

## Advances in metabarcoding techniques bring us closer to reliable monitoring of the marine benthos

Steyaert, Margaux ; Priestley, Victoria ; Osborne, Owen; Herraiz, Alba ; Arnold, Richard; Savolainen, Vincent

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1 Title: Advances in metabarcoding techniques bring us closer to reliable monitoring of the  
2 marine benthos

3 Authors: Margaux Steyaert<sup>1,2</sup>, Victoria Priestley<sup>1</sup>, Owen Osborne<sup>1,3</sup>, Alba Herraiz<sup>1</sup>, Richard  
4 Arnold<sup>4</sup>, Vincent Savolainen<sup>1\*</sup>

5 <sup>1</sup> Department of Life Sciences, Imperial College London, Silwood Park Campus, SL5 7PY,  
6 United Kingdom.

7 <sup>2</sup> Current address: Department of Zoology, University of Oxford, Zoology Research and  
8 Administration Building, 11a Mansfield Road, Oxford, OX1 3SZ.

9 <sup>3</sup> Current address: School of Natural Sciences, Bangor University, Bangor, Gwynedd, LL57  
10 2DG, United Kingdom.

11 <sup>4</sup> Thomson Environmental Consultants, Surrey Research Park, GU2 7AG, United Kingdom.

12 \*Corresponding author: v.savolainen@imperial.ac.uk

## 13 **Abstract**

14 1. Reliable and accurate biodiversity census methods are essential for monitoring ecosystem  
15 health and assessing potential ecological impacts of future development projects. Although  
16 metabarcoding is increasingly used to study biodiversity across ecological research,  
17 morphology-based identification remains the preferred approach for marine ecological impact  
18 assessments. Comparing metabarcoding to morphology-based protocols currently used by  
19 ecological surveyors is essential to determine whether this DNA-based approach is suitable  
20 for the long-term monitoring of marine ecosystems.

21 2. We compared metabarcoding and morphology-based approaches for the analysis of  
22 invertebrates in low diversity intertidal marine sediment samples. We used a recently  
23 developed bioinformatics pipeline and two taxonomic assignment methods to resolve and  
24 assign amplicon sequence variants (ASVs) from Illumina amplicon data. We analysed the  
25 community composition recovered by both methods and tested the effects, on the levels of  
26 diversity detected by the metabarcoding method, of sieving samples prior to DNA extraction.

27 3. Metabarcoding of the mitochondrial marker cytochrome c oxidase I (COI) gene recovers  
28 the presence of more taxonomic groups than the morphological approach. We found that  
29 sieving samples results in lower alpha diversity detected and suggests a community  
30 composition that differs significantly from that suggested by un-sieved samples in our  
31 metabarcoding analysis. We found that whilst metabarcoding and morphological approaches  
32 detected similar numbers of species, they are unable to identify the same set of species across  
33 samples.

34 4. *Synthesis and Applications* We show that metabarcoding using the COI marker provides a  
35 more holistic, community-based, analysis of benthic invertebrate diversity than a traditional  
36 morphological approach. We also highlight current gaps in reference databases and  
37 bioinformatic pipelines for the identification of intertidal benthic invertebrates that need to be  
38 addressed before metabarcoding can replace traditional methods. Ultimately, with these  
39 limitations taken into consideration, resolving community-wide diversity patterns with  
40 metabarcoding could improve the management of non-protected marine habitats in the U.K.

41 **Keywords:** Biodiversity, Biomonitoring, Marine Benthos, Metabarcoding, Ecological  
42 Surveying.

## 43 **Introduction**

44 Understanding and quantifying the diversity of organisms is fundamental in the assessment of  
45 ecosystem health. Detecting significant shifts in species composition can lead to important  
46 changes in environmental policy, conservation efforts or the management of wild resources.  
47 As ecosystems are increasingly under pressure from climate and land-use change, it is vital  
48 that we understand which species are present or absent in habitats that interact with human  
49 civilization (Bardgett & van der Putten, 2014; Cardinale et al., 2012). In turn, by  
50 understanding trends in species composition, we can better quantify the value of ecosystems  
51 and the services they provide (Hautier et al., 2015). Reliable and fast methods for surveying  
52 species diversity are therefore highly sought after within both academia and the public sector  
53 (Keck et al., 2017; Baird et al., 2012).

54 Marine invertebrates have long been used to categorise and assess the health of marine  
55 ecosystems as shifts in their composition often reflect on wider patterns of human impact or  
56 natural disturbances (Borja, 2019; Chain et al., 2016). These organisms are considered  
57 important ecosystem bioindicators and have been utilised to screen the level of pollution and  
58 other anthropogenic impacts on marine habitats (Pérez et al., 2019; Poikane et al., 2016;  
59 Chiarelli & Roccheri, 2014). Macrobenthic invertebrates form a vital component of current  
60 biomonitoring programs, such as the European Union Water and Marine Strategy Framework  
61 Initiative (Hoey et al., 2019). Littoral and estuarine zones are key habitats often harbouring  
62 economically and ecologically important species but are increasingly impacted by the  
63 expansion of human development and pollution. In the United Kingdom, impact assessments  
64 following Water Framework Directive guidelines are required to be submitted when planning  
65 infrastructure development projects along the coastline in order to assess the level of impact  
66 such activities may cause (Environmental Agency, 2016). Such surveys routinely include an  
67 evaluation of macrobenthic invertebrate diversity, along with analyses of sediment particle  
68 size and isotopes. To our knowledge, all UK-based ecological consultancy companies  
69 currently offering marine consultancy services, including Environmental Impact Assessment  
70 (EIA) or Habitat Regulations Appraisals (HRA) surveys, only use traditional census methods  
71 in their identification of marine benthic invertebrates. These methods rely on examining  
72 morphological traits using light microscopy to taxonomically identify species and have been  
73 widely used to study macroinvertebrate diversity. A significant advantage to a morphological

74 approach is its ability to distinguish organisms that are present in a sample from biological  
75 remnants of transient species as well as enabling a direct count of individuals.

76 DNA metabarcoding enables the bulk identification of multiple species within an ecological  
77 sample by simultaneously amplifying individual ‘DNA barcodes’ (that is, DNA fragments  
78 that can be used for species identification), which are then sequenced and identified using  
79 HTS. This genetic method can allow for the identification of organisms that are too small or  
80 too degraded for light microscopy protocols, as well as cryptic taxa or species that exhibit  
81 phenotypic plasticity (Elbrecht & Leese, 2015). Finally, metabarcoding allows species  
82 diversity to be observed over a large spatial and temporal window, since genetic material  
83 from both present and transient organisms can be detected (Leray & Knowlton, 2015;  
84 Thomsen & Willerslev, 2015). However, there are still several limiting factors that prevent it  
85 from completely replacing traditional methods (Kelly et al., 2017; Lejzerowicz et al., 2015).  
86 These include a lack of available reference sequences in genetic databases, primer bias for  
87 amplification, copy number variation in target loci as well as unstandardized sample  
88 processing and sequence data analysis steps (Elbrecht et al., 2017; Drummond et al., 2015).

