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1 **Diversity of Wadden Sea macrofauna and meiofauna communities highest in DNA from extractions preceded by**

2 **cell lysis.**

3

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16

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20

21 **ABSTRACT**

22 Metabarcoding of genetic material in environmental samples has increasingly been employed as a means to assess

23 biodiversity, also of marine benthic communities. Current protocols employed to extract DNA from benthic samples

24 and subsequent bioinformatics pipelines differ considerably. The present study compares three commonly

25 deployed metabarcoding approaches against a morphological approach to assess benthic biodiversity in an

26 intertidal bay in the Dutch Wadden Sea. Environmental DNA was extracted using three different approaches;

27 extraction of extracellular DNA, extraction preceded by cell lysis of a sieved fraction of the sediment, and extraction

of DNA directly from small amounts of sediment. DNA extractions after lysis of sieved sediment fractions best recovered the macrofauna diversity whereas direct DNA extraction of small amounts of sediment best recovered the meiofauna diversity. Extractions of extracellular DNA yielded the lowest number of OTUs per sample and hence an incomplete view of benthic biodiversity. An assessment of different bioinformatic pipelines and parameters was conducted using a mock sample with a known species composition. The RDP classifier performed better than BLAST for taxonomic assignment of the samples in this study. Novel metabarcodes obtained from local specimens were added to the SILVA 18S rRNA database to improve taxonomic assignment. This study provides recommendations for a general metabarcoding protocol for marine benthic surveys in the Wadden Sea.

1. Introduction

Benthic organisms play a crucial role in marine nutrient cycling and in primary and secondary productivity in the ocean and shelf seas (Austen et al., 2002; Covich et al., 2004; Levin et al., 2001; Snelgrove, 1997; Thrush et al., 2006). Anthropogenic stresses on the seafloor such as trawling, oil, gas and sand extraction but also warming and ocean acidification (Anadón et al., 2007; Halpern et al., 2008) are inducing changes in benthic ecosystems. Subsequent disruption of key ecosystem services and community stability from an accelerated loss of biodiversity is currently a major concern (Daily et al., 2000; Danovaro et al., 2008; Hooper et al., 2012; Solan, 2004). The ever expanding economic exploitation necessitates implementation of policies to ensure habitat protection. This, in turn, requires regular monitoring of marine benthic ecosystems. Benthic biodiversity is a widely used indicator of ecosystem health (Snelgrove, 1997). Ideally the monitoring approach should assess biodiversity at the relevant temporal and spatial scales in a consistent and reliable way.

Current estimations of biodiversity are subject to high levels of uncertainty, especially in marine ecosystems (Costello, 2015; Hajibabaei et al., 2011; Hortal et al., 2015; May, 1988), suggesting that current methods are insufficient. Assessing the composition of the marine benthos by traditional methods such as morphological identification of individual specimens is time-consuming, labour-intensive as well as costly, and requires a taxonomic knowledge that is increasingly scarce, particularly for invertebrates (Bucklin et al., 2011; Cardoso et al., 2011; Cowart et al., 2015). Morphological identification is typically limited to large specimens and consequently the meiofauna and immature individuals usually remain unidentified (Balsamo et al., 2012; Boyd et

55 al., 2000; Chariton et al., 2015; Compton et al., 2013; Danovaro et al., 2000; Spilmont, 2013; Zeppilli et al., 2015).
56 During recent years, DNA sequencing has emerged as an alternative and efficient method for species identification,
57 most recently in the form of next-generation sequencing (NGS) and metabarcoding (Taberlet et al., 2012a). In
58 principle, metabarcoding facilitates the assessment of biodiversity in a consistent and replicable manner across
59 different ecosystems (Baird and Hajibabaei, 2012). This potentially allows comparisons of in situ biodiversity studies
60 (Bik et al., 2012; Cowart et al., 2015; Ji et al., 2013).
61 Several studies have successfully implemented metabarcoding approaches to assess marine benthic
62 biodiversity (Brannock et al., 2014; Chariton et al., 2010, 2015; Fonseca et al., 2010; Guardiola et al., 2015).
63 However, several aspects of metabarcode-based assessments of benthic diversity potentially bias the outcome and
64 this has been tested insufficiently. One aspect is the DNA extraction approach and the corresponding fractioning of
65 sediment samples. Common DNA extraction methods for marine sediment samples can be divided in three
66 categories; direct DNA extraction from small amounts of sediments, DNA extraction from fractioned sediments,
67 and extraction of extracellular DNA. Direct DNA extraction from small amounts of non-fractioned sediments has
68 been applied for the identification of both macrofauna and meiofauna communities (e.g., Chariton et al., 2015;
69 Sinniger et al., 2016). This method retrieves both the extracellular DNA present in the sample as well as the
70 intracellular DNA through a lysis step. The DNA extraction of a particular size-fraction, obtained after a sieve or
71 elutriation step is restricted to intracellular DNA from faunal species (and their incidentally gut contents) within this
72 size fraction (e.g., Fonseca et al., 2014; Leray and Knowlton, 2016). Recently, DNA extraction of only extracellular
73 DNA in sediment samples, has been applied as an alternative approach (Bienert et al., 2012; Guardiola et al., 2015;
74 Taberlet et al., 2012b). Extracellular DNA adsorbed on minerals has been shown to be protected against
75 degradation and is therefore expected to reflect longer-term biodiversity (Dell'Anno and Corinaldesi, 2004).
76 Therefore this approach, in principle, is less susceptible to short-term temporal heterogeneity (Alawi et al., 2014;
77 Taberlet et al., 2012a). The extraction of extracellular DNA has been applied for the identification of both
78 macrofauna and meiofauna communities (Guardiola et al., 2015; Pearman et al., 2015). To date, no comparisons
79 have been undertaken with regards to which of the three DNA extraction methods is best suited for the assessment
80 of marine benthic biodiversity.

81 Another aspect that potentially biases the outcome of metabarcode based benthic biodiversity
82 assessments is the taxonomic assignment of operational taxonomic units (OTUs) among the sequenced
83 metabarcodes. Species identification, not just OTU diversity, relies heavily upon the completeness of reference
84 DNA databases. Many studies solely rely on the DNA sequences that are available in public databases such as
85 GenBank™ and SILVA. The DNA sequences, and hence the OTU composition, detected during a study are often
86 interpreted without in-depth knowledge of the species diversity at the study site or the reference databases.
87 However, the current databases are incomplete and strongly biased towards model organisms. Consequently, the
88 identification is mainly confined to specimens belonging to well-known taxa (Pompanon and Samadi, 2015).
89 Although some studies have compared the efficiency of different metabarcoding methods (Brannock and Halanych,
90 2015; Lekang et al., 2015), and some studies investigated the effectiveness of metabarcoding with artificial
91 compiled samples (Dell'Anno et al., 2015; Leray and Knowlton, 2015), only a few studies verified different
92 molecular approaches against morphological identification for marine benthic samples; usually due to a lack of the
93 necessary taxonomic knowledge and time constraints (Creer et al., 2016). Positive controls, mock samples with
94 known species composition, are typically absent in benthic biodiversity assessments, even though mock
95 communities represent an excellent approach to validate the specific experimental and bioinformatics pipeline of a
96 study (Creer et al., 2016).
97 This study compared three methods that are commonly employed in metabarcoding studies against a
98 morphological approach to assess marine benthic macrofauna and meiofauna diversity in an intertidal area in the
99 western Dutch Wadden Sea. Public reference databases were complemented with sequences obtained from
100 morphologically identified local benthic species, representing all abundant macrofauna species known for the
101 sampling area. The focus of this study was on the effectiveness of different DNA extraction methods; as employed
102 in current biodiversity studies, to capture the benthic macrofauna and/or meiofauna diversity. Also, a comparison
103 was conducted between two commonly used methods to assign OTUs to their nearest taxon (i.e., BLAST and the
104 RDP classifier) in public and local reference databases. A mock sample of marine benthic biodiversity was analysed
105 to assess the quality of taxonomic assignment (Leray and Knowlton, 2016).
106
107 **2. Material and methods**

108

109 **2.1. Reference library**

110 *2.1.1. Sampling*

111 Benthic macrofauna species were sampled from the western part of the Dutch Wadden Sea during the period
112 between February 2014 and March 2016 following Beukema and Cadée (1997). Specimens were identified by an
113 experienced taxonomist according to Hartmann-Schröder (1996) and Hayward and Ryland (1995) based on
114 morphological characteristics. Molluscs, crustaceans and polychaetes were identified to the species level, whereas
115 oligochaetes and Nemertea were identified to the phylum level. After identification, up to three specimens per
116 species were stored individually in separate tubes in 96% ethanol at room temperature.

117

118 *2.1.2. Molecular analyses*

119 Genomic DNA was extracted using the GenElute™ Mammalian Genomic DNA miniprep kit (Sigma-Aldrich Inc.)
120 following the manufacturer's protocol, except for the length of the initial cell lysis step which was increased to 18
121 hours to enhance DNA yield. A 650 base pair (bp) part of the 18S rRNA gene was amplified using the
122 oligonucleotides F-566 and R-1200 as primer pair (Hadziavdic et al., 2014). All polymerase chain reactions (PCR)
123 were performed in a 50µl reaction volume, containing 0.5µM of each primer, 0.2µM dNTPs, 2U BioTherm™+ Taq
124 DNA Polymerase (BioTherm™ Inc.), 1x PCR buffer (BioTherm™ Inc.) and 2µl of DNA extract. PCR reactions were
125 subjected to five minutes at 95°C, followed by 35 cycles comprised of 45 seconds at 95°C, 60 seconds at 60°C and
126 60 seconds at 72°C, respectively, and one final extension step of seven minutes at 72°C. Subsample of the PCR
127 products (5µl) were checked by electrophoresis through a 2% agarose gel at 75volt for 50 minutes after ethidium
128 bromide staining. The size of the 18S rRNA PCR product matched the expected 650 bp for all species, except
129 arthropod species. In arthropods the 18S rRNA PCR products were ~1000 bp. The PCR products were Sanger
130 sequenced in both directions with the ABI3730XL sequencer from Life Technologies by BaseClear (Leiden,
131 Netherlands).

