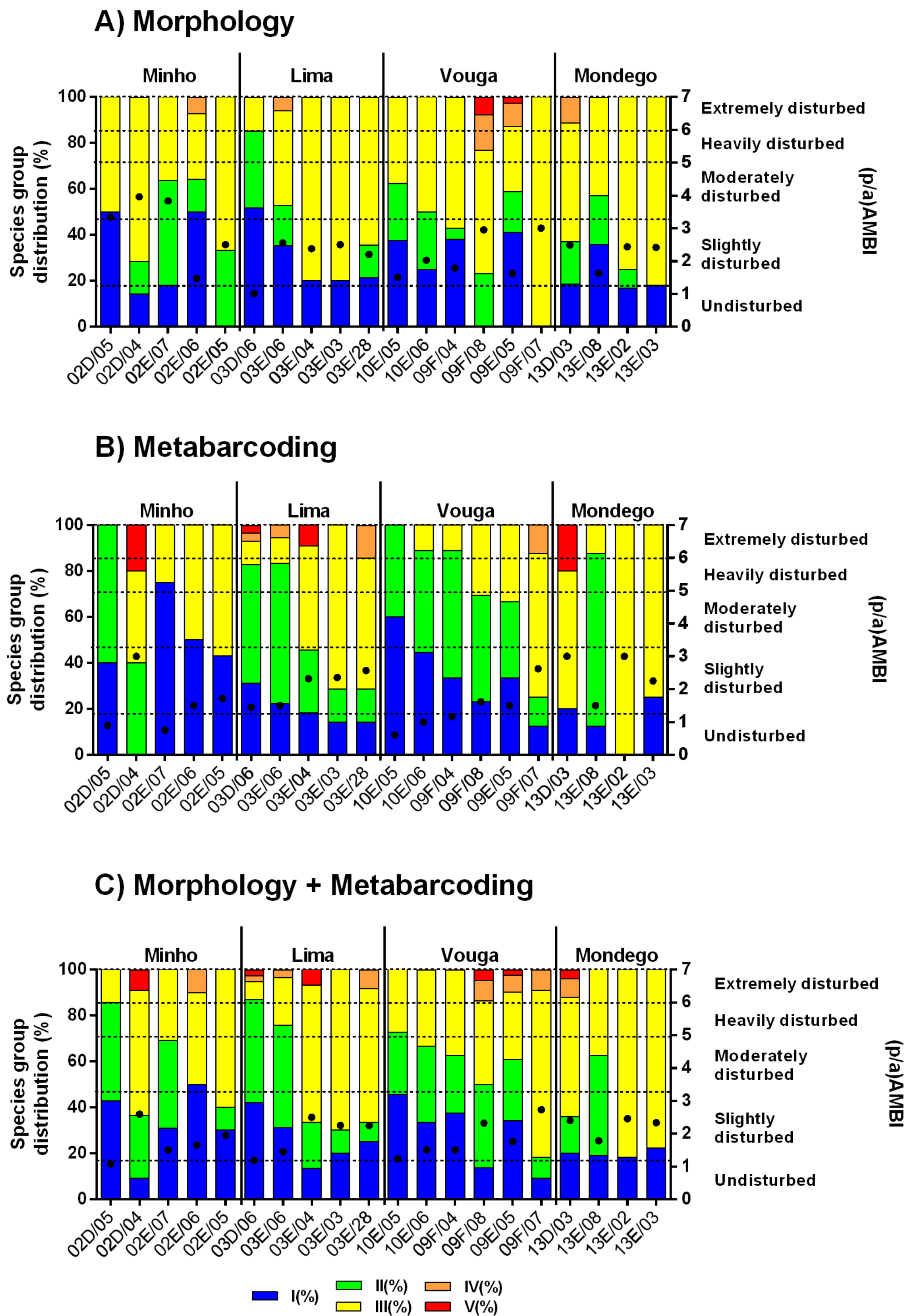
469 The % of each ecological group, based on marine invertebrate species, varied across sampling
470 sites, and slight differences were found between both methodologies (Fig. 7). Since no clear
471 relationship was found between the number of reads and the number of specimens found, for each
472 species detected with both methodologies (Supplementary Material: Table S6, Fig. S2), we opted
473 to calculate the % of each ecological group and AMBI indices based solely on the
474 absence/presence of species ((p/a)AMBI).

475 By using morphology-based identification (Fig. 7A), class III ecological group dominated in most
476 sampling sites (in 15 out of 20), resulting in most of the sites being attributed with a classification
477 of slightly disturbed, with the exception of 03D/06 from Lima, which was classified as
478 undisturbed, and 02D/05, 02D/04 and 02E/07 from Minho, which were classified as moderately
479 disturbed.

480 For the metabarcoding dataset (Fig. 7B), the distribution of the species among the different
481 ecological groups was more variable. Even so, class III ecological group dominated or co-
482 dominated in 11 of the sampled sites, while in the other 9, the dominant or co-dominant ecological
483 groups included class II (10 out of 20) and to a lesser extent class I (4 out of 20) ecological groups.
484 Most of the sampling sites were still classified as slightly disturbed (15), while 5 sites as
485 undisturbed (02D/05 and 02E/07 in Minho and 10E/05, 10E/06 and 09F/04 in Vouga).

486 (p/a)AMBI values calculated using morphology and metabarcoding-based assessments were
487 poorly correlated (Supplementary Material: Fig. S3), probably due to the differences in the % of
488 the ecological groups detected between both methodologies. When joining the two datasets (Fig.
489 7C), class III ecological group still dominated or co-dominated in most of the sampling sites (11),
490 class II in 7 sites and class I in 6 sites. All sampling sites were classified as slightly disturbed,
491 with the exception of sites 02D/05, in Minho, and 03D/06, in Lima, which were classified as
492 undisturbed. No significant correlations were found between (p/a)AMBI indices and PC1 and
493 PC2 axes scores of environmental variables from Fig. 2, with the exception of (p/a)AMBI values
494 of the combined datasets which were significantly correlated with PC2 (Supplementary Material:
495 Fig. S4).

bioRxiv preprint doi: https://doi.org/10.1101/2022.05.10.491303; this version posted May 11, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.



496

497 **Figure 7.** % contribution of each ecological group at each sampling site based on presence and
498 absence of each marine invertebrate species identified using morphology (A), metabarcoding (B)
499 and the combination of both approaches (C). Dots on each bar represent (p/a)AMBI indices scores
500 obtained at each sampling site.

501

502

bioRxiv preprint doi: https://doi.org/10.1101/2022.05.10.491303; this version posted May 11, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

503 Canonical correspondence analysis of the relationships between physical and chemical
504 parameters of the water and the structure of marine invertebrate species based on morphology
505 (Fig. 8A), metabarcoding (Fig. 8B) and in the combination of both approaches (Fig. 8C), showed
506 that the first two axes explained ca. 32%, 30% and 30% of the total variance, respectively. For
507 both datasets (morphology and metabarcoding) and when combined, there was a clear separation
508 of invertebrate communities from the oligohaline site 03E/28 (Lima), from the remaining sites.
509 The distribution of the sites accordingly to the invertebrate community structure derived from
510 morphology, metabarcoding and from the combination of both approaches was very similar along
511 the environmental gradient defined by axis 1 and axis 2. Despite the small differences found, in
512 all datasets, the variables that most significantly affected community structure were salinity,
513 conductivity and Si (details of r coefficients and p values in Supplementary Material: Table S7).
514 Other variables that significantly affected community structure included transparency, maximum
515 depth and nitrates concentrations (Supplementary Material: Table S7).
516
517
518

bioRxiv preprint doi: https://doi.org/10.1101/2022.05.10.491303; this version posted May 11, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Figure 8. Canonical correspondence analysis diagrams for the ordination of physical and chemical parameters and invertebrate species at the 20 sampled sites, based on morphology (A), metabarcoding (B) and morphology plus metabarcoding (C).

bioRxiv preprint doi: https://doi.org/10.1101/2022.05.10.491303; this version posted May 11, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

523 **4. Discussion**

524 Although the use of DNA metabarcoding to monitor aquatic benthic invertebrate communities
525 has been growing steeply in the last few years (Duarte et al. 2021), few studies targeted
526 transitional ecosystems (Chariton et al. 2015, Lobo et al. 2017b, Aylagas et al. 2018, Steyaert et
527 al. 2020); therefore, they require further and more exhaustive proof-of-concept studies. In the
528 current study, we addressed this research gap by building upon the WFD biomonitoring surveys
529 of the Portuguese Environmental Agency (APA), to assess the ecological condition of 20 sites
530 from four main transitional ecosystems in the west coast of Portugal – the estuaries of Lima,
531 Minho, Vouga and Mondego Rivers. In fact, at least to our knowledge, only one previous study
532 involved an official biomonitoring program to benchmark metabarcoding against the
533 morphology-based approach for characterizing benthic invertebrate communities in transitional
534 ecosystems (Aylagas et al. 2018, in the Basque country, Spain). In Portugal, this is the first time
535 that a study of this kind is conducted in transitional waters, but see Martins et al. (2020) for
536 freshwater ecosystems.

