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Worldwide analysis of sedimentary DNA reveals major gaps in taxonomic knowledge of deep-sea benthos

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Provisional

1 **Worldwide analysis of sedimentary DNA reveals major gaps**
2 **in taxonomic knowledge of deep-sea benthos**

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22 **rDNA**

23 **Abstract**

24 Deep-sea sediments represent the largest but least known ecosystem on earth. With
25 increasing anthropogenic pressure, it is now a matter of urgency to improve our
26 understanding of deep-sea biodiversity. Traditional morpho-taxonomic studies
27 suggest that the ocean floor hosts extraordinarily diverse benthic communities.
28 However, due to both its remoteness and a lack of expert taxonomists, assessing deep-
29 sea diversity is a very challenging task. Environmental DNA (eDNA) metabarcoding
30 offers a powerful tool to complement morpho-taxonomic studies. Here we use eDNA
31 to assess benthic metazoan diversity in 39 deep-sea sediment samples from bathyal
32 and abyssal depths worldwide. The eDNA dataset was dominated by meiobenthic taxa
33 and we identified all animal phyla commonly found in the deep-sea benthos; yet, the
34 diversity within these phyla remains largely unknown. The large numbers of
35 taxonomically unassigned molecular operational taxonomic units (OTUs) were not
36 equally distributed among phyla, with nematodes and platyhelminthes being the most
37 poorly characterized from a taxonomic perspective. While the data obtained here

38 reveal pronounced heterogeneity and vast amounts of unknown biodiversity in the
39 deep sea, they also expose the difficulties in exploiting metabarcoding datasets
40 resulting from the lack of taxonomic knowledge and appropriate reference databases.
41 Overall, our study demonstrates the promising potential of eDNA metabarcoding to
42 accelerate the assessment of deep-sea biodiversity for pure and applied deep-sea
43 environmental research but also emphasises the necessity to integrate such new
44 approaches with traditional morphology-based examination of deep-sea organisms.

45 **Introduction**

46 Prior to global industrialisation, the deep sea was protected from human influence by
47 its remoteness. However, the impacts of human activities have increased rapidly in
48 recent decades (Glover and Smith, 2003; Benn et al., 2010), mainly through waste
49 disposal (e.g. Thiel, 2003; Watters et al., 2010; Miyake et al., 2011; Ramirez-Llodra
50 et al., 2013) and the expansion of fishing and hydrocarbon extraction to bathyal
51 depths on continental margins (e.g. Koslow et al., 2000; Roberts, 2002; Clark, 2009).
52 The mining of metal-rich minerals in environments ranging from abyssal plains to
53 hydrothermal vents and seamounts is a serious prospect in the fairly near future (e.g.
54 Wedding et al., 2013; Fisher et al., 2014; Schlacher et al., 2014; Van Dover, 2014).
55 Such combinations of different direct anthropogenic stressors will likely exacerbate
56 multiple interacting stressors arising from climatic changes acting at a global scale
57 (Mora et al., 2013; Jones et al., 2014), creating major threats to the largest
58 environment on Earth (Ramirez-Llodra et al., 2011). Environmental stresses on whole
59 ecosystems led to a loss of biodiversity observed worldwide, with consequences to
60 ecosystem functioning (Worm et al., 2006; Hooper et al., 2012). It is, therefore,
61 essential to acquire baseline information on deep-sea diversity in order to establish
62 reference data reflecting near pristine or less impacted habitats. Such baseline studies
63 are crucial to the assessment of changes in deep-sea ecosystems resulting from the
64 increasing human activity.

65 The World Register of Marine Species (<http://www.marinespecies.org>) lists 23,708
66 metazoan species found in the deep sea (Fig. 1). Arthropods, chordates, molluscs,
67 annelids and echinoderms dominate this inventory of deep-sea species. Although such
68 richness is certainly an underestimate, there is no consensus on how many deep-sea
69 species exist (Miljutin et al., 2010), in part because of uncertainty concerning their
70 distribution patterns in a vast and chronically undersampled environment (McClain
71 and Hardy, 2010). The lack of publicly available molecular data, with only about one
72 fifth of the inventoried species (4918) being associated with such data in publicly
73 accessible databases (Fig. 1), coupled with the particularly challenging taxonomic
74 identification of meiofauna (Herman and Heip, 1988; Giere, 2008), illustrate clearly
75 the difficulty in assessing metazoan diversity in deep-sea sediments mainly inhabited
76 by small-sized animals (e.g., Thiel, 1975; 1983).

77 The development of DNA barcoding has substantially improved taxonomic
78 knowledge in some groups that are difficult to identify morphologically (Blaxter,
79 2004). Investigation of the molecular signatures of benthic fauna in environmental
80 samples was therefore the logical development of DNA barcoding approaches
81 (Markmann and Tautz, 2005). In recent years, *en mass* sequencing of environmentally
82 derived DNA has expanded rapidly with the availability of high-throughput
83 sequencing technologies, commonly referred to as metabarcoding (Taberlet et al.,
84 2012b). We consider environmental DNA (eDNA) to comprise not only DNA from

85 living species, including their eggs and larvae, but also DNA from fragments of dead
86 organisms, gut contents and extracellular DNA (Taberlet et al., 2012a). Since marine
87 sediments host a tremendous diversity of eukaryotic organisms, metabarcoding is
88 particularly useful because of its potential to explore the biodiversity of all taxa in
89 parallel (Bik et al. (2012b). Such an approach has revealed novel biodiversity in
90 various coastal environments (e.g. Chariton et al., 2010; Fonseca et al., 2010; Bik et
91 al., 2012a; Lallias et al., 2014; Pawlowski et al., 2014; Cowart et al., 2015). However,
92 despite the dynamic expansion of eDNA studies, little metabarcoding information is
93 available for benthic diversity at bathyal and abyssal depths (Pawlowski et al., 2011).
94 High-throughput sequencing of deep-sea sedimentary eDNA has revealed a high level
95 of previously unknown diversity among benthic foraminifera (Lecroq et al., 2011) and
96 other deep-sea protists (Stock et al., 2013). Moreover, the capacity of deep-sea
97 sediments to preserve DNA (Corinaldesi et al., 2011) has allowed inferences to be
98 drawn about the past biodiversity of planktonic and benthic eukaryotes (Lejzerowicz
99 et al., 2013). Metabarcoding has also been used to explore biogeographic patterns of
100 microbial eukaryotes in the deep sea (Bik et al., 2012c; Guardiola et al., 2015). Thus,
101 in many different ways, limited studies on eDNA clearly show the strong potential of
102 metabarcoding in deep-sea biodiversity research where samples are scarce and
103 expensive to collect.

104 Here, we use eDNA metabarcoding to identify gaps in our taxonomic knowledge of
105 deep-sea biodiversity. We address this issue using en masse sampling of sediments
106 from deep-sea environments distributed worldwide from upper bathyal (a few
107 hundred meters depth) to abyssal (4 to 5 km below sea surface) depths. We use our
108 global dataset to (a) test the potential of eDNA metabarcoding to assess deep-sea
109 biodiversity and (b) identify the taxonomic breadth of hitherto unknown benthic
110 diversity.