132

133 *2.1.3. Alignment of Sanger sequences*

134 Forward and reverse sequences obtained by the Sanger procedure were aligned using Geneious™ (version. R9,
135 Kearse et al., 2012). Alignments were obtained using the default Geneious alignment function with a gap open
136 penalty at 12 and gap extension penalty at 3. The cost matrix was set at a 65% similarity (5.0/4.0). The consensus
137 sequence was obtained with a highest quality threshold. All sequences were supplemented with their taxonomic
138 data and stored as a local reference database.
139
140 *2.1.4. Mock sample*
141 One mock test sample was composed by combining DNA extractions from ten local species, representing three
142 different phyla (Table 1). The DNA extracts of the selected species were quantified on a Qubit 3.0 fluorimeter
143 (Qiagen, Inc.) and were pooled in equimolar quantities. The mock sample served as a positive control throughout
144 the 18S species identification process.
145
146 **2.2. Field experiment**
147 *2.2.1. Sampling*
148 Samples were collected during low tide at nine locations separated by less than 50 m in Mokbaai, an intertidal bay
149 at the southern tip of the isle of Texel in the western part of the Dutch Wadden Sea (53°20′00′N; 4°46′50″E). Three
150 sediment cores were collected at each location: two cores (termed A and B below) had a 177 cm2 surface area and
151 a 25 cm sampling depth (equivalent to roughly 4.5 liters of sediment). The third sample (termed sample C below)
152 was collected using a smaller core at 5.60 cm2 surface area and a 10 cm sampling depth (equivalent to 50ml of
153 sediment). Samples A were stored in clean plastic buckets at 5°C. Samples B were sieved through a 1mm round
154 mesh in the field and stored at 5°C in plastic bags. Samples C were stored at -80°C in clean plastic pots.
155
156 *2.2.2. Molecular analyses*
157 Three different DNA extraction methods were employed.
158 1) The extracellular DNA extraction method was adapted from Taberlet, et al., (2012b). The entire sampled
159 sediment from sample A (4,5 L sediment; n=9) was dissolved in 5 L saturated phosphate buffer (Na2HPO4,
160 0.12M, pH ≈8) and mixed for 15 minutes. Two 50 mL aliquots of dissolved sediment were collected and

161 centrifuged at 10 000 g for ten minutes. A volume of 400µL of supernatant was recovered and the DNA
162 extracted using the Powersoil™ DNA isolation kit (MoBio Inc.) following the manufacturer's instructions
163 leaving out the initial lysis step.
164 2) For the sieved lysis method, the sieved fraction from sample B (4,5 L sediment; n=9) was cryodesiccated
165 and ground in liquid nitrogen. Subsequently, DNA was extracted from the ground residue using the
166 Powermax Soil™ DNA isolation kit (MoBio Inc.) following the manufacturer's instructions.
167 3) The direct DNA extraction method where all samples C (50 ml sediment; n=9) were cryodesiccated and
168 ground in liquid nitrogen. DNA was extracted from 10 g of ground sediment following the procedure
169 described in method 2.
170
171 The extracted DNA was quantified using a Qubit™ 3.0 fluorimeter (Qiagen, Inc.). The six samples with the highest
172 DNA yields were selected among the nine extracts from each DNA extraction method. The DNA extracts from these
173 18 samples as well as the compiled mock sample, were used as template to amplify the 650 bp fragment of the 18S
174 rRNA gene using the oligo-nucleotides F-566 and R-1200 as PCR primers. The F-566 oligo-nucleotide was extended
175 at the 5'-end with a ten nucleotide multiplex identifier (MID) designed by 454 Roche Life Sciences (Corp.). Each
176 sample was labelled with a unique MID. All PCRs were performed in triplet in a 25µL volume reaction, containing
177 0.5µM of each primer, 0.2µM dNTP, 800ng/µL BSA, 1U Phusion® High-Fidelity DNA Polymerase (Thermo Scientific
178 Inc.), 1X Phusion® HF buffer (Thermo Scientific Inc.) and 3 µL of DNA extract. The thermal cycle programme
179 included an initial cycle of 30 seconds at 98°C; followed by 29 cycles, comprised of 10 seconds at 98°C, 20 seconds
180 at 68°C and 30 seconds at 72°C, followed by a single cycle of seven minutes at 72°C. The PCR products were run
181 through a 2% agarose gel at 75volt for 50 minutes. The PCR products were visualized by ethidium bromide staining.
182 Three out of the 18 samples failed during PCR and were discarded. The remaining amplification products were
183 purified with the Qiaquick™ purification kit (Qiagen Inc.) and quantified with a Qubit™ 3.0 fluorimeter (Qiagen Inc.).
184 All samples were pooled in equimolar quantities together with a positive control, the mock sample, and a blank PCR
185 control. The pooled sample was then subjected to a final purification using a MinElute™ PCR Purification column
186 (Qiagen Inc.) as described by the manufacturer. Pyrosequencing was performed on the pooled sample in a single
187 lane using Roche 454 GS-FLX Titanium platform with the Lib-L kit, by Macrogen Inc.

188

189 *2.2.3. Bioinformatics*

190 Raw sequences were quality trimmed and filtered using the FASTX-Toolkit

191 (http://hannonlab.cshl.edu/fastx_toolkit/) using the fastq_quality_trimmer and fastq_quality_filter scripts. Reads

192 shorter than 250 bps were discarded and bases with Phred quality scores less than 30 were end-trimmed. Reads

193 with a quality score ≤ 30 at >50% of the positions were discarded. Quality filtered reads were de-multiplexed based

194 on the MID sequences in QIIME (Caporaso et al., 2010) using the split_libraries.py script. Reads were first

195 dereplicated at a 100% similarity and the unique sequences were clustered using a 95% similarity cut off in

196 VSEARCH (Rognes et al., 2016). Taxonomic assignments were performed against the SILVA 18S rRNA database

197 (release 119, www.arb-silva.de; Pruesse et al., 2007), our local reference database or both, using two different

198 assignment algorithms. Two alternative BLAST searches were performed, the first using the default blastn settings

199 in which the e-value is 10, word _size is 10, match/mismatch scores are set on respectively 1 and -2 and gap costs

200 are linear. A custom blastn search was performed using an extended word_size of 30, match/mismatch scores were

201 set at 1 and -4 and the penalty for opening a gap was set at 12 and for the extension at 2. The second taxonomic

202 assignment was performed using the RDP Classifier (Wang et al., 2007) using the assign_taxonomy.py script in

203 QIIME with a minimum confidence of 0.5. Also, a random set of 10 OTU's were manually blasted as an empirical

204 control. A neighbour-joining tree was built from the 18S rRNA V4-V5 barcodes of the species that were found in the

205 mock sample.

206

207 *2.2.4. Morphological analyses*

208 Samples A were sieved through a 1 mm mesh sieve. Sieved fractions were preserved in a 4% formaldehyde solution

209 and stained using Bengal rose. Species in the sieve residue were sorted by hand and identified while alive following

210 the procedure described above (section 2.1.1.).

211

212 **2.2.5. Data analysis**

213 OTUs assigned to either the Annelida and Mollusca phylum and the Arthropod class Malacostraca were categorized

214 collectively as macrofauna. Although larvae or juveniles within these groups technically could be meiofauna, the

215 adult stages were used as the reference point for this classification. OTUs from the phyla Gastrotricha,
216 Gnathostomulida, Nematoda, Platyhelminthes and Xenacoelomorpha as well as the Arthropod classes Hexanauplia
217 and Ostracoda were categorized as meiofauna. Presence/absence ratios for macrofauna were estimated at the
218 genus level from both the morphological (samples A) and the molecular data (samples A, B and C). Species diversity
219 at the intertidal Wadden Sea is extremely low and a 95% cut off in combination with identification of macrofauna
220 at the genus level has been found reliable by analysing sequence variance from our reference database. As
221 meiofauna species were not sampled for our reference data base, presence/absence ratios for meiofauna were not
222 estimated beyond the family level from the molecular data.
223
224 **3. Results**
225 **3.1. Validation taxonomic assignment – reference library – mock sample**
226 After sequence quality control, a total of 6,946 reads were assigned to the mock sample, resulting in 27 OTUs
227 clustering at a threshold of >95%. Initial taxonomic assignment using the custom BLASTn search and the RDP
228 classifier against the SILVA SSU rRNA reference database, respectively missed five and four out of the ten species
229 present in the mock sample. After complementing the database with new 18S rRNA DNA sequences of macrofauna
230 species that are common in the Wadden Sea, the RDP classifier recovered all mollusc and annelid mock species
231 (Table 1). Also the custom BLASTn search recovered these species. However, in addition five false positives were
232 detected: the polychaetes *Abarenicola affinis, Platynereis dumerilii* and *Notomastus tenuis* and the molluscs *Phaxas*
233 *pellucidus* and *Scissula similis*. The barcode sequences of these false positives were all similar but not identical to
234 the mock sample species (Figure 1). The taxonomic assignment at the genus level of the 10 randomly picked OTU's,
235 which were manually blasted, all corresponded to those assigned by the RDP classifier. However, one OTU was
236 different at the genus level compared to both BLASTn searches, a match was found at the family level.
237 The arthropod species that were included in the mock community, *Urothoe poseidonis* and *Corophium*
238 *arenarium*, were not recovered and only a few reads were assigned to *Bathyporeia sarsi*. In all cases, a few OTUs
239 were assigned to the Siphonostomatoida, a group of parasitic copepods that might have been present in the
240 macrofauna from which the mock sample was generated.
241

3.2. Comparison extraction methods

3.2.1. Taxonomic composition

The 454-based amplicon sequencing generated 142,118 raw reads of which 104,101 were retained after quality control. One sample out of the 15 was discarded since it yielded only 765 18S rRNA sequences; the according multiplex identifier (MID) was suspected to interfere during amplification (Berry et al., 2011). The number of recovered 18S rRNA sequences varied considerably among the remaining samples but did not differ significantly among methods (one-way Anova, $F_{2,11} = 1.47$, $p = 0.272$). The OTU diversity was highest with the direct DNA extraction method (section 2.3.3., method 3), with an average number of 82 (SD = 20) OTUs per sample (Table 2). The extracellular DNA extraction method (section 2.3.3., method 1) recovered 80 (SD = 27) OTUs per sample, whereas the DNA extraction method performed on sieved sediment (section 2.3.3., method 2) recovered on average 18 (SD = 7) OTUs. This was significantly lower than the number of OTUs recovered with the two other DNA extraction methods (one-way Anova, $F_{2,11} = 19.3$, $p < 0.001$). The sieved lysis DNA extraction method recovered the highest percentage of metazoan OTUs, 91% versus 31% and 48% in case of the extracellular and direct DNA extraction method, respectively (one-way Anova, $F_{2,11} = 32.83$, $p < 0.001$).