537

538 **4.1. Metabarcoding outperformed morphology-based assessments of benthic invertebrate**
539 **species**

540 Compared to the morphology-based approach, metabarcoding was able to recover an equal or
541 higher species richness and phylogenetic diversity in 10 out of the 20 sampling sites
542 (Supplementary Material: Fig. S1). The significance of this result is reinforced by the fact that
543 this greater species detection ability was accomplished using only one replicate in metabarcoding,
544 compared to 3 replicates in morphology-based assessments. However, when comparing exactly
545 the same replicate, where both approaches were concurrently employed, metabarcoding always
546 outperformed the morphology-based approach, with the exception of 2 sampling sites (02E/06
547 and 13E/02), where the differences were minute (3 and 4 *versus* 2 species, for morphology and
548 metabarcoding, respectively) (Fig. 5). In some cases, the differences were fairly high, the most
549 notable for sampling site 03E/06, where a total of 22 species were recovered through
550 metabarcoding (12 and 14, for COI and 18S, respectively), in comparison to only one species
551 detected through morphology (the polychaete trumped worm *Lagis koreni*). Several
552 metabarcoding studies have reported the occasional detection of species that were apparently
553 lacking through the morphological analyses of the same samples (Cowart et al. 2015, Aylagas et
554 al. 2016, 2018, Hollatz et al. 2017, Lobo et al. 2017b, Cahill et al. 2018, Steyaert et al. 2020).
555 Although organisms were carefully sorted from the debris before DNA extraction, eventually
556 small portions of associated inorganic (sediment) and organic detritus (algae) were also picked.
557 These may contain tiny organisms, body fragments or tissues, and even gut contents or DNA from
558 ecologically associated species that can be detected through metabarcoding, due to the high
559 sensitivity of the technique (Aylagas et al. 2016, Hollatz et al. 2017, Lobo et al. 2017b). In fact,

bioRxiv preprint doi: https://doi.org/10.1101/2022.05.10.491303; this version posted May 11, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

560 a closer look into the diversity found in this particular sample (03E/06) revealed the presence of
561 three hydrozoans, three entoprocts, one nematode and one platyhelminth species in the
562 metabarcoding dataset, which could have been easily unnoticed through the morphological
563 inspection. Interestingly, although undetected through morphology, the DNA of *Abra alba* was
564 also detected in this sample, a species that is often reported to live in close association with *Lagis*
565 *koreni* (Thiébaut et al. 1997, Bacouillard et al. 2020). Similarly, *Lagis koreni* was detected only
566 through metabarcoding in a sample (03D/06) where high densities of *Abra alba* were recorded by
567 morphological inspection.
568 In addition, the fraction of species detected by both methods was quite low: 23% if we considered
569 all the dataset (Fig. 3); 17% to 25%, at the estuary level and 0 to 40%, at site level (Fig. 4 and
570 Supplementary Material: Fig. S1). Our results are congruent with a recent meta-analysis where
571 the authors concluded that species inventories of macroinvertebrates obtained with DNA
572 metabarcoding showed pronounced differences to traditional methods, missing some taxa, but at
573 the same time detecting overlooked diversity (Keck et al. 2022).
574 Despite the differences recorded, the dominant taxonomic groups recovered were common to both
575 methodologies, namely Annelida:Polychaeta, Arthropoda:Crustacea and Mollusca:Bivalvia,
576 which are well known to dominate benthic communities in Portuguese transitional ecosystems
577 (e.g., Sousa et al. 2006, França et al. 2009, Neto et al. 2010, Rodrigues et al. 2011, Lobo et al.
578 2017b). Exceptionally, Cnidaria:Hydrozoa dominated in the metabarcoding datasets of some sites
579 of the Vouga estuary (10E/06, 09F/04, 09F/08) (Fig. 5). Many of these species have polyp stages
580 and can experience easy fragmentation of their body or tissues, which can be easily detected
581 through metabarcoding, but go unnoticed through the morphological approach, as already above-
582 mentioned.
583
584 **4.2. The use of a multi-marker strategy increased recovered species through metabarcoding**
585 Overall, only 35 species were identified by both methodologies, out of a global sum of 154
586 recorded in this study. However, this number would be even lower if only one genetic marker was
587 employed, instead of two. For instance, from the 35 species detected with both approaches, 17
588 were recovered exclusively in the COI dataset and 7 in the 18S dataset (Fig. 3). In a recent review
589 where 90 publications where analysed (Duarte et al. 2021), partial segments of the COI gene have
590 been by far the most used for targeting benthic invertebrate communities in marine ecosystems,
591 including transitional ecosystems, while 18S has been less used, whereas the concurrent use of
592 both markers is less common (e.g. Cowart et al. 2015, Wangensteen et al. 2018a,b, Leite et al.
593 2021).
594 The COI marker is by far the marker for which a higher coverage exists in genetic databases, in
595 particular for dominant groups of benthic marine invertebrates (40 to 80%, for the AMBI
596 checklist, Weigand et al. 2019; 16 to 53%, for Mollusca, Crustacea and Polychaeta occurring in

bioRxiv preprint doi: https://doi.org/10.1101/2022.05.10.491303; this version posted May 11, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

597 Atlantic Iberia, Leite et al. 2020). While species level resolution can be substantially higher using
598 COI, the primer binding sites can be highly variable (see more discussion about this issue in the
599 next sub-section) failing to anneal with DNA templates from species for which the primers have
600 a lower affinity. On the other hand, 18S has been reported to have low variability in primer
601 binding sites (Tang et al. 2012, Brown et al. 2015), but can lack resolution in species level
602 detections (Cowart et al. 2015) and may amplify many small sized species (< 1mm), such as
603 harpacticoid copepods, ostracods and gastrotrichs, that are not included in current morphology-
604 based bioassessments (Lejzerowicz et al. 2015). However, in our study both markers frequently
605 complemented each other, with benthic invertebrate species, which were representative of
606 different ecological groups and present in the morphology dataset, detected exclusively by 18S:
607 the polychaetes *Capitella capitata* (V), *Eumida sanguinea* (II) and *Nephtys incisa* (II) and the
608 bivalves *Cerastoderma edule* (III), *Mytilus edulis* (III), *Pharus legumen* (I) and *Spisula*
609 *subtruncata* (I). Although reference sequences exist for all of them in BOLD, these species would
610 have gone unnoticed through metabarcoding if only COI was employed, which can have
611 important ecological implications. Our results are consistent with previous findings for marine
612 invertebrate communities, where a multi-marker strategy can significantly improve the number
613 of recovered species (Wangesteen et al. 2018a,b, Leite et al. 2021), therefore contributing to
614 increase the comprehensiveness and reliability of benthic ecosystems assessments using DNA
615 metabarcoding.
616
617 **4.3. Probable reasons for the failed detection of benthic invertebrates through**
618 **metabarcoding**
619 Given that 55 invertebrate species were detected exclusively through morphology, we
620 investigated the reasons for this in greater detail. We have found straightforward reasons for failed
621 detection through metabarcoding for most of the species: i) 18 species (20%) do not have DNA
622 sequences on BOLD (COI) and/or SILVA (18S); ii) 2 species (~2%) had less than 9 reads (the
623 minimum threshold set in the bioinformatic pipeline) (*Asterias rubens* and *Mactra stultorum*) and
624 iii) 26 species (29%) were not recorded in the replicate that was also used for metabarcoding (i.e.,
625 R3 of each sampling site), but were identified through morphology in the remaining replicates;
626 hence they were counted as species detected through morphology although they did not occur in
627 the replicate used for DNA metabarcoding (Fig. 6).
628 For 9 species (ca. 10%) we did not find any apparent reason for failed molecular detection, namely
629 the amphipods *Ampelisca brevicornis*, and *Microdeutopus chelifer*, the isopods *Cyathura*
630 *carinata*, *Sphaeroma serratum*, and *Eurydice pulchra,* the decapod *Liocarcinus holsatus*, the
631 bivalves *Fabulina fabula* and *Scrobicularia plana* and the polychaete *Diopatra marocensis*
632 (Supplementary Material: Table S5). All of them are represented with COI sequences in BOLD,
633 and 7 of them with 18S sequences in SILVA. In addition, several specimens of each of these

