Chronic trace metals effects of mine tailings on estuarine assemblages revealed by environmental DNA

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Abstract

Mine tailing disasters have occurred worldwide and contemporary release of tailings of large proportions raise concerns of the chronic impacts that trace metals may have on the aquatic biodiversity. Environmental metabarcoding (eDNA) offers an yet poorly explored opportunity for biological monitoring of impacted aquatic ecosystems from mine tailings and contaminated sediments. eDNA has been increasingly recognized to be an effective method to detect previously unrecognized small-sized Metazoan taxa, but their ecological responses to environmental pollution has not been assessed by metabarcoding. Here we evaluated chronic effects of trace metal contamination from sediment eDNA of the Rio Doce estuary, 1.7 years after the Samarco mine tailing disaster, which released over 40 million m^3 of iron tailings in the Rio Doce river basin. We identified 123 new sequence variants (eOTUs) of benthic taxa and an assemblage composition dominated by Nematoda, Crustacea and Platyhelminthes; typical of other estuarine ecosystems. We detected environmental filtering on the meiofaunal assemblages and multivariate analysis revealed strong influence of Fe contamination, supporting chronic impacts from mine tailing deposition in the estuary. This was in contrast to environmental filtering of meiofaunal assemblages of non-polluted estuaries. Here we suggest that the eDNA metabarcoding technique provides an opportunity to fill up biodiversity gaps in coastal marine ecology and may become a valid method for long term monitoring studies in mine tailing disasters and estuarine ecosystems with high trace metals content.

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42 **Introduction**

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44 Environmental assessment studies rely on accurate detection of biodiversity of an extremely

45 diverse and small-sized benthic fauna. For decades, morphological methods are the basis to

46 impact assessment (IA) protocols at the cost of neglecting an enormous number of meiofaunal

47 species that could not be accurately identified (Bhadury et al., 2006; Fonseca et al., 2010). There

48 have been considerable advances in recent years by applying DNA-sequence based techniques,

49 commonly referred as metabarcoding, to identify and quantify meiofaunal biodiversity (Creer et

50 al., 2010; Bik et al. 2012; Brannock et al., 2014). Metabarcoding techniques can now be applied

51 to access levels of richness and spatial patterns of diversity on marine Metazoans with use of

52 homologous genes (nuclear 18S rRNA), and help uncover the extreme high biodiversity of

53 meiofaunal benthic assemblages (Fonseca et al., 2010). These modern approaches offer fast

54 assessments of marine Metazoan meiofaunal assemblages and are particularly useful for the

55 identification of new species in areas with poorly reported biodiversity, which may be of special

56 interest in IA studies.

57 Estuarine coastal ecosystems offer an opportunistic case to evaluate biodiversity-environmental

58 relationships through environmental DNA (eDNA) since many estuaries are widely impacted by

59 pollutants with deleterious effects to benthic assemblages (Lotze et al., 2006; Chariton et al.,

60 2015; Hadlich et al., 2018; Lana and Bernardino, 2018). The Samarco mine tailing disaster that

61 occurred in Brazil on November 2015, released near 43 million m^3 of tailings in the Rio Doce

62 river basin, which were transported for over 600 km until reaching the estuary and the Atlantic

63 Ocean (Carmo et al., 2017; Magris et al., 2019). The tailings severely impacted the Rio Doce

64 riverine and estuarine ecosystems causing rapid sediment accumulation, burial and death of

65 benthic organisms, and rapidly (1-2 days) increased sediment heavy metal accumulation by

66 orders of magnitude from pre-impact conditions (Gomes et al., 2017). Although the released

67 tailings had trace metal concentrations that were within the Brazilian legislation (Segura et al.,

68 2016); the iron tailings deposited in the estuarine soils were heavily associated with trace metals

69 which are potentially bioavailable given the redox conditions of estuarine soils (Queiroz et al.,

70 2018).

71 Trace metal accumulation in coastal ecosystems are reported to be highly associated with

72 changes in benthic assemblages and to increase human health risks due to potential

73 bioaccumulation in food webs (Venturini et al., 2002; Muniz et al., 2004; Rainbow, 2007;

74 Hauser-Davis et al., 2015). As a result, impact assessment studies that followed the Samarco

75 disaster were also based on traditional morphological biodiversity assessments (Gomes et al.,

76 2017). The potential chronic pollution effects in the Rio Doce estuary will likely demand long

77 term monitoring programs for this ecosystem. To that end, technical and taxonomic expertise

78 will be of key importance to monitor the estuarine biodiversity, but these efforts are typically

79 limited to the macrofaunal and megafaunal benthic taxa. Therefore, monitoring this

80 environmental disaster by increasing its biodiversity assessment to a broader range of cryptic and

81 meiobenthic taxa may bring valuable information on the extension of impacts.

82 In this study, we used an eDNA metabarcoding approach to evaluate the benthic biodiversity in

83 the Rio Doce estuary 1.7 years after the initial impacts of the Samarco disaster. We hypothesized

84 that spatial patterns of chronic metal contamination in the estuary would be significantly

85 associated with patterns of meiofaunal environmental taxonomic units (eOTUs), evidencing the

86 potential use of this technique for long term impact assessment of the estuary. We targeted

87 benthic meiofaunal eukaryote organisms by amplifying and sequencing the V9 hypervariable

88   region of the 18S ribosomal gene from purified eDNA. In addition, sediment variables (particle

89   size, organic carbon content) and trace metals concentrations were used to test for spatial

90   changes in benthic assemblages in response to contamination levels in the estuary.

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93   **Materials & Methods**

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95   Study site

96   The Rio Doce estuary (19°38′ to 19°45′S, 39°45′ to 39°55′W; Figure 1), is located on the Eastern

97   Marine Ecoregion of Brazil that has two well-defined seasons, dry winter (April to September)

98   and wet summer (October to March), with an average monthly rainfall of 145 mm and

99   temperatures of 24 to 26 °C (Bernardino et al., 2018; Bissoli and Bernardino, 2018). The Rio

100   Doce estuary has been altered by historical human occupation, but ecosystem health of the

101   estuary was poorly known before the Samarco disaster that occurred in November 2015

102   (Bernardino et al., 2016; Gomes et al., 2017). The initial impacts of the Samarco disaster in the

103   estuary were reported by Gomes et al. (2017), and a standard monitoring of benthic assemblages

104   and contamination levels were established in 2017 with a disaster-response program funded by

105   Brazilian government agencies (Fapes, Capes and CNPq). The first monitoring campaign

106   occurred in August 2017 (SISBIO sampling license N 24700-1), approximately 1.7 years after

107   the initial impacts were observed in the estuary, when we aimed to quantify the potential chronic

108   effects of trace metal pollution that were first observed in November 2015.