Most metazoan OTUs were assigned to annelids within the extracellular DNA extraction method (32%) and the sieved lysis DNA extraction method (72%), whereas with the direct DNA extraction method most OTUs (36%) were assigned to nematodes (Figure 2). The sieved lysis DNA extraction method recovered four metazoan phyla; Annelida, Arthropoda, Mollusca and Nematoda. The two DNA extraction methods starting with unsieved sediment samples both recovered four additional meiofaunal phyla; Gastrotricha, Gnathostomulida, Platyhelminthes and Xenacoelomorpha. The extracellular DNA extraction method also recovered the phylum Cnidaria.

3.2.2. Macrofauna

The three DNA extraction methods recovered similar macrofaunal diversity (Figure 3). Annelids were the most diverse group with 88%, 81% and 75% of the OTUs for the extracellular DNA extraction method, the sieved lysis method and the direct DNA extraction method respectively. Only few arthropod OTUs were recovered with the sieved lysis method and none with the unsieved methods. The traditional classification method, based on morphology, identified 16 different macrofaunal genera, belonging to three different phyla (Figure 4). Annelids

269 were most diverse as ten genera were recovered whereas three mollusc and arthropod genera were recovered in
270 each phyla. The molecular methods recovered most annelid genera except the *Marenzelleria* sp. which were not
271 detected by any of the DNA extraction methods. Most mollusc genera identified with the traditional morphological
272 methods were also recovered with the molecular methods. However, *Limecola* sp. was not recovered with the
273 direct DNA extraction method and *Peringia* sp. was not recovered with the extracellular DNA extraction method.
274 Among the three arthropod genera detected using traditional morphological methods, only *Bathyporeia* sp. was
275 recovered, with the sieved lysis DNA extraction method.
276 Presence/absence estimations obtained with the sieved lysis method correlated significantly (Pearsons, $r = 0.54$, $p =$
277 0.014) with presence/absence estimations based on morphological identifications (Figure 5). The extracellular and
278 direct DNA extraction methods both underestimated the presence/absence ratios of the genera *Eteone* sp. and
279 *Hediste* sp. whereas the presence/absence ratios of the genera *Lanice* sp. and *Cerastoderma* sp. were
280 overestimated. Remarkable differences were found in the presence/absence ratios for *Heteromastus* sp., which
281 was recovered in all samples with the extracellular DNA extraction method but in none of the samples with the
282 direct DNA extraction method.
283
284 *3.2.3. Meiofauna*
285 The meiofaunal taxons recovered with the extracellular DNA extraction method and the direct DNA extraction
286 method belonged to the arthropods, nematodes, flat-worms, gnathostomulids and gastrotrichs (Figure 6). The total
287 number of OTUs for all meiofaunal orders did not differ between the two methods (t-test, $t_6 = 2.202$, $p = 0.07$) but
288 the number of nematode OTUs differed significantly (t-test, $t_6 = 3.594$, $p = 0.011$). Compared to the direct DNA
289 extraction method, the extracellular DNA extraction method recovered only one-third of the nematode OTUs and
290 entirely failed to recover OTUs from the order Araeolaimida. Presence/absence estimations for all meiofaunal
291 orders obtained with the extracellular and the direct DNA extraction methods were highly different (paired-t, $t_{13} =$
292 2.939, $p = 0.012$) (Figure 7). Presence/absence estimations for meiofauna orders obtained with the direct DNA
293 extraction method were overall higher. This was especially true for nematode orders that were detected in 57% of
294 the sediment samples with the direct DNA extraction method and in 16% of the samples with the extracellular DNA

295 extraction method. Presence/absence ratios for the nematode orders differed significantly (paired-t, $t_4 = 4.489$, $p =$
296 0.011) between these two DNA extraction methods.
297
298 ## 4. Discussion
299 ### 4.1. Validation of methods
300 Assessing biodiversity from metabarcoding data is undergoing an exponential increase; in part due to the ability of
301 these approaches to capture diversity in complex and diverse communities (i.a. Chariton et al, 2015; Lejzerowicz et
302 al., 2015; Sinniger et al., 2016). Accordingly, metabarcoding-based assessments of biodiversity need
303 standardization to allow comparison between studies. A mock sample was employed to assess the consistency of
304 species identification from the metabarcode sequences. The analysis of the mock sample exposed data gaps in the
305 SILVA 18S rRNA reference database with respect to Wadden Sea fauna. The quality of taxonomic assignments is, to
306 a large extent, depending on the completeness of the reference database. The sensitivity and accuracy of
307 taxonomic assignment increases when more species are present in the reference database allowing OTU
308 assignment to higher taxonomic levels (Carugati et al., 2015; Creer et al., 2016; Pompanon and Samadi, 2015;
309 Richardson et al., 2017; Thomsen and Willerslev, 2015). In our study, only few reads were assigned to the
310 arthropod species *Bathyporeia sarsi* and no reads were assigned to the species *Corophium arenarium* and *Urothoe*
311 *poseidonis* or any other species within the class Malacostraca. This outcome persisted even after the addition of
312 sequences from these species to the SILVA reference database. The overall absence of Malacostraca barcodes in
313 the mock sample as well as in the environmental samples suggests methodological problems for certain arthropod
314 species rather than misidentifications during the bioinformatic process. The V4-V5 region of the 18S rRNA locus
315 targeted in our study, has been reported to allow identification of OTUs across a wide taxonomic range (Hadziavdic
316 et al., 2014; Hugerth et al., 2014). This specific region is also known to exhibit length polymorphism (Hadziavdic et
317 al., 2014; Hugerth et al., 2014; Nickrent and Sargent, 1991). The amplicon size was in the range of 600bp to 650bs
318 for the majority of our targeted species. However, the arthropod amplicons were longer, around ~1,000 bp. Longer
319 amplicons may be underrepresented by PCR and Roche 454 sequencing. This may potentially explain the absence
320 of arthropod OTUs (Berry et al., 2011; Engelbrektson et al., 2010; Herbold et al., 2015).

321 BLAST is typically the default method for taxonomic assignment in benthic metabarcoding studies (Cowart
322 et al., 2015; Dell'Anno et al., 2015; Lejzerowicz et al., 2015; Sinniger et al., 2016). However, this study, and in
323 particular the analysis of the mock sample, revealed some incorrect assignments when using BLAST. Although all
324 species that were included in the mock sample were recovered, five additional species were detected with BLAST.
325 These five additional species were closely related to species in the mock sample but did not have identical barcodes
326 for the subjected 18S rRNA region. This suggests that the BLAST taxonomic assignment in combination with our
327 OTU clustering method was not strict enough, even at more stringent settings. The RDP classifier is not commonly
328 used for marine benthic biodiversity studies but it performed well for the taxonomic assignment of metazoan
329 genera in other studies (Chariton et al., 2015; Cole et al., 2009; Porter et al., 2014). During this study, the RDP
330 classifier was able to recover the exact species present in the mock sample and caused no misidentifications.
331 Although many more taxonomic assignment and aligning methods have been developed next to BLAST and the RDP
332 classifier (i.a. Liu et al., 2008; Coissac, Riaz and Puillandre, 2012; Richardson, et al., 2017), it is beyond the scope of
333 this study to compare all these methods. Our results indicate that the RDP classifier is an adequate tool for the
334 taxonomic assignment of Wadden Sea benthic fauna studies.
335
336 **4.2. Comparison of extraction methods**
337 The recovery of DNA from marine benthic communities is a crucial first step in molecular-based assessments of
338 biodiversity. This study presents one of the first comparative analysis of benthic assessment based on
339 morphological and different molecular approaches with respect to different DNA extraction methods. Our results
340 indicate that DNA extraction methods preceded bya lysis step are efficient in terms of recovering marine benthic
341 macrofauna and meiofauna biodiversity. The utility of an additional sieving step prior to DNA extraction depends
342 on which portion of biodiversity is of interest, in this study, sieving improved the detection of macrofaunal
343 diversity.
344 All three DNA extraction methods recovered most of the macrofauna families that were detected with the
345 traditional morphological identification method, as reported earlier (Guardiola et al., 2015; Lejzerowicz et al., 2015;
346 Pearman et al., 2016). However, the sieved lysis method was the only method from which presence/absence ratios
347 of macrofaunal genera correlated to the rations found in the morphological approach. Previous studies using a

348 sieving, or an elutriation step, already showed good results for large metazoan species (Brannock and Halanych,
349 2015; Vanreusel et al., 2010; Yu et al., 2012). However, this is the first study that shows a one-to-one correlation
350 with morphological approaches. The sieved lysis method in this study included organisms retained on a 1mm sieve
351 and hence recovered only macrofaunal taxons. Mesh sizes used for separating benthic fauna from sediment
352 substrates through sieving, or elution, can be adapted to broaden the size range of the sampled species and also
353 include meiofaunal species (Brannock and Halanych, 2015; Creer et al., 2016). Presence/absence ratios of
354 macrofauna genera based on the morphological approach did not correlate to the ratios based on the extracellular
355 DNA extraction method and the direct DNA extraction method. Although some studies assumed that the
356 extracellular DNA excreted by macrofauna species reflects the biodiversity present, this study could not support
357 this assumption.
358 Meiofaunal diversity was represented best by the direct DNA extraction method compared to the
359 extracellular DNA extraction method. In particular nematods seemed underrepresented with the extracellular DNA
360 extraction method. Nematodes are the most abundant and diverse meiofaunal group in the Wadden Sea (Blome,
361 Schleier and Van Bernem, 1999; Heip et al., 1985; Witte and Zijlstra, 1984) and the extracellular method failed to
362 capture this important group. The low nematode diversity detected with the extracellular DNA extraction method
363 has been reported earlier and is possibly characteristic for this particular method (Guardiola et al., 2016, 2015).
364 The methods employed here were selected from methods currently employed in metabarcoding studies.
365 The methods did not only differ in DNA extraction strategy, but also in the sample volume. The sample volume and
366 the sieving procedure for the sieved lysis method was similar to the morphological method and the high correlation
367 as found in this study between the results of these methods was expected. The sample volume of the extracellular
368 DNA extraction method was also similar to the morphological method, however, this method was infeasible to
369 reflect the macrofauna diversity and numbers of metazoan OTUs were relatively low. The direct DNA extraction
370 method showed the highest OTU diversity, even though this method processed only 1/90 of the sample volume of
371 the other two methods.
372 The numbers of metazoan OTUs detected were lowest with the extracellular DNA extraction method. The
373 low recovery rates may be due to the relatively long DNA fragment (650bp used) targeted in this study, since the
374 length of the amplicon affects the recovery rate of partly degraded extracellular DNA (Coissac et al., 2012;