bioRxiv preprint doi: https://doi.org/10.1101/2022.05.10.491303; this version posted May 11, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

634 species were present in the analysed samples, with the exception of *D. marocensis*, *E. pulchra*, *L.*
635 *holsatus* and *S. plana* which were represented by only one specimen. However, the robustness of
636 metabarcoding has been well demonstrated in several studies, where sequences from species
637 represented by only one specimen (Lobo et al. 2017b) or from single larvae spiked in
638 environmental samples (Pochon et al. 2013, Zhan et al. 2013), have been detected despite the co-
639 occurrence of a large array of other species. Thus, we consider that the most possible reasons for
640 the absence of these species from the metabarcoding dataset may either include inefficient DNA
641 extraction or mismatches between the selected primers and target templates. Another possibility
642 is occurrence of cryptic diversity, which is common in marine invertebrates (e.g., polychaetes:
643 Lobo et al. 2016, Teixeira et al. 2020; amphipods: Lobo et al. 2017a, Vieira et al. 2020;
644 gastropods: Borges et al. 2016), meaning that the species is thought to be present in the reference
645 libraries, but the specimens collected belong to a divergent lineage that is not represented in the
646 library. They would be identified through morphology, but would be missed by metabarcoding.
647 However, we investigated this possibility and found that reference sequences were all from
648 specimens collected in the region, therefore the chances of missing a cryptic lineage are low.
649 Although, morphological traits have been reported to influence DNA extraction of marine
650 invertebrates (e.g., body size, presence of chitine or $CaCO_3$), in particular from preservative
651 ethanol, in bulk DNA samples species detection does not seem to be much affected (Derycke et
652 al. 2021). In addition, we used a non-destructive method for DNA extraction, involving the
653 temporary immersion of the bulk specimens in an extraction buffer without previous
654 homogenization (Carew et al. 2018, Leite et al. 2021), since homogenization of bulk samples
655 previously to DNA extraction may favour the amplification of non-target taxa (e.g., non-
656 invertebrate metazoans, fungi, protists, bacteria) (Lejzerowicz et al. 2015, Aylagas et al. 2018).
657 From the initial raw reads, 55% and 39% matched with sequences from marine invertebrate
658 species, for COI and 18S, respectively, but, when considering only quality-filtered reads, these
659 percentages increased to as high as 86% and 67%. These rates compare well with other studies,
660 so we may conclude that there was a relatively high success in recovering sequences of the target
661 group (invertebrates) (e.g., up to 66.5% of COI quality-filtered reads, in the study of Aylagas et
662 al. 2018).
663 Although we cannot completely discard an inefficient DNA extraction, the most possible
664 explanation is indeed possible mismatches that may exist between the primers used and target
665 templates. Marine invertebrate communities dominating benthic estuarine ecosystems are very
666 complex and highly diverse, belonging to phylogenetically distant taxonomic groups (e.g.,
667 Annelida, Mollusca, Crustacea) (Lobo et al. 2013, Zhang et al. 2021). Thus, primers used in the
668 PCR reaction may have higher affinity for DNA templates of particular species, which will be
669 preferentially detected compared to other species also present in the sample. In a recent study,
670 DNA metabarcoding was also unable to detect 19 out of 57 morphospecies, for the best

bioRxiv preprint doi: https://doi.org/10.1101/2022.05.10.491303; this version posted May 11, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

671 performing primer set, and the authors concluded that the most probable reason was indeed the
672 absence of match between the primers employed and the species present in the samples (Derycke
673 et al. 2021). Interestingly, in previous studies, where species were identified in morphology-based
674 assessments and the same primers were used for COI (Lobo et al. 2017b, Leite 2021), no reads
675 were also generated for *Cyathura carinata* in the metabarcoding datasets. In addition, Lobo and
676 co-authors (2017) used four different primers for the COI region, including one pair targeting the
677 complete barcode region and with which sequences from these species have been successfully
678 generated previously (Lobo et al. 2013). In another study using mock communities, *Cyathura*
679 *carinata* was detected by a single read for a single primer set out of 5 tested sets (Hollatz et al.
680 2017). However, for the remaining 8 species we did not find the same evidence in previous studies
681 using the same primers (Hollatz et al. 2017, Lobo et al. 2017b, Derycke et al. 2021, Leite 2021).
682 Species which appear to be particularly recalcitrant to metabarcoding should be signalled and
683 their detection success carefully examined in future studies, as it can lead to systematic false
684 negatives in metabarcoding-based biomonitoring. Possible recalcitrant species are one additional
685 reason by which a multi-marker approach may be a better solution to recover as much as possible
686 the diversity in benthic invertebrate samples using DNA metabarcoding. Given that PCR-free
687 approaches are still too expensive (e.g., Dowle et al. 2016, Giebner et al. 2020), the design of
688 primers customized to specific taxonomic groups (e.g., dominant groups such as Mollusca,
689 Annelida, and Arthropoda), may provide an alternative to avoid biases of broad-coverage primers
690 (Westfall et al. 2020).
691
692 **4.4. Morphological and metabarcoding-based indices did not match completely in all sites,**
693 **but both datasets responded similarly to the environmental gradient**
694 AMBI indices based on presence-absence of species calculated using morphology and
695 metabarcoding-based taxonomic assessments did not match completely in all sites, contrarily to
696 the observed in previous studies (Aylagas et al. 2016, 2018, Lobo et al. 2017b). The most probable
697 reason for this were the differences found, between both methodologies, in the percentage of
698 contribution of each ecological group recovered (Fig. 7). While the majority of the species
699 detected belong to ecological group III, indicating dominance of species tolerant to pollution, an
700 increase in the % of ecological groups I (sensitive species) and II (indifferent species), was
701 observed in metabarcoding-based assessments. The higher resolution of metabarcoding,
702 providing species level identifications for smaller organisms or difficult taxa in morphology-
703 based assessments, such as Platyhelminthes (Group II), Bryozoa (Group II) and Cnidaria (Group
704 I and II), may have contributed to this outcome. Because of this, 6 sites were evaluated with a
705 better ecological condition with metabarcoding-based assessments in Minho and Vouga (02D/05,
706 02D/04, 02E/07 and 10E/05, 10E/06 and 09F/04), while the opposite was rarely found, with the
707 exception of one site in Lima, which has switched from undisturbed to slightly disturbed

bioRxiv preprint doi: https://doi.org/10.1101/2022.05.10.491303; this version posted May 11, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