89 Metabarcoding is currently gaining considerable popularity, with many studies having  
90 successfully recovered the presence and diversity of marine species using this novel tool  
91 (Pearman et al., 2018; Yamamoto et al., 2017; Chain et al., 2016; Chariton et al., 2015).  
92 Several studies have directly compared traditional and metabarcoding approaches to  
93 surveying local marine benthic diversity (Aylagas et al., 2018; Cahill et al., 2018; Lobo et al.,  
94 2017; Lejzerowicz et al., 2015). A metabarcoding approach to sampling benthic  
95 macroinvertebrates has been shown to outperform traditional methods in the level of diversity  
96 recovered within an ecological sample (Lobo et al., 2017). However, datasets resulting from  
97 both methods are often difficult to compare directly as individuals are identified to different  
98 taxonomic levels (Cahill et al., 2018; Aylagas et al., 2016). Whilst there is a growing  
99 consensus that the future of biomonitoring now lies with high-throughput sequencing (HTS)  
100 methods such as metabarcoding and the targeting of environmental DNA (Aylagas et al.,  
101 2018; Pawlowski et al., 2018; Baird & Hajibabaei, 2012), further research must be  
102 undertaken prior to integrating these approaches into public sector biomonitoring. This  
103 includes comparing metabarcoding and morphology-based census methods currently used by  
104 companies offering ecological surveying services to evaluate how these protocols may differ  
105 from or complement one another.

106 In this study, we present a comparison between metabarcoding and morphological  
107 approaches for the assessment of species diversity in intertidal marine benthos samples. We  
108 follow a morphology-based protocol routinely used to survey marine macrobenthic diversity  
109 by a leading UK ecological consultant, Thomson Environmental Consultants. In order to  
110 directly compare each method's ability to detect and identify species, we perform both  
111 analyses on sets of environmental cores sampled from the same locations in an estuarine  
112 ecosystem. We hypothesise that our metabarcoding approach will 1) detect the presence of all  
113 macroinvertebrate species identified in the morphological approach and 2) will recover a  
114 larger range and diversity of organisms, including specimens only detectable via  
115 environmental DNA traces. We evaluate the effect of sieving versus not sieving samples  
116 prior to DNA extractions in order to assess the amount of organismal diversity represented by  
117 size fractions smaller than 0.5mm (a commonly used minimum size for morphology-based  
118 identification). Overall, this study benchmarks biomonitoring methods and provides further  
119 insight into the potential suitability of DNA based identification methods for the surveying of  
120 marine benthos communities.

## 121 **Materials and methods**

### 122 *Sample collection*

123 A total of 20 1-litre benthic samples were collected at 10 sites along the intertidal region of  
124 the Harwich International Port estuary (Norfolk, UK), in April 2017 (Figure 1, Table S1).  
125 This site is regularly surveyed by Thomson Environmental Consultants as part of an  
126 Environmental Impact Assessment project they carry out for the Harwich Haven Authority.  
127 All benthic cores were collected at low tide. For each sampling site, two cores (one for  
128 metabarcoding analysis and one for morphological identification) were extracted within  
129 10cm of each other by inserting an extraction tube (surface area of 0.01 m<sup>2</sup>) to a depth of  
130 10cm. Cores collected for the metabarcoding analysis were placed in individual sterile Whirl-  
131 Pak® (Nasco, USA) bags and kept on ice during transport. Sterile gloves were always worn  
132 and replaced between each collection so as to limit cross contamination. Cores were stored at  
133 -80°C approximately 6 hours after field collection.

134 *Sample processing, homogenisation and DNA extraction*

135 Overall, all sample processing, DNA extractions, sequence amplification, library prep and  
136 sequencing stages were undertaken at Imperial College (see schematic overview in Figure  
137 S1). Prior to sample homogenisation and DNA extractions, cores were thawed at 4°C for 24  
138 hours. The extraction apparatus was washed with nuclease-free water and detergents. Core  
139 samples number 1, 3, 5, 7 and 9 were individually sieved using a 0.5mm sterile mesh sieve.  
140 Organisms and biological matter were then separated from sediment using a decantation step  
141 whereby approximately 200g of benthos, along with 500ml of purified nuclease-free water,  
142 were first added to a 1L graduated cylinder, covered with Parafilm, and then were vigorously  
143 shaken before being decanted through the sieve. Empty shells were checked for sessile  
144 organisms and discarded prior to homogenisation. Remaining organic matter and organisms  
145 were collected and crushed using a sterile pestle and mortar. Core samples number 2, 4, 6, 8  
146 and 10 were homogenised using a bulk blending approach. Cores were individually mixed in  
147 a sterile 1.5L glass blender (Klarstein, 700W) on the highest setting for 10 minutes. DNA  
148 was then extracted from two individual 8.5g technical replicate sub samples from each mixed  
149 or crushed core using the Mo Bio PowerMax® Soil DNA Isolation Kit (Qiagen), following  
150 the manufacturer's instructions. We extracted DNA from two technical replicates in order to  
151 conduct parallel polymerase chain reaction (PCR) and sequencing runs of each sample.  
152 Results from these technical replicates are then merged in the bioinformatics pipeline.  
153 Glassware were autoclaved and worktops bleached between each extraction to avoid cross  
154 contamination. The extracted DNA samples were then purified and concentrated using an  
155 ethanol precipitation protocol (Supplementary Text 2).

156 *Morphological identification protocol*

157 Thomson Environmental Consultants processed and analysed 20 cores following the National  
158 Marine Biological Analytical Quality Control Scheme (NMBAQC) Processing Requirements  
159 protocol for the identification of invertebrate species using light microscopy (Worsfold and  
160 Hall, 2010). Cores were filtered using a 0.5 mm meshed sieve. All organisms retained by the  
161 sieve were counted and identified to species level where possible by taxonomic experts.

162 *Library preparation & sequencing*

163 A 313 base pairs (bp) fragment of the COI gene was targeted using two universal primers  
164 with attached overhang Illumina adapters (*mlCOIintF* and *lgHCO2198*; Geller et al., 2013;  
165 Leray et al., 2013; Table S3). The amplicon region targeted by this degenerate primer pair

166 has been shown to be one of the most effective for metazoan metabarcoding, especially for  
167 the identification of marine macroinvertebrates (Ransome et al., 2017; Aylagas et al., 2016;  
168 Leray et al., 2013; Leray et al., 2015). Library preparation was carried out following  
169 recommendations made in Illumina's 16S Metagenomic Sequencing Library Preparation  
170 protocol (Illumina; Supplementary Text 1). This library was then sequenced on an Illumina  
171 Miseq platform using a MiSeq reagent kit v3 (2x300 cycle).