109

110   Sample collection and DNA isolation

111   Environmental DNA was obtained from two biological replicates of estuarine undisturbed

112   surface (0-5 cm) sediments samples at 22 sites on the Rio Doce estuary in August 2017 (Figure

113   1). The top 5 cm (~300g wet weight) sediment was sampled with DNA-free sterile material and

114   immediately frozen in liquid nitrogen. In the laboratory, all glassware was cleaned and

115   autoclaved between samples to avoid cross contamination. Sediment samples were elutriated in

116   DNA-free material to concentrate benthic metazoans and eDNA was extracted following

117   protocols of Brannock and Halanych (2015), stored at -20 °C and sent to the Genomic Services

118   Laboratory at Hudson Alpha Institute for Biotechnology (Huntsville, Alabama) for

119   metabarcoding sequencing. Briefly, the total DNA from 200 g (ww) of frozen sediments were

120   extracted from each replicate separately with a Mobio PowerSoil(R) kit according to

121   manufacturer's protocol with a 2 min bead-beating step. DNA integrity was evaluated using

122   electrophoresis on 1% agarose gels and DNA purity was assessed with a NanoDrop

123   spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Accurate DNA

124   quantification was obtained using a Qubit® 3.0 Fluorometer (Life Technologies-Invitrogen,

125   Carlsbad, CA, USA). Only 20 stations had enough bulk DNA after extraction and 7 samples out

126   of the expected 40 replicates did not yield high quantities of purified eDNA. In total 33 sediment

127   eDNA samples from the Rio Doce estuary were then submitted to amplicon library preparation

128   and Illumina sequencing (Table 1).

129   Sediment samples were obtained for trace metals, grain size and total organic matter analysis and

130   frozen (-20 °C). Grain size was analyzed by sieving and pipetting techniques (Suguio, 1973).

131   Total Organic Matter (TOM) content was quantified gravimetrically as the weight loss after

132   combustion (500 °C for 3 h). In each station, metal contamination was evaluated from two

133   independent replicate samples. For the total trace metal contents, ~ 1g of dry sediment samples

134   were digested by an acid mixture $(HCl + HNO_3 + HF$; USEPA, 1996) in a microwave digestion

135   system. Following digestion, concentrations of trace metals (Al, Ba, Cr, As, Fe, Zn, Mn, Pb, Cd,

136   Co) in all samples were determined using an inductively coupled plasma optical emission

137   spectroscopy (ICP-OES; Thermo Scientific - iCAP 6200).

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139   Illumina sequencing and bioinformatic pipelines

140   eDNA samples were sent to the Genomic Services Laboratory at Hudson Alpha Institute for

141   Biotechnology (Huntsville, Alabama) for amplicon sequencing. The Eukaryotic-specific V9

142   hypervariable region of 18S SSU rRNA gene was amplified using primers Illumina_Euk_1391f

143   forward primer [GTACACACCGCCCGTC] and Illumina_EukBr reverse primer

144   [TGATCCTTCTGCAGGTTCACCTAC] (Caporaso et al., 2010). The V9 region has been

145   previously has been shown to be accurately identify eukaryotes from environmental samples and

146   has an amplicon length suited to most commercially available Illumina platforms (Amaral-

147   Zettler et al., 2009; Brannock and Halanych 2015). Library size distribution was accessed using

148   a 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). Amplicons were sequenced on MiSeq

149   (Illumina, San Diego, CA, USA) using the Reagent Kit v3 (300bp PE).

150   Demultiplexed raw single-end reads for each sample were processed and analyzed using the

151   2018.8 distribution of the QIIME2 software suite to estimate the observed taxa across replicates

152   (Bolyen et al., 2018). Fastq files were first imported as QIIME2 artifacts with the appropriate

153   import plugin. Single-end reads were then denoised via DADA2 (Callahan et al., 2016) with the

154   dada2 denoise-single plugin, where the --p-trunc parameter was set to 270 to remove low-quality

155   bases and the --p-trim was set to 20 to remove primer sequence. The taxonomic composition of

156   the amplicon sequence variants, generated after running the dada2 plugin, were assigned using

157   the machine learning Python library scikit-learn (Pedregosa et al., 2011). A pre-trained Naïve

158   Bayes classifier, trained on Silva 132 database (Quast et al., 2013) clustered at 99% similarity,

159   was downloaded from QIIME2 website [https://docs.qiime2.org/]. The feature-classifier plugin

160   was used to generate de classification results, and the taxonomic profiles of each sample were

161   visualized using the taxa barplot plugin.

162

163   Statistical analysis

164   Only Metazoan variant calls were selected for ecological analysis. Comparisons of community

165   composition were based on replicate averages of eOTU reads from benthic taxonomic groups.

166   Benthic taxa were grouped for taxonomic comparisons into main taxa including Gastrotricha,

167   Platyhelminthes, Nematoda, Annelida, Crustacea, Mollusca and Cnidaria. Other invertebrate taxa

168   including Gnathostomulida, Micrognathozoa, Tardigrada, Rotifera and Bryozoa were grouped

169   into "Other invertebrates". Unassigned or other taxa (e.g. Insecta) were represented as "Other

170   Metazoa". Taxonomic (eOTUs) accumulation curves (Chao1) were compared across datasets by

171   using: i. full eOTU matrices (Table S1), ii. dominant eOTUs with over 0.1% of total Metazoan

172   reads (Table S2); and iii) the baseline benthic morphological diversity from the Rio Doce estuary

173   (Gomes et al., 2017). Chao 1 curves were based on presence-absence eOTU matrices integrated

174   between replicates from each station and were estimated in Primer-e V6 (Clarke and Gorley,

175   2006).

176   The spatial consistency of metal contamination with benthic assemblage composition was tested

177   with a Canonical Analysis of Principal coordinates (CAP; Anderson and Willis, 2003)

178   complemented with multidimensional scaling (Anderson, 2001; McArdle & Anderson, 2001;

179   Oksanen et al., 2018). Before the CAP analysis was run, the existence of highly correlated

180   variables (trace metals) was assessed and trace metals with significant correlation with Fe

181   contents were removed. The resulting multivariate analysis was only run with sediment contents

182   of Fe, As and Pb, given their non-significant auto-correlations (Table S3). In addition, these trace

183   metals (Fe and Pb) markedly increased (5 to 20-fold) in concentration with the impact (Gomes et

184   al., 2017) and were often above the recommended limits within the Brazilian legislation (Guerra

185   et al., 2017). Given that the concentration of other trace metals were highly correlated with Fe,

186   Fe contents likely represent the overall effect of mine tailings deposited in the estuary (Queiroz

187   et al., 2018).