708 (03D/06). Nevertheless, it must be stressed that for most sites (13), the same degree of
709 perturbation (slightly disturbed) was found with both methodologies (Fig. 7). A recent meta-
710 analysis concluded that, the taxonomic inferences for macroinvertebrates derived from the two
711 methods can be very different, with metabarcoding complementing rather than providing identical
712 estimates compared to traditional approaches (Keck et al. 2022). Our results seem to support this
713 conclusion. Whereas traditional morphology-based identification will specifically target
714 macroinvertebrate taxa higher than 0.5 or 1 mm, metabarcoding will recover a higher diversity of
715 organisms, including body and tissue fragments and early developmental stages unnoticed by the
716 traditional approach. Also, smaller taxonomic groups that can be sensitive to environmental
717 stressors, but that are largely ignored in current biomonitoring due to the inherent difficulties in
718 the morphological identification (e.g., prokaryotes, protists, metazoan meiofauna) can be also
719 detected. In addition, for 4 species (*Corophium multisetosum*, *Crangon crangon*, *Hediste*
720 *diversicolor* and *Tritia reticulata*) we were able to detect multiple molecular operational units
721 (MOTUs) in the COI metabarcoding dataset (data not shown, since we worked at species level),
722 and some with maximum genetic divergences as high as 18%. For instance, for *Hediste*
723 *diversicolor*, 23 different MOTUs were found distributed among the 20 estuarine sites (data not
724 shown). To what extent these different genetic lineages will have differential sensitivities to
725 pollution and environmental stress remains to be tested, and more benchmarking is definitely
726 needed, with as many samples as possible collected under different environmental contexts. This
727 would suggest the interesting possibility that through the use a broader set of informative taxa in
728 the biotic indexes, metabarcoding can potentially augment the resolution of bioassessments
729 enabling a better discrimination among sites, giving a more holistic vision of an entire ecosystem,
730 that otherwise would be considered identical (Pawlowski et al. 2018). Other possibilities would
731 be to develop new indices based on new indicator groups, as developed, for example, for bacteria
732 (e.g., microgAMBI, Aylagas et al. 2017, Borja 2018) or to use taxonomy-free approaches and
733 machine learning predictive models (Apothéloz-Perret-Gentil et al. 2017, Cordier et al. 2017).
734 Interestingly, despite the differences found in our study, the outcome of CCA analyses was
735 somehow similar for morphology and metabarcoding-based assessments, and also when
736 combining both datasets (Fig. 8). Salinity (and consequently conductivity and pH) was the
737 variable that better explained the differences in benthic invertebrates' composition among sites,
738 which was patent in all diversity assessments (Supplementary Material: Table S7). In previous
739 assessments conducted in these estuaries, salinity has been pointed out to be among the factors
740 that most clearly influence benthic assemblages (Minho: Sousa et al. 2008; Lima: Sousa et al.
741 2007; Vouga: Rodrigues et al. 2011; Mondego: Teixeira et al. 2008a,b). Nitrates, which changed
742 in the opposite direction to the salinity gradient, displaying higher values at oligohaline sites (2
743 to 6 times higher in Lima and 13 to 24 times higher in Mondego), also influenced community
744 structure assessed with both methods (more in the morphology dataset), but this was not reflected

bioRxiv preprint doi: https://doi.org/10.1101/2022.05.10.491303; this version posted May 11, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

745 in the AMBI indices. It has been reported that in estuarine ecosystems the natural variability, as
746 well as natural events (e.g., extreme climatic events), play an important role in the response of
747 biotic indices (Chainho et al. 2007, Teixeira et al. 2008b, Neto et al. 2010). In a previous study
748 conducted in Mondego estuary, both natural and anthropogenic variability were satisfactorily
749 detected, but only when accounting the information provided by three different indices (AMBI,
750 Margalef and Shannon-Wiener) (Teixeira et al. 2008b). Still, a great proportion of the variability
751 remained unexplained in the CCA analyses (>50%, for all datasets). Sediment features, are
752 recognized as another highly important factor influencing benthic assemblages' composition and
753 species abundance within salinity zones (Sousa et al. 2007, Teixeira et al. 2008b). Part of this
754 unexplained variation may therefore derive from the specific sediment characteristics within each
755 site, but which were not assessed in this specific APA's biomonitoring campaign.
756

757 **5. Conclusions**

758 In conclusion, our results support that metabarcoding provide higher estimates of diversity than
759 the morphology-based approach, and the use of a multi-locus strategy increased recovered
760 diversity through metabarcoding. In addition, sequence gaps on genetic databases, but also PCR
761 failure seem to be the main reasons for the absence of species detection in the metabarcoding
762 dataset. Although morphological and metabarcoding-based indices did not match completely in
763 all sites, similar responses to the environmental gradient were obtained with both methods. Thus,
764 our results support that rather than moving towards a DNA-based approach independent of
765 morphology-based methods, a harmonized approach should be employed, where, when possible,
766 both methods should be integrated to complement each other, in order to improve and expedite
767 benthic monitoring. Specimen identification based on morphological taxonomy continues to be
768 invaluable, providing the prime foundation in all biodiversity assessments, importantly enabling
769 an estimation of organisms' abundances and assessment of prevalent life stages, which is actually
770 conducted in 1 to 2 events per 6-year management cycle (Hering et al. 2010). On the other hand,
771 DNA-based monitoring can be less expensive and more responsive to immediate regulatory and
772 management needs, such as the required for monitoring pollution events or restoration activities,
773 which can be employed in a yearly basis or even two-times/year and possibly with higher spatial
774 density. We anticipate that metabarcoding can also increase the quality of the assessments
775 (representativeness and precision), allowing identifications of all specimens in a sample including
776 larval stages and juveniles, but also small organisms from taxonomic groups that cannot be
777 identified to species level using the traditional approach and that are largely ignored in routine
778 biomonitoring and may be sensitive as well to environmental stress (e.g., nematodes, ciliates,
779 foraminifera), as the current targeted BQEs. In addition, cryptic lineages can also be detected, as
780 we were able to do so for four common bioindicator species, promising a greater taxonomic
781 resolution and improvement of the delineation of tolerance/sensitivity groups commonly used in

bioRxiv preprint doi: https://doi.org/10.1101/2022.05.10.491303; this version posted May 11, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

782 biotic indices, such as AMBI. DNA-based monitoring can also generate standardized data more
783 amenable to audit and less vulnerable to variability in taxonomic expertise among studies,
784 facilitating direct comparison among independent diversity assessments and that can be more
785 easily articulated at regional, European and even at global scale.
786

787 **Declaration of Interest Statement**

788 The authors declare that they have no known competing financial interests or personal
789 relationships that could have appeared to influence the work reported in this paper.
790

791 **Acknowledgments**

792 This work was funded by the project "River2Ocean – Socio-ecological and biotechnological
793 solutions for the conservation and valorization of aquatic biodiversity in the Minho Region"
794 (NORTE-01-0145-FEDER-000068), co-financed by the European Regional Development Fund
795 (ERDF), through Programa Operacional Regional do Norte (NORTE 2020), by the "Contrato-
796 Programa" UIDB/04050/2020 funded by national funds through the FCT I.P (Foundation for
797 Science and Technology) and the project MESCLA – "Melhorar e Complementar os Critérios de
798 Classificação do Estado das Massas de Água de Transição e Costeiras" (POSEUR-03-2013-FC-
799 000001). Financial support granted by the FCT to SD (CEECIND/00667/2017), BRL
800 (PD/BD/127994/2016), MALT (SFRH/BD/131527/2017), and PEV (through the project NIS-
801 DNA: PTDC/BIA-BMA/29754/2017) is also acknowledged. The authors also want to thank to
802 Àngel Borja for helping in the decision of the ecological categories of species that are not yet
803 contemplated in the AMBI list.
804