375 Corinaldesi et al., 2008; Sinniger et al., 2016; Taberlet et al., 2012b). OTU detection based on extracellular DNA is
376 biased by species specific differences in DNA release and the fate of extracellular DNA. Many factors influence
377 environmental DNA release. The annelid *Lanice* sp. produces relatively high amounts of slime and is well
378 represented in the extracellular DNA pool whereas the gastropod *Peringia* sp. is enclosed by a shell and may
379 therefore be less prominent in assessments based on extracellular DNA (Barnes and Turner, 2016).
380 The suitability of different DNA extraction methods depends on the specific research objective. The sieved
381 lysis method appears best suited to characterise marine macrofaunal biodiversity. However, the direct DNA
382 extraction method provided a more complete characterization of the marine benthic diversity. Unsieved sediment
383 samples included intracellular DNA of species present in the small sediment core as well as environmental DNA
384 from surrounding species which makes this specific method versatile (Barnes and Turner, 2016; Delmont et al.,
385 2011). Targeting shorter DNA fragments, as now done for Illumina sequencing, might increase the diversity found
386 with the extracellular DNA extraction method. However, taxonomic resolution will decrease inherently (Elbrecht &
387 Leese, 2015). Although 454 sequencing, as used in the study, is no longer operational, the results of these studies
388 will still be relevant. Nanopore technologies are quickly emerging and have the ability to sequence the longer
389 amplicons as used in this study.
390 This study demonstrated the feasibility of metabarcoding as a means to assess marine benthic biodiversity
391 in the Dutch intertidal Wadden Sea. Metabarcoding allowed for a rapid, replicable and nearly complete approach
392 for the study of benthic communities. However, the outcome of the classic morphological approach and the
393 outcome of metabarcoding studies are not necessarily identical. A more comprehensive discussion about the
394 interpretation of metabarcoding studies can be found in Cowart et al., (2015) and Lejzerowicz et al., (2015). Besides
395 macrofauna, also meiofauna key indicators for ecosystem health (Balsamo et al., 2012; Carugati et al., 2015;
396 Spilmont, 2013) can now easily be included in marine benthic studies. Still, caution is needed when designing and
397 interpreting metabarcoding studies. This study shows that results, i.e. the biodiversity recovered, may vary with the
398 DNA extraction method and the combination of amplicon and reference database used. Studies need to clearly
399 describe the methods and the reference databases used in order to enable comparisons with other studies. The
400 need for incorporating a mock sample to test for optimal bioinformatics methods is shown here.
401

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406

407 **Data availability**

408 Sequencing data reported here have been deposited in the European Nucleothide Archieve (ENA) (accession
409 number: xxx)

410

411 **5. Cited Literature**

412 Alawi, M., Schneider, B., Kallmeyer, J., 2014. A procedure for separate recovery of extra- and intracellular DNA from
413 a single marine sediment sample. J. Microbiol. Methods 104, 36–42. doi:10.1016/j.mimet.2014.06.009

414

415 Anadón, R., Danovaro, R., Dippner, J.W., Drinkwater, K.F., Hawkins, S.J., O'Sullivan, G., Oguz, T., Reid, P.C., 2007.
416 Impacts of Climate Change on the European Marine and Coastal Environment Ecosystems Approach. Eur. Sci.
417 Found. 84.

418

419 Austen, M., Lambshead, P., Hutchings, P., Boucher, Gl., Snelgrove, P., Heip, C., King, G., Koike, I., Smith, C., 2002
420 Biodiversity links above and below the marine sediment-water interface that may influence community stability.
421 Biodiversity and conservation 11, 113-136. doi:10.1023/A:1014098917535

422

423 Baird, D.J., Hajibabaei, M., 2012. Biomonitoring 2.0: A new paradigm in ecosystem assessment made possible by
424 next-generation DNA sequencing. Mol. Ecol. 21, 2039–2044. doi:10.1111/j.1365-294X.2012.05519.x

425

426 Balsamo, M., Semprucci, F., Frontalini, F., Coccioni, R., 2012. Meiofauna as a tool for marine ecosystem
427 biomonitoring. Mar. Ecosyst. A. Cruzado (Ed.), InTech, 77–104. doi:10.5772/34423

428

429 Barnes, M.A., Turner, C.R., 2016. The ecology of environmental DNA and implications for conservation genetics.
430 Conserv. Genet. 17, 1–17. doi:10.1007/s10592-015-0775-4
431
432 Berry, D., Mahfoudh, K. Ben, Wagner, M., Loy, A., 2011. Barcoded primers used in multiplex amplicon
433 pyrosequencing bias amplification. Appl. Environ. Microbiol. 77, 7846–7849. doi:10.1128/AEM.05220-11
434
435 Beukema, J.J., Cadée, G.C., 1997. Local differences in macrozoobenthic response to enhanced food supply caused
436 by mild eutrophication in a Wadden Sea area. Food is only locally a limiting factor. Limnol. Oceanogr. 42, 1424–
437 1435. doi:10.4319/lo.1997.42.6.1424
438
439 Bienert, F., De Danieli, S., Miquel, C., Coissac, E., Poillot, C., Brun, J.J., Taberlet, P., 2012. Tracking earthworm
440 communities from soil DNA. Mol. Ecol. 21, 2017–2030. doi:10.1111/j.1365-294X.2011.05407.x
441
442 Bik, H.M., Porazinska, D.L., Creer, S., Caporaso, J.G., Knight, R., Thomas, W.K., 2012. Sequencing our way towards
443 understanding global eukaryotic biodiversity. Trends Ecol. Evol. 27, 233–243. doi:10.1016/j.tree.2011.11.010
444
445 Blome, D., Schleier, U., Van Bernem, K.H., 1999. Analysis of the small-scale spatial patterns of free-living marine
446 nematodes from tidal flats in the East Frisian Wadden Sea. Mar. Biol. 133, 717–726. doi:10.1007/s002270050513
447
448 Boyd, S.E., Rees, H.L., Richardson, C.A., 2000. Nematodes as Sensitive Indicators of Change at Dredged Material
449 Disposal Sites. Estuar. Coast. Shelf Sci. 51, 805–819. doi:10.1006/ecss.2000.0722
450
451 Brannock, P.M., Halanych, K.M., 2015. Meiofaunal community analysis by high-throughput sequencing: Comparison
452 of extraction, quality filtering, and clustering methods. Mar. Genomics 23, 67–75.
453 doi:10.1016/j.margen.2015.05.007
454

455 Brannock, P.M., Waits, D.S., Sharma, J., Halanych, K.M., 2014. High-throughput sequencing characterizes intertidal
456 meiofaunal communities in Northern Gulf of Mexico (Dauphin Island and Mobile Bay, Alabama). Biol. Bull. 227,
457 161–174. doi:10.1086/BBLv227n2p161

458

459 Bucklin, A., Steinke, D., Blanco-Bercial, L., 2011. DNA Barcoding of Marine Metazoa. Ann. Rev. Mar. Sci. 3, 471–508.
460 doi:10.1146/annurev-marine-120308-080950

461

462 Caporaso, J.G., Kuczynski, J., et al, 2010. QIIME allows high throughput community sequencing data. Nat. Methods
463 7, 335–336. doi:10.1038/nmeth0510-335

464

465 Cardoso, P., Erwin, T.L., Borges, P.A.V., New, T.R., 2011. The seven impediments in invertebrate conservation and
466 how to overcome them. Biol. Conserv. 144, 2647–2655. doi:10.1016/j.biocon.2011.07.024

467

468 Carugati, L., Corinaldesi, C., Dell’Anno, A., Danovaro, R., 2015. Metagenetic tools for the census of marine
469 meiofaunal biodiversity: An overview. Mar. Genomics 24, 11–20. doi:10.1016/j.margen.2015.04.010

470

471 Chariton, A.A., Court, L.N., Hartley, D.M., Colloff, M.J., Hardy, C.M., 2010. Ecological assessment of estuarine
472 sediments by pyrosequencing eukaryotic ribosomal DNA. Front. Ecol. Environ. 8, 233–238. doi:10.1890/090115

473

474 Chariton, A.A., Stephenson, S., Morgan, M.J., Steven, A.D.L., Colloff, M.J., Court, L.N., Hardy, C.M., 2015.
475 Metabarcoding of benthic eukaryote communities predicts the ecological condition of estuaries. Environ. Pollut.
476 203, 165–174. doi:10.1016/j.envpol.2015.03.047

477

478 Coissac, E., Riaz, T., Puillandre, N., 2012. Bioinformatic challenges for DNA metabarcoding of plants and animals.
479 Mol. Ecol. 21, 1834–1847. doi:10.1111/j.1365-294X.2012.05550.x