## 172 *Sequence analysis*

173 The open-source software package DADA2 (version 1.12) was used to quality check, filter,  
174 trim and remove chimeras from the raw demultiplexed reads following the online DADA2  
175 Pipeline Tutorial 1.12 (<https://benjjneb.github.io/dada2/tutorial.html>) in R Studio 1.2.5019  
176 (Callahan et al., 2016). DADA2 infers exact amplicon sequence variants (ASVs) from large  
177 amplicon datasets by creating and using a parametric error matrix. This enables biological  
178 sequences to be inferred prior to steps in the metabarcoding pipeline that can introduce errors  
179 from PCR and sequencing. Using ASV methods to analyse metabarcoding datasets have been  
180 shown to provide higher resolution of community composition than traditional Operational  
181 Taxonomic Unit (OTU) methods which are based on clustering sequencing reads based on a  
182 pre-determined dissimilarity threshold (Callahan et al., 2017; Needham et al., 2017).  
183 Taxonomy was then assigned to the resulting list of ASVs from DADA2 using the insect R  
184 package (version 1.3.0.9000) following the online tutorial [https://cran.r-](https://cran.r-project.org/web/packages/insect/vignettes/insect-vignette.html)  
185 [project.org/web/packages/insect/vignettes/insect-vignette.html](https://cran.r-project.org/web/packages/insect/vignettes/insect-vignette.html)) and a reference dataset made  
186 using the MIDORI-UNIQUE database specific to the *mlCOIintF\_F/jghCO2198* primer  
187 amplicon region (Wilkinson et al., 2018). This latter package assigns taxon identification  
188 using classification trees. An alternative taxonomy assignment on the ASV list was also  
189 carried out using the online BLASTn tool (standard nucleotide BLAST) and the online  
190 nucleotide collection (Altschul et al., 1990).

191 The R package LULU 0.1.0, along with the command line package VSEARCH 2.14.2, were  
192 used to curate the ASV list from the DADA2 pipeline (Froezlev et al., 2017; Rognes et al.,  
193 2016). The online LULU R package tutorial was followed, along with recommended default  
194 settings (<https://github.com/tobiasgf/lulu>). LULU evaluates the co-occurrence of ASVs  
195 amongst samples and removes potential erroneous variants, resulting in more realistic  
196 diversity estimates and metrics (Froeslev et al., 2017). A step by step breakdown of the  
197 DADA2, insect and LULU pipelines is available as supplementary information (R

198 Harwich\_metabarcoding\_DADA2\_LULU\_script). The ‘phyloseq’ R package 1.30.0 was  
199 used to visualise the taxonomic composition and estimate the alpha diversity of samples,  
200 following the online phyloseq tutorial  
201 ([https://vaalot.github.io/tutorials/Phyloseq\\_tutorial.html](https://vaalot.github.io/tutorials/Phyloseq_tutorial.html)) (McMurdie & Holmes, 2013). The  
202 packages ‘vegan’ (version 2.5.6) and ‘DESeq2’ (version 1.26.0) were then used to analyse  
203 beta diversity across sieved and un-sieved samples. These packages were used to run a beta-  
204 dispersion ‘betadisper’ to test for homogeneity of dispersion amongst sieved and un-sieved  
205 samples, a permutational ANOVA ‘adonis’ (with 999 random permutations) to test for  
206 significant differences in community composition between sieved and un-sieved samples, and  
207 to plot a Principle Coordinates Analysis (PCoA) to visualise community similarity between  
208 the two processing steps. A step by step breakdown of the ‘phyloseq’, ‘vegan’ and ‘DESeq2’  
209 analyses is available as supplementary information (R  
210 Harwich\_metabarcoding\_data\_analysis\_script).

## 211 **Results**

### 212 *Sequencing results and the effect of sieving on detecting species diversity*

213 Sequencing resulted in a total of 22,146,908 raw reads (Table 1). Quality filtering, merging  
214 and chimera removal steps resulted in a total of 621,170 merged reads across all 20 technical  
215 replicates (Table 1). Overall, 87% of reads were removed during a strict DADA2 filtering  
216 step so as to avoid spurious results further on in the pipeline (Table 1). A total of 1,662  
217 ASVs were identified across samples, including 1,405 belonging to marine taxonomic  
218 groups. Of these, 307 ASVs were identified across 13 metazoan marine phyla.

219 More ASVs were detected in un-sieved samples (1,509) than in sieved samples (734) (Figure  
220 2). Sequence alpha rarefaction curves level off across all sieved and un-sieved samples,  
221 indicating that the majority of the estuarine diversity has been sampled by our metabarcoding  
222 approach (Figure 2). A permutational ANOVA test determined that the community structure  
223 in sieved and un-sieved samples were significantly different ( $F = 1.24, p < 0.05$ ). Annelida  
224 was found to be the dominant phylum in both sieved and un-sieved samples (representing  
225 64.9% and 64.6% of reads, respectively) (Figure 3). Molluscs made up a larger portion of un-  
226 sieved sample reads than sieved sample reads (27.2% and 19.4%, respectively). Annelid

227 species *Amphichaeta sannio*, *Paranais litoralis*, *Phyllodoce groenlandica*, copepod *Delavalia*  
228 *palustris* and colonial hydroid *Clava multicornis* were only found in un-sieved samples. The  
229 common cockle *Cerastoderma edule* and flatworm *Zonorhynchus seminascatus* species were  
230 only found in sieved samples. Two chordate taxa, *Homo sapiens* (humans) and *Astyanax*  
231 (blind cave fish), were detected across several samples. The presence of human DNA is  
232 either due to contamination during the sampling, processing or extraction steps or potentially  
233 due to the presence of sewage in the sampling location at Harwich International port. We  
234 believe the presence of blind cave fish DNA is due to lab contamination as another research  
235 project focusing on this species was taking place within the same laboratory during the time  
236 of this study's metabarcoding analysis.

### 237 *Morphological analysis*

238 A total of 2,144 specimens were identified across the 10 core samples. Sample 9 had the  
239 highest number of organisms (583 individuals) and sample 8 had the least (73 individuals)  
240 (Table S2). Specimens representing six different phyla, 14 orders and 24 families of  
241 macroinvertebrates were identified. A total of 25 species from six different phyla were  
242 identified (Table 2). On average, 78% of individual specimens (1,414 individuals) were  
243 identified down to species level across samples, with sample 2 having the highest  
244 identification rate and samples 8 having the lowest (97% and 44% respectively; Table S2).  
245 Platyhelminthes, nemertean and nematodes were only identified to phylum level.  
246 Unidentified animal eggs were also detected in one sample. Annelid worms (*Tharyx*,  
247 *Tubificoides* and *Streblospio*) dominated total specimen counts and were present in all  
248 samples (Table S2).

