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1 **Microbial indicators along a metallic contamination gradient in tropical coastal**
2 **sediments**

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21

22 **Abstract**

23 The structure and diversity of microbial community inhabiting coastal sediments reflect
24 the exposition to contaminants. Aiming to assess the changes in the microbiota from
25 Sepetiba Bay (SB, Brazil) sediments, correlations between the 16S rRNA gene data (V4-
26 V5 region), metal contamination factors (CF), and the ecological risk classification
27 provided by the Quality Ratio (QR) index were considered. The results show that
28 microbial diversity differs significantly between the less (SB external sector) and the
29 most (SB internal sector) polluted sectors. Also, differences in the microbial community
30 structure regarding the ecological risk classifications validated the QR index as a reliable
31 tool to report the SB chronic contamination. Microbial indicator genera resistant to metals
32 (*Desulfatiglans*, *SEEP-SRBI*, *Spirochaeta 2*, among others) presented mainly anaerobic
33 metabolisms. These genera are related to the sulfate reducing and methanogenic
34 metabolisms probably participating in the natural attenuation processes but also
35 associated with greenhouse gas emissions. In contrast, microbial indicator genera
36 sensitive to metals (*Rubripirellula*, *Blastopirellula*, *Aquibacter*, among others) presented
37 mainly aerobic metabolisms. It is suggested that future works should investigate the
38 metabolic functions to evaluate the influence of metallic contaminants on microbial
39 community inhabiting SB sediment.

40

41 **Environment implication**

42 The paper aims to decipher the impact of metal contamination on microbial communities
43 in Sepetiba Bay (RJ-Brazil) sediments in order to assess the environmental quality status
44 that we believe falls within the journal scope.

45 The ecological risk of sediments, based on Quality Ratio (QR) index, combined with
46 metal content and 16S rRNA gene metabarcoding data, revealed that the most polluted
47 sites own sulfate-reducing and methanogenic metabolisms, participating thus to
48 greenhouse gas emissions. The microbial community changed according to metal content
49 and QR status, allowing the identification of microbial taxa metal-tolerant/resistant and
50 metal-sensitive, which represent bioindicator taxa for environmental management.

51

52

53 **Keywords:** Risk assessment, metal pollution, microbial ecology, coastal sediments,
54 Quality Ratio index, co-occurrence network

55 1 Introduction:

56

57 Worldwide, the anthropogenic activities that lead to the economic and urban development
58 around coastal areas result in the release of contaminants such as metals, that accumulate in
59 sediments impacting the microbial community diversity, structure, and functional dynamics (Duran
60 et al., 2015a,b; Pringault et al., 2008). Prokaryotic organisms (Bacteria and Archaea) are the most
61 abundant component of the microbial community in the aquatic environment (Danovaro et al.,
62 2010; Whitman et al., 1998). Because of their fast response to environmental disturbances and
63 metabolic diversity (Flemming and Wingender, 2010; Harrison et al., 2007), they play a relevant
64 role in the nutrient remineralization processes (Caruso et al., 2015; Dell'Anno et al., 2003), making
65 them available to the entire trophic web, and also in the transformation/detoxification of
66 contaminants (Chen et al., 2018; Nogales et al., 2011). Environmental changes caused by
67 anthropogenic disturbances (e.g., metal pollution) could damage the ecosystem services provided by
68 the microbial community (Birrer et al., 2019).

69 In pollution scenarios, microbial indicators could be used to provide information on the
70 changes of pollutants over time and space (Hamza-Chaffai, 2014) because contaminants exert a
71 selective pressure in the microbial community resulting in the alteration of their structure and
72 composition (Ben Salem et al., 2019; DeLong et al., 2006; Duran et al., 2015; Flemming and
73 Wingender, 2010). However, despite the valuable information they provide regarding the
74 environmental health status (Astudillo-García et al., 2019), the development of microbial indicators
75 as tools for monitoring environmental quality need further improvements (Caruso et al., 2015).

76 Since the 1960s, the Sepetiba Bay (SB, Rio de Janeiro – Brazil) is chronically contaminated
77 by metals (mainly Zn and Cd) because of the Companhia Siderúrgica Mercantil Ingá SA activity.
78 Now a day, SB receives metallic contaminants from industrial, harbor, and tourism activities as well
79 as from urban expansion. The direct release of domestic effluent into the rivers of the SB watershed,
80 covering a population of almost 8 million inhabitants is the major source of pollution (Barcellos et
81 al., 1997; IBGE, 2011; Lacerda and Molisani, 2006; Monte et al., 2015). Therefore, careful
82 monitoring of the SB environment quality with appropriate and accurate tools is urgently requested,
83 once the Brazilian legislation proposes only *ex-situ* bioassays tool for monitoring dredged coastal
84 sediments (CONAMA, 2012).