480

481 Cole, J.R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R.J., Kulam-Syed-Mohideen, A.S., McGarrell, D.M., Marsh,
482 T., Garrity, G.M., Tiedje, J.M., 2009. The Ribosomal Database Project: Improved alignments and new tools for rRNA
483 analysis. Nucleic Acids Res. 37. doi:10.1093/nar/gkn879
484
485 Compton, T.J., Holthuijsen, S., Koolhaas, A., Dekinga, A., ten Horn, J., Smith, J., Galama, Y., Brugge, M., van der Wal,
486 D., van der Meer, J., van der Veer, H.W., Piersma, T., 2013. Distinctly variable mudscapes: Distribution gradients of
487 intertidal macrofauna across the Dutch Wadden Sea. J. Sea Res. 82, 103–116. doi:10.1016/j.seares.2013.02.002
488
489 Corinaldesi, C., Beolchini, F., Dell'Anno, A., 2008. Damage and degradation rates of extracellular DNA in marine
490 sediments: Implications for the preservation of gene sequences. Mol. Ecol. 17, 3939–3951. doi:10.1111/j.1365-
491 294X.2008.03880.x
492
493 Costello, M.J., 2015. Biodiversity: The known, unknown, and rates of extinction. Curr. Biol. 25, R368–R371.
494 doi:10.1016/j.cub.2015.03.051
495
496 Covich, A, Austen, M., Barlocher, F., Chauvet, E., Cardinale, B., Biles, C., Inchausti, P., Dangles, O., Solan, M.,
497 Gessner, M., Statzner, B., Moss, B., 2004. The role of biodiversity in the functioning of freshwater and marine
498 benthic ecosystems. Bioscience 54, 767-775.doi:10.1641/0006-3568(2004)054[0767:TRO-BIT]2.0.CO;2
499
500 Cowart, D.A., Pinheiro, M., Mouchel, O., Maguer, M., Grall, J., Miné, J., Arnaud-Haond, S., 2015. Metabarcoding is
501 powerful yet still blind: A comparative analysis of morphological and molecular surveys of seagrass communities.
502 PLoS One 10, 1–26. doi:10.1371/journal.pone.0117562
503
504 Creer, S., Deiner, K., Frey, S., Porazinska, D., Taberlet, P., Thomas, W.K., Potter, C., Bik, H.M., 2016. The ecologist's
505 field guide to sequence-based identification of biodiversity. Methods Ecol. Evol. 7, 1008–1018. doi:10.1111/2041-
506 210X.12574
507

508 Daily, G.C., Söderqvist, T., Aniyar, S., Arrow, K., Dasgupta, P., Ehrlich, P.R., Folke, C., Jansson, A., Jansson, B.-O.,
509 Kautsky, N., Levin, S., Lubchenco, J., Mäler, K.-G., Simpson, D., Starrett, D., Tilman, D., Soderqvist, T., Maler, K.-G.,
510 Walker, B., 2000. The Value of Nature and the Nature of Value. Walk. Source Sci. New Ser. 289, 395–396.
511 doi:10.1890/154-9295-10.4.171
512
513 Danovaro, R., Gambi, C., Dell'Anno, A., Corinaldesi, C., Fraschetti, S., Vanreusel, A., Vincx, M., Gooday, A.J., 2008.
514 Exponential Decline of Deep-Sea Ecosystem Functioning Linked to Benthic Biodiversity Loss. Curr. Biol. 18, 1–8.
515 doi:10.1016/j.cub.2007.11.056
516
517 Danovaro, R., Gambi, C., Manini, E., Fabiano, M., 2000. Meiofauna response to a dynamic river plume front. Mar.
518 Biol. 137, 359–370. doi:10.1007/s002270000353
519
520 Dell'Anno, A., Carugati, L., Corinaldesi, C., Riccioni, G., Danovaro, R., 2015. Unveiling the biodiversity of deep-sea
521 nematodes through metabarcoding: Are we ready to bypass the classical taxonomy? PLoS One 10, 1–18.
522 doi:10.1371/journal.pone.0144928
523
524 Dell'Anno, A., Corinaldesi, C., 2004. Degradation and Turnover of Extracellular DNA in Marine Sediments : Ecological
525 and Methodological Considerations Degradation and Turnover of Extracellular DNA in Marine Sediments :
526 Ecological and Methodological Considerations. Appl. Environ. Microbiol. 70, 4384–4386.
527 doi:10.1128/AEM.70.7.4384
528
529 Delmont, T.O., Robe, P., Clark, I., Simonet, P., Vogel, T.M., 2011. Metagenomic comparison of direct and indirect
530 soil DNA extraction approaches. J. Microbiol. Methods 86, 397–400. doi:10.1016/j.mimet.2011.06.013
531
532 Elbrecht, V., Leese, F., 2015. Can DNA-based ecosystem assessments quantify species abundance? Testing primer
533 bias and biomass-sequence relationships with an innovative metabarcoding protocol. PLoS ONE 10, 1-16.
534 doi:10.1371/journal.one.0130324

535

536 Engelbrektson, A., Kunin, V., Wrighton, K.C., Zvenigorodsky, N., Chen, F., Ochman, H., Hugenholtz, P., 2010.
537 Experimental factors affecting PCR-based estimates of microbial species richness and evenness. ISME J. 4, 642–647.
538 doi:10.1038/ismej.2009.153

539

540 Fonseca, V.G., Carvalho, G.R., Sung, W., Johnson, H.F., Power, D.M., Neill, S.P., Packer, M., Blaxter, M.L.,
541 Lambshead, P.J.D., Thomas, W.K., Creer, S., 2010. Second-generation environmental sequencing unmasks marine
542 metazoan biodiversity. Nat. Commun. 1, 98. doi:10.1038/ncomms1095

543

544 Fonseca, V.G., Carvalho, G.R., Nichols, B., Quince, C., Johnson, H.F., Neill, S.P., Lambshead, J.D., Thomas, W.K.,
545 Power, D.M., Creer, S., 2014. Metagenetic analysis of patterns of distribution and diversity of marine meiobenthic
546 eukaryotes. Glob. Ecol. Biogeogr. 23, 1293–1302. doi:10.1111/geb.12223

547

548 Guardiola, M., Uriz, M.J., Taberlet, P., Coissac, E., Wangensteen, O.S., Turon, X., 2015. Deep-sea, deep-sequencing:
549 Metabarcoding extracellular DNA from sediments of marine canyons. PLoS One 10.
550 doi:10.1371/journal.pone.0139633

551

552 Guardiola, M., Wangensteen, O.S., Taberlet, P., Coissac, E., Uriz, M.J., Turon, X., 2016. Spatio-temporal monitoring
553 of deep-sea communities using metabarcoding of sediment DNA and RNA. PeerJ 4, e2807. doi:10.7717/peerj.2807

554

555 Hadziavdic, K., Lekang, K., Lanzen, A., Jonassen, I., Thompson, E.M., Troedsson, C., 2014. Characterization of the 18s
556 rRNA gene for designing universal eukaryote specific primers. PLoS One 9. doi:10.1371/journal.pone.0087624

557

558 Hajibabaei, M., Shokralla, S., Zhou, X., Singer, G.A.C., Baird, D.J., 2011. Environmental barcoding: A next-generation
559 sequencing approach for biomonitoring applications using river benthos. PLoS One 6.
560 doi:10.1371/journal.pone.0017497

561

562 Halpern, B.S., Walbridge, S., Selkoe, K.A., Kappel, C. V., Micheli, F., D'Agrosa, C., Bruno, J.F., Casey, K.S., Ebert, C.,
563 Fox, H.E., Fujita, R., Heinemann, D., Lenihan, H.S., Madin, E.M.P., Perry, M.T., Selig, E.R., Spalding, M., Steneck, R.,
564 Watson, R., 2008. A Global Map of Human Impact on Marine Ecosystems. Science 80, 948–952.
565 doi:10.1126/science.1149345
566
567 Hartmann-Schröder, G., 1996. Annelida, Borstenwürmer, Polychaeta. 2nd revised ed. Die Tierwelt Deutschalands
568 und der angrenzenden Meeresteile nach ihren Merkmalen und nach ihrer Lebensweise, 58. Gustav Fischer, Jena.
569 648pp.
570
571 Hayward, P., Ryland, J. (eds). 1995. Handbook of the marine fauna of north-west Europe. Oxford University Press,
572 Oxford, 800pp.
573
574 Heip, C., Vincx, M., Vranken, G., 1985. The Ecology of Marine Nematodes.pdf. Oceanogr. Mar. Biol. An Annu. Rev.
575 23, 399–489.
576
577 Herbold, C.W., Pelikan, C., Kuzyk, O., Hausmann, B., Angel, R., Berry, D., Loy, A., 2015. A flexible and economical
578 barcoding approach for highly multiplexed amplicon sequencing of diverse target genes. Front. Microbiol. 6.
579 doi:10.3389/fmicb.2015.00731
580
581 Hooper, D.U., Adair, E.C., Cardinale, B.J., Byrnes, J.E.K., Hungate, B.A., Matulich, K.L., Gonzalez, A., Duffy, J.E.,
582 Gamfeldt, L., O'Connor, M.I., 2012. A global synthesis reveals biodiversity loss as a major driver of ecosystem
583 change. Nature 1–5. doi:10.1038/nature11118
584
585 Hortal, J., de Bello, F., Diniz-Filho, J.A.F., Lewinsohn, T.M., Lobo, J.M., Ladle, R.J., 2015. Seven Shortfalls that Beset
586 Large-Scale Knowledge of Biodiversity. Annu. Rev. Ecol. Evol. Syst. 46, 523–549. doi:10.1146/annurev-ecolsys-
587 112414-054400
588