### 249 *Comparing morphological and metabarcoding datasets across samples*

250 All seven phyla detected in the morphological analysis were found in the metabarcoding  
251 analysis (Table 2). Overall, a total of 24 marine species were identified across metabarcoding  
252 samples and 25 species were identified by the morphological approach (Table 2, although  
253 some more taxa were identified at higher taxonomic level). However, only 11 species were  
254 identified by both methods (Table 3). Several taxa that were identified by the morphological  
255 approach and not identified by the metabarcoding approach were found to have  
256 representative sequences in the MIDORI-UNIQUE reference dataset used in this study (Table  
257 3). However, *Exogone naidina*, *Sphaerosyllis tetralix*, *Eusarsiella zostericola*, *Abra tenuis*,  
258 *Tharyx robustus* and *Tharyx killariensis* had no representative sequences in the MIDORI-

259 UNIQUE reference dataset. The BLASTn search using the DADA2 output file identified  
260 eight species that had been detected by the morphological approach, but not by the  
261 metabarcoding approach described above. Both morphology and DNA identified Annelida as  
262 the most common phylum (83% of specimens identified by the morphological approach and  
263 65% of total metazoan reads in metabarcoding; Figure 5.). Morphological identified  
264 specimens from 15 families whilst the metabarcoding insect/RDP classifier and BLASTn  
265 taxonomy assignment recovered 10 and 9 families, respectively.

## 266 **Discussion**

267 Sieving is a method commonly utilized in metabarcoding studies surveying marine  
268 macrobenthic invertebrates to partition bulk samples, and smaller size fractions have often  
269 been found to be the most diverse (Pearman et al., 2018; Wangenstein et al., 2018; Ransome  
270 et al., 2017). We find that sieving samples in the metabarcoding analysis results in a  
271 reduction in the number of species identified (Table 2) as well as a reduction in amplicon  
272 sequence variant (ASV) richness and alpha diversity estimates (Figure 2), indicating that a  
273 large portion of reads originate either from whole organisms that are smaller than 0.5 mm or  
274 from environmental DNA. We find that whilst ASV richness and diversity estimates are  
275 higher in un-sieved samples, sieved samples had the highest number of reads (62,995 reads in  
276 sieved samples versus 44,221 reads in un-sieved samples). This is to be expected as the  
277 removal of sediment and fine inorganic matter would have concentrated the amount of  
278 biological tissue used for DNA extraction.

279 Our study shows that whilst all samples harbour low meiofauna diversity in general, sieving  
280 prior to DNA extraction also had a significant effect on the community composition  
281 recovered in metabarcoding samples. This is also to be expected as benthic meiofauna, which  
282 range between 40µm up to 500µm and form an important part of intertidal diversity (Coull &  
283 Chandler, 2001), would have been washed out in sieved samples.

284 Arthropods form a large and important component of marine zooplankton and benthos, often  
285 acting as key intermediates in food webs (Pearman & Irigoien, 2015). In the identification of  
286 marine arthropods, the morphological analysis only identified the presence of *Eusarsiella*

287 *zostericola*, a non-native myodocopid ostracod in samples 3, 7, 8 and 10. In comparison,  
288 metabarcoding recovered the presence of myodocopid ostracods, as well as calanoid  
289 copepods of the *Acartia* genus, across all samples. Furthermore, there were considerably  
290 more arthropod sequence reads across un-sieved samples than in sieved samples (1,578 and  
291 483 reads, respectively), including reads identified as the copepod species *Delavalia palustris*  
292 in the unsieved sample 4. These organisms can range in size smaller or larger than 0.5mm,  
293 meaning some will have been washed away in sieving steps in both the morphological  
294 approach and in some metabarcoding samples.

295 The ability of metabarcoding to detect minute organisms is advantageous as it allows us to  
296 better understand the true diversity of intertidal marine benthos, unlike standard morphology-  
297 based approach, which is limited to surveying organisms larger than 500  $\mu\text{m}$ . The presence  
298 and diversity of meiofauna communities have been shown to reflect patterns of  
299 environmental degradation and levels of pollution (Morad et al., 2017). Recovering the  
300 presence of both meio- and macro-fauna is therefore important when assessing the health of  
301 degraded areas such as the Harwich International Port. However, whilst metabarcoding  
302 allows for a more holistic community-based approach, we recommend that careful  
303 consideration be taken when deciding to implement sieving in metabarcoding protocols.

304 We find that overall the metabarcoding analysis recovered almost double the number of  
305 animal phyla than the morphological method (13 metazoan phyla in the metabarcoding  
306 analysis vs 7 metazoan phyla in the morphological approach). Metabarcoding was able to  
307 recover the presence of several marine species in phyla not targeted by the morphological  
308 approach, including the hydrozoan *Clava multicornis* and the kinorhynch *Pycnophyes*  
309 *kielensis*. Whilst metabarcoding recovered the presence of more taxonomic groups than the  
310 morphological approach, it appears the overall diversity of the Harwich International Port  
311 estuary is very low and has been effectively sampled as rarefaction curves level off in all  
312 samples.

313 Annelids form a major part of estuarine benthic ecosystems and are often the most abundant  
314 phylum of macroinvertebrates detected by COI metabarcoding studies (Haenel et al., 2017;  
315 Aylagas et al., 2016a). Furthermore, annelids have been shown to dominate estuarine mud-  
316 flat environments and are often used as indicator taxa for characterizing intertidal estuarine  
317 environments (Conde et al., 2013). Both methods detected the dominance of annelid worms

318 across all samples (Figure 5). The species found to have the highest number of reads across  
319 all metabarcoding samples is the carnivorous polychaete *Nephtys hombergii*. In fact, the two  
320 sequence variants with the highest abundance of reads across samples were both identified as  
321 *Nephtys hombergii*, indicating the presence of potential intraspecific genetic diversity of the  
322 gene region targeted by the *mlCOI\_intF/jghCO2198* primer pair. Both morphological and  
323 metabarcoding approaches recover the presence of this species in samples 1 to 8, and not in  
324 samples 9 and 10. Similarly, both methods detected the presence of polychaete worms of the  
325 family Cirratulidae in the third transect (samples 9 and 10). Whilst both methods were able to  
326 recover matching ecological distributions of these two taxa, not all species identified in both  
327 analyses were detected in the same samples. For example, the metabarcoding analysis  
328 recovered the presence of the common polychaete *Hediste diversicolor* and saltwater clams  
329 *Macoma balthica* and *Nucula nitidosa* in several samples, more than the morphological  
330 approach. In contrast, the morphological analysis recovered polychaete worms of *Capitella*  
331 and *Streblospio* genera across more samples than the metabarcoding approach.