805 **References**

806 Apothéloz-Perret-Gentil L, Cordonier A, Straub F, Iseli J, Esling P, Pawlowski J (2017)
807 Taxonomy-free molecular diatom index for high-throughput eDNA biomonitoring.
808 Molecular Ecology Resources 17: 1231-1242. https://doi.org/10.1111/1755-0998.12668
809 Aylagas E, Borja A, Rodríguez-Ezpeleta N (2014) Environmental status assessment using DNA
810 metabarcoding: towards a genetics based marine biotic index (gAMBI). PLoS ONE 9:
811 e90529. https://doi.org/10.1371/journal.pone.0090529
812 Aylagas E, Borja A, Irigoien X, Rodríguez-Ezpeleta N (2016) Benchmarking DNA
813 metabarcoding for biodiversity-based monitoring and assessment. Frontiers in Marine
814 Science 3: 96. https://doi.org/10.3389/fmars.2016.00096
815 Aylagas E, Borja A, Muxika I, Rodríguez-Ezpeleta N (2018) Adapting metabarcoding-based
816 benthic biomonitoring into routine marine ecological status assessment networks.
817 Ecological Indicators 95: 194-202. https://doi.org/10.1016/j.ecolind.2018.07.044

bioRxiv preprint doi: https://doi.org/10.1101/2022.05.10.491303; this version posted May 11, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

818 Aylagas E, Borja Á, Tangherlini M, Dell'Anno A, Corinaldesi C, Michell CT, Irigoien X,
819 Danovaro R., Rodríguez-Ezpeleta N (2017) A bacterial community-based index to assess
820 the ecological status of estuarine and coastal environments. Marine Pollution Bulletin
821 114: 679-688. https://doi.org/10.1016/j.marpolbul.2016.10.050
822 Bacouillard L, Baux N, Dauvin JC, Desroy N, Geiger KJ, Gentil F, Thiébaut É (2020) Long-term
823 spatio-temporal changes of the muddy fine sand benthic community of the Bay of Seine
824 (eastern English Channel). Marine Environmental Research 161: 105062.
825 https://doi.org/10.1016/j.marenvres.2020.105062
826 Borges LMS, Hollatz C, Lobo J, Cunha AM, Vilela AP, Calado G, Coelho R, Costa AC, Ferreira
827 MSG, Costa MH, Costa FO (2016) With a little help from DNA barcoding: investigating
828 the diversity of Gastropoda from the Portuguese coast. Scientific Reports 6: 20226.
829 https://doi.org/10.1038/srep20226
830 Borja A (2018) Testing the efficiency of a bacterial community-based index (microgAMBI) to
831 assess distinct impact sources in six locations around the world. Ecological Indicators 85:
832 594-602. https://doi.org/10.1016/j.ecolind.2017.11.018
833 Borja A, Franco J, Pérez V (2000). A marine biotic index to establish the ecological quality of
834 soft-bottom benthos within European estuarine and coastal environments. Marine
835 Pollution Bulletin 40: 1100-1114. https://doi.org/10.1016/S0025-326X(00)00061-8
836 Borja A, Barbone E, Basset A, Borgersen G, Brkljacic M, Elliott M, Garmendia JM, Marques JC,
837 Mazik K, Muxika I, Neto JM, Norling K, Rodríguez JG, Rosati I, Rygg B, Teixeira H,
838 Trayanova A (2011) Response of single benthic metrics and multi-metric methods to
839 anthropogenic pressure gradients, in five distinct European coastal and transitional
840 ecosystems. Marine Pollution Bulletin 63: 499-513.
841 https://doi.org/10.1016/j.marpolbul.2010.12.009
842 Brown EA, Chain FJJ, Crease TJ, MacIsaac HJ, Cristescu ME (2015) Divergence thresholds and
843 divergent biodiversity estimates: can metabarcoding reliably describe zooplankton
844 communities? Ecology and Evolution 5: 2234-2251. https://doi.org/10.1002/ece3.1485
845 Cahill AE, Pearman JK, Borja A, Carugati L, Carvalho S, Danovaro R, Dashfield S, Romain D,
846 Féral J-P, Olenin S, Šiaulys A, Somerfield PJ, Trayanova A, Uyarra MC, Chenuil A
847 (2018) A comparative analysis of metabarcoding and morphology-based identification of
848 benthic communities across different regional seas. Ecology and Evolution 8: 8908-8920.
849 https://doi.org/10.1002/ece3.4283
850 Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, Madden TL (2009)
851 BLAST+: architecture and applications. BMC Bioinformatics 10: 421.
852 https://doi.org/10.1186/1471-2105-10-421
853 Campbell A, Nicholls J (2008) Fauna e Flora do litoral de Portugal e Europa. FAPAS - Fundo
854 Para a Protecção dos Animais Selvagens, 320 pp.

bioRxiv preprint doi: https://doi.org/10.1101/2022.05.10.491303; this version posted May 11, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

855 Carew ME, Kellar CR, Pettigrove VJ, Hoffmann AA (2018) Can high-throughput sequencing
856 detect macroinvertebrate diversity for routine monitoring of an urban river? Ecological
857 Indicators 85: 440-450. https://doi.org/10.1016/j.ecolind.2017.11.002
858 Chainho P, Costa JL, Chaves ML, Dauer DM, Costa MJ (2007) Influence of seasonal variability
859 in benthic invertebrate community structure on the use of biotic indices to assess the
860 ecological status of a Portuguese estuary. Marine Pollution Bulletin 54: 1586-1597.
861 https://doi.org/10.1016/j.marpolbul.2007.06.009
862 Chariton AA, Stephenson S, Morgan MJ, Steven ADL, Colloff MJ, Court LN, Hardy CM (2015)
863 Metabarcoding of benthic eukaryote communities predicts the ecological condition of
864 estuaries. Environmental Pollution 203: 165-174.
865 http://dx.doi.org/10.1016/j.envpol.2015.03.047
866 Cordier T, Esling P, Lejzerowicz F, Visco J, Ouadahi A, Martins C, Cedhagen T, Pawlowski J
867 (2017) Predicting the ecological quality status of marine environments from eDNA
868 metabarcoding data using supervised machine learning. Environmental Science and
869 Technology 51: 9118-9126. https://doi.org/10.1021/acs.est.7b01518
870 Comeau AM, Douglas GM, Langille MGI (2017) Microbiome helper: a custom and streamlined
871 workflow for Microbiome Research. mSystems 2: e00127-16
872 https://doi.org/10.1128/msystems.00127-16
873 Cowart DA, Pinheiro M, Mouchel O, Maguer M, Grall J, Miné J, Arnaud-Haond S (2015)
874 Metabarcoding is powerful yet still blind: a comparative analysis of morphological and
875 molecular surveys of seagrass communities. PLoS ONE 10: e0117562.
876 https://doi.org/10.1371/journal.pone.0117562
877 Cristescu ME (2014) From barcoding single individuals to metabarcoding biological
878 communities: towards an integrative approach to the study of global biodiversity. Trends
879 in Ecology and Evolution 29: 566-571. https://doi.org/10.1016/j.tree.2014.08.001
880 Derycke S, Maes S, Van den Bulcke L, Vanhollebeke J, Wittoeck J, Hillewaert H, Ampe B,
881 Haegeman A, Hostens K, De Backer A (2021) Detection of macrobenthos species with
882 metabarcoding is consistent in bulk DNA but dependent on body size and sclerotization
883 in eDNA from the ethanol preservative. Frontiers in Marine Science 8: 637858.
884 https://doi.org/10.3389/fmars.2021.637858
885 Dowle EJ, Pochon X, Banks JC, Shearer K, Wood SA (2016) Targeted gene enrichment and high-
886 throughput sequencing for environmental biomonitoring: a case study using freshwater
887 macroinvertebrates. Molecular Ecology Resources 16: 1240–1254.
888 https://doi.org/10.1111/1755-0998.12488
889 Duarte S, Leite BR, Feio MJ, Costa FO, Filipe AF (2021) Integration of DNA-based approaches
890 in aquatic ecological assessment using benthic macroinvertebrates. Water 13: 331.
891 https://doi.org/10.3390/w13030331

bioRxiv preprint doi: https://doi.org/10.1101/2022.05.10.491303; this version posted May 11, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