85 Recently, Nascimento et al. (2019) developed the Quality Ratio (QR) index as an alternative
86 monitoring tool. The QR index, based on the integration of physical/geochemical variables listed as
87 essential for monitoring the quality of dredged coastal sediment, as established in Brazilian
88 legislation (CONAMA - Conselho Nacional do Meio Ambiente, 2012), and the *in situ* enzymatic
89 biomarker response of the microbial community, allows classifying the potential ecological risk of

90 sediments for benthic biota. A cluster analysis considering the QR variables divided the SB samples
91 into two groups (internal and external sectors), and the index showed that the SB sediments present
92 low ($QR \geq 10^{-1}$), moderate ($10^{-2} \leq QR < 10^{-1}$), and high ($QR \leq 10^{-3}$) risks according to their location
93 (Moreira et al., 2021). The stations most exposed to human activities, showing the highest metallic
94 contaminant levels, exhibited QR high risk. Also, the QR index attests that the benthic microbial
95 community shows alterations in the energy metabolism (represented by the Esterase – EST - and
96 Dehydrogenase – DHA - activities) when exposed to multiple contaminant, indicating that a shift in
97 the microbial community diversity / structure might occur. The present study aimed to determine
98 the microbial diversity and structure along a gradient of metallic contamination in SB sediments.
99 We hypothesize that the variations in the community structure might represent the chronic exposure
100 to metals identifying specific microbial taxa, which might represent potential bioindicators for
101 metal contamination. Such bioindicators represent potential tools reporting the influence of metallic
102 contaminants on microbial assemblages and on natural attenuation processes. Since we observed
103 that the number of bacterial cells is not correlated with the degree of contamination of metals,
104 defined as the sum of contamination factor of metals (ΣCF) (Moreira et al., 2021), we believe that
105 the microbial community inhabiting the stations with the lowest QR values (high risks for biota) are
106 composed by taxa resistant to contamination, thus differing from the microbial taxa inhabiting the
107 stations with high QR (low risk).

108

109 **2. Materials and Methods**

110

111 2.1 Site description

112 Sepetiba Bay is located between latitudes 22°55'00" S and 23°03'60" S and longitudes
113 43°56'30" W and 43°36'20" W, 60 km distant from Rio de Janeiro city (Brazil). It is highly
114 influenced by the tidal cycle (Signorini, 1980), ranging from 419 to 447 Km² with an average depth
115 of 6 m, 6-day water renewal time, and a volume of 2.56×10^9 m³, approximately (Barcellos et al.
116 1997; Molisani et al. 2004; Smoak et al. 2012).

117 The São Francisco Channel is the main tributary of the 10 rivers that make up the drainage
118 basin, being responsible for 86% of the freshwater inputs (Barcellos et al. 1997; Molisani et al.
119 2004; Smoak et al. 2012). According to IBGE (2010), the 10 municipalities occupying the SB basin
120 release sewage produced by more than 7.611.000 inhabitants into the SB tributary rivers with no
121 proper treatment. In addition, the bay presents a history of chronic metal contamination (mainly Cd
122 and Zn) from the activity of the former Campania Siderúrgica Mercantil Ingá SA. Since the
123 beginning of its operation, more than 2 million tons of tailings were dumped in the Bay. The
124 sampling sites SB11 and SB12 located at Saco do Engenho (Figure 1) were the most affected sites

125 due to their proximity to the tailings storage yard, which used to overflow during the rainy season
126 (Barcellos et al. 1997; Lacerda et al. 1987; Molisani et al. 2004, 2006a; Monte et al. 2015; Ribeiro,
127 Figueiredo, Santos, et al. 2013).

128 The area surrounding the bay also houses an industrial complex with industries belonging to
129 the petrochemical, metallurgical, textile, and smelting sectors, and a harbor station with significant
130 importance in the Brazilian economic scenario (IBGE, 2010). In addition, the territorial occupation
131 around the bay is expanding rapidly, including residential demand, tourism, recreation and fishing,
132 which results in an increased load of multiple pollutants in the SB.