188   The CAP was run based on presence or absence matrices with full Metazoan eOTUs and with the

189   reduced assemblage composed of dominant reads (>0.1% of reads; Table S2). The CAP eOTU

190   matrices were then compared with environmental (trace metal concentrations, sediment OM, %

191   sand and salinity) spatial patterns based on Euclidean distances matrix to determine vectors that

192   contributed to differences among samples (Mazzuco et al., 2019). Graphical and analytical

193   processing were performed in R project (R Core Team, 2016) with the packages: 'stats' and

194   'vegan' (Oksanen et al., 2018).

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197   **Results**
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199   The Rio Doce estuary exhibited low salinities at the time of sampling (0.1 to 3.7). Sediments

200   were dominated by sand particles (>62% sand), with the exception of site 2 which showed less

201   sand-sized particles (12%; Table 1). Sediment total organic matter (TOM) varied from 1.5 to

202   16.8%, with the highest organic content at stations 3, 13 and 22 (16.8, 13.8 and 10.2%;

203   respectively). Several estuarine areas had TOM in a similar range of 2 to 6.2% (Table 1), and

204   sediment organic content was significantly correlated to Fe content (Pearson $r= 0.5043$, $p=$

205   0.023; Table S4). The concentration of trace metals in the estuarine sediments also varied

206   markedly along the studied area (Table 1). Fe concentrations ranged from 18,814 to 54,982

207 mg.kg^{-1} and were highly correlated with several other trace metals including Al, Cd, Cr, Co, Cu,

208 Mn and Zn (Table S3; Table S5).

209 We obtained a total of 9,836,039 sequence reads, of which 6,840,886 were of high quality. The

210 number of sequence reads per station ranged from 35,915 (St 16) to 359,718 (St 4), with an

211 average of 207,285 total sequence variants per station. Stations that had only one replicate

212 sequenced had a lower (e.g. stations 14 and 16) or a similar number of reads (e.g. stations 19 and

213 20) of sites that had two replicates sequenced. On average, 55.4% of reads corresponded to

214 aquatic or marine Metazoan taxa (Table S1). The eOTU richness per station ranged over three-

215 fold from 16 to 54 eOTUs (Table 1). Assemblages were dominated by Nematoda (34 eOTUs),

216 Platyhelminthes (19), Crustacea (18), Gastrotricha and Annelida (12 eOTUs each; Table S1;

217 Figure 2). Most sites had over 80% of sequence variant reads represented by two to three

218 meiofaunal taxa, including the dominant Gastrotricha, Nematoda and Crustacea. The number of

219 unassigned Metazoan taxa was large (> 50%) at stations 16 and 17; whereas it remained less than

220 20% in most sites.

221 The eDNA species accumulation curves did not reach an asymptote with addition samples

222 suggesting an yet incomplete biodiversity characterization of the estuary (Figure 3). Several

223 eOTUs (N=88) were represented by less than 0.1% of sequence variant reads. When we removed

224 the eOTUs that had less than 0.1% of sequence reads, the species accumulation stabilized at 32

225 eOTUs with 5 to 7 samples, with no additional gain of taxa. The species accumulation asymptote

226 with dominant eOTUs was reached in about half the number of samples necessary in

227 morphology-based studies (12 to 14 samples; Figure 3). The number of meiofaunal eOTUs

228 (eOTU richness) were largely uncorrelated to sediment grain size ($p= 0.161$), Fe ($p= 0.647$) and

229 organic matter content ($p= 0.6395$; Table S4; Figure 4).

230   The multivariate patterns of dominant meiofaunal (S= 32) eOTU composition were significantly

231   related to Fe contents in sediments (F= 2.89, p=0.018, Figure 5; Table 2). The CAP axes 1 and 2

232   explained 44% and 21% of multivariate variability; respectively (Table 2). Fe contents in

233   sediments was associated to the multivariate distribution of meiofaunal eOTUs including the

234   Nematoda *Mesodorylaimus nigritulus* and *Epitobrilus stefanskii*, Harpacticoid copepods, the

235   Platyhelminthes *Cirrifera dumosa* and *Bothrioplana sinensis*, and Ostracods (*Chrissia*

236   *dongqianhuensis*). Monhysteridae and *Desmodorida* spp. nematode worms were negatively

237   correlated to Fe concentrations (CAP1 score= -0.25 to -0.18). Pb and As contamination were not

238   correlated to Fe concentrations in sediments and were not significantly associated with the

239   meiofaunal multivariate composition (Table 2).

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242   **Discussion**
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244   Our study demonstrates that environmental DNA can be an effective method to indicate chronic

245   contamination effects on benthic assemblages of the Rio Doce estuary, supporting our

246   hypothesis. This first eDNA survey in the Rio Doce estuary also revealed a previously

247   unrecognized benthic biodiversity, even with significant impacts by trace metal levels 1.7 years

248   after the initial impacts. Although there is no baseline eDNA assessment from the Rio Doce

249   estuary, the impacted sediments potentially supported over 32 dominant meiofaunal taxa

250   (eOTUs), with a spatial distribution significantly related to Fe (and correlated metals)

251   contamination.

252   The Rio Doce eDNA composition was similar to other estuarine and marine sediments assessed

253   by metabarcoding methods (Fonseca et al., 2010; Faria et al., 2018). Nematoda, Gastrotricha and

254   Crustaceans were highly dominant in the estuary with local changes in relative abundance across

255   sites sampled. The marked spatial variability in assemblage composition within the estuary

256   indicates that benthic assemblages were spatially structured; which is a similar pattern commonly

257   observed in morphology-based assessments. Environmental filtering in benthic assemblages may

258   result from a combination of sediment and water variables, with grain size, salinity and food

259   availability being critical to species turnover and replacement in estuarine benthos (Menegotto et

260   al., 2019). Although our study design does not allow for an hierarchical spatial analysis of

261   variables that determined the observed environmental filtering, the detection of spatial variance

262   in assemblages from eDNA samples suggests that the biodiversity assessment is likely

263   representing living benthic organisms instead of predominantly ancient or allochthone DNA.

264   There is now strong evidence supporting that eDNA techniques can detect complex spatial

265   variability in estuarine and coastal marine ecosystems (Chariton et al., 2015; Faria et al., 2018);

266   and our data additionally supports its use to biodiversity assessment in a heavily impacted

267   estuary.