111 **Methods**

112 *Sampling and DNA extractions*

113 Sediments were collected during various cruises worldwide, mostly at abyssal and
114 bathyal depths (see Table S1 and Fig. 2), and stored at -80°C. The sampling methods
115 differed depending on the cruises (multicores, box cores, grab samples). Subsamples
116 were obtained following a standardised method from the more or less undisturbed
117 surficial sediments collected by the sampling gear. The samples consisted of fine mud,
118 except for the Maud Rise samples that included a larger sand fraction. Only surface
119 sediments (approximately within the first 3 cm) were processed. For each location, 4
120 DNA extractions were performed using MOBIO PowerMax extraction kits with less
121 than 10 g of sediments (corresponding to a volume of between 5 and <10 ml
122 depending on the nature of the sediments). In addition, for Northwest Pacific, Arctic,
123 Southern Ocean and South Atlantic samples, 8 replicates of less than 1 g of sediments
124 (roughly corresponding to 0.7 to 1 ml each) were extracted using MOBIO PowerSoil
125 extraction kits.

126 *PCR amplification and 454 sequencing*

127 Preparation of amplicon libraries for 454 sequencing of the V1-V2 region of the
128 nuclear small ribosomal subunit (18S) followed the protocols described previously
129 (Fonseca et al., 2010). In order to maximise inclusion of metazoans in the primer mix,

130 primer R22 (Blaxter et al., 1998) was modified as follows: R22mod
131 5'CCTGCTGCCTTCCTTRGA3', the primer F04 was left unmodified. Compared to
132 the original R22 primer and based on sequences of various phyla from GenBank,
133 R22mod was shortened to remove mismatches in some groups (cnidarians,
134 echinoderms, priapulids and kinorhynchs) and one ambiguity (R) was added to
135 accommodate the presence of a thymidine (T) instead of a cytidine (C) at this position
136 in brachiopods, bryozoan, kinorhynchs, rotifers and within several other groups. The
137 PCR amplifications were performed directly using the combined 454 adaptors, link,
138 MID (Molecular Identifier) tags and primers. MID tags were inserted only in the
139 forward primers as sequencing was made unidirectionally and 8bp MID tags were
140 used to distinguish between independent samples (Table S2). In order to reduce
141 chimera artefacts created during the PCR (Fonseca et al., 2012), we reduced the PCR
142 cycles to 23-25 cycles. The positive amplifications were identified on a 1.5% agarose
143 gel stained with ethidium bromide. Five amplifications were performed in parallel for
144 each core and extraction method. PCR products for each sample were pooled on a 2 %
145 agarose gel, and then excised bands were purified using the QIAquick PCR
146 purification kit (Qiagen). The purified products were quantified using a Bioanalyzer
147 (Agilent) and sequenced on a 454 Roche GSFLX sequencer on either quartets, or half
148 plates at the sequencing platform of Liverpool University.

149 *Sequence analyses*

150 Sequences were analysed using the QIIME 1.7 (Caporaso et al., 2010). Raw reads
151 were assigned to samples based on MID tags and checked for quality using
152 `split_libraries.py`. All sequences shorter than 200 bp were discarded, minimum quality
153 score was set at 25 and maximum homopolymer run was set at 6 bp. No mismatches
154 in primer or MID tags were tolerated. In order to reduce the potential bias introduced
155 by intragenomic variability and sequencing errors, OTUs were clustered at 97%
156 identities. Cluster seeds were selected as representative sequences for each OTUs
157 using `pick_rep_set.py` in QIIME 1.7. Sequences were aligned using `align_seqs.py` and
158 the aligned s108 SILVA database as template. Chimeric sequences were removed
159 using `identify_chimeric_seqs.py` and ChimeraSlayer in QIIME 1.7 and single
160 singletons (i.e. single sequences present in a single sample) were removed from the
161 dataset.

162 The 18S sequences were then compared, using the BLAST method with an E- value
163 threshold of $1e^{-100}$, against a reference database consisting of a "customised" version
164 of the Silva database s108 release formatted for use within the QIIME pipeline
165 (Caporaso et al., 2010), using `assign_taxonomy.py` in QIIME 1.7. The E-value
166 threshold was empirically identified following the observation that the default
167 parameter (E-value threshold of 0.001) provided an unrealistic number of identified
168 OTUs, which after verification were often only poorly related to the assigned taxa,
169 even at phylum level. Several OTUs were independently compared to the GenBank
170 database and the E-values below $1e^{-100}$ did not allow reliable identification at phylum
171 level in many cases. As a result, we chose to use a strict E-value threshold to limit
172 "folkloric" taxonomic assignments. The database was customised by correcting some
173 obvious misidentification (e.g., a copepod crustacean sequence labelled as octocoral
174 cnidarian) and adding recent deep-sea sequences obtained from public databases and
175 individual sequencing of deep-sea organisms. All "uncultured (marine) eukaryote" or
176 environmental samples identified above the phylum level were also removed from the

177 reference database. Raw 454 reads and reference database are available on the
178 European Nucleotide Archive (Acc. No. PRJEB 13170).

179 After taxonomic assignment, a phylogenetic tree was built with the unassigned
180 sequences and the branch corresponding to metazoans was identified by independent
181 blasts against the GenBank database. All the sequences forming the branch identified
182 with confidence as metazoan were isolated and merged to the metazoan dataset with
183 the following taxonomic assignment “Eukaryota;Metazoa; no blast hit”. However,
184 because of the conservative approach chosen, and based on the limited phylogenetic
185 resolution provided by the fragment analysed, the most basal metazoan OTUs (such
186 as those assigned to sponges) may not have been included in the “metazoan branch”.
187 The decision to restrict the analyses to metazoans reflects the fact that the original
188 primers were specific to metazoans and the observation that one major group of deep-
189 sea protists, the foraminifera, was not found in our dataset, thus providing a biased
190 estimation of deep-sea eukaryotic biodiversity.

191 Alpha diversity was measured using the simple “observed OTU” metric in order to
192 estimate the depth of sequencing for the sediments analyses. OTU networks, linking
193 the OTUs to the different biogeographic provinces in which they were found, were
194 built using `make_otu_network.py` in QIIME 1.7 and drawn in Cytoscape 2.7.0
195 (Shannon et al., 2003) using the unweighted spring embedded layout. Beta diversity
196 analyses were conducted using the unweighted UNIFRAC method (Lozupone and
197 Knight 2005) implemented in QIIME 1.7. This method takes into account the
198 phylogenetic information in the dataset. The unweighted approach allows the use of
199 only qualitative data (i.e. presence/absence) and reduces bias from quantitative results.
200 While this approach increases the importance of rare taxa, the quantitative bias
201 potentially induced by the biomass (Bohmann et al., 2014; Hirai et al., 2015) was
202 estimated too high considering the minimal amounts of sediments used. Unrarefied
203 data allowed us to consider the total diversity recovered from the samples analysed.
204 However, in order to make more objective comparisons between heterogeneous
205 samples, Principal Coordinates Analyses of beta diversity was performed based on
206 hundred rarefied datasets at 4488 metazoan reads per province. The same beta-
207 diversity distances matrices were used to build UPGMA trees. Bootstrap support was
208 calculated based on the 100 rarefied datasets. Biogeographic comparisons are based
209 on the lower bathyal and abyssal provinces described in Watling et al. (2013).
210 According to the latter (Watling et al., 2013), the Mediterranean Sea and the North
211 Atlantic both belong to the same bathyal province BY4. However, based on the
212 differences in our sampling locations and data, as well as the suggestion made by
213 Watling et al. (2013) that this province may require subdivision based on differences
214 in environmental parameters such as temperature, we decided to treat both locations
215 separately and refer to the bathyal Mediterranean as BY4 and the bathyal NW
216 Atlantic as BY4b. Additionally, since Watling et al. (2013) did not divide upper
217 bathyal regions (300-800 m depth) into provinces, we identified our upper bathyal
218 regions by adding a “Z” to the name (i.e. BY1Z, BY9Z and BY11Z).