589 Hugerth, L.W., Muller, E.E.L., Hu, Y.O.O., Lebrun, L.A.M., Roume, H., Lundin, D., Wilmes, P., Andersson, A.F., 2014.
590 Systematic design of 18S rRNA gene primers for determining eukaryotic diversity in microbial consortia. PLoS One
591 9. doi:10.1371/journal.pone.0095567
592
593 Ji, Y., Ashton, L., Pedley, S.M., Edwards, D.P., Tang, Y., Nakamura, A., Kitching, R., Dolman, P.M., Woodcock, P.,
594 Edwards, F.A., Larsen, T.H., Hsu, W.W., Benedick, S., Hamer, K.C., Wilcove, D.S., Bruce, C., Wang, X., Levi, T., Lott,
595 M., Emerson, B.C., Yu, D.W., 2013. Reliable, verifiable and efficient monitoring of biodiversity via metabarcoding.
596 Ecol. Lett. 16, 1245–1257. doi:10.1111/ele.12162
597
598 Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S.,
599 Duran, C., Thierer, T., Ashton, B., Meintjes, P., Drummond, A., 2012. Geneious Basic: An integrated and extendable
600 desktop software platform for the organization and analysis of sequence data. Bioinformatics 28, 1647–1649.
601 doi:10.1093/bioinformatics/bts199
602
603 Lejzerowicz, F., Esling, P., Pillet, L., Wilding, T.A., Black, K.D., Pawlowski, J., 2015. High-throughput sequencing and
604 morphology perform equally well for benthic monitoring of marine ecosystems. Sci. Rep. 5, 13932.
605 doi:10.1038/srep13932
606
607 Lekang, K., Thompson, E., Troedsson, C., 2015. A comparison of DNA extraction methods for biodiversity studies of
608 eukaryotes in marine sediments. Aquat. Microb. Ecol. 75, 15–25. doi:10.3354/ame01741
609
610 Leray, M., Knowlton, N., 2016. Censusing marine eukaryotic diversity in the twenty-first century. Philos. Trans. R.
611 Soc. Lond. B. Biol. Sci. 371, 20150331. doi:10.1098/rstb.2015.0331
612
613 Leray, M., Knowlton, N., 2015. DNA barcoding and metabarcoding of standardized samples reveal patterns of
614 marine benthic diversity. Proc. Natl. Acad. Sci. 112, 2076–2081. doi:10.1073/pnas.1424997112
615

616 Levin, L., Boesch, D., Covich, A., Dahm, C., Erseus, C., Ewel. K., Kneib, R., Moldenke, A., Palmer, M., Snelgrove, P.,
617 Strayer, D., Weslawski, J., 2001. The function of marine critical transition zones and the importance of sediment
618 biodiversity. Ecosystems 4, 430-451. doi:10.1007/s10021-001-0021-4
619
620 Liu, Z., Desantis, T.Z., Andersen, G.L., Knight, R., 2008. Accurate taxonomy assignments from 16S rRNA sequences
621 produced by highly parallel pyrosequencers. Nucleic Acids Res. 36. doi:10.1093/nar/gkn491
622
623 May, R.M., 1988. How Many Species Are There on Earth? Science 80, 1441–1449.
624 doi:10.1126/science.241.4872.1441
625
626 Nickrent, D.L., Sargent, M.L., 1991. An overview of the secondary structure of the V4 region of eukaryotic small-
627 subunit ribosomal RNA. Nucleic Acids Res. 19, 227–235. doi:10.1093/nar/19.2.227
628
629 Snelgrove, P.V.R., 1997. The Importance of Marine Sediment Biodiversity in Ecosystem Processes. Ambio 26, 578–
630 583.
631
632 Pearman, J., Irigoien, X., Carvalho, S., 2015. Extracellular DNA amplicon sequencing reveals high levels of benthic
633 eukaryotic diversity in the central Red Sea. Marine genomics 26, 29-39. doi:10.1016/j.margen.2015.10.008
634
635 Pearman, J.K., Anlauf, H., Irigoien, X., Carvalho, S., 2016. Please mind the gap - Visual census and cryptic biodiversity
636 assessment at central Red Sea coral reefs. Mar. Environ. Res. 118, 20–30. doi:10.1016/j.marenvres.2016.04.011
637
638 Pompanon, F., Samadi, S., 2015. Next generation sequencing for characterizing biodiversity: promises and
639 challenges. Genetica 143, 133–138. doi:10.1007/s10709-015-9816-7
640

641 Porter, T.M., Gibson, J.F., Shokralla, S., Baird, D.J., Golding, G.B., Hajibabaei, M., 2014. Rapid and accurate
642 taxonomic classification of insect (class Insecta) cytochrome c oxidase subunit 1 (COI) DNA barcode sequences
643 using a naïve Bayesian classifier. Mol. Ecol. Resour. 14, 929–942. doi:10.1111/1755-0998.12240
644
645 Pruesse, E., Quast, C., Knittel, K., Fuchs, B.M., Ludwig, W., Peplies, J., Glöckner, F.O., 2007. SILVA: A comprehensive
646 online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids
647 Res. 35, 7188–7196. doi:10.1093/nar/gkm864
648
649 Richardson, R.T., Bengtsson-Palme, J., Johnson, R.M., 2017. Evaluating and optimizing the performance of software
650 commonly used for the taxonomic classification of DNA metabarcoding sequence data. Mol. Ecol. Resour. 17, 760–
651 769. doi:10.1111/1755-0998.12628
652
653 Rognes, T., Flouri, T., Nichols, B., Quince, C., Mahé, F., 2016. VSEARCH: a versatile open source tool for
654 metagenomics. PeerJ 4, e2584. doi:10.7717/peerj.2584
655
656 Sinniger, F., Pawlowski, J., Harii, S., Gooday, A.J., Yamamoto, H., Chevaldonné, P., Cedhagen, T., Carvalho, G., Creer,
657 S., 2016. Worldwide Analysis of Sedimentary DNA Reveals Major Gaps in Taxonomic Knowledge of Deep-Sea
658 Benthos. Front. Mar. Sci. 3, 1–14. doi:10.3389/fmars.2016.00092
659
660 Solan, M., 2004. Extinction and Ecosystem Function in the Marine Benthos. Science 80, 1177–1180.
661 doi:10.1126/science.1103960
662
663 Spilmont, N., 2013. The Future of Benthic Indicators: Moving up to the Intertidal. Open J. Mar. Sci. 03, 76–86.
664 doi:10.4236/ojms.2013.32A008
665
666 Taberlet, P., Coissac, E., Pompanon, F., Brochmann, C., Willerslev, E., 2012a. Towards next-generation biodiversity
667 assessment using DNA metabarcoding. Mol. Ecol. 21, 2045–2050. doi:10.1111/j.1365-294X.2012.05470.x

668

669 Taberlet, P., Prud'Homme, S.M., Campione, E., Roy, J., Miquel, C., Shehzad, W., Gielly, L., Rioux, D., Choler, P.,
670 Clément, J.C., Melodelima, C., Pompanon, F., Coissac, E., 2012b. Soil sampling and isolation of extracellular DNA
671 from large amount of starting material suitable for metabarcoding studies. Mol. Ecol. 21, 1816–1820.
672 doi:10.1111/j.1365-294X.2011.05317.x

673

674 Thomsen, P.F., Willerslev, E., 2015. Environmental DNA - An emerging tool in conservation for monitoring past and
675 present biodiversity. Biol. Conserv. 183, 4–18. doi:10.1016/j.biocon.2014.11.019

676

677 Thrush, S, Gray, J., Hewitt, J., Ugland, K., 2006. Predicting the effects of habitat homogenization on marine
678 biodiversity. Ecological applications 16, 1636-1642. doi: 10.1890/1051-0761(2006)016[1636:PTEOHH]2.0.CO;2

679

680 Vanreusel, A., Fonseca, G., Danovaro, R., Da Silva, M.C., Esteves, A.M., Ferrero, T., Gad, G., Galtsova, V., Gambi, C.,
681 Da Fonsêca Genevois, V., Ingels, J., Ingole, B., Lampadariou, N., Merckx, B., Miljutin, D., Miljutina, M., Muthumbi, A.,
682 Netto, S., Portnova, D., Radziejewska, T., Raes, M., Tchesunov, A., Vanaverbeke, J., Van Gaever, S., Venekey, V.,
683 Bezerra, T.N., Flint, H., Copley, J., Pape, E., Zeppilli, D., Martinez, P.A., Galeron, J., 2010. The contribution of deep-
684 sea macrohabitat heterogeneity to global nematode diversity. Mar. Ecol. 31, 6–20. doi:10.1111/j.1439-
685 0485.2009.00352.x

686

687 Wang, Q., Garrity, G.M., Tiedje, J.M., Cole, J.R., 2007. Naïve Bayesian classifier for rapid assignment of rRNA
688 sequences into the new bacterial taxonomy. Appl. Environ. Microbiol. 73, 5261–5267. doi:10.1128/AEM.00062-07

689

690 Witte, J.I.J., Zijlstra, J.J., 1984. the Meiofauna of a Tidal Flat in the Western Part of the Wadden Sea and Its Role in
691 the Benthic Ecosystem. Mar. Ecol. Ser. 14, 129–138. doi:10.3354/meps014129

692

693 Yu, D.W., Ji, Y., Emerson, B.C., Wang, X., Ye, C., Yang, C., Ding, Z., 2012. Biodiversity soup: Metabarcoding of
694 arthropods for rapid biodiversity assessment and biomonitoring. Methods Ecol. Evol. 3, 613–623.
695 doi:10.1111/j.2041-210X.2012.00198.x
696
697 Zeppilli, D., Sarrazin, J., Leduc, D., Arbizu, P.M., Fontaneto, D., Fontanier, C., Gooday, A.J., Kristensen, R.M.,
698 Ivanenko, V.N., Sørensen, M. V., Vanreusel, A., Thébault, J., Mea, M., Allio, N., Andro, T., Arvigo, A., Castrec, J.,
699 Danielo, M., Foulon, V., Fumeron, R., Hermabessiere, L., Hulot, V., James, T., Langonne-Augen, R., Le Bot, T., Long,
700 M., Mahabror, D., Morel, Q., Pantalos, M., Pouplard, E., Raimondeau, L., Rio-Cabello, A., Seite, S., Traisnel, G.,
701 Urvoy, K., Van Der Stegen, T., Weyand, M., Fernandes, D., 2015. Is the meiofauna a good indicator for climate
702 change and anthropogenic impacts? Mar. Biodivers. 45, 505–535. doi:10.1007/s12526-015-0359-z

703 **TABLES/FIGURES**

704 **Table 1 | Species added to the mock sample and their presence in the molecular dataset after taxonomic**
705 **assignment.** The species indicated with an * where those added to the mock sample. The SILVA and SILVA + local
706 column show the outcome of taxonomic assignment for either BLAST or the RDP-classifier based on respectively the
707 SILVA SSU rRNA database or the same database complemented with sequences from local species. Species were
708 either retrieved (+) or not found (-). The colours indicate if the species was correctly identified (green), misidentified
709 (red).