268   Most eOTUs represented new occurrences for the estuary, but yet with several unassigned taxa,

269   stressing the complementarity value of molecular and morphological approaches to ecological

270   and impact assessment studies (Leasi et al., 2018). We recovered a total of 123 environmental

271   OTUs (eOTUs) in the Rio Doce estuarine sediments, increasing by over 20-fold the previous

272   richness of benthic taxa based on morphological identifications (Gomes et al., 2017). The species

273   accumulation curves did not reach an asymptote with addition of eDNA samples, and most

274   eOTUs (N=88) were represented by less than 0.1% of sequence variant reads, suggesting an yet

275   incomplete biodiversity assessment of the Rio Doce estuary even with high levels of trace

276   metals. However, estuaries are highly connected to continental and marine ecosystems and it is

277   unlikely that species accumulation curves would reach an asymptote with a single biodiversity

278   assessment (Chariton et al., 2015; Nascimento et al., 2018). The rapid increase and stabilization

279   of the number of dominant meiofaunal OTUs with the addition of new samples suggests a

280   reasonable beta-diversity assessment of the Rio Doce estuary with the effort taken. Sites that had

281   only one sequenced replicate due to low DNA stocks attained similar or lower OTUs richness if

282   compared to other stations, but the sediment volumes used in this study (> 200g) were well over

283   the necessary to avoid technical bias in the detection of Metazoan diversity (Brannock and

284   Halanych, 2015; Nascimento et al., 2018).

285   This single eDNA survey was efficient in assembling benthic meiofaunal assemblages in the Rio

286   Doce estuary. The species accumulation curves indicate that half of the sampling effort would be

287   necessary to characterize the dominant meiofaunal groups in the estuary if compared to the

288   species accumulation rate of previous morphological assessments. eDNA metabarcoding can be

289   more efficient at characterizing marine taxa (Lobo et al., 2017), and our data supports its use on

290   long term studies where taxonomic and technical limitations cannot be controlled (Bista et al.,

291   2017). The lack of controlled methods may be a crucial problem to the biodiversity monitoring

292   that followed the Samarco disaster on the Rio Doce, given the extremely large scale and diversity

293   of impacted ecosystems. It is estimated that over 2,000 ha of terrestrial, limnetic and estuarine

294   ecosystems along the Rio Doce basin were directly impacted by the disaster (Carmo et al., 2017);

295   with additional potential effects on nearby coastal zones (Magris et al., 2019). Given the large

296   area, the diversity and natural complexity of ecosystems to be monitored, it is likely that the

297   number of biological samples needed to reach reasonable statistical power to detect biodiversity

298   impacts would be prohibitive (Fairweather, 1991). Therefore, the massive amount of data

299   obtained in metabarcoding techniques could have a profound contribution to environmental

300   monitoring in this scenario, which would also increase dramatically the discovery of cryptic

301   species on a range of aquatic and terrestrial ecosystems.

302   Multivariate analysis revealed that Fe contents (and other correlated metals) are partially

303   structuring spatial patterns of dominant benthic meiofaunal assemblages in the Rio Doce estuary

304   1.7 years after the disaster. The sediment Fe contents were significant predictors of changes in

305   dominant meiofaunal eOTUs including nematodes, copepods, ostracods and flatworms. These

306   groups corresponded to over 2/3 of meiofaunal OTUs in the estuary and revealed that trace metal

307   contents are driving spatial patterns of the Rio Doce estuarine biodiversity. Our data suggest that

308   benthic assemblages were highly sensitive to chronic metal contamination in polluted estuaries,

309   and partially explains a lower effect of sediment grain size and organic matter on local

310   meiofauna (e.g. Faria et al., 2018; Menegotto et al., 2019). This could indicate that the Rio Doce

311   estuarine assemblages were strongly impacted after the disaster through the exclusion of

312   intolerant species, although we lack baseline eDNA to fully support that hypothesis.

313   The extremely high Fe contents allied to covariance of several potentially toxic trace metals that

314   are adhered to iron oxides present in the tailings strongly suggest that the tailings have led to

315   major changes in the estuarine benthic biodiversity since the initial impact (Queiroz et al., 2018).

316   The Rio Doce basin was previously polluted by historical mining and urban activities, so the

317   trace metals that rapidly accumulated in the estuary were likely transported downstream attached

318   to Fe oxides from the released tailings. The initial impacts in the Rio Doce estuary were

319   observed immediately with the arrival of tailings, which led to significant (2-20 times) increases

320   in sediment Fe, Mn, Cr, Ni, Cu, Zn and As (Gomes et al., 2017). Sediment concentrations of Fe,

321   Pb and selected trace metals in the Rio Doce in August 2017 continued to be 2-20 times higher

322   compared to preserved (Piraquê-Açu-Mirim estuary) or polluted estuaries such as the Vitoria

323   Bay, located in a major metropolitan and industrial area ~100km to the south (Hadlich et al.,

324   2018).

325   The statistical lack of As and Pb effects on the multivariate distribution and composition of

326   meiofaunal assemblages have important implications for future environmental monitoring in the

327   estuary. One plausible cause is that not all elements that are accumulated in the sediments are

328   bioavailable and have toxicity to the estuarine biota. Given the amplitude of trace metals

329   accumulated in the Rio Doce sediments since the mining tailing impacts that occurred in 2015, it

330   is very likely that a combination of these contaminants lead to further changes in the estuarine

331   benthos. The sediment concentrations of Pb in August 2017 were over 20 times higher than

332   baseline values (Gomes et al., 2017); and several other trace metals also increased with time

333   since the impact. Queiroz et al. (2018) reported a significant correlation between Fe, Pb and

334   other trace metals in tailing deposits after the initial impacts in the Rio Doce estuary in 2015. The

335   iron oxides from tailings deposited in the estuary have a strong capacity of metal retention

336   (Cornell and Schwertmann 2003; Yin et al. 2016); and they are likely to be released due

337   dissimilatory iron reduction under estuarine conditions (Bonneville et al. 2009; Queiroz et al.,

338   2018; Xia et al. 2019). Although anoxic estuarine soils favor the formation of sulfides (e.g.

339   Pyrite; AVS) which have strong affinity and role in the chelation of metals (Machado et al.,

340   2010; Nóbrega et al., 2013), the Rio Doce estuary contrast to several other estuaries due to low

341   salinity, low tidal influence and an apparent limited sulfate availability and sulfate reduction that

342   reduce the formation of sulfides (Queiroz et al., 2018). As a result, the observed relationship of

343   meiofaunal assemblages with Fe contents (and other trace metals) suggest that the tailings have

344   some toxicity to benthic organisms even though a number of contaminants may not achieve

345   alarming concentrations.

346   The effects of trace metal contents on the Rio Doce benthic assemblages resemble impacts in

347   other areas that are highly polluted with trace metals, but these effects could be confounded with

348   the constant environmental changes that typically occur in these ecosystems (Krull et al., 2014;

349   Martins et al., 2015). Our approach of selecting dominant meiofaunal OTUs to multivariate

350   analysis led to positive detection of Fe contents effects and associated trace metals. This

351   approach was justifiable given that we detected 88 eOTUs with less than 0.1% of sequence

352   variant reads, which could be potentially associated with allochthone DNA from connected river

353   or ocean ecosystems and would not be under influence of local contaminants. The use of

354   indicator taxa or functional groups to eDNA biodiversity assessment studies is becoming practice

355   in ecological studies (e.g. Bista et al., 2017) and our approach offers an important

356   methodological approach for detection of trace metals effects in aquatic biota that need to be

357   further investigated in other case studies.