332 With metabarcoding, we recovered the presence of important UK indicator species such as  
333 *Hediste diversicolor* and *Scrobicularia plana*. However, as abundance is measured here by  
334 the number of sequence variants in metabarcoding, it is not possible to know whether a  
335 species is found in high abundance due to a large number of individual organisms detected or  
336 as a result of DNA extracted from a large number of cells. This highlights a current pitfall of  
337 metabarcoding methods, which cannot provide yet accurate estimates of abundance, which is  
338 needed in some common benthic indices (Borja, 2019; Conde et al., 2013).

339 Recent studies have suggested that analysing high-throughput amplicon sequencing data  
340 using amplicon sequence variants (ASVs), which involves resolving the sequenced region  
341 down to the level of individual nucleotides by estimating and applying modelled error rates,  
342 provides a more accurate representation of diversity than using traditional sequencing OTU  
343 clusters (Glassman & Martiny, 2018; Callahan et al., 2017). Whilst our study finds that using  
344 metabarcoding sequences results in a species count comparable with the morphological  
345 method (Table 2), not all the species found in the morphological approach are identified by  
346 the metabarcoding approach and vice versa (Table 3). Of the 36 taxonomic groups (species,  
347 genera or family group) identified in the morphological analysis, only 11 of these were  
348 detected by our metabarcoding analysis using the RDP classifier and the MIDORI-UNIQUE  
349 reference dataset. Sixteen of the taxa identified in the morphological analysis and not in the

350 metabarcoding analysis had representative sequences in the MIDORI-UNIQUE reference  
351 dataset. Our alternative BLASTn search, using the ASV sequences from the DADA2 step and  
352 the online nucleotide collection, was able to recover the presence of half of these missing  
353 taxa. Several species and genera, which are detected in the morphological analysis and have  
354 representative sequences in both the MIDORI-UNIQUE and online nucleotide database,  
355 remained un-identified by both the *insect* and BLASTn taxonomy assignment methods (Table  
356 3). It is possible that these taxonomic groups were mis-identified in the morphological  
357 approach. It is also possible that were not identified as a result of the primer pair used in this  
358 study's metabarcoding approach. Only a limited set of representative annelid specimens were  
359 used to create the primer set used in this study and these originated from specimens collected  
360 for the Moorea Biocode project, an initiative which is based in French Polynesia and focuses  
361 on assembling specimens from tropical ecosystems (Leray et al., 2013). Previous  
362 metabarcoding research has described the difficulty of deriving species level taxonomic  
363 assignment for marine benthic fauna due to the paucity of reference barcode sequences in  
364 public databases along with the presence of mis-identified and erroneous sequences (Leray et  
365 al., 2015). In this study we show that whilst representative sequences are available for the  
366 majority of fauna found in our samples, potential primer bias likely played a part in the  
367 failure to recover the same set of species as the morphological approach. The use of multiple  
368 "barcode" genes, and the use of a more degenerate set of primers (for example the recently  
369 developed Leray-XT primer pair), are ways of reducing marker bias and allowing for  
370 improved representation of the species composition within an ecological sample  
371 (Wangensteen et al., 2018; Alberdi et al., 2017; Drummond et al., 2015).

372 With the advent of cheaper and faster HTS methods, metabarcoding has become  
373 economically viable and therefore attractive for businesses and governments to use as part of  
374 their ecological assessment protocols. Metabarcoding has already been used to detect shifts in  
375 macroinvertebrate composition around oil-drilling platforms and in response to land use  
376 change (Laroche et al., 2017; Beng et al., 2016; Lanzén et al., 2016). There is a now a  
377 growing consensus that the future of marine benthic biomonitoring lies with HTS methods,  
378 such as metabarcoding and the targeting of environmental DNA (Carvalho et al., 2019;  
379 Aylagas et al., 2018; Baird & Hajibabaei, 2012). Our study presents a comparison of a  
380 metabarcoding approach to a morphological protocol regularly used by a leading  
381 environmental consultancy firm. We demonstrate that metabarcoding allows for a more  
382 holistic, cross-community, approach that recovers the presence of meio- and macro-faunal

383 taxa across many more phyla groups than a morphological approach. Our findings show that  
384 the use of different taxonomy-assignment methods and reference databases can lead to  
385 inconsistent species-level identification in the metabarcoding analysis. Whilst bioinformatic  
386 pipelines and analysis tools for HTS are constantly evolving and improving, there is still a  
387 need for exploratory studies of understudied taxa such as marine benthic meio- and macro-  
388 fauna. A way to tackle the current paucity of reference databases would be to encourage  
389 environmental consultancy firms and the research community to archive and barcode  
390 specimens collected during traditional morphometric surveys, so that localised curated  
391 reference datasets may be built over time to facilitate future metabarcoding efforts.

392 **Data Accessibility**

393 Raw FASTQ files are available online

394 ([https://datadryad.org/stash/share/XfL\\_GJyDgvFKW113V8ihvWHXQXkxLyLCLJWh1-](https://datadryad.org/stash/share/XfL_GJyDgvFKW113V8ihvWHXQXkxLyLCLJWh1-b2sQQ)  
395 [b2sQQ](https://datadryad.org/stash/share/XfL_GJyDgvFKW113V8ihvWHXQXkxLyLCLJWh1-b2sQQ)).

396 **Authors' contributions**

397 MS, VP and VS designed the project. VS and RA supervised the research. MS collected and  
398 analysed data. AH helped with the laboratory work. OGO helped with bioinformatics  
399 pipelines. MS wrote the initial manuscript with subsequent contributions from all authors.