219 **Results**

220 *Taxonomic composition*

221 After quality checking, and removal of chimera and single singletons (i.e. single reads
222 present in a single sample, rather than reads being single in each of a few samples),

223 the resulting dataset contained 530976 reads (from 3819 to 97456 reads per location),
224 38% of which could be confidently assigned to metazoans using a combination of
225 BLAST and the phylogenetic approach (Table 1). The remaining reads were assigned
226 to various other eukaryotic groups (e.g. Stramenopiles, Fungi). The distribution of
227 reads within the different metazoan groups was highly variable between locations
228 with a large dominance of annelids at nearly half of the sampling sites. Nematodes
229 were also sequenced abundantly in several samples followed by arthropods and a
230 large proportion of reads that could not be confidently assigned by BLAST methods
231 to any specific phylum (Fig 3).

232 OTU richness data showed less variation between locations compared to abundance
233 data (Fig 3). At a clustering threshold of 97%, metazoan OTUs represented between
234 8.4% (S Brazil Basin, abyssal) and 34.8 % (Antarctic Peninsula) of the total
235 eukaryotic richness for each site. Metazoan diversity was largely dominated by
236 nematodes, which formed the most diverse group in all samples (from 25.4% to
237 48.9%). The next most diverse phyla were arthropods (mainly copepods) and annelids,
238 followed by platyhelminthes (Fig 3a). These four groups comprised nearly 88% of the
239 total number of assigned OTUs. The remaining OTUs were assigned to 19 other phyla.
240 Among the unassigned OTUs that could not be reliably recognised using BLAST with
241 the strict E-value threshold selected here, individual BLAST of some OTUS against
242 GenBank database suggests that they likely belong to the phyla Mesozoa and
243 Tardigrada. Combined, the taxa inferred to be present based on our data represented
244 almost all higher-level diversity of marine Metazoa (with the exception of a few
245 minor phyla such as Acanthocephala, Entoprocta and Phoronida).

246 A substantial diversity of orders and families was recovered within each of the three
247 major phyla (nematodes, annelids, arthropods), based on BLAST data. At order level,
248 the diversity was relatively equally distributed among nematodes, with less than a
249 quarter of OTUs belonging to the order Enoplida (Fig. 4a). The arthropods were
250 clearly dominated by harpacticoid copepods (68%) (Fig. 4b), while more than 50% of
251 annelids belonged to the infraclass Scolecida or orders Spionida and Terebellida (Fig.
252 4c). Within orders, a wide diversity of families has been observed in the three major
253 phyla (Fig. 4a-b-c).

254 Phylogenetic analyses of metazoan OTUs (97% identity threshold) showed the
255 uneven distribution of unidentified OTUs (in red in fig. 5) throughout the resulting
256 tree. Although the unknown OTUs were found in almost all taxonomic groupings,
257 several clusters were composed mainly of unassigned OTUs. Our analyses confirmed
258 the impressive diversity of nematodes, representing almost half of the tree (Fig. 5),
259 although a significant part of this diversity may originate from intragenomic
260 polymorphisms (Dell'Anno et al., 2015). Other monophyletic clusters were formed by
261 the superphylum Deuterostomia, the phylum Gastrotricha, the class Ostracoda and the
262 subclass Copepoda. The Copepoda comprised mainly harpacticoids as shown by a
263 comparison between OTUs assigned to copepods and sequences of harpacticoids
264 available in the database (Fig. S2).

265 Several clades were formed by OTUs belonging to different taxonomic groups (Fig.
266 5). The annelids grouped with the molluscs, the kinorhynchans grouped with mites and
267 potential tardigrades while the echinoderms clustered with hemichordates and
268 chordates forming a deuterostome clade. Within deuterostomes, detailed observation
269 showed that echinoderms, hemichordates, vertebrates and tunicates formed

270 independent sub-clusters with tunicates appearing clearly distinct at the base of this
271 group (data not shown). Early metazoans (sponges, placozoans and cnidarians) also
272 formed a monophyletic group branching between the deuterostomes and the clade
273 comprising loriciferans, aceolomorphs and putative mesozoans. Nemerteans were
274 either located within the two clades containing annelids or formed an independent
275 cluster nearby. Within this “nemertean only” cluster, it is interesting to note the
276 presence of a few “platyhelminthes” OTUs assigned to the flatworm “*Nematoplana*
277 sp.”. However, most likely the reference sequence from the public database originates
278 from a misidentified specimen (sequence GenBank D85093). Except for this likely
279 artefactual identification, all platyhelminthes clustered together in a monophyletic
280 group. Interestingly, aside from the platyhelminthes cluster, which already includes a
281 significant number of unassigned OTUs, another large clade is composed exclusively
282 of unassigned OTUs. While no reliable BLAST identification could be obtained, these
283 OTUs appear to be related to acoelomorphs. Although meiobenthic tunicates can be
284 found, the presence of DNA from vertebrates (fish and cetacean) and most likely
285 planktonic tunicates illustrates well the potential of eDNA to amplify not only
286 organisms physically present in the sediments but also both indigenous and
287 allochthonous extracellular DNA.

288 *Biogeographic patterns*

289 When considering all provinces regardless of the depth, out of the 1570 metazoan
290 OTUs recovered, only 3 OTUs were shared among all 13 provinces (a harpacticoid
291 copepod, a nematode and an unassigned OTU), 18 additional OTUs were found in 10
292 to 12 provinces (7 nematodes, 3 copepods and another undefined arthropod, 2
293 annelids, 1 hemichordate and 4 unassigned OTUs). No evidence was found for a
294 higher proportion of predominantly planktonic groups, such as Ctenophora and
295 Chaetognatha, among “cosmopolitan” OTUs, although some tunicates, hydrozoans
296 and halocyprid ostracods observed in the abyssal provinces might have originated
297 from the water column. Such findings correspond with the overall low representation
298 of OTUs originating from the water column.

299 The spring embedded network visualisation of OTUs distributes the provinces on the
300 network in order to minimize the differences in lengths of the edges connecting OTUs
301 to the provinces (i.e. in a similar way as if the edges would be springs connecting
302 balls corresponding to OTUs and provinces). Although easily saturated when
303 including large amounts of OTUs, this method of visualising the distribution showed
304 that the geographic distribution of OTUs appeared not random for several taxonomic
305 groups. In annelids, the OTUs from abyssal provinces tended to cluster together,
306 separated from the upper bathyal provinces by the lower bathyal provinces. Moreover,
307 the two polar lower bathyal provinces grouped near the abyssal ones while the two
308 polar upper bathyal provinces appeared more isolated (Fig. 6a).