358
359   **Conclusions**
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361   In conclusion, our eDNA survey of benthic meiofaunal assemblages in the Rio Doce estuary

362   detected environmental filtering with strong influence of contamination by Fe and other

363   correlated trace metals, supporting chronic mine tailing impacts in the estuary. Our study is also

364   in agreement with previous assertions that ecological inferences from eDNA analysis may

365   increase the performance of biodiversity assessments in marine ecosystems by capturing a range

366   of cryptic taxa, thus greatly improving current short and long-term impact assessment studies.

367   The use of eDNA to the Samarco mine tailing disaster would benefit monitoring assessments

368   with standard techniques and dramatically increase our knowledge of the biodiversity of cryptic

369   aquatic species. The continued sampling and monitoring would also increase the precision of the

370   eDNA assessments, especially if allied to detailed morphological work.

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Acknowledgements

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375 We thank students that helped on field sampling.

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559

Figure 1

Map of the study site

Map of sediment sampling stations at the Rio Doce estuary, Brazil in August 2017

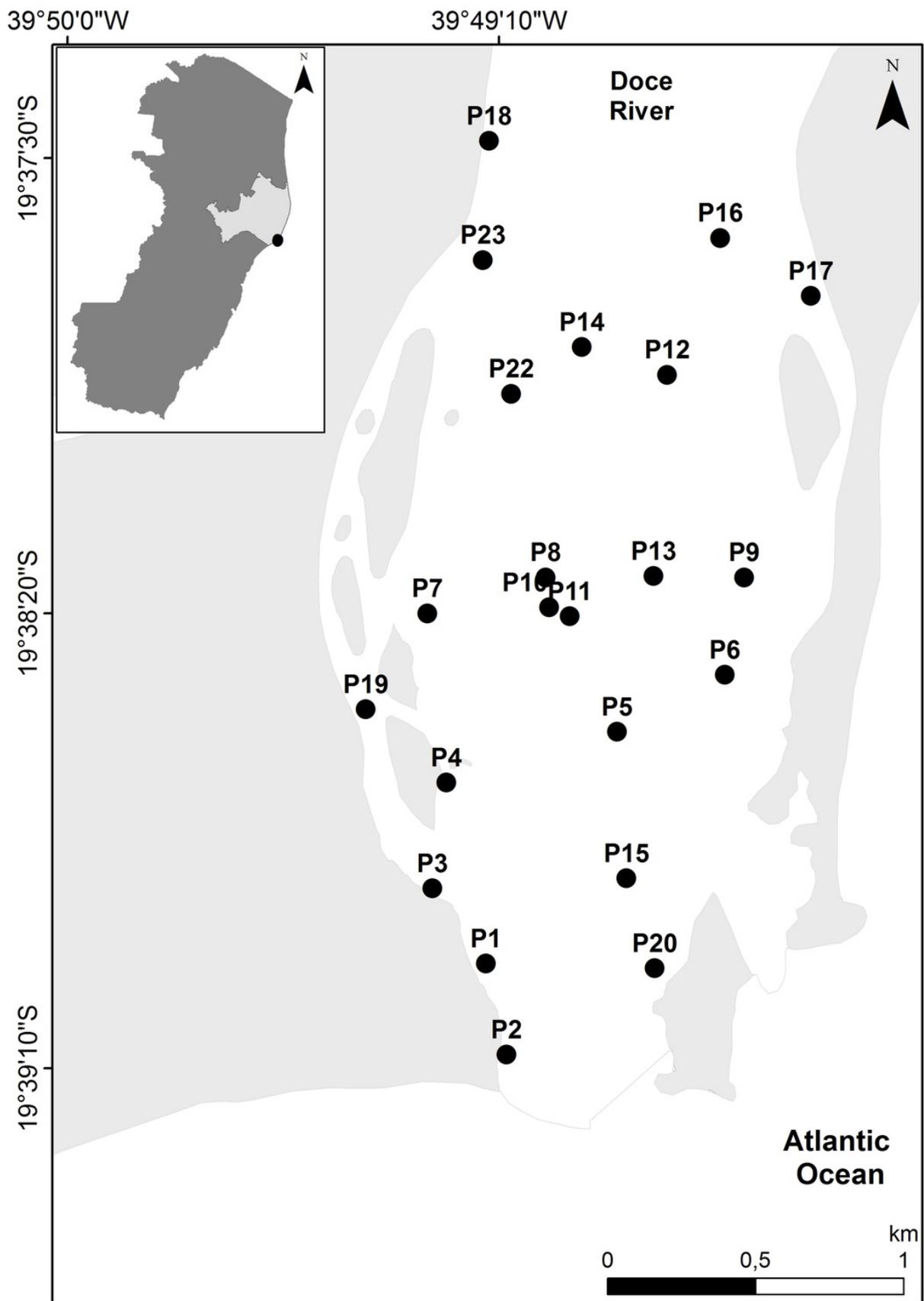



Figure 2

Benthic assemblage composition of the Rio Doce estuary

Benthic meiofaunal assemblage composition based on eDNA samples from the Rio Doce estuary in August 2017.