Taxonomy				SILVA + local	
Phylum	**Class**	**Family**	**Species**	**Blastn**	**RDP**
Annelida	**Polychaeta**	**Arenicolidae**	**Arenicola marina***	+	+
Annelida	Polychaeta	Arenicolidae	Abarenicola affinis	+	-
Annelida	**Polychaeta**	**Capitellidae**	**Heteromastus filiformis***	+	+
Annelida	Polychaeta	Capitellidae	Notomastus tenuis	+	-
Annelida	**Polychaeta**	**Orbiniidae**	**Scoloplos armiger***	+	+
Annelida	**Polychaeta**	**Nereididae**	**Hediste diversicolor***	+	+
Annelida	Polychaeta	Nereididae	Platynereis dumerilii	+	-
Arthropoda	**Malacostraca**	**Bathyporidae**	**Bathyporeia sarsi***	+	+
Arthropoda	**Malacostraca**	**Corophiidae**	**Corophium arenarium***	-	-
Arthropoda	**Malacostraca**	**Urothoidae**	**Urothoe poseidonis***	-	-
Arthropoda	Hexanauplia	Siphonostomatoida		+	+
Mollusca	**Bivalvia**	**Cardiidae**	**Cerastoderma edule***	+	+
Mollusca	Bivalvia	Pharidae	Phaxas pellucidus	+	-
Mollusca	Bivalvia	Tellinidae	Scissula similis	+	-
Mollusca	**Bivalvia**	**Tellinidae**	**Limecola balthica***	+	+
Mollusca	**Gastropoda**	**Hydrobiidae**	**Peringia ulvae***	+	+

710

711

712 **Table 2 | Numbers of OTUs for each DNA extraction method.** OTU numbers are calculated as mean value per sample

713 for the extracellular DNA extraction method (n=3), the sieved lysis method (n=5) and the direct method (n=6).

Method	Eukaryota	Metazoa	Proportion metazoan
Extracellular	80 ± 27 SD	27 ± 17 SD	31%
Sieved lysis	18 ± 7 SD	17 ± 6 SD	91%
Direct extraction	82 ± 20 SD	41 ± 19 SD	48%

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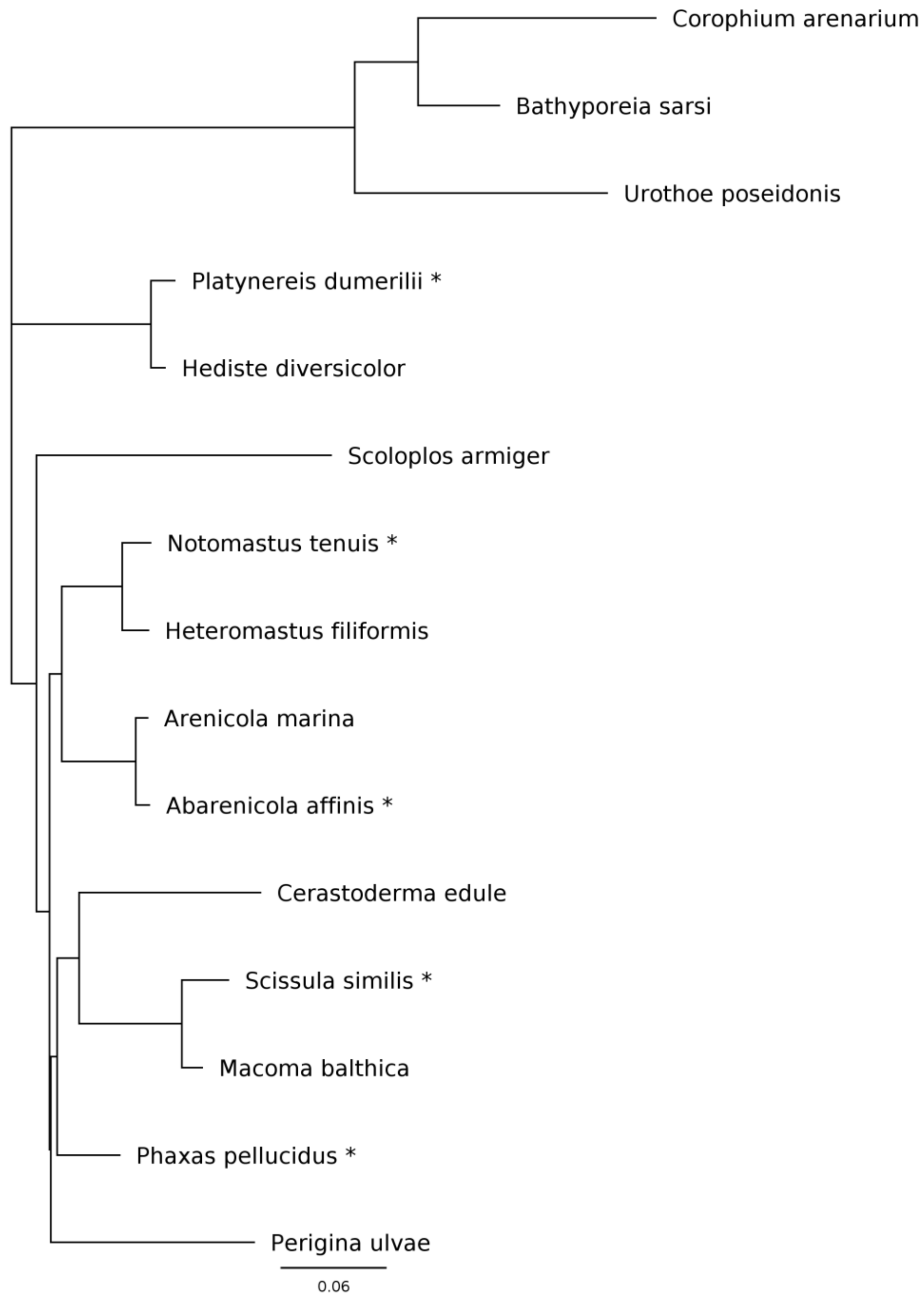

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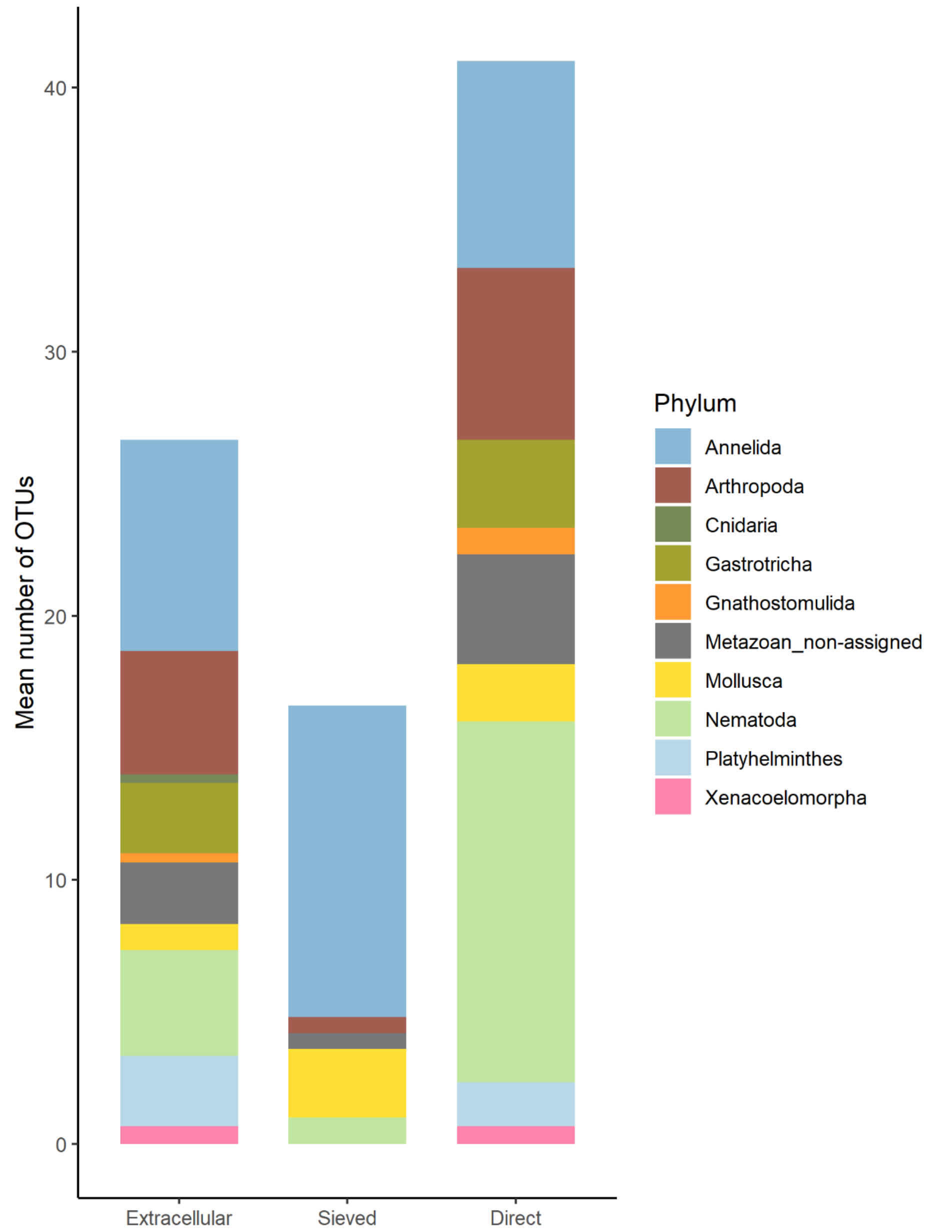
717 **Figure 1 | Neighbor-Joining tree of species found in the mock sample.** The neighbour-joining tree is based on the

718 18S rRNA barcodes from the V4-V5 region (Hadziavdic et al., 2014). Species indicated with* where present in the

719 mock sample.