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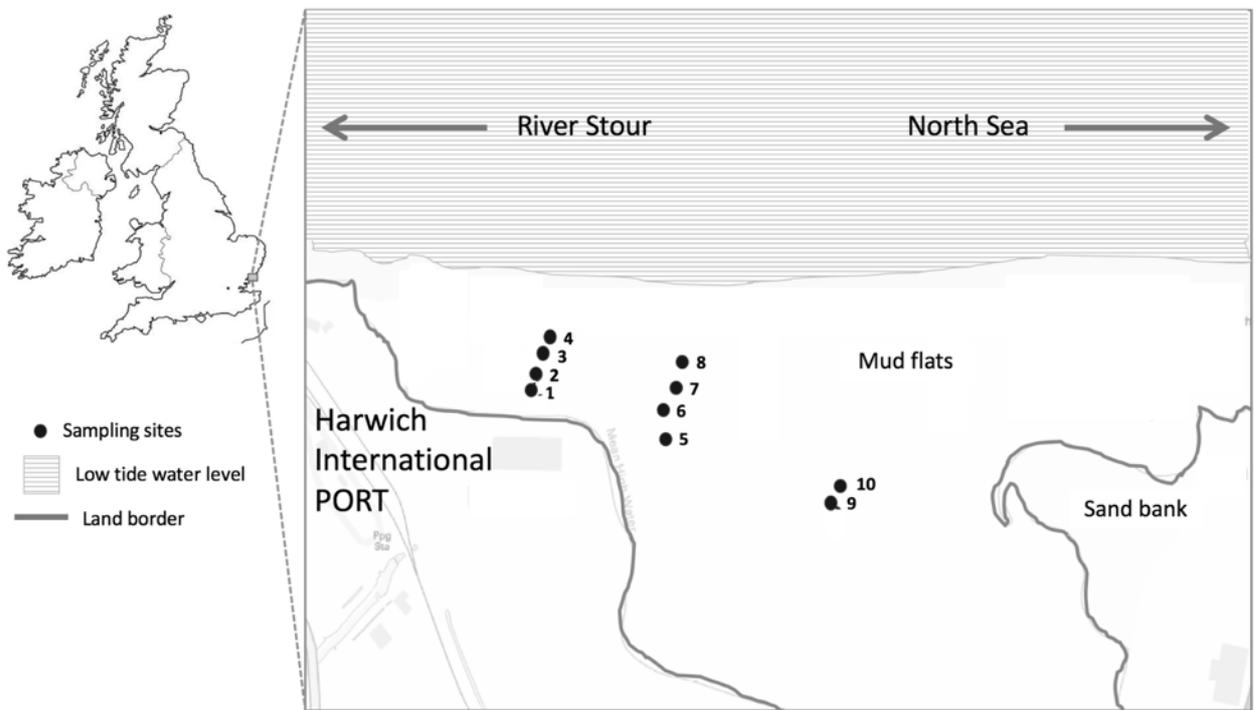
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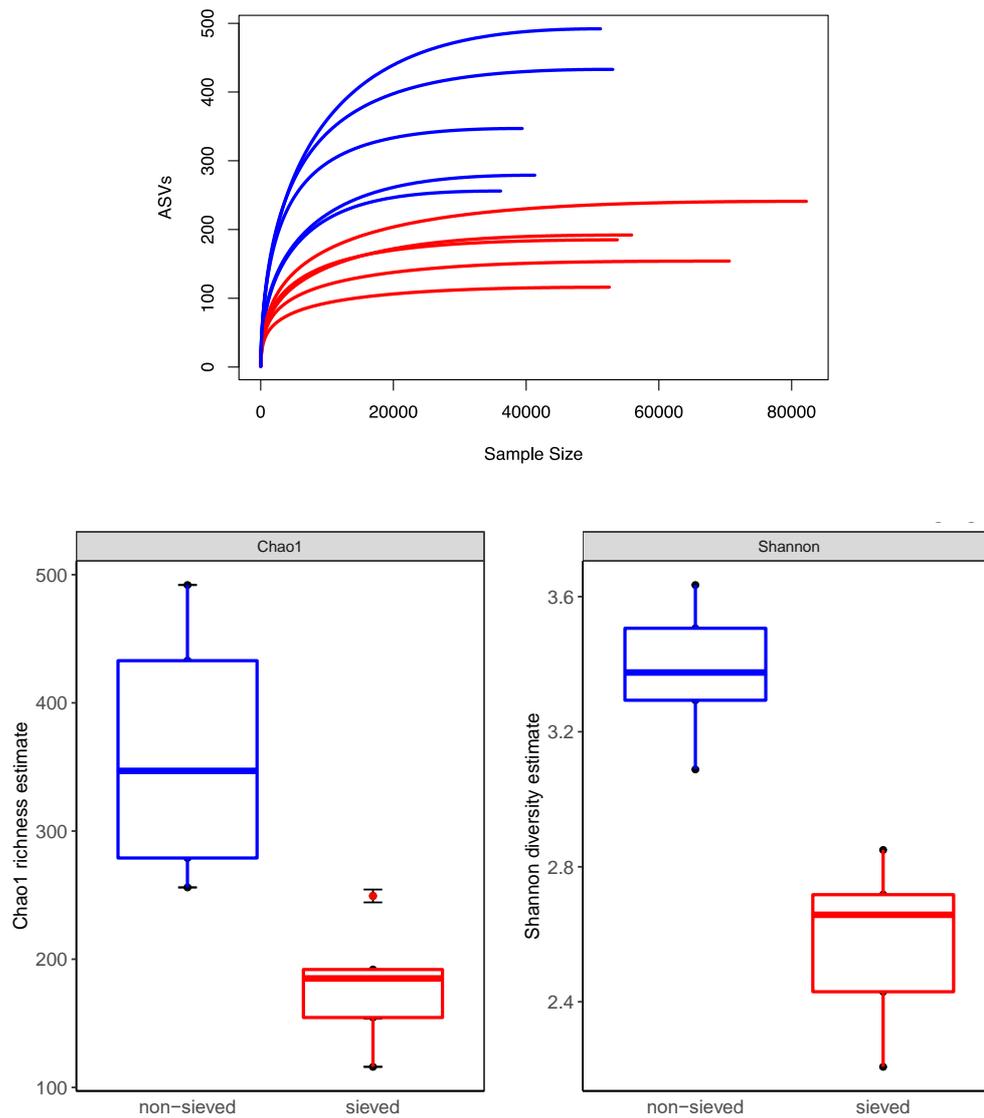
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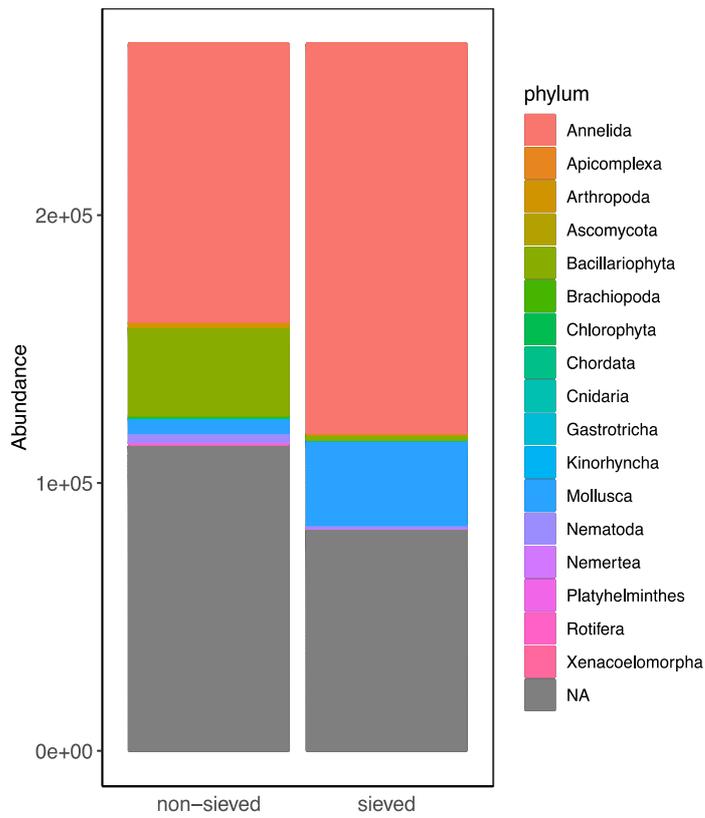
641 **Figure 1.** Map showing the location of extracted benthic cores at the Harwich International  
642 Port estuary, Essex, U.K. GPS coordinates for all ten sample sites can be found in Table S1.