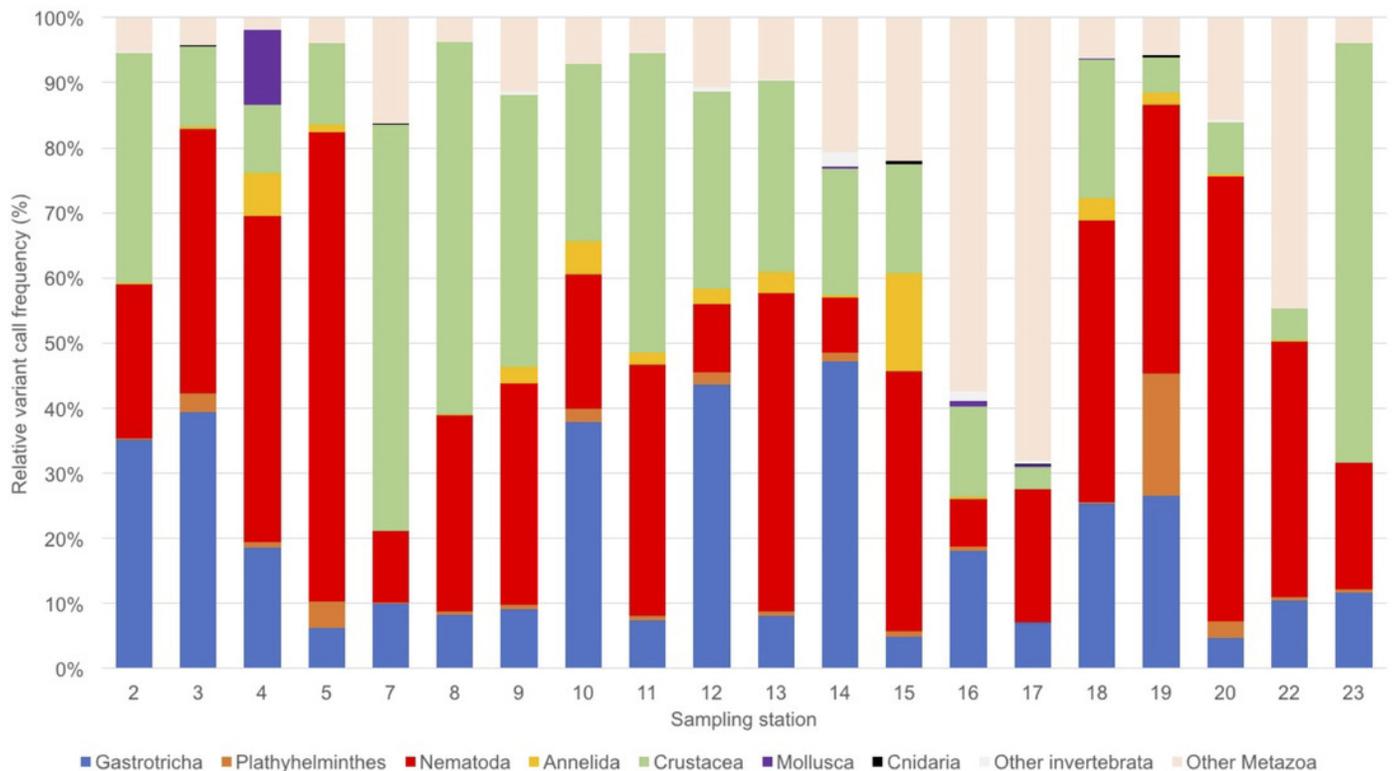



Figure 3

Taxa accumulation curves from eDNA samples

Taxa accumulation curves (Chao1 index) based on full eOTU matrices (eDNA, blue dotted line), dominant eOTUS (>0.1% sequence reads; eDNA_dom black dotted line) and on morphology-based macrofaunal pre-impact assessments (author's data published on Gomes et al., 2017) in the Rio Doce estuary.

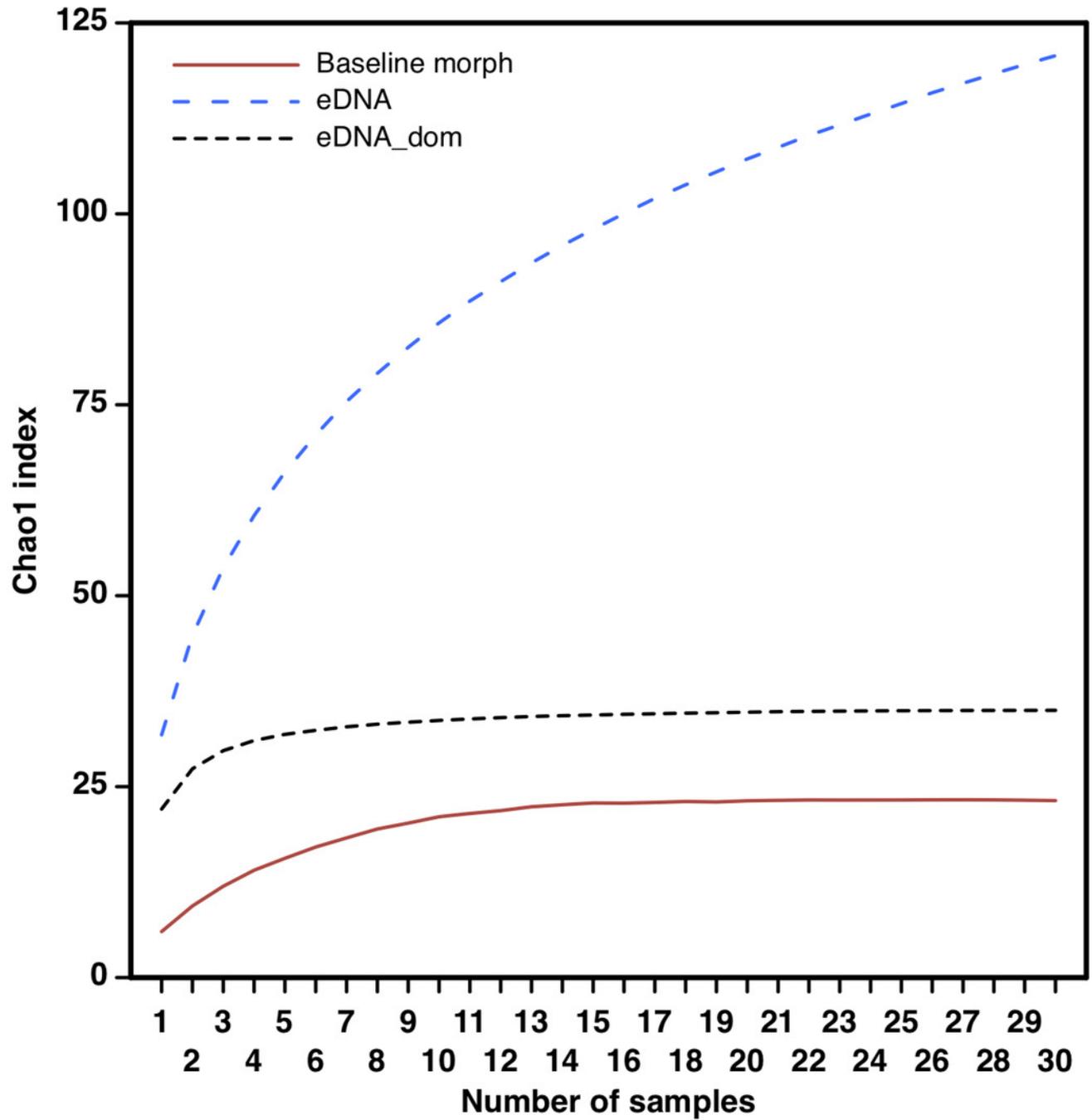



Figure 4

Correlation of eOTU richness with sediment Fe and TOM content

Correlation of eOTU richness with sediment Fe and TOM content across all sampling stations in the Rio Doce estuary in August 2017