309 Such patterns are not visible for all phyla, but the pattern observed in the Nematoda
310 (Fig. 6b, based on OTUs shared by 6 provinces or more) tends to suggest relationships
311 between abyssal provinces, although more appropriate sampling is required to explore
312 biogeography in details. Compared to the patterns obtained with annelid OTUs, the
313 upper bathyal Arctic also grouped close to the South Atlantic and Weddell Sea
314 abyssal provinces. Moreover, the polar bathyal provinces clustered near or even
315 within abyssal provinces in the nematodes data. The network made from arthropod
316 MOTUs (Fig. 6c, including all OTUS) showed a different pattern with the Southern

317 Ocean provinces clustering together, as well as the Arctic and abyssal South Atlantic
318 provinces. The single abyssal Pacific province (NW Pacific, on the edge of the Japan
319 Trench) appears relatively isolated from the other abyssal provinces and the
320 Mediterranean, NW Atlantic and two Andaman Sea provinces all appear quite
321 isolated from each other and from the other provinces.

322 While networks facilitate the visualisation of the OTUs distributions, they are limited
323 to taxonomic groups with limited numbers of OTUs (otherwise the network will
324 saturate) and are directly affected by the sequencing depth (not rarefied). Principal
325 coordinate analyses (PCoA) on rarefied dataset of all metazoan OTUs provided a
326 more robust comparison of the different locations (Fig. 7). Unfortunately, the
327 rarefaction threshold of 4488 metazoan reads per location did not allow the inclusion
328 of several locations in the analyses (e.g. most abyssal locations including all the
329 equatorial and South Atlantic locations, see table 1). Nevertheless, all the locations in
330 Andaman Sea, the province with the most locations sampled, clustered relatively
331 closely together. The two Mediterranean locations also grouped within this cluster.
332 The situation was different for the lower bathyal Southern Ocean, for which the two
333 sampled locations considered did not group together. One possible reason for the
334 difference observed between the Lazarev Sea and the Maud Rise, may be related to
335 the sediment characteristics, as Maud Rise sediments sampled were sandier than the
336 fine muddy sediments from Lazarev Sea. Moreover, compared to the distances
337 between the locations within Mediterranean Sea or within Andaman Sea, the Maud
338 Rise was much more distant from the Lazarev Sea, increasing the possibility of
339 different ecosystems being sampled (Giere, 2008).

340 **Discussion**

341 *Most deep-sea diversity is unknown*

342 Pioneering investigations in the 1960s (e.g., Hessler and Sanders, 1967; Sanders and
343 Hessler, 1969), together with more recent studies (e.g., Snelgrove and Smith, 2002;
344 Brandt et al., 2007; Rex and Etter, 2010) on bathyal and abyssal fauna, have
345 challenged the long-held notion that the deep sea hosts a low diversity of metazoan
346 organisms. Our results, based on the total DNA from the sediments (including
347 organismal and extraorganismal DNA), reveal a large proportion of unassigned OTUs
348 (Fig. 5). Although there is not necessarily a direct correspondence between DNA
349 sequence data and morphological species diversity, these results do suggest that
350 significant unknown diversity exists in deep-sea sediments at different taxonomic
351 levels, supporting the idea of a highly diverse deep-sea fauna. Moreover, the irregular
352 distribution of unassigned OTUs in the phylogenetic tree provides clear evidence that
353 some taxonomic groups are particularly understudied. These less sequenced groups
354 include cryptic and/or fragile organisms such as the acoelomorphs and loriciferans,
355 which are rarely seen in deep-sea samples, as well as several groups of nematodes.

356 Based on the rate at which new taxonomic descriptions are being published, it has
357 been proposed recently that most biodiversity on Earth might be described in the
358 relatively near future (e.g. Appeltans et al., 2012; Costello et al., 2013). A distinction
359 does need to be made between species that have been described taxonomically and
360 those that have only been sequenced (Fig. 1). Nevertheless, by suggesting that
361 important unknown genetic diversity exists within several deep-sea metazoan phyla
362 (Fig. 4), our results tend to challenge these ambitious predictions and support the view

363 that a large part of the planet's biodiversity remains to be discovered in the deep sea
364 (e.g. Grassle and Maciolek, 1992; Poore and Wilson, 1993; Brandt et al., 2007;
365 George et al., 2014). Many of these OTUs could not be assigned to any taxonomic
366 group and some could therefore represent new higher taxa. Unfortunately, no DNA
367 information is available for much of the known deep-sea metazoan diversity (Fig. 1)
368 and their novelty is therefore impossible to confirm. These uncertainties should not
369 undermine the potential of metabarcoding to better understand the diversity of poorly
370 known communities. Indeed, the total information obtained from a large number of
371 taxa in parallel provides a good estimator of environmental community diversity that
372 has many practical applications for ecosystem assessment and monitoring (Chariton et
373 al., 2010; Czernik et al., 2013; Stephenson et al., 2013; Chariton et al., 2014; Lallias
374 et al., 2014; Pawlowski et al., 2014; Willerslev et al., 2014; Guardiola et al., 2015;
375 Lejzerowicz et al., 2015; Pochon et al., 2015; Boschen et al., 2016).

376 Almost all marine benthic phyla were found in the sediments analysed and even with
377 a conservative OTU clustering threshold of 97% and a limited number of samples, a
378 wide diversity of OTUs was identified within the dominant phyla (Fig. 5, Fig. S2).
379 The prevalence of nematodes and other meiofaunal groups is immediately apparent
380 and confirms that meiofauna are an important component of the deep-sea benthic
381 biodiversity. Such community composition is consistent with the slower rate of
382 decline in the abundance and biomass of metazoan meiofauna from bathyal to abyssal
383 regions compared to that of larger animals (macrofauna and megafauna) (e.g., Thiel,
384 1975; Rex et al., 2006; Rex and Etter, 2010). However, the absence of some
385 macrobenthic taxa (e.g. peracarid crustaceans) and particularly of megabenthos such
386 as decapod crustaceans, sea cucumbers and fish, which are very common in the deep
387 sea, can be partially explained by the limited volume of analysed sediment samples
388 (i.e. less than 10 ml per core). The megafaunal (vertebrate) sequences found in our
389 data clearly originate from extracellular DNA and illustrate well the potential of
390 environmental DNA to inform not only on the organisms physically present in the
391 sample but also on DNA traces of large sized species.

392 In comparison with previous studies, we retrieved a lower proportion of metazoans
393 than when the meiobenthos is isolated by decantation and sieving (45-1000 μ m size
394 fraction) prior to DNA extraction (Creer et al., 2010; Fonseca et al., 2010; Bik et al.,
395 2012a; Bik et al., 2012c; Fonseca et al., 2014). This finding is consistent with recent
396 comparisons between different sampling sizes (Brannock and Halanych, 2015).
397 However, in terms of metazoan diversity, our approach of analysing the DNA
398 extracted directly from sediments does not appear to have retrieved significantly
399 different patterns of diversity, as the dominant phyla are similar using both
400 approaches. Brannock and Halanych (2015) recommend the use of elutriated samples
401 (with meiofauna extracted from the sediments) to increase the amount of metazoan
402 reads recovered. However, such approach requires larger volumes of sediments,
403 which are not always available in deep-sea research. Extracting DNA from raw
404 sediments has the advantage of including more extracellular DNA, but also the risk of
405 including DNA from non-benthic organisms. Another study based on abyssal
406 sedimentary DNA suggested that the DNA of planktonic species might account for
407 more than 30% of all eDNA preserved in seafloor sediments (Pawlowski et al. 2011),
408 although our new results do not reflect these findings. The length of the amplified
409 fragment likely explains the higher proportion of benthic diversity observed here. The
410 DNA fragment sequenced in this study was significantly longer (approximately 450

411 bp) than the 150-bp-long V9 region used in Pawlowski et al. (2011). Comparison
412 between different genetic markers or data obtained with different primer pairs should
413 be considered with caution (Hadziavdic et al., 2014). For example, different markers
414 will likely have different evolution rates that will additionally vary between
415 taxonomic groups, leading to confusing taxonomic interpretations of the results
416 obtained. However, overall, targeting larger DNA fragments will favour the
417 amplification of DNA from living organisms, or that of recently dead individuals
418 whose genomic content still persists in good condition in the environment.
419 Considering the logistic difficulties to sample in the deep sea, and the additional bias
420 induced during the meiofaunal isolation process (Bik et al., 2012c), sequencing the
421 total sedimentary eDNA represents a good compromise for exploring the biodiversity
422 of small-sized, deep-sea metazoans.