892 European Commission (2000) Directive 2000/60/EC of the European Parliament and of the
893 Council of 23 October 2000 establishing a framework for Community action in the field
894 of water policy. https://eur-lex.europa.eu/eli/dir/2000/60/oj
895 European Commission (2008) Directive 2008/56/EC of the European Parliament and of the
896 Council of 17 June 2008 establishing a framework for community action in the field of
897 marine environmental policy (Marine Strategy Framework Directive). https://eur-
898 lex.europa.eu/eli/dir/2008/56/oj
899 European Commission (2011) Common Implementation Strategy for the Water Framework
900 Directive (2000/60/EC). Guidance document No. 14 – Guidance document on the
901 Intercalibration Process 2008-2011. https://circabc.europa.eu/ui/group/9ab5926d-bed4-
902 4322-9aa7-9964bbe8312d/library/1bfd8ec3-bac3-4ea0-9d8c-e70c0f9ea3db/details
903 Fais M, Duarte S, Vieira PE, Sousa R, Hajibabaei M, Canchaya CA, Costa FO (2020) Small-scale
904 spatial variation of meiofaunal communities in Lima estuary (NW Portugal) assessed
905 through metabarcoding. Estuarine, Coastal and Shelf Science. 238: 106683.
906 https://doi.org/10.1016/j.ecss.2020.106683
907 França S, Vinagre C, Pardal MA, Cabral HN (2009) Spatial and temporal patterns of benthic
908 invertebrates in the Tagus estuary, Portugal: comparison between subtidal and an
909 intertidal mudflat. Scientia Marina 73: 307-318.
910 https://doi.org/10.3989/scimar.2009.73n2307
911 Fontes JT, Vieira PE, Ekrem T, Soares P, Costa FO (2021) BAGS: an automated Barcode, Audit
912 & Grade System for DNA barcode reference libraries. Molecular Ecology Resources 21:
913 573-583. https://doi.org/10.1111/1755-0998.13262
914 Giebner H, Langen K, Bourlat SJ, Kukowka S, Mayer C, Astrin JJ, Misof B, Fonseca VG (2020)
915 Comparing diversity levels in environmental samples: DNA sequence capture and
916 metabarcoding approaches using 18S and COI genes. Molecular Ecology Resources 20:
917 1333-1345. https://doi.org/10.1111/1755-0998.13201
918 Hajibabaei M (2012) The golden age of DNA metasystematics. Trends in Genetics 28: 535-537.
919 https://doi.org/10.1016/j.tig.2012.08.001
920 Hammer Ø, Harper DAT, Ryan PD (2001) PAST: Paleontological statistics software package for
921 education and data analysis. Palaeontologia Electronica 4, 9 pp. http://palaeo-
922 electronica.org/2001_1/past/issue1_01.htm
923 Hayward PJ, Nelson-Smith T, Shields C (1996). Collins pocket guide – sea shore of Britain &
924 Europe. HarperCollins Publishers, 352 pp.
925 Hayward PJ, Ryland JS (2017) Handbook of the marine fauna of North-West Europe. Oxford
926 University Press. https://doi.org/10.1093/acprof:oso/9780199549443.001.0001
927 Hering D, Borja A, Carstensen J, Carvalho L, Elliott M, Feld CK, Heiskanen A-S, Johnson RK,
928 Moe J, Pont D, Solheim AL, van de Bund W (2010) The European Water Framework

bioRxiv preprint doi: https://doi.org/10.1101/2022.05.10.491303; this version posted May 11, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

929 Directive at the age of 10: a critical review of the achievements with recommendations
930 for the future. Science of the Total Environment 408: 4007-4019.
931 https://doi.org/10.1016/j.scitotenv.2010.05.031
932 Hering D, Borja A, Jones JI, Pont D, Boets P, Bouchez A, Bruce K, Drakare S, Hänfling B,
933 Kahlert M, Leese F, Meissner K, Mergen P, Reyjol Y, Segurado P, Vogler A, Kelly M
934 (2018) Implementation options for DNA-based identification into ecological status
935 assessment under the European Water Framework Directive. Water Research 138: 192-
936 205. https://doi.org/10.1016/j.watres.2018.03.003
937 Hollatz C, Leite BR, Lobo J, Froufe H, Egas C, Costa FO (2017) Priming of a DNA
938 metabarcoding approach for species identification and inventory in marine macrobenthic
939 communities. Genome 60: 260-271. https://doi.org/10.1139/gen-2015-0220
940 Houba VJG, Novozamsky I, Uittenbogaard J, Lee JJ van der (1987) Automatic determination of
941 "total soluble nitrogen" in soil extracts. Landwirtschaftliche Forschung 40: 295-302.
942 Illumina (2013) 16S Metagenomic sequencing library preparation manual - preparing 16S
943 ribosomal RNA gene amplicons for the Illumina MiSeq System, 28 pp.
944 Ivanova NV, De Waard JR, Hebert PDN (2006) An inexpensive, automation-friendly protocol
945 for recovering high-quality DNA. Molecular Ecology Notes 6: 998-1002.
946 https://doi.org/10.1111/j.1471-8286.2006.01428.x
947 Keck F, Blackman RC, Bossart R, Brantschen J, Couton M, Hürlemann S, Kirschner D, Locher
948 N, Zhang H, Altermatt F (2022) Meta-analysis shows both congruence and
949 complementarity of DNA and eDNA metabarcoding to traditional methods for biological
950 community assessment. Molecular Ecology 31: 1820-1835.
951 https://doi.org/10.1111/mec.16364
952 Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD (2013) Development of a dual-
953 index sequencing strategy and curation pipeline for analyzing amplicon sequence data on
954 the MiSeq Illumina sequencing platform. Applied and Environmental Microbiology 79:
955 5112-5120. https://doi.org/10.1128/AEM.01043-13
956 Krom MD (1980) Spectrophotometric determination of ammonia: a study of a modified Berthelot
957 reaction using salicylate and dichloroisocyanurate. Analyst 105: 305-316,
958 https://doi.org/10.1039/an9800500305
959 Kroon H (1993) Determination of nitrogen in water: comparison of a continuous-flow method
960 with on-line UV digestion with the original Kjeldahl method. Analytica Chimica Acta
961 276: 287-293. https://doi.org/10.1016/0003-2670(93)80396-3
962 Leese F, Bouchez A, Abarenkov K, Altermatt F, Borja A, Bruce K, Ekrem T, Čiampor Jr F,
963 Čiamporová-Zaťovičová Z, Costa FO, Duarte S, Elbrecht V, Fontaneto D, Franc A,
964 Geiger MF, Hering D, Kahlert M, Stroil BK, Kelly M, Keskin E, Liska I, Mergen P,
965 Meissner K, Pawlowski J, Penev L, Reyjol Y, Rotter A, Steinke D, van der Wal B,

bioRxiv preprint doi: https://doi.org/10.1101/2022.05.10.491303; this version posted May 11, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