133

134 2.2 Sampling

135 Surface sediment sampling was performed with a stainless steel Van Veen, in 26 stations
136 distributed along the SB coastline (Figure 1). The localization of sampling stations aimed to study
137 the contribution of continental drainage and the contamination gradient, both in the internal (eastern
138 region - SB01 to SB12) and in the external sectors (western region - SB13 to SB26) of the bay
139 (Figure 1). SB26 was considered because the site represents the former SB dredged sediment
140 disposal area. Metal contents from the current oceanic disposal area for dredged sediments (Dump
141 area, Figure 1) were used as ambient background since it corresponds to an unpolluted area because
142 of the rapid dispersal of dredged sediments (Moreira et al., 2021) following the recommendation
143 from (Gałuszka, 2007). Samples were collected with a sterile metal-free (10% HCl treated) plastic
144 spatula, then fractionated, and stored according to the analysis protocols to be performed.

145 Aliquots for molecular analysis (V4-V5 region of 16S rRNA gene analysis) were stored in
146 sterile Whirl-Pak[®] sample bags and kept cool until arrival at the laboratory where the samples were
147 frozen at -20 °C, until DNA extraction. Aliquots used for determining the physical-chemical and
148 geochemical parameters including total organic carbon (TOC), fine grain content, and metal content
149 were also stored at -20 °C in metal-free containers. The aliquot samples for determining the esterase
150 (EST) and dehydrogenase (DHA) activities were processed and stored as described in Moreira et al.
151 (2021).

152

153 2.3 Quality Ratio (QR) index

154 The QR index integrates microbiological, physical-chemical and geochemical sedimentary
155 parameters (Nascimento et al., 2019), aiming to classify sediments according to their potential
156 ecological risks to the benthic microbiota. This index considers TOC, fine-grain content (% silt +
157 clay) and the degree of contamination (ΣCF) that are classic physical-chemical parameters

158 associated with contaminant retention. The quantification of these parameters is established by
159 Brazilian legislation for the monitoring of dredged coastal sediments.

160 The quantified metals (Hg, Cd, As, Pb, Cr, Cu, and Zn) are those considered of high
161 toxicological risk for the biota by the Brazilian legislation CONAMA (2012). In this study metal
162 contents determined by Moreira et al. (2021) are used. The metals were quantified by ICP-MS
163 (Agilent Technologies-7900) after acid extraction ($3\text{HNO}_3 + 1\text{HCl}$) following the US EPA 3051A
164 (US EPA, 2007) as previously described (Moreira et al., 2021).

165 The metal contamination factors were calculated for each sample station as follows

$$166 \quad \text{CF} = \frac{C}{C_0} \quad (1)$$

167 Where C is the total metal content (mg/Kg) in the sediment sample, and C_0 is the
168 background content (mg/Kg) of the metal (Dump site – Figure 1).

169 The degree of contamination (Cd) corresponds to the sum of the contamination factors of all
170 quantified metals for a determined station

$$171 \quad \text{Cd} = \sum \text{CF} \quad (2)$$

172 Then, the above-mentioned physical/geochemical parameters integrate the Geochemical
173 Term (GT)

$$174 \quad \text{GT} = \frac{\text{TOC} * \sum \text{CF}}{\text{fine grain content}} \quad (3)$$

175 Where TOC corresponds to total organic carbon (% w.w⁻¹) and fine grain content is
176 the percentage of fine grain (% silt + clay).

177 Then, the Microbial Term (MT) is determined as follow

$$178 \quad \text{MT} = \frac{\text{DHA}}{\text{EST}} \quad (4)$$

179 Where DHA ($\mu\text{gFDA.g}^{-1}$) and EST ($\mu\text{gINT-F.g}^{-1}$) represent the esterase and dehydrogenase
180 activities respectively. The activities of EST and DHA enzymes are related to the process of energy
181 production for the survival of the microbial cells.