423 *Biogeographic patterns*

424 Broad spatial distributions are reported among small-sized eukaryotic taxa such as
425 rotaliid foraminifera (Pawlowski et al., 2007; Gooday and Jorissen, 2012), nematodes
426 (Vanreusel et al., 2010; Zeppilli et al., 2011) and harpacticoid copepods (Menzel et al.,
427 2011), as well as certain macrofaunal and megafaunal taxa (Sibuet, 1979; Allen,
428 2008). However, in some cases, detailed morphological and/or molecular re-
429 examination of putative cosmopolitan species resulted in the recognition of cryptic
430 species having much smaller distribution ranges (Moura et al., 2008; Brandão and
431 Yasuhara, 2013; Krapp-Schickel and De Broyer, 2014; Yasuhara et al., 2014). The
432 lower numbers of cosmopolitan taxa in our study originate either from higher than
433 expected biodiversity or undersampling of the vast ocean-floor environment.
434 Unfortunately, we cannot discriminate between these two hypotheses because the
435 samples on which this study is based were collected opportunistically, with different
436 numbers of samples and different sequencing depths at each location.

437 Deep-sea habitat heterogeneity at larger spatial scales is poorly understood but is
438 believed to play an important role in the maintenance of benthic biodiversity on the
439 ocean floor (e.g. Levin et al., 2001; Van Gaever et al., 2009; Vanreusel et al., 2010;
440 Durden et al., 2015). Our data suggest a considerable degree of taxonomic
441 differentiation, and hence biogeographic patterning, between the soft-sediment
442 benthic communities that are represented by our eDNA samples (Figs 6,7). For
443 example, the lower bathyal (1920-2160 m) polychaete data from the Lazarev Sea and
444 Maud Rise (BY9) and those derived from upper bathyal (290-500 m) samples taken
445 on the unusually deep Antarctic shelf (BY9Z) are strikingly different. This is
446 inconsistent with the extended bathymetric ranges often observed among species
447 living around the Antarctic continent (Brandt et al., 2007), but is not surprising given
448 the distinctive nature of benthic communities on the western side of the Antarctic
449 Peninsula (Scotia area; (De Broyer and Koubbi, 2014)). The composition of soft-
450 sediment communities in the lower and particularly the upper bathyal Andaman Sea
451 provinces will almost certainly be influenced by the oxygen minimum zone in this
452 region (Cedhagen et al., 2013). However, further biogeographic interpretation of our
453 data would be inappropriate given the fact that our samples were obtained
454 opportunistically from scattered locations. More extensive sampling, preferably
455 targeted in relation to environmental gradients (e.g. depth, productivity, bottom-water
456 oxygen levels), will be required in order to assess the full potential of eDNA

457 metabarcoding to explore the heterogeneity of deep-sea metazoan communities and
458 their biogeographic patterns.

459 *Future challenges of deep-sea eDNA metabarcoding.*

460 From a molecular perspective, the main challenge for deep-sea metabarcoding studies
461 is to find optimal molecular markers for metazoan species delimitation. Metazoa
462 comprise highly diversified phyla with different rates of evolution (Johnson et al.,
463 2014) and selecting a region of rRNA genes that would have a similar taxonomic
464 resolution for all species is virtually impossible. For example, based on 18S rDNA
465 data publicly available, different species within the deep-sea mollusc genus
466 *Bathymodiolus* share between 99.2 and 100 % identities, while species of the
467 crustacean genus *Paramunida* share between only 91.5 and 99.4% identities and
468 different genera within the cnidarian family Parazoanthidae share between 97.8 and
469 99.7% identities. Consequently both 97% and 99% identity clustering thresholds will
470 merge OTUs representing very different taxonomic ranks depending on the taxa
471 concerned. Even within organisms, a recent study of deep-sea nematodes by
472 Dell'Anno et al. (2015) demonstrates how significant intragenomic polymorphism can
473 impact the interpretation of metabarcoding data. Due to the arbitrary nature of species
474 definitions and evolutionary differences between metazoan taxa, such issue will
475 remain crucial despite efforts made to improve the taxonomic assignments of high-
476 throughput sequencing data (Quince et al., 2011; Morgan et al., 2013).

477 The widely used COI gene has been proposed as an alternative metabarcoding marker
478 to compensate for the lack of resolution of 18S rDNA at species, genus, or even
479 higher taxonomic ranks in meiobenthos taxa (Tang et al., 2012). However, finding
480 conserved COI priming sites in all metazoans is even more problematic than for 18S
481 rDNA (Deagle et al., 2014). Moreover, the level of codon saturation provided by COI
482 precludes us from identifying OTUs without an accurate and complete reference
483 dataset. A test study conducted in parallel with this research has shown that less than
484 10% of the reads obtained for a standard COI fragment could be identified by BLAST
485 (unpublished data). These results support published data on seagrass meadows where
486 93% of the COI OTUs recovered remained unassigned (Coward et al., 2015).
487 Therefore, although the high-resolution power of COI for identifying species provides
488 a significant advantage, the substantial inadequacy of available reference sequences is
489 even more acute than for rRNA genes.

490 From a taxonomic perspective, the main challenge for deep-sea biodiversity research
491 is to expand the reference database. Deep-sea diversity remains largely unknown
492 (Costello et al., 2010; Danovaro et al., 2010) and even when identified using
493 molecular taxonomic approaches, high-level assignment cannot be achieved in many
494 cases. Moreover, a complete and reliable reference database is not only needed for
495 taxonomic assignment but is also essential for post-sequencing processing of the data
496 (Edgar et al., 2011; Quince et al., 2011; Fonseca et al., 2014). As shown in Figure 5,
497 the level of taxonomic identification depends on the group. For example, the copepod
498 clade, several groups of nematodes, and one group of annelids include only a modest
499 proportion of unassigned OTUs. However, other groups are almost entirely composed
500 of unassigned OTUs, suggesting the absence of reference sequences in the database.
501 Overall, the amount and clustering of the unassigned OTUs observed here suggest the
502 existence of largely uncharacterized taxonomic groups and highlights the potential
503 extent of the unknown diversity in the deep sea.