966 Vitecek S, Zimmermann J, Weigand AM (2018) Chapter Two - Why we need sustainable
967 networks bridging countries, disciplines, cultures and generations for aquatic
968 biomonitoring 2.0: a perspective derived from the *DNAqua-Net* COST Action. Advances
969 in Ecological Research 58: 63-99. https://doi.org/10.1016/bs.aecr.2018.01.001
970 Leite BR (2021) Monitoring coastal benthic colonization of artificial substrates with the support
971 of DNA metabarcoding approaches. PhD thesis in Biology Specialization in Integrated
972 Management of the Sea, University of Minho, Braga, Portugal, 212 pp.
973 Leite BR, Vieira PE, Teixeira MAL, Lobo-Arteaga J, Hollatz C, Borges LMS, Duarte S, Troncoso
974 JS, Costa FO (2020) Gap-analysis and annotated reference library for supporting
975 macroinvertebrate metabarcoding in Atlantic Iberia. Regional Studies in Marine Science
976 36: 101307. https://doi.org/10.1016/j.rsma.2020.101307
977 Leite BR, Vieira PE, Troncoso JS, Costa FO (2021) Comparing species detection success between
978 molecular markers in DNA metabarcoding of coastal macroinvertebrates. Metabarcoding
979 and Metagenomics 5: e70063. https://doi.org/10.3897/mbmg.5.70063
980 Lejzerowicz F, Esling P, Pillet L, Wilding TA, Black KD, Pawlowski J (2015) High-throughput
981 sequencing and morphology perform equally well for benthic monitoring of marine
982 ecosystems. Scientific Reports 5: 13932. https://doi.org/10.1038/srep13932
983 Leray M, Yang JY, Meyer CP, Mills SC, Agudelo N, Ranwez V, Boehm JT, Machida RJ (2013)
984 A new versatile primer set targeting a short fragment of the mitochondrial COI region for
985 metabarcoding metazoan diversity: application for characterizing coral reef fish gut
986 contents. Frontiers in Zoology 10: 34. https://doi.org/10.1186/1742-9994-10-34
987 Lincoln RJ (1979) British Marine Amphipoda: Gammaridea. British Museum (Natural History).
988 Lobo J, Costa PM, Teixeira MAL, Ferreira MSG, Costa MH, Costa FO (2013) Enhanced primers
989 for amplification of DNA barcodes from a broad range of marine metazoans. BMC
990 Ecology 13: 34. https://doi.org/10.1186/1472-6785-13-34
991 Lobo J, Teixeira MAL, Borges LMS, Ferreira MSG, Hollatz C, Gomes PT, Sousa R, Ravara A,
992 Costa MH, Costa FO (2016) Starting a DNA barcode reference library for shallow water
993 polychaetes from the southern European Atlantic coast. Molecular Ecology Resources
994 16: 298-313. https://doi.org/10.1111/1755-0998.12441
995 Lobo J, Ferreira MS, Antunes IC, Teixeira MAL, Borges LMS, Sousa R, Gomes PA, Costa MH,
996 Cunha MR, Costa FO (2017a) Contrasting morphological and DNA barcode-suggested
997 species boundaries among shallow-water amphipod fauna from the southern European
998 Atlantic coast. Genome 60: 147-157. https://doi.org/10.1139/gen-2016-0009
999 Lobo J, Shokralla S, Costa MH, Hajibabaei M, Costa FO (2017b) DNA metabarcoding for high-
1000 throughput monitoring of estuarine macrobenthic communities. Scientific Reports 7:
1001 15618. https://doi.org/10.1038/s41598-017-15823-6

bioRxiv preprint doi: https://doi.org/10.1101/2022.05.10.491303; this version posted May 11, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1002 Martins FMS, Porto M, Feio MJ, Egeter B, Bonin A, Serra SRQ, Tarbelet P, Beja P (2020)
1003 Modelling technical and biological biases in macroinvertebrate community assessment
1004 from bulk preservative using multiple metabarcoding markers. Molecular Ecology 30:
1005 3221-3238. https://doi.org/10.1111/mec.15620
1006 Neto JM, Teixeira H, Patrício J, Baeta A, Veríssimo H, Pinto R, Marques JC (2010) The response
1007 of estuarine macrobenthic communities to natural- and human-induced changes:
1008 dynamics and ecological quality. Estuaries and Coasts 33: 1327-1339.
1009 https://doi.org/10.1007/s12237-010-9326-x
1010 Pawlowski J, Kelly-Quinn M, Altermatt F, Apothéloz-Perret-Gentil L, Beja P, Boggero A, Borja
1011 A, Bouchez A, Cordier T, Domaizon I, Feio MJ, Filipe AF, Fornaroli R, Graf W, Herder
1012 J, van der Hoorn B, Jones JI, Sagova-Mareckova M, Moritz C, Barquín J, Piggott JJ,
1013 Pinna M, Rimet F, Rinkevich B, Sousa-Santos C, Specchia V, Trobajo R, Vasselon V,
1014 Vitecek S, Zimmerman J, Weigand A, Leese F, Kahlert M (2018) The future of biotic
1015 indices in the ecogenomic era: Integrating (e)DNA metabarcoding in biological
1016 assessment of aquatic ecosystems. Science of The Total Environment 637-638: 1295-
1017 1310. https://doi.org/10.1016/j.scitotenv.2018.05.002
1018 Pochon X, Bott NJ, Smith KF, Wood SA (2013) Evaluating detection limits of Next-Generation
1019 Sequencing for the surveillance and monitoring of international marine pests. PLoS ONE
1020 8: e73935. https://doi.org/10.1371/journal.pone.0073935
1021 Pruesse E, Peplies J, Glöckner FO (2012) SINA: accurate high throughput multiple sequence
1022 alignment of ribosomal RNA genes. Bioinformatics 28: 1823-1829
1023 https://doi.org/10.1093/bioinformatics/bts252
1024 Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO (2013) The
1025 SILVA ribosomal RNA gene database project: improved data processing and web-based
1026 tools. Nucleic Acids Research 41: D590-D596. https://doi.org/10.1093/nar/gks1219
1027 Ratnasingham S (2019) mBRAVE: The Multiplex Barcode Research and Visualization
1028 Environment. Biodiversity Information Science and Standards 3: e37986.
1029 https://doi.org/10.3897/biss.3.37986
1030 Ratnasingham S, Hebert PDN (2007) BOLD: The Barcode of Life Data System. Molecular
1031 Ecology Notes 7: 355-364. https://doi.org/10.1111/j.1471-8286.2007.01678.x
1032 Rodrigues AM, Quintino V, Sampaio L, Freitas R, Neves R (2011) Benthic biodiversity patterns
1033 in Ria de Aveiro, Western Portugal: environmental-biological relationships. Estuarine,
1034 Coastal and Shelf Science 95: 338-348. https://doi.org/10.1016/j.ecss.2011.05.019
1035 Rognes T, Flouri T, Nichols B, Quince C, Mahé F (2016) VSEARCH: a versatile open-source
1036 tool for metagenomics. PeerJ 4: e2584. https://doi.org/10.7717/peerj.2584
1037 Rosenberg R, Blomqvist M, Nilsson HC, Cederwall H, Dimming A (2004) Marine quality
1038 assessment by use of benthic species-abundance distributions: a proposed new protocol

bioRxiv preprint doi: https://doi.org/10.1101/2022.05.10.491303; this version posted May 11, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1039 within the European Union Water Framework Directive. Marine Pollution Bulletin 49:
1040 728-739. https://doi.org/10.1016/j.marpolbul.2004.05.013
1041 Salas F, Neto JM, Borja A, Marques JC (2004) Evaluation of the applicability of a marine biotic
1042 index to characterize the status of estuarine ecosystems: the case of Mondego estuary
1043 (Portugal). Ecological Indicators 4: 215-225.
1044 https://doi.org/10.1016/j.ecolind.2004.04.003
1045 Schmieder R, Edwards R (2011) Quality control and preprocessing of metagenomic datasets.
1046 Bioinformatics 27: 863-864. https://doi.org/10.1093/bioinformatics/btr026
1047 Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley
1048 BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF
1049 (2009) Introducing mothur: Open-source, platform-independent, community-supported
1050 software for describing and comparing microbial communities. Applied and
1051 Environmental Microbiology 75: 7537-7541. https://doi.org/10.1128/AEM.01541-09
1052 Solan M, Cardinale BJ, Downing AL, Engelhardt KAM, Ruesink JL, Srivastava DS (2004)
1053 Extinction and ecosystem function in the marine benthos. Science 306: 1177-1180.
1054 https://doi.org/10.1126/science.1103960
1055 Sousa R, Dias S, Antunes JC (2006) Spatial subtidal macrobenthic distribution in relation to
1056 abiotic conditions in the Lima estuary, NW of Portugal. Hydrobiologia 559: 135-148.
1057 https://doi.org/10.1007/s10750-005-1371-2
1058 Sousa R, Dias S, Antunes C (2007) Subtidal macrobenthic structure in the lower Lima estuary,
1059 NW of Iberian Peninsula. Annales Zoologici Fennici 44: 303-313.
1060 https://www.jstor.org/stable/23736773
1061 Sousa R, Dias S, Freitas V, Antunes C (2008) Subtidal macrozoobenthic assemblages along the
1062 River Minho estuarine gradient (north-west Iberian Peninsula). Aquatic Conservation:
1063 Marine and Freshwater Ecosystems 18: 1063-1077. https://doi.org/10.1002/aqc.871
1064 Steinke D, deWaard SL, Sones JE, Ivanova NV, Prosser SWJ, Perez K, Braukmann TWA, Milton
1065 M, Zakharov EV, deWaard JR, Ratnasingham S, Hebert PDN (2022) Message in a bottle–
1066 Metabarcoding enables biodiversity comparisons across ecoregions. GigaScience 11:
1067 giac040. https://doi.org/10.1093/gigascience/giac040
1068 Steyaert M, Priestley V, Osborne O, Herraiz A, Arnold R, Savolainen V (2020) Advances in
1069 metabarcoding techniques bring us closer to reliable monitoring of the marine benthos.
1070 Journal of Applied Ecology 57: 2234-2245. https://doi.org/10.1111/1365-2664.13729
1071 Stoeck T, Bass D, Nebel M, Christen R, Jones MDM, Breiner H-W, Richards TA (2010) Multiple
1072 marker parallel tag environmental DNA sequencing reveals a highly complex eukaryotic
1073 community in marine anoxic water. Molecular Ecology 19: 21-31.
1074 https://doi.org/10.1111/j.1365-294X.2009.04480.x