182 Then, Quality Ratio (QR) is calculated as follows

$$183 \quad \text{QR} = \frac{\text{MT}}{\text{GT}} \quad (5)$$

184 The physical-chemical, geochemical and microbiological data from Moreira et al. (2021)
185 were used to calculate the QR index. The methods for determining the DHA and EST activities are
186 described in detail in Moreira et al. (2021). The SB sediments were classified into 3 classes of
187 potential risk to biota according to QR index (Moreira et al., 2021) as:

188 $\text{QR} \geq 10^{-1}$ indicates low risk

189 $10^{-2} \leq \text{QR} < 10^{-1}$ indicates moderate risk

190 and $QR \leq 10^{-3}$ indicates high risk

191

192 2.4 DNA extraction, PCR amplification and sequencing

193 The DNeasy® PowerSoil® Kit (QIAGEN) was used for DNA extraction following the
194 manufacturer's protocol, using aliquots of 0.25-0.40g of wet sediment.

195 For each sample, the V4-V5 region of the 16S rRNA gene was amplified using a 25 μ L
196 solution composed of 12.5 μ L of AmpliTaq® Gold 360 Master Mix (Applied Biosystems), 1 μ L of
197 each primer 515F (5'-GTGYCAGCMGCCGCGGTA-3') (Parada et al., 2016) and 928R (5'-
198 CCCCgycaattcmtttragt-3') (Wang and Qian, 2009), 2.0 μ L of DNA and 8.5 μ L of
199 sterilized MilliQ water. PCR amplification was carried out in an Eppendorf 5333 Mastercycler
200 Thermal Cycler thermocycler (Germany) as follows: initial DNA denaturing at 95°C for 10
201 minutes, followed by 30 cycles of 30 seconds at 95°C, 30 seconds at 60°C, 40 seconds at 72°C and
202 extension for 10 minutes at 72°C. The PCR products were run for 15 minutes at 150 V in 1.0%
203 (w/v) agarose gel electrophoresis in 1 \times Tris-Acetate-EDTA buffer and stained with ethidium
204 bromide (0.2 μ g mL⁻¹) as previously described (Bordenave et al., 2004). DNA bands were detected
205 under UV light for checking the size of PCR products using DNA GeneRuler 1 kb as DNA
206 molecular size marker.

207 Illumina sequencing was performed by the NED team (UMR1388 GenPhySE) and the GeT
208 core facility (Toulouse, France), using Illumina MiSeq technology. The complete protocol is
209 available on the website of the platform ([https://sites.google.com/site/olivierzembwebsite/16s-
210 sequencing](https://sites.google.com/site/olivierzembwebsite/16s-sequencing)). QIIME 2 2019.4 (Quantitative Insights Into Microbial Ecology; Caporaso et al. 2010)
211 pipeline was applied for demultiplexing, denoising (using dada2 package), and rarefying sequences.
212 The taxonomy assignment (97% similarity) was performed comparing reference sequences,
213 clustered in representative amplicon sequence variants (ASVs), to SILVA (v. 132) database of
214 known 16S rRNA gene (Beccati et al., 2017; Quast et al., 2013).

215 The complete dataset was deposited in the NCBI Sequence Read Archive (SRA) database
216 under SRA accession number SAMN20003651 to SAMN20003676, BioProject number
217 PRJNA743207.

218

219 2.5 Data analysis

220 The community data (ASVs table) was transformed into relative abundances, and those
221 greater than 1% were kept for the analysis. Statistical and community composition/diversity
222 analysis were performed on R software (R Core Team, 2019), using specific packages. Since not all

223 data (physical, geochemical, enzymatic, and ASVs) presented normal distribution (Shapiro Wilk
224 test, $p < 0.05$), non-parametric analyzes were applied.

225 Alpha diversity indices (including species richness and Shannon index) of microbial
226 community were calculated based on the ASVs table using Vegan package (Oksanen et al. 2019).
227 For Spearman correlation, Mann-Whitney's U test, and Permutational Analysis of Variance
228 (perMANOVA - Vegan package; Oksanen et al. 2019), the physical/geochemical data (TOC, fine
229 grain content, metal contamination factor – CF - and the metallic degree of contamination - Σ CF),
230 the microbiological parameters (EST and DHA) and the Shannon diversity index were standardized
231 by ranging for variables with arbitrary zero, as proposed by Milligan and Cooper (1985). Canonical
232 Correspondence Analysis (CCA), an ordination method where species distribution (microbial
233 composition) is constrained by incorporating environmental variables (physical-chemical
234 parameters), was performed for determining the correlation of microbial community composition
235 (Phylum level) with physical-chemical and microbiological parameters. CCA uses two data
236 matrices, one of species occurring in various samples and the other of the environmental
237 characteristics of those samples. The relationships between the microbial species distributions and
238 the physical-chemical parameters are thus an integral part of the ordination. CAA was performed
239 using Vegan Package (Oksanen et al. 2019). The standardization of physical, geochemical, and
240 microbiological variables were kept and community data was Hellinger transformed.