504 DNA barcoding and morphology-based taxonomy have sometimes been perceived as
505 antagonistic approaches (e.g. Ebach and Holdrege, 2005; Trewick, 2008; Boero,
506 2010). However, our results clearly emphasize the absolute necessity to increase
507 taxonomic effort, including morphological analyses as proposed originally for DNA
508 barcoding approaches (Hebert et al., 2003), in order to fully exploit the gigantic
509 amounts of DNA data obtained by metabarcoding. On the one hand, morphological
510 examination of the specimens that compose benthic communities is not always
511 possible and even when possible is often extremely time consuming and usually
512 requires the expertise of specialist taxonomists. Such limitations apply especially in
513 the deep sea, where samples are difficult to obtain and often limited in size. On the
514 other hand, as discussed above and in Dell'Anno et al. (2015), interpretation of
515 metabarcoding data is limited by the reference database available. Therefore, rather
516 than being in competition, the two approaches complement each other in providing a
517 concerted framework that can be used to obtain the most accurate estimation of
518 marine biodiversity on our planet for both pure and applied environmental research.

519 **Conflict of interest**

520 The authors declare that the research was conducted in the absence of any commercial
521 or financial relationships that could be construed as a potential conflict of interest.

522 **Author contributions**

523 FS, JP, SC planned the experiments; FS conducted the experiments, JP, SC, HY, SH,
524 PC, TC contributed to the experiments, FS, SC, JP, SH analysed the data, FS, JP, AG,
525 SC wrote the manuscript; SC, GC, SH, PC critically reviewed the manuscript.

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910 Legend to the figures

911 **Figure 1:** Proportions of deep-sea species with available DNA sequences on
 912 GenBank. The numbers are based on valid species described and registered in the
 913 World Register of Deep-Sea Species. The category “Other” groups Porifera,
 914 Bryozoa, Nematoda, Brachiopoda, Platyhelminthes, Sipuncula, Echiura,
 915 Chaetognatha, Nemertea, Ctenophora, Tardigrada, Hemichordata, Cephalorhyncha,
 916 Dicyemida, Acanthocephala, Entoprocta, Gastrotricha, Phoronida (listed by order of
 917 described species number). . Data accessed 17.10.2014.

918 **Figure 2:** Map of the sampled regions. 1. Arctic (BY1 and BY1Z), 2. NW Atlantic
 919 (BY4b), 3. Mediterranean Sea (BY4), 4. Pernambuco Abyssal Plain (AB2), 5. Brazil
 920 Basin (AB3), 6. Argentinean Basin (AB5), 7. Southern Ocean (AB6 and BY9), 8.
 921 Antarctic Peninsula (BY9Z), 9. Andaman Sea (BY11), 10. NW Pacific (AB13).

922 **Figure 3:** Taxonomic composition. a) Species richness, contribution of OTUs from
 923 different phyla to the total metazoan species richness for each province. b)
 924 Quantitative distribution of the reads from each phyla to the total amount of metazoan
 925 reads for each province. The number of metazoan reads obtained for each location is
 926 indicated above each column. The last column represents the proportions based on all
 927 locations.

928 **Figure 4:** Order and family diversity. Proportions at order (inside chart) and family
 929 (outside chart) levels for a) nematodes, b) arthropods and c) annelids. Different shades
 930 of the same colour in the outside charts indicate different families within each order.
 931 Detailed legends on the family charts are available in Fig. S1.

932 **Figure 5:** Phylogenetic distance tree obtained from all metazoan OTUs. OTUs
933 unassigned using BLAST are named in red. Major taxa identified through
934 independent BLASTs are highlighted. Names within quotation marks indicate taxa
935 corresponding to OTUs that could not be reliably identified by BLAST but that were
936 subsequently identified using the complete GenBank database and phylogenetic
937 distance with reference sequences. Branch lengths are representative of the genetic
938 distances between sequences.

939 **Figure 6:** A) Annelid OTU network. Small dots represent OTUs and larger discs
940 represent provinces. Lines connect OTUs to the provinces they were found. Lines are
941 colored according to depth: blue = abyssal, red = lower bathyal, green = upper bathyal.
942 B) Network of nematode OTUs shared by 6 provinces or more. C) Network of all
943 arthropod OTUs.

944 **Figure 7:** Principal Coordinate Analyses (PCoA) plot of the beta diversity distances
945 obtained from the unweighted UNIFRAC analyses on 100 independent resampling of
946 4488 metazoan reads per province (most abyssal samples did not reach this threshold
947 and were discarded from the analyses). Symbol colour correspond to the
948 biogeographic regions, overlapping ellipses (most often masked by the symbols)
949 represent the interquartile range. Grey triangles = BY9 (Southern Ocean with Lazarev
950 Sea and Maud Rise), pink pentagon = BY1 (Arctic), petrol blue triangles = BY4
951 (Mediterranea), green squares = BY11 (bathyal Andaman Sea), salmon squares =
952 BY11Z (upper bathyal Andaman Sea), yellow diamond = BY9Z (upper bathyal
953 Antarctic peninsula), blue hexagon = BY4b (NW Atlantic), purple circle = AB13
954 (Abyssal NW Pacific).

955

956 **Supplementary material**

957 **Table S1 :** Sampling location and information.

958 **Table S2:** List and sequences of the MID tags used.

959 **Figure S1:** Order and family diversity (as in Fig. 4). Proportions at order (inside
960 chart) and family (outside chart) levels for a) nematodes, b) arthropods and c)
961 annelids.

962 **Figure S2:** Phylogenetic tree of copepods. Reference sequences obtained from
963 GenBank are represented in red. OTUs obtained in this study are in black.

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972 **Table 1:** Amounts and proportions of reads/OTUs for each location. Maximum and
 973 minimum values are indicated in green and red respectively.

Location	Biogeographic region	Eukaryota		Metazoa		Metazoan proportion (%)	
		Reads	OTUs	Reads	OTUs	Reads	OTUs
Andaman S1	BY11	35390	1886	19210	396	54.28	21.00
Andaman S3	BY11	41689	2278	18291	465	43.87	20.41
Andaman S4	BY11Z*	14583	1515	4936	233	33.85	15.38
Andaman S5	BY11Z*	16642	1267	7793	214	46.83	16.89
Mediterranea 890	BY4	26059	1866	8374	280	32.13	15.01
Mediterranea 950	BY4	10881	954	7002	189	64.35	19.81
NW Atlantic	BY4b*	42974	856	4488	123	10.44	14.37
Japan Trench	AB13	56946	1674	21550	248	37.84	14.81
Pernambuco	AB2	20564	1482	2324	165	11.30	11.13
Abyssal Plain	AB3	7651	934	446	100	5.83	10.71
N Brazil Basin	AB3	42480	2383	2658	201	6.26	8.43
S Brazil Basin	AB5	6829	576	1412	165	20.68	28.65
Argentinean Basin	AB5	6829	576	1412	165	20.68	28.65
Antarctic Peninsula	BY9Z*	97456	722	67582	251	69.35	34.76
Weddell Sea	BY9	13295	979	2986	90	22.46	9.19
Lazarev Sea	BY9	11878	924	6543	110	55.09	11.90
Maud Rise	BY9	43974	2371	18600	316	42.30	13.33
Arctic lower bathyal	BY1	37866	2169	6191	258	16.35	11.89
Arctic upper bathyal	BY1Z*	3819	626	1522	132	39.85	21.09
Total		530976	25462	201978	1568	38.04	6.16

974 * indicates regions not listed in Watling et al. 2013, "Z" refers to upper bathyal depths,
 975 while BY4 was split to allow distinction between North Atlantic and Mediterranean
 976 Sea.