bioRxiv preprint doi: https://doi.org/10.1101/2022.05.10.491303; this version posted May 11, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1075 Tang CQ, Leasi F, Obertegger U, Kieneke A, Barraclough TG, Fontaneto D (2012) The widely
1076 used small subunit 18S rDNA molecule greatly underestimates true diversity in
1077 biodiversity surveys of the meiofauna. Proceedings of the National Academy of Sciences
1078 109: 16208–16212. https://doi.org/10.1073/pnas.1209160109
1079 Teixeira H, Salas F, Borja A, Neto JM, Marques JC (2008a) A benthic perspective in assessing
1080 the ecological status of estuaries: the case of the Mondego estuary (Portugal). Ecological
1081 Indicators 8: 404-416. https://doi.org/10.1016/j.ecolind.2007.02.008
1082 Teixeira H, Salas F, Neto JM, Patrício J, Pinto R, Veríssimo H, García-Charton JA, Marcos C,
1083 Pérez-Ruzafa A, Marques JC (2008b) Ecological indices tracking distinct impacts along
1084 disturbance-recovery gradients in a temperate NE Atlantic Estuary – Guidance on
1085 reference values. Estuarine, Coastal and Shelf Science 80: 130-140.
1086 https://doi.org/10.1016/j.ecss.2008.07.017
1087 Teixeira H, Neto JM, Patrício J, Veríssimo H, Pinto R, Salas F, Marques JC (2009) Quality
1088 assessment of benthic macroinvertebrates under the scope of WFD using BAT, the
1089 Benthic Assessment Tool. Marine Pollution Bulletin 58: 1477-1486.
1090 https://doi.org/10.1016/j.marpolbul.2009.06.006
1091 Teixeira MAL, Vieira PE, Pleijel F, Sampieri BR, Ravara A, Costa FO, Nygren A (2020)
1092 Molecular and morphometric analyses identify new lineages within a large *Eumida*
1093 (Annelida) species complex. Zoologica Scripta 49: 222-235.
1094 https://doi.org/10.1111/zsc.12397
1095 Ter Braak CJF, Verdonschot PFM (1995) Canonical correspondence analysis and related
1096 multivariate methods in aquatic ecology. Aquatic Sciences 57: 255-289.
1097 https://doi.org/10.1007/BF00877430
1098 Thiébaut E, Cabioch L, Dauvin J-C, Retière C, Gentil F (1997) Spatio-temporal persistence of the
1099 *Abra alba-Pectinaria koreni* muddy-fine sand community of the eastern Bay of Seine.
1100 Journal of the Marine Biological Association of the United Kingdom 77: 1165-1185.
1101 https://doi.org/10.1017/S0025315400038698
1102 Van den Bulcke L, De Backer A, Ampe B, Maes S, Wittoeck J, Waegeman W, Hostens K,
1103 Derycke S (2021) Towards harmonization of DNA metabarcoding for monitoring marine
1104 macrobenthos: the effect of technical replicates and pooled DNA extractions on species
1105 detection. Metabarcoding and Metagenomics 5: e71107.
1106 https://doi.org/10.3897/mbmg.5.71107
1107 Vieira PE, Desiderato A, Holdich DM, Soares P, Creer S, Carvalho GR, Costa FO, Queiroga H
1108 (2020) Deep segregation in the open ocean: Macaronesia as an evolutionary hotspot for
1109 low dispersal marine invertebrates. Molecular Ecology 28: 1784-1800.
1110 https://doi.org/10.1111/mec.15052

bioRxiv preprint doi: https://doi.org/10.1101/2022.05.10.491303; this version posted May 11, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1111 Vinagre PA, Pais-Costa AJ, Marques JC, Neto JM (2015) Setting reference conditions for
1112 mesohaline and oligohaline macroinvertebrate communities *sensu* WFD: Helping to
1113 define achievable scenarios in basin management plans. Ecological Indicators 56: 171-
1114 183. https://doi.org/10.1016/j.ecolind.2015.04.008
1115 Wangensteen OS, Cebrian E, Palacín C, Turon X (2018a) Under the canopy: community-wide
1116 effects of invasive algae in marine protected areas revealed by metabarcoding. Marine
1117 Pollution Bulletin 127: 54-66. https://doi.org/10.1016/j.marpolbul.2017.11.033
1118 Wangensteen OS, Palacín C, Guardiola M, Turon X (2018b) DNA metabarcoding of littoral hard-
1119 bottom communities: high diversity and database gaps revealed by two molecular
1120 markers. PeerJ 6: e4705. https://doi.org/10.7717/peerj.4705
1121 Weigand H, Beermann AJ, Čiampor F, Costa FO, Csabai Z, Duarte S, Geiger MF, Grabowski M,
1122 Rimet F, Rulik B, Strand M, Szucsich N, Weigand AM, Willassen E, Wyler SA, Bouchez
1123 A, Borja A, Čiamporová-Zaťovičová Z, Ferreira S, Dijkstra K-DB, Eisendle U, Freyhof
1124 J, Gadawski P, Graf W, Haegerbaeumer A, van der Hoorn BB, Japoshvili B, Keresztes
1125 L, Keskin E, Leese F, Macher JN, Mamos T, Paz G, Pešic V, Pfannkuchen DM,
1126 Pfannkuchen MA, Price BW, Rinkevich B, Teixeira MAL, Várbíró G, Ekrem T (2019)
1127 DNA barcode reference libraries for the monitoring of aquatic biota in Europe: Gap-
1128 analysis and recommendations for future work. Science of the Total Environment 678:
1129 499-524. https://doi.org/10.1016/j.scitotenv.2019.04.247
1130 Westfall KM, Therriault TW, Abbot CL (2020) A new approach to molecular biosurveillance of
1131 invasive species using DNA metabarcoding. Global Change Biology 26: 1012-1022.
1132 https://doi.org/10.1111/gcb.14886
1133 WoRMS Editorial Board (2021) World Register of Marine Species. Available from
1134 http://www.marinespecies.org at VLIZ. Accessed on 2021-11-23.
1135 http://doi.org/10.14284/170
1136 Zhan A, Hulák M, Sylvester F, Huang X, Adebayo AA, Abbott CL, Adamowicz SJ, Heath DD,
1137 Cristescu ME, MacIsaac HJ (2013) High sensitivity of 454 pyrosequencing for detection
1138 of rare species in aquatic communities. Methods Ecology and Evolution 4: 558-565.
1139 https://doi.org/10.1111/2041-210X.12037
1140 Zhang Y, Wang J, Lv M, Gao H, Meng LF, A Y, Seim I, Zhang H, Liu S, Zhang L, Liu X, Xu X,
1141 Yang H, MI0K+ consortium, Fan G (2021) Diversity, function and evolution of marine
1142 invertebrate genomes. bioRxiv, https://doi.org/10.1101/2021.10.31.465852
1143