241 Venn Diagrams (VennDiagram package - Chenn 2018) were built to analyze the distribution
242 of ASVs according to the habitat (SB sectors) and the ecological risk of sediment (QR
243 classifications).

244 Heatmaps showing the phyla and class relative abundances distribution per station were
245 performed using the pheatmap package (Kolde, 2019). The same package was used to perform
246 cluster correlations based on the 50 most abundant genera, to determine the correlations (Spearman)
247 between microbial genera and metal CF. The hierarchical clustering of correlations was based on
248 Spearman correlation.

249 A Linear discriminant analysis effect size (LEfSe; Segata et al., 2011) was performed to
250 detect bioindicator genera among the 50 most abundant, according to the habitat and the potential
251 ecological risk of sediments. For this, the non-parametric Kruskal-Wallis sum-rank test was
252 performed to detect taxa with significant differential abundance ($p < 0.05$) considering contrasting
253 conditions (e.g., internal and external sectors; high, moderate and low ecological risks). The
254 biological consistency was investigated by performing a pairwise Wilcoxon test ($p < 0.05$). A
255 Linear Discriminant Analysis (LDA) threshold score of 2.0 and 1000 bootstrap interactions were
256 applied.

257 In order to determine correlations between ASVs and metal contamination a clustered
258 correlation (ClusCor) analysis was performed as described previously (Duran et al., 2015). Full
259 description of ClusCor is provided by (Weinstein et al. 1997). A program for making clustered
260 correlation is available (<http://discover.nci.nih.gov.gate1.inist.fr>).

261 To determine the ASV co-occurrence and mutual exclusion relationships, a network was
262 built using a Cytoscape plugin CoNet 1.1.1 beta (Co-occurrence Network inference;
263 <http://psbweb05.psb.ugent.be/conet/>; Faust et al., 2012). The input matrix consisted of ASVs > 1%
264 data from all the sample stations and a metadata table consisting of metal contamination factors
265 standardized by ranging for variables with arbitrary zero as already mentioned. The analysis was
266 performed with Spearman correlation method and 0.05 p-value threshold. The co-occurrence
267 network was visualized with Cytoscape 3.8.2 (Shannon et al., 2003).

268

269 **3 Results and Discussion**

270 3.1 Ecological quality status assessed by Quality Ratio

271 A previous study (Moreira et al., 2021), comparing the stations on the SB sectors based on
272 environmental variables, grouped the stations as follow: the internal sector (SB01 to SB12, Figure
273 1) presents higher levels of fine-grain, TOC, and metal contents than the external sector (SB13 to
274 SB26). Accordingly, the QR index classified the SB sediments into three levels of risk for the
275 benthic biota (Supplementary material – Table S2): 2 stations with Low risk ($QR \geq 10^{-1}$), 9 stations
276 with Moderate risk ($10^{-2} \leq QR < 10^{-1}$), and 15 stations with High risk ($QR \leq 10^{-3}$). Higher
277 ecological risks (i.e. the lowest QR values) in the internal sector stations are explained by the
278 intense anthropogenic activities in the area, mainly from the domestic effluent input brought by the
279 main tributary rivers. Also, the QR index indicated that SB11 and SB12 stations exhibited higher
280 risk in comparison to the other stations located in the internal sector. Such observation is probably
281 explained by the close location of stations SB11 and SB12 to a former mining site exploiting zinc
282 ores in the 1960s, which resulted in metals contamination. SB26 presents moderate risk despite
283 being the former SB dredged sediment deposition area. We hypothesize that stations with a high
284 risk for biota (low QR) are predominantly colonized by microbial taxa resistant to metal
285 contamination.

286

287 3.2 Microbial community diversity along the contamination gradient

288 After trimming, the retained 188,782 sequences, with size 376.6 ± 3.5 bp, were dispatched
289 into 3,444 amplicon sequence variants (ASVs, Table 1), which were reduced to 518 ASVs
290 following rarefaction applying a 1% abundance threshold.