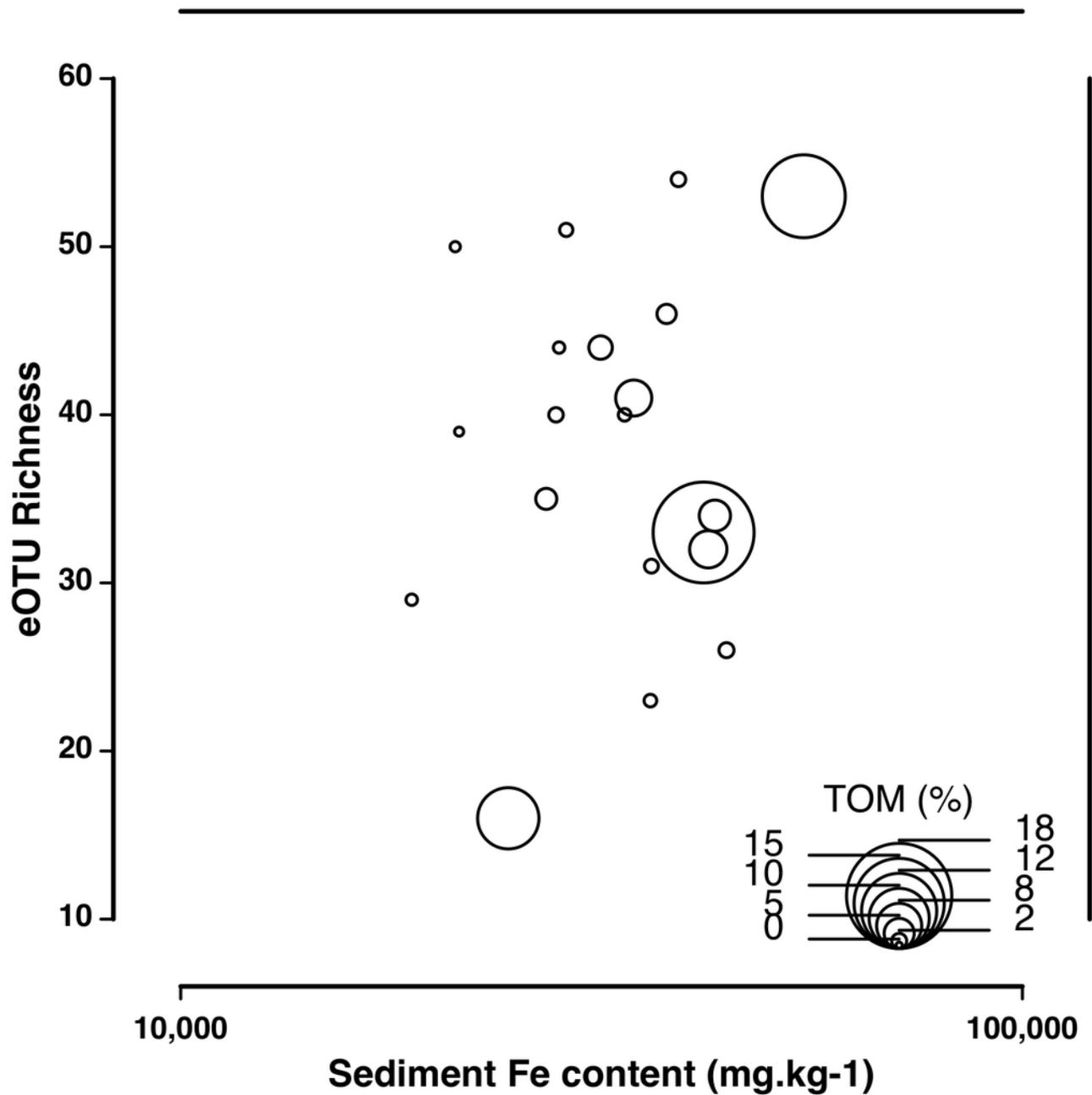
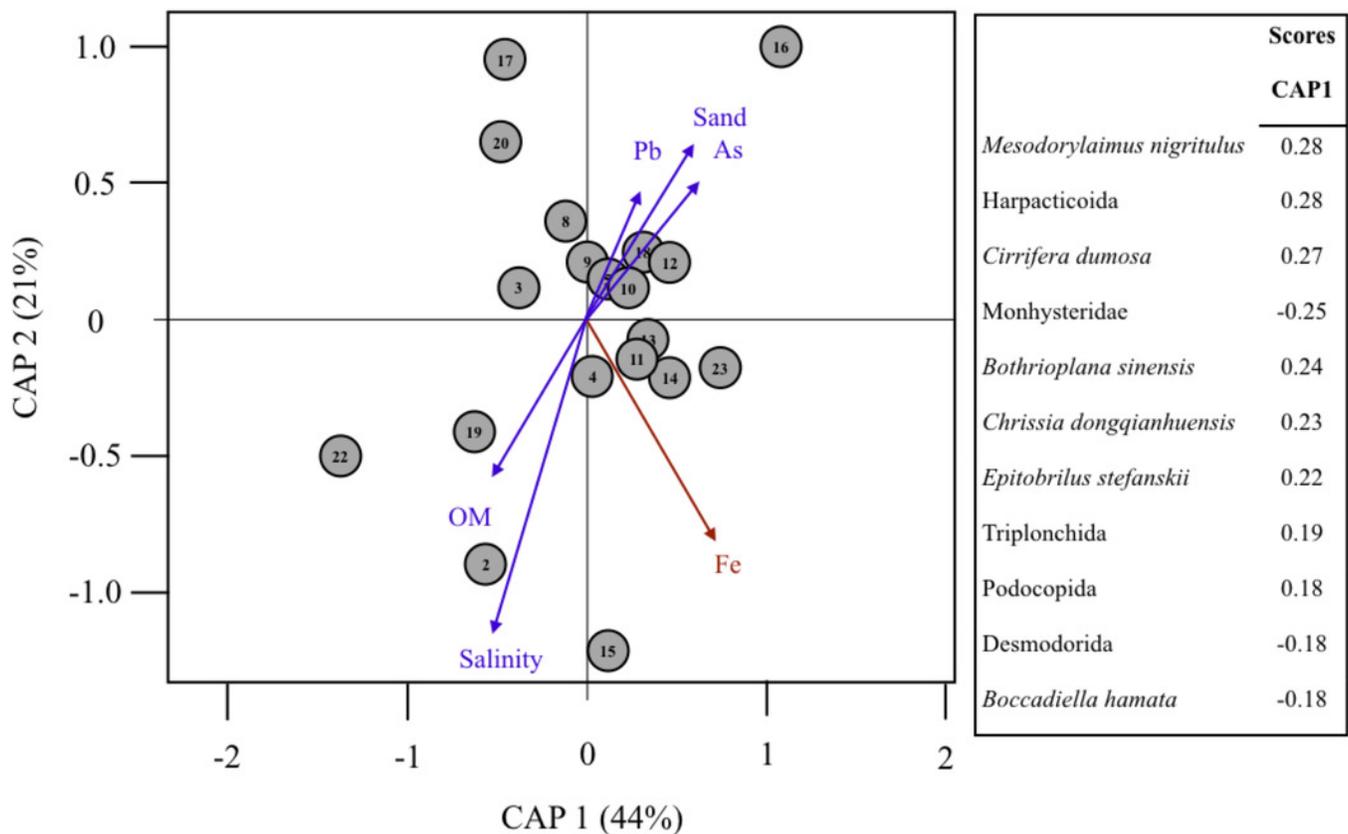