643 **Table 1.** Summary of the number of raw reads, number of reads post filtering, merging and  
644 chimera removal steps in the DADA2 pipeline and the number of ASVs prior to and post LULU  
645 curation across sieved and un-sieved samples.

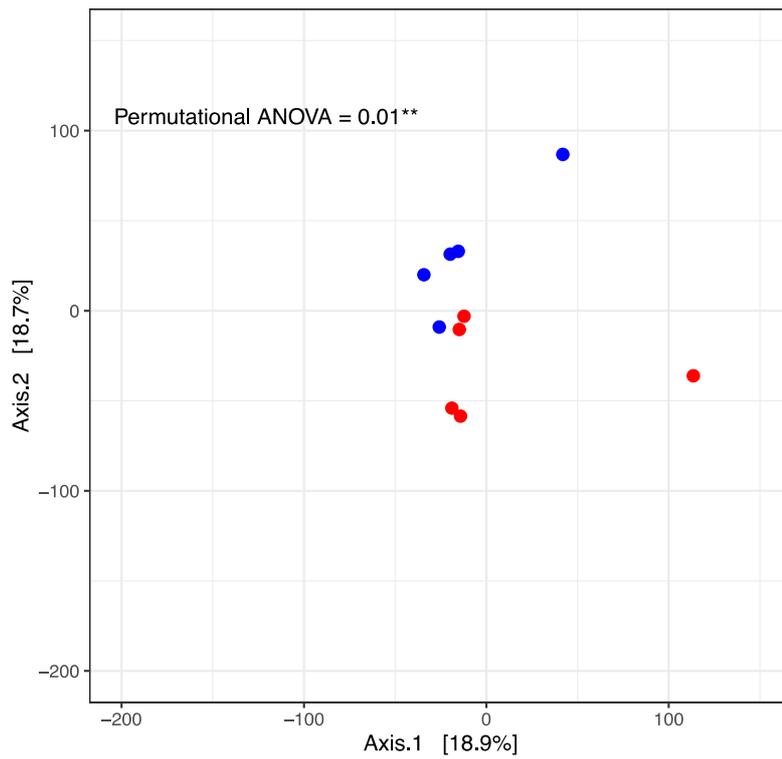
Sample	Processing step	Raw reads	Number of reads post DADA2 filtering step	Number of reads post DADA2 merging step	Number of reads post DADA2 chimera removal step	Number of ASVs prior to LULU curation step	Number of ASVs post LULU curation step
1	Sieved	1,053,425	82,449	80,381	77,456	208	154
2	Not sieved	1,135,799	58,899	57,842	57,247	349	279
3	Sieved	1,034,144	70,674	69,324	67,298	143	116
4	Not sieved	1,131,688	48,468	47,114	46,476	432	347
5	Sieved	1,1129,646	61,482	59,960	58,199	231	185
6	Not sieved	870,689	47,965	46,003	43,464	326	257
7	Sieved	1,175,309	94,870	93,408	91,223	313	241
8	Not sieved	1,258,270	68,755	66,637	65,155	547	433
9	Sieved	1,119,207	60,351	59,293	58,359	225	192
10	Not sieved	1,165,277	56,958	56,958	56,293	620	492



662 **Figure 2.** a) Rarefaction curves of ASV diversity in sieved (red) and non-sieved (blue)  
 663 samples and b) boxplot of Chao1 and Shannon estimates of ASV richness and diversity in  
 664 sieved (red) and non-sieved (blue) samples.



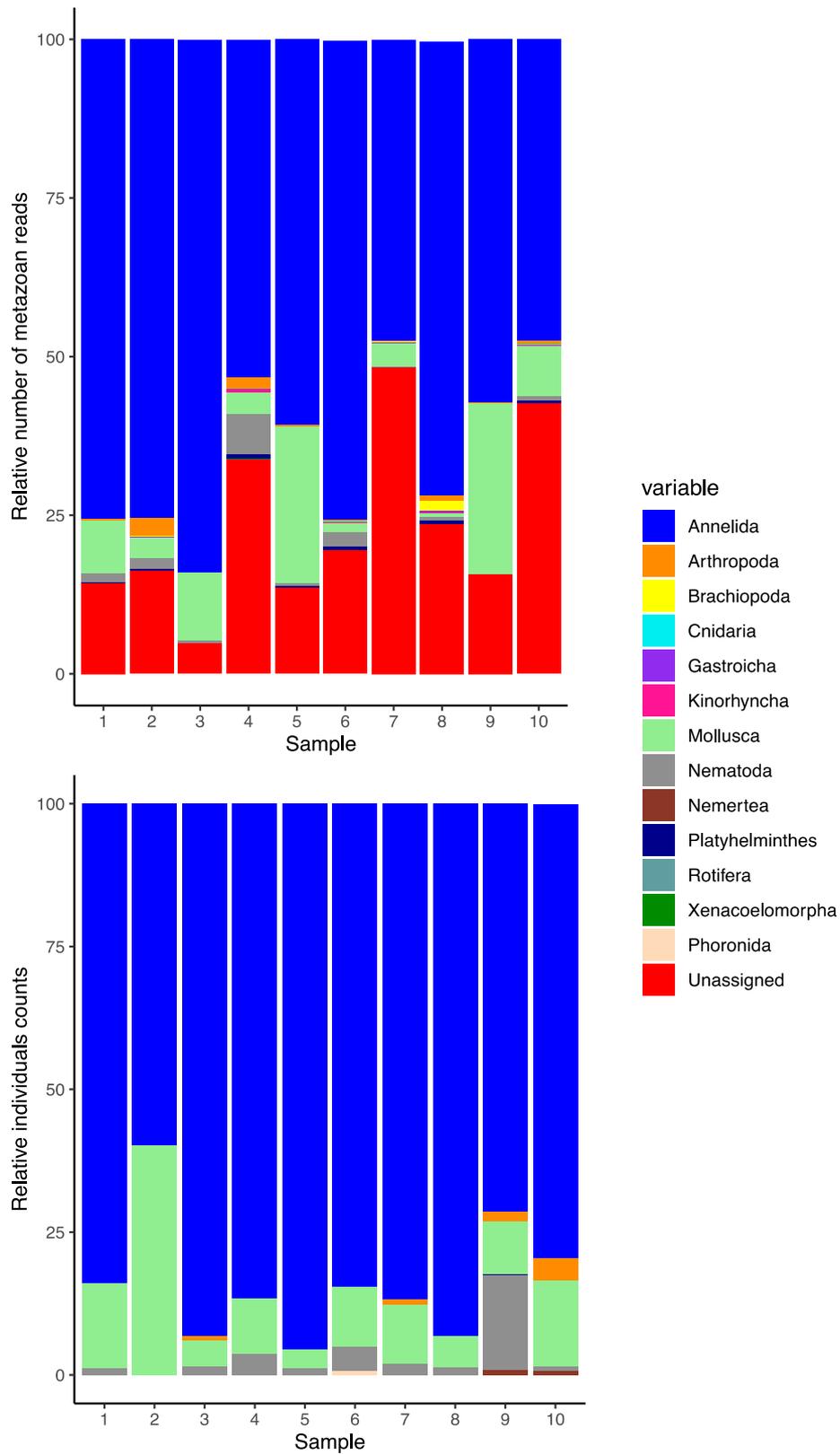
665 **Figure 3.** Barplot comparing relative abundances of normalised numbers of reads per phyla  
 666 across non-sieved and sieved samples.