291 The Venn diagram with the remaining 518 ASVs showed that both SB sectors shared 243
292 ASVs (Figure 2). Considering (i) the chronic contamination of the study area (Monte et al., 2015;
293 Moreira et al., 2021), (ii) the natural selection process responsible for the elimination of microbial
294 taxa sensitive to contaminants at the beginning of the exposure period (Harrison et al., 2007), (iii)
295 the rapid response of prokaryotes to variations in environmental conditions and their physiological
296 diversity (Aylagas et al., 2017; Caruso et al., 2015), and (iv) their adaptability from the exchange
297 (Flemming and Wingender, 2010; Harrison et al., 2007; Lewis, 2007) and expression of genes that
298 confer adaptive advantages (Beelen and Doelman, 1997; Dick, 2018; Harrison et al., 2007) in
299 polluted environments, it is assumed that more than 70% of the SB microbial taxa (ASVs shared
300 between sectors + ASVs exclusive to the internal sector) is adapted to the pollution (Figure 2a). The
301 effect of contaminants on microbial community structure is not only dependent on the concentration
302 of contaminants, but also on their bioavailability according to environmental parameters controlling
303 their accumulation in sediments (Burton, 2002; Gough, 2007; Løkke et al., 2013; Mumtaz et al.,
304 2010), which can also directly influence microbial communities (Bai et al., 2012; Coclet et al.,
305 2019; Yang et al., 2016). Thus, it is worth considering the physical and geochemical parameters
306 such as the fine-grain and TOC contents - main metal-binding matrices (Di Toro et al., 1990; Marx
307 and Kamber, 2010) to obtain a comprehensive view of the contaminant effects on microbial
308 communities. Besides, several studies highlighted the need to integrate the biological response to
309 contamination exposure (represented here by the EST and DHA enzyme activities) to the physical
310 and geochemical parameters to assess the ecological quality status (Burton, 2002; Rosado et al.,
311 2015). The observed higher number of microbial ASV richness in stations classified as moderate
312 (408 ASVs) and high (491 ASVs) risks (Figure 2b) by the QR index (Moreira et al., 2021) in
313 comparison to that observed in stations defined as low risk by QR index (285 ASVs) suggested that
314 the microbial communities are adapted to the contamination level. Thus, the specific ASVs likely
315 represent specialist ASVs adapted to the contamination level: 96 ASVs for the high risk (i.e. high
316 contamination level), 19 ASVs for the moderate risk (i.e. moderate contamination level), and 1
317 ASV for low risk (i.e. low contamination level).

318 The stations of the SB external sector, exhibiting lower contamination degree (ΣCF_{mean} SB
319 external sector stations = 49 ± 6.8 ; Table S1), had significantly higher (Mann Whitney's U test; $w =$
320 143 , $p = 0.002$) microbial ASV richness (S_{mean} external sector stations = 159 ± 10 ; Table 1) and
321 significantly higher (Mann Whitney's U test; $w = 134$, $p = 0.01$) Shannon's diversity index (H_{mean}
322 external sector stations = 4.7 ± 0.3 ; Table 1) than the stations of the SB internal sector, exhibiting
323 high contamination degree (ΣCF_{mean} SB internal sector stations = 536 ± 246 ; Table S1). Despite the
324 significant differences, it is noticeable that the range between the Shannon diversity index of the
325 two sectors (Shannon diversity index range = Internal: 4.1 to 4.8, External: 3.9 to 5.0) is small,

326 which means that both sectors are diverse with the homogeneous distribution. However, it cannot be
327 excluded that other environmental parameters such as salinity and dissolved oxygen, affect the ASV
328 diversity (Beaulne et al., 2020; Ben Salem et al., 2019; Bourhane et al., 2022; Terrisse et al., 2017;
329 Zhang et al., 2016). Indeed, it has been shown that the external sector at the proximity of the SB
330 entrance is influenced by the ocean currents (Ribeiro et al., 2013). Conversely, in the internal sector,
331 there is a reduction in water circulation speed resulting in the reduction of oxygenation (Ribeiro et
332 al., 2013; Signorini, 1980) further favored by the high organic load from the continental drainage
333 and the mangrove productivity (Carvalho et al., 2021, 2020). The QR index classified the internal
334 sector as high risk ($QR \leq 10^{-3}$) corresponding to significantly (Mann Whitney's U test; $w = 8$, $p =$
335 0.0001) higher contamination degree (ΣCF_{mean} SB internal sector stations = 536 ± 246 ; Table S1)
336 and significantly (Mann Whitney's U test; $w = 16$, $p = 0.0005$) higher geochemical term (GT_{mean} SB
337 internal sector = 9989 ± 3442 ; Table S2). Despite these differences, both sectors showed similar
338 (Mann Whitney's U test; $w = 52$, $p = 0.1$) microbial terms (MT_{mean} SB internal sector = 3.78 ± 1.3 ;
339 MT_{mean} SB external sector = 5.2 ± 1.7 ; Table S2), suggesting that the microbial activities were not
340 affected by the presence of contaminants in the SB internal sector, probably because the microbial
341 communities were adapted to cope with contaminants.