Figure 5

Multivariate analysis of assemblage composition and environmental filtering in the Rio
Doce in August 2017

Canonical analyses of principal coordinates (CAP) ordination of samples according to
multivariate distribution of dominant eOTUS (>0.1% total SV reads) in the Rio Doce estuary.
The strength and direction of environmental effects (Spearman correlation values with p<
0.5 in red) on biological assemblages is represented by arrows of variable size.
Environmental variables were based on Table 1 (Fe, Pb, As, Salinity, TOM and %Sand). Taxa
scores indicate OTUs mostly correlated to site differences. Proportion of variance explained
by axis 1 and 2 are in parenthesis. Symbol numbers indicate sampling station.

Sediment, eDNA and environmental variables in the Rio Doce estuary

Salinity, Sediment total organic matter (TOM, %), particle size (% sand), concentration of trace metals (Fe, As and Pb), and Number of sequence variant reads (reads SV) and richness per station. All data sampled in August 2017 or 1.7 years after the Samarco disaster. Trace metals averaged from N=2 replicates (SE). N. number of eDNA replicate samples sequenced per station. Reads SV. Total marine/aquatic meiofaunal sequence variants.

1 *Table 1. Salinity, Sediment total organic matter (TOM, %), particle size (% sand), concentration*
2 *of trace metals (Fe, As and Pb), and Number of sequence variant reads (reads SV) and richness*
3 *per station. All data sampled in August 2017 or 1.7 years after the Samarco disaster. Trace metals*
4 *averaged from N=2 replicates (SE). N. number of eDNA replicate samples sequenced per station.*
5 *Reads SV. Total marine/aquatic meiofaunal sequence variants.*

Station (N)	Salinity	TOM (%)	%sand	Fe (mg.kg⁻¹)	As (mg.kg⁻¹)	Pb (mg.kg⁻¹)	Reads SV	Total SV richness
2 (2)	1.0	6.2	12	42,343 (2,468)	2.3 (0.1)	56.9 (4.8)	256,072	32
3 (2)	0.6	16.8	72	41,808 (1,278)	10.1 (14)	77.8 (2.7)	265,363	33
4 (2)	0.3	2.1	90	33,681 (2,429)	4.5 (1.6)	173.3 (7.8)	359,718	40
5 (2)	0.3	2.2	95	28,710 (3,686)	1.6 (2.2)	115.4 (2.8)	293,669	51
7 (2)	0.4	2.1	64	36,142 (134)	0.1 (0.2)	74.5 (7.5)	101,127	23
8 (2)	0.2	1.5	96	21,419 (3,212)	0.1 (0)	134.8 (5.8)	238,735	39
9 (2)	1.0	1.9	91	28,155 (1,391)	28.8 (34.3)	111.4 (40.7)	254,335	44
10 (2)	0.2	3.5	89	27,184 (227)	0.1 (0)	83.1 (5.9)	226,548	35
11 (2)	0.2	5.2	70	43,116 (2,768)	0.1 (0)	67.3 (4.3)	272,299	34
12 (1)	0.1	2.4	84	39,029 (11,713)	13.3 (16.2)	174.4 (28)	132,722	54
13 (2)	0.2	13.8	91	54,983 (4,157)	3.9 (5.5)	117.3 (12.9)	236,707	53
14 (1)	1.6	2.4	86	27,920 (7,793)	0.1 (0)	30.3 (11.8)	71,648	40
15 (2)	3.7	6	85	34,532 (1,980)	16.7 (15.6)	78.2 (4.6)	320,192	41
16 (1)	0.3	3.9	90	31,539 (1,001)	0.0 (0)	33.1 (2.1)	35,915	44
17 (2)	0.1	1.7	90	21,191 (42)	2.1 (0.2)	192.9 (15.1)	54,355	50
18 (2)	0.2	3.2	88	37,781 (1,120)	11.2 (2.7)	160.8 (6.2)	222,481	46
19 (1)	0.4	2.3	93	36,244 (801)	3.7 (0.5)	118.0 (3.5)	103,987	31
20 (1)	1.9	1.9	62	18,814 (94)	0.1 (0)	14.2 (1.5)	139,712	29
22 (1)	1.3	10.2	91	24,501 (3,804)	0.1 (0)	16.0 (6.9)	69,713	16
23 (1)	0.3	2.5	89	44,506 (1,079)	4.9 (2.2)	99.1 (7.6)	133,700	26

Results of the canonical analysis of principal coordinates

Results of the Canonical Analysis of Principal coordinates (CAP) testing the contribution of sediment (TOM%, sand content), water salinity and concentrations of trace metals in sediments (As, Fe, Pb) to the multivariate distribution of meiofaunal (eDNA) assemblages in the samples from Rio Doce estuary. Spearman correlation values for each sediment variable are described for in CAP axis 1-2. Note: proportion of variability explained by CAP axes are highlighted, F for statistic, significant results ($p < 0.05$) are in bold.

1 Table 2. Results of the Canonical Analysis of Principal coordinates (CAP) testing the
2 contribution of sediment (TOM%, sand content), water salinity and concentrations of trace
3 metals in sediments (As, Fe, Pb) to the multivariate distribution of meiofaunal (eDNA)
4 assemblages in the samples from Rio Doce estuary. Spearman correlation values for each
5 sediment variable are described for in CAP axis 1-2. Note: proportion of variability explained by
6 CAP axes are highlighted, F for statistic, significant results ($p < 0.05$) are in bold.

	All eOTUS (N=123)				Dominant eOTUS (N=32)			
	axis1 0.33	axis2 0.29	F	*p*	axis1 0.44	axis2 0.21	F	*p*
Salinity	-0.63	0.09	1.24	0.223	-0.30	-0.71	1.60	1.77
OM	-0.55	-0.18	0.89	0.485	-0.33	-0.36	0.71	0.592
Sand	0.71	0.26	1.01	0.413	0.37	0.40	1.17	0.272
As	0.02	0.26	0.63	0.815	0.39	0.31	0.79	0.506
Fe	-0.29	0.46	1.45	0.135	0.43	-0.51	2.89	**0.018**
Pb	0.70	-0.08	1.11	0.303	0.19	0.28	1.59	0.160

9
10