Figure 01.JPEG

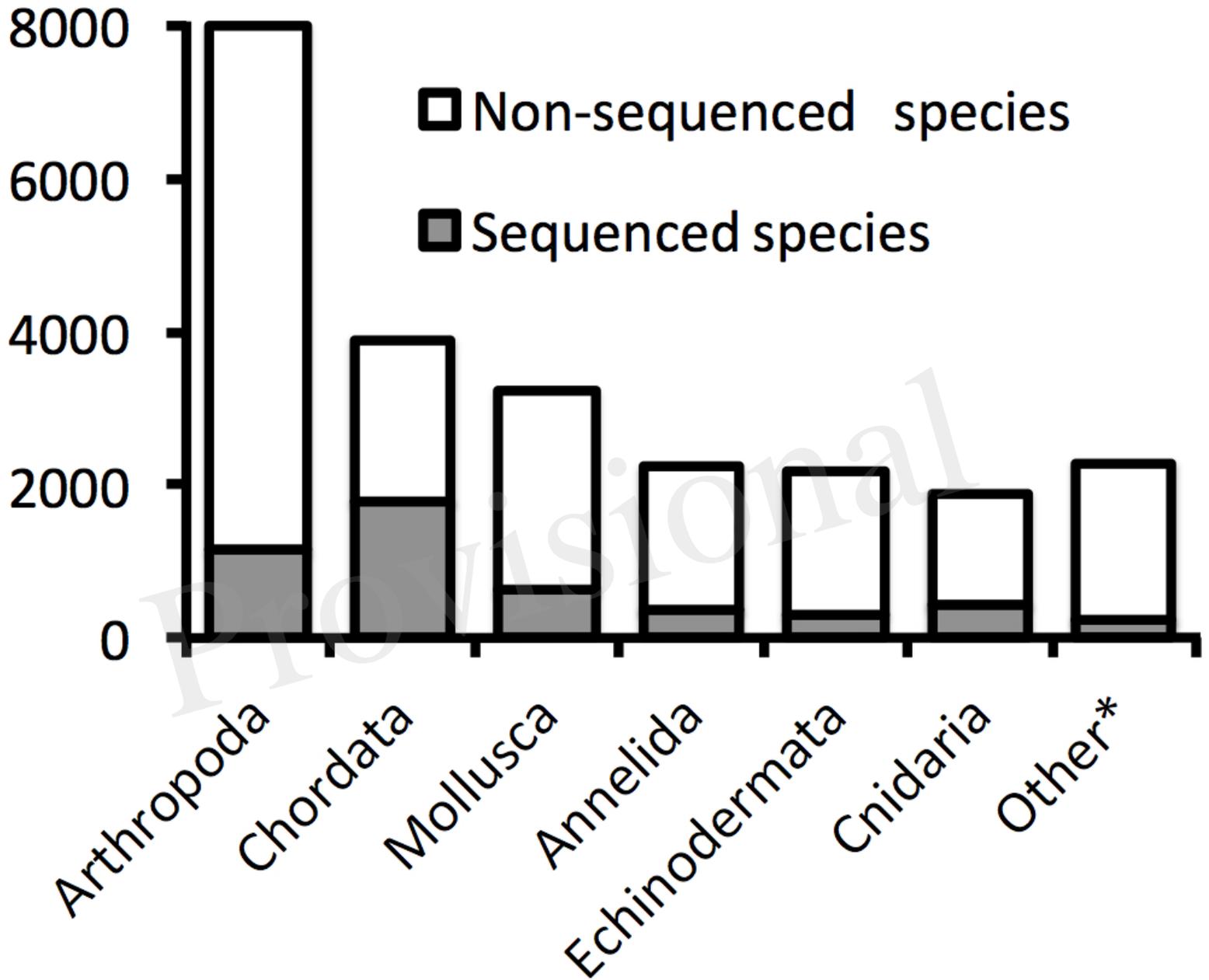


Figure 02.JPEG

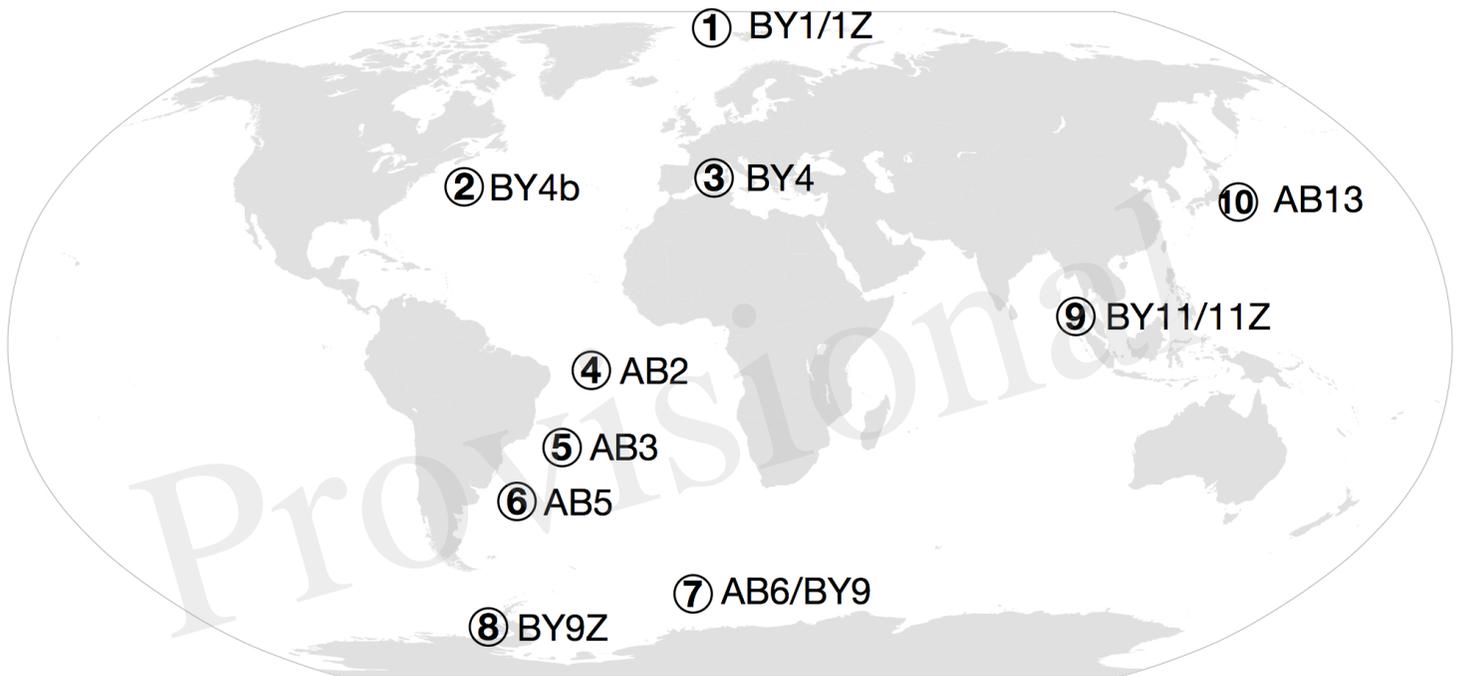