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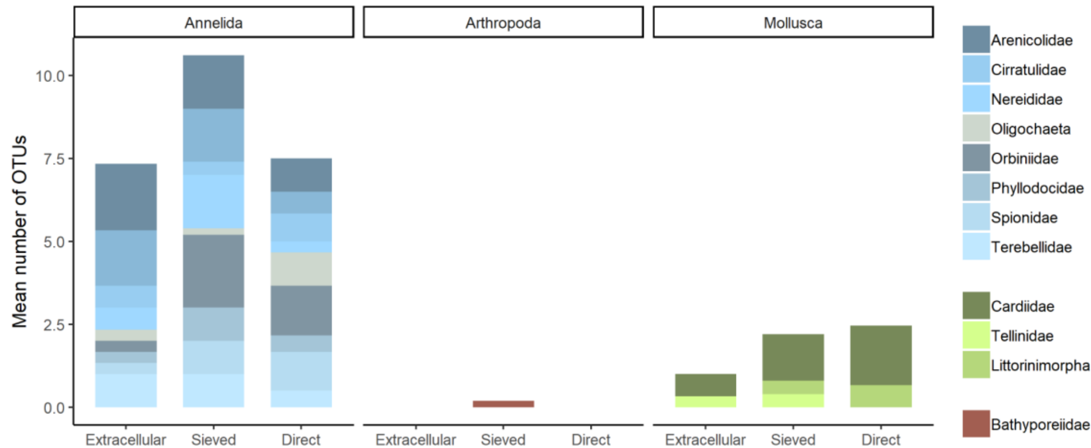
721 **Figure 2 | Taxonomic composition.** For each extraction method, the average number of OTUs per phylum is shown.



722

723 **Figure 3 | Order and family diversity for macrofauna.** The number of OTUs for macrofaunal orders from the phyla
724 Annelida, Mollusca and Arthropoda are shown for the different extraction methods. The mean number of
725 macrofauna OTUs per sample were 8, 14 and 10 for respectively the extracellular, sieved and direct DNA extraction
726 method.

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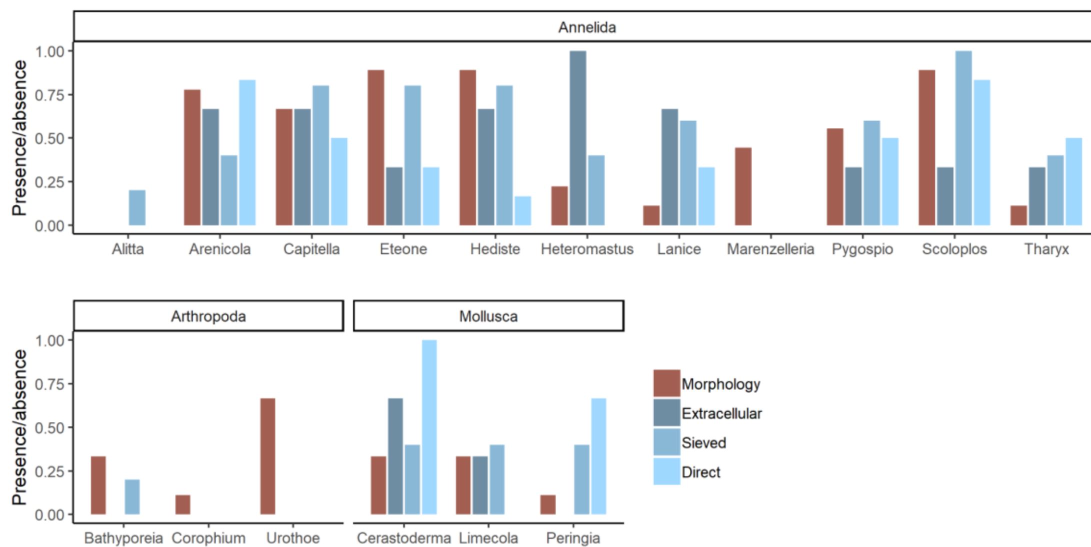

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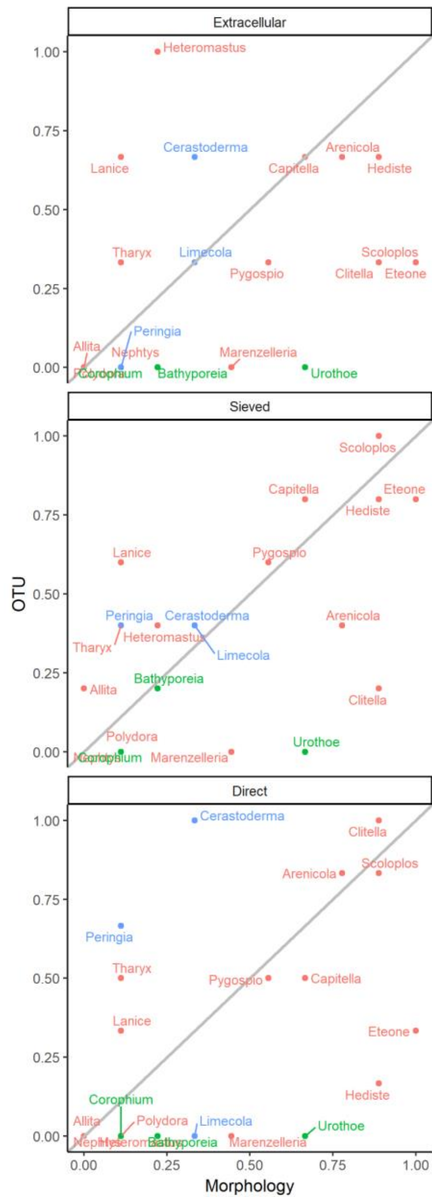
729 **Figure 4 | Presence/absence ratios for macrofauna genera.** Presence/absence ratios are reported for macrofauna
730 genera in the Annelida, Arthropoda and Mollusca phyla. Ratios are calculated as detection rate within the samples
731 for either the extracellular DNA extraction method, sieved lysis method, direct DNA extraction method or
732 morphological method.



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734 **Figure 5 | Scatterplots of presence/absence ratios.** Presence/absence ratios for macrofauna genera assessed by

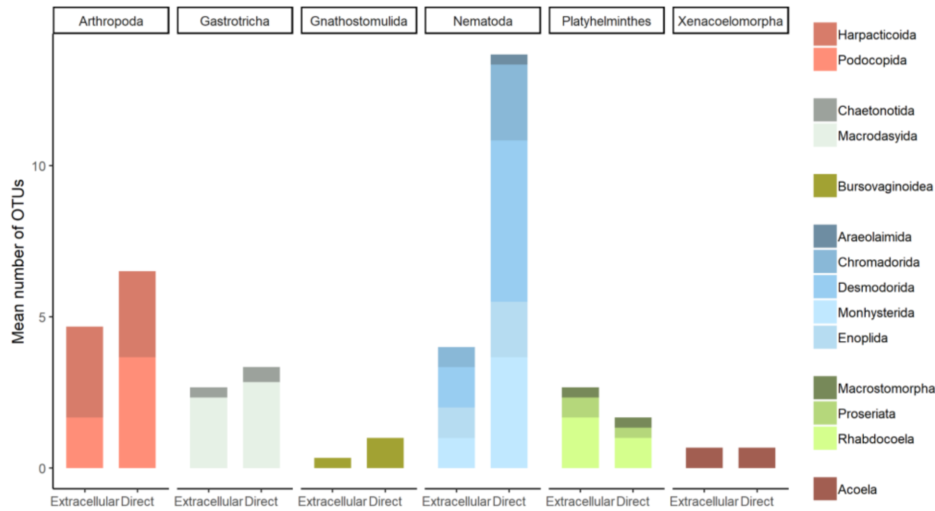
735 either classical taxonomy versus the extracellular DNA extraction method, the sieved lysis method or the direct

736 DNA extraction method. Presence/absence ratios for the morphological approach are calculated as detection rates

737 from all morphological identified samples (n=9). The presence/absence ratios for the molecular approaches are

738 calculated as detection rates within the samples of the particular molecular method. The colours represent

739 different phyla; red = Annelida, blue = Mollusca, green = Arthropoda.



740

741 **Figure 6 | Phylum and order diversity for meiofauna.** The number of OTUs for the meiofaunal orders from the

742 phyla Arthropoda, Nematoda, Gastrotricha, Gnathostomulida, Platyhelminthes and Xenacoelomorpha. The mean

743 number of OTUs per sample were 15 and 26 for respectively the extracellular and direct DNA extraction method.

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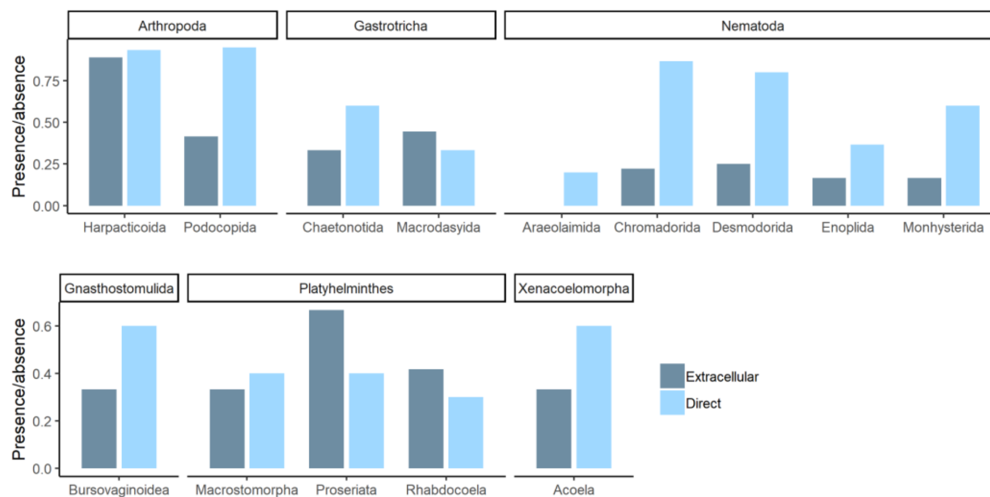

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746 **Figure 7 | Presence/absence ratios for meiofauna orders.** Presence/absence ratios are reported for meiofaunal

747 orders from the Arthropoda, Gastotricha, Nematoda, Gnasthostomulida, Platyhelminthes, Xenacoelomorpha phyla.

748 Ratios are calculated as detection rate within the samples for either the extracellular or direct DNA extraction

749 method.