667 **Figure 4.** Principle Coordinates Analysis (PCoA) plot of ASV abundances across sieved (red)  
668 and unsieved (blue) samples, using Euclidean distances. The result of a permutational ANOVA  
669 test comparing the communities of sieved and unsieved samples is displayed within the plot.

670 **Table 2.** Summary table of the number of marine species, per phylum, identified across all  
671 metabarcoding and morphological samples, as well as in un-sieved and sieved metabarcoding  
672 samples. Cells with dashes indicate the phyla in question were not targeted by the  
673 morphological approach. Cells with species count as 0 indicate that whilst no species was  
674 identified, the presence of this phylum was detected via the identification of an ASV to a higher  
675 taxonomic rank (e.g. class, order or family level).

Phylum	Number of species detected across all metabarcoding samples	Number of species identified across all morphological samples	Number of species in sieved metabarcoding samples	Number of species in un-sieved metabarcoding samples
Annelida	11	16	9	11
Apicomplexa	0	-	0	0
Arthropoda	1	1	0	1
Bacillariophyta	0	-	0	0
Bryozoa	0	-	0	0
Chlorophyta	1	-	1	1
Chordata	1	-	0	1
Cnidaria	1	-	1	1
Echinodermata	0	-	0	0
Gastrotricha	0	-	0	0
Kinorhyncha	1	-	0	1
Mollusca	6	8	5	5
Nematoda	1	0	1	1
Nemertea	0	0	0	0
Platyhelminthes	1	0	1	0
Rotifera	0	-	0	0
Xenacoelomorpha	0	-	0	0
Total number of sp.	24	25	18	22



676 **Figure 5.** Abundance barplots displaying the composition of organisms identified at phylum  
 677 level in both the morphological and metabarcoding analyses. Colour legend applies to both  
 678 graphs.

679 **Table 3.** Summary table of the taxa identified in the morphological analysis and if those taxa  
680 were i) identified by the metabarcoding approach, ii) if not, what the closest taxonomic level  
681 to that taxa is, iii) whether the taxa is represented in the reference dataset used in this study and  
682 finally iv) whether the taxa in question is detected using BLASTn, a different taxonomy  
683 assignment tool.

Taxa detected by morphological approach	Is this taxon detected by the metabarcoding approach using the RDP classifier dataset?	If not detected, what is closest taxonomic level to this taxon that is detected?	Is this taxon represented in the MIDORI-UNIQUE reference dataset?	Is this taxon detected using BLASTn and the NCBI nucleotide collection reference database?
<i>Pholoe</i>	No	Phyllodocida order	Yes	No
<i>Phyllodoce mucosa</i>	No	<i>Phyllodoce</i> genus	Yes	Yes
<i>Eteone (Type 1)</i>	No	Phyllodocidae family	Yes	Yes
<i>Glycera tridactyla</i>	No	Phyllodocidae family	Yes	No
<i>Exogone naidina</i>	No	Phyllodocidae family	No (only genus present)	No
<i>Sphaerosyllis tetralix</i>	No	Phyllodocida order	No (only genus present)	No
<i>Hediste diversicolor</i>	Yes	-	Yes	Yes
<i>Nephtys hombergii</i>	Yes	-	Yes	Yes
<i>Pygospio elegans</i>	No	Spionidae family	Yes	Yes
<i>Streblospio</i>	No	Spionidae family	Yes	Yes
<i>Cirratulidae</i>	Yes	-	Yes	Yes
<i>Aphelochaeta</i>	No	Cirratulidae family	Yes	No
<i>Cirriformia tentaculata</i>	No	Cirratulidae family	Yes	Yes
<i>Tharyx</i>	No	Terebellida family	No (only family present)	No
<i>Tharyx robustus</i>	No	Terebellida family	No (only family present)	No
<i>Tharyx killariensis</i>	No	Terebellida family	No (only family present)	No
<i>Cossura pygodactylata</i>	No	No close taxonomic level detected	No (only genus present)	No
<i>Capitella</i>	Yes	-	Yes	No
<i>Galathowenia</i>	No	No close taxonomic level detected	Yes	No
<i>Melinna palmata</i>	No	Ampharetidae family	n (only genus present)	No
<i>Manayunkia</i>	No	Spionidae family	Yes	No
<i>Tubificoides amplivasatus</i>	No	<i>Tubificoides</i> genus	Yes	Yes
<i>Tubificoides benedii</i>	Yes	-	Yes	Yes
<i>Tubificoides pseudogaster (agg.)</i>	Yes	-	Yes	Yes
<i>Eusarsiella zostericola</i>	No	Myodocopida order	No (only genus present)	No
<i>Peringia ulvae</i>	Yes	-	Yes	Yes
<i>Limapontia depressa</i>	No	Limapontiidae family	Yes	Yes

<i>Nuculidae</i>	Yes	-	Yes	Yes
<i>Nucula nitidosa</i>	No	<i>Nuxcula</i> genus	Yes	Yes
<i>Mytilidae</i>	No	Bivalvia class	Yes	No
<i>Cardiidae</i>	Yes	-	Yes	Yes
<i>Cerastoderma edule</i>	Yes	-	Yes	Yes
<i>Limecola balthica</i>	No	<i>Limecola</i> genus	Yes	Yes
<i>Abra tenuis</i>	No	Cardiida order	No (only genus present)	No
<i>Scrobicularia plana</i>	Yes	-	Yes	Yes
<i>Phoronis</i>	No	Phoroniformea sub-phylum	Yes	No