Figure 03.JPEG

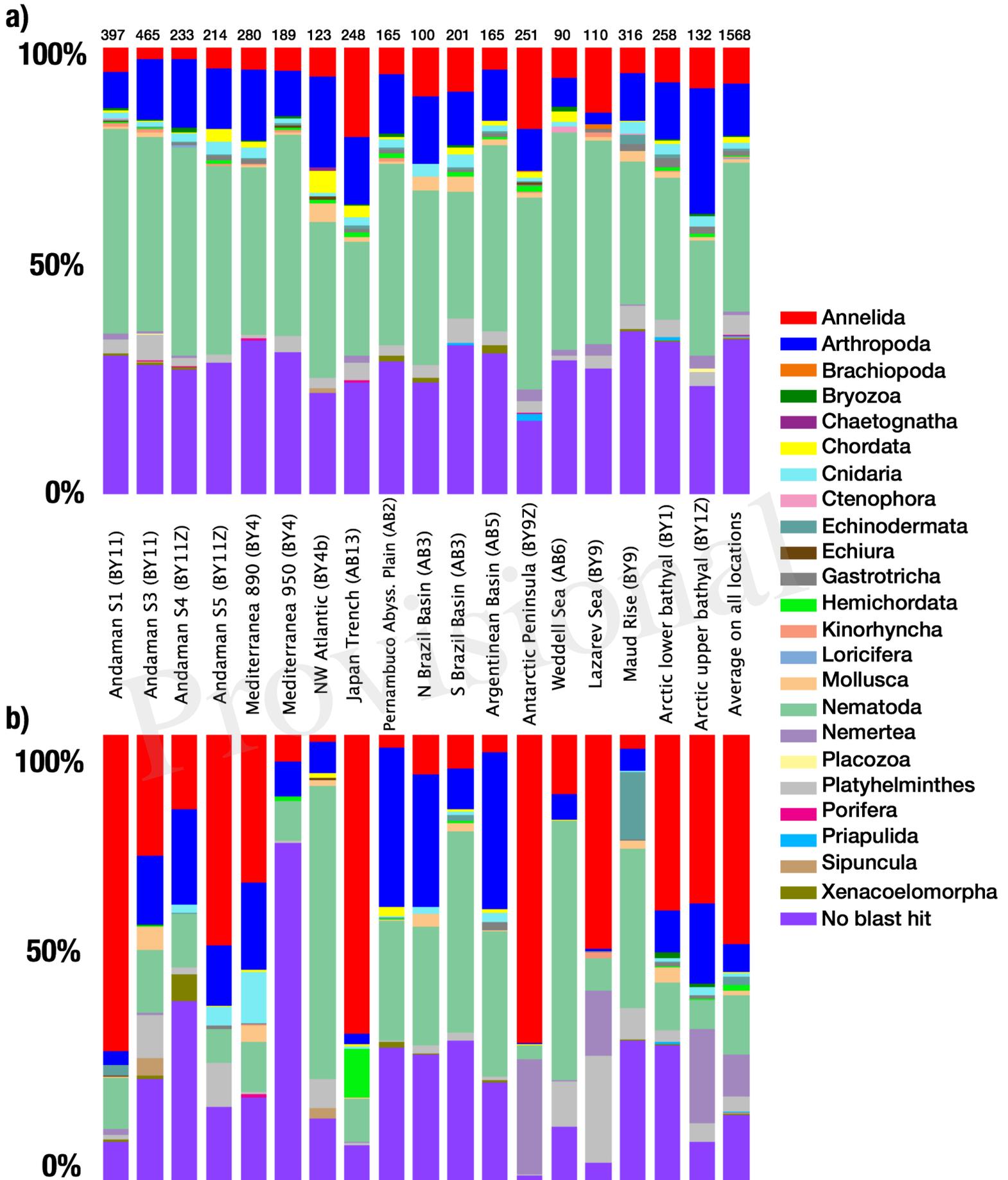


Figure 04.JPEG

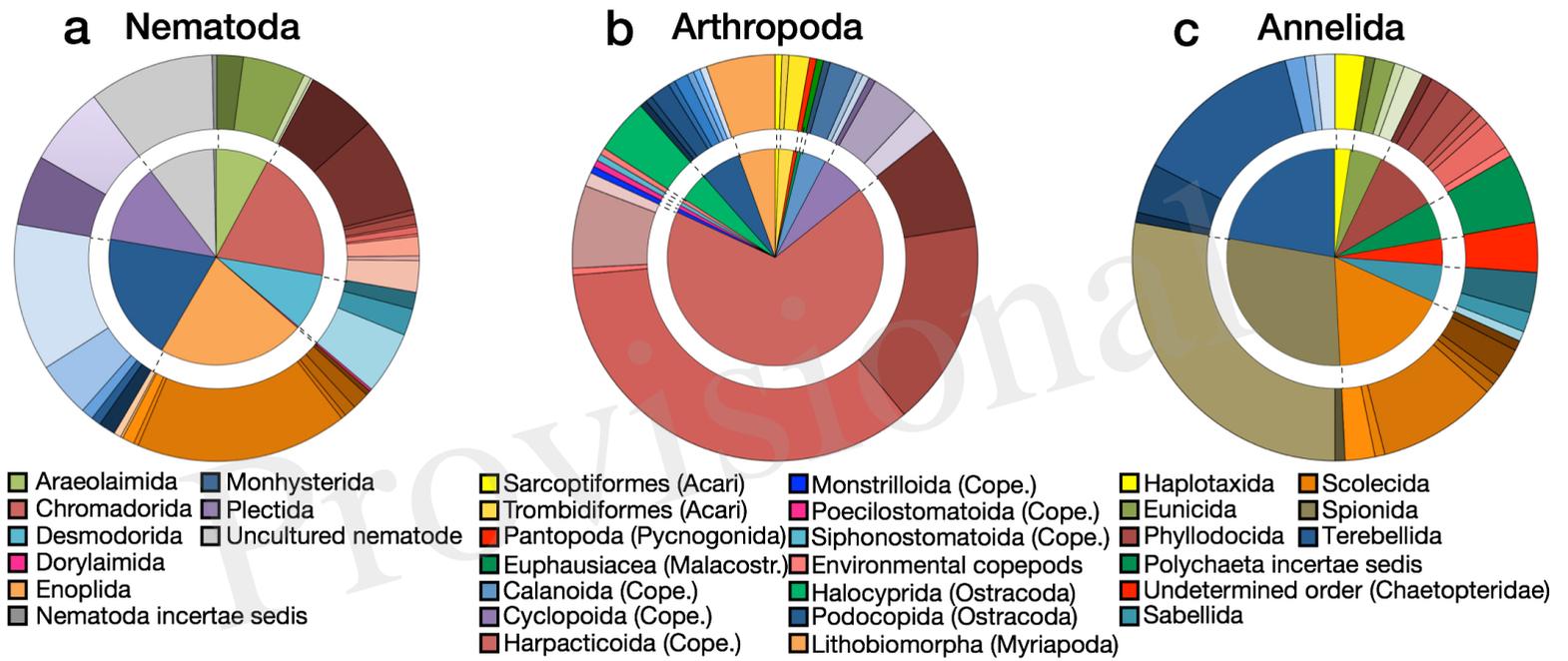


Figure 05.JPEG