342 The lower microbial diversity found in the most polluted sector is consistent with previous
343 reports (Bruneel et al., 2006; Li et al., 2020). However, the microbial diversity in the contaminated
344 SB internal sector remains high compared with other contaminated sites (Chen et al., 2018; Paissé
345 et al., 2008; Stauffert et al., 2013; Wang et al., 2007), which is in agreement with the microbial
346 diversity levels observed in chronically impacted environments. In a chronic contamination
347 scenario, the microbial diversity decreases at the beginning of the pollutant exposure period;
348 consequently, the microbial community becomes as diverse as a microbial community from the
349 non-impacted environment over time (Gillan et al., 2005; Jeanbille et al., 2016b; Mossa et al., 2017;
350 Paissé et al., 2008), which corresponds to the microbial adaptation to the pollutant also known as
351 legacy effect (Jurburg et al., 2017). It is likely that in the initial phase of exposure, there is a
352 selection of pollutant-resistant microorganisms (Bouskill et al., 2010), which can be subsequently
353 amplified by mechanisms exchanging genetic materials (e.g., horizontal gene transfer) (Brochier-
354 Armanet and Moreira, 2015; Lewis, 2007), resulting thus to the stability of the microbial
355 community (Harrison et al., 2007; Nascimento et al., 2020; Waite et al., 2016).

356 CCA analysis, explaining almost 40% of the microbial community distribution, corroborated
357 the differences observed between the microbial communities from each SB sector (Figure 3). The
358 microbial communities from the internal sector were correlated with metal CF, TOC, fine-grain
359 content, and EST activity, while the microbial communities from the external sector were linked
360 with DHA activity. It is important to highlight that high levels of fine-grain and TOC (nutrient

361 supply) favor the accumulation of metals (high Σ CF) (Förstner and Wittmann, 1981). The stress
362 provoked by metal accumulation in sediment leads to an increase in EST activity that, consequently,
363 would make the metals bioavailable after the breakdown of organic matter (Nascimento et al., 2019;
364 Waite et al., 2016). When absorbed by microbial cells, metals would compromise DHA activity
365 (Coppolecchia et al., 2011; Sabadini-Santos et al., 2014; Waite et al., 2016), which would result in
366 an imbalance between the enzymatic biomarkers. This imbalance designates the increased energy
367 demand of the microbial community, represented by the low MT (MT = DHA / EST) when exposed
368 to multiple chronic contaminants (Moreira et al., 2021; Nascimento et al., 2019).

369

370 3.2.1 Microbial community structure in response to chronic exposure to metals

371

372 The 518 ASVs were classified into 23 phyla and 48 classes. Proteobacteria (53.5%) was the
373 most abundant phylum (Figure 4a), with a predominance of the Gammaproteobacteria (29%),
374 Deltaproteobacteria (21%), and Alphaproteobacteria (3.4%) classes. The other representative phyla
375 were Bacteroidetes, Firmicutes, Acidobacteria, and Chloroflexi (17.0%, 9%, 7.6%, and 5.8%,
376 respectively).

377 The predominance of Proteobacteria, more specifically of the Gammaproteobacteria and
378 Deltaproteobacteria classes, suggests that they play an important role in biogeochemical cycles in
379 the sediments (Ben Salem et al., 2019). ASVs affiliated to Gammaproteobacteria present the highest
380 relative abundances in stations located in the internal sector (Figure 4b), which is consistent with
381 the fact that they are linked with sediments contaminated by metals (Bouskill et al. 2010). The
382 positive correlation between the abundance of Gammaproteobacteria ASVs and the Cd and Zn
383 contents (Spearman; $r > 0.39$, $p < 0.05$ - Figure 4c) further supports the hypothesis that
384 Gammaproteobacteria can tolerate the presence of metals.

385 Microbial communities composition from SB proved to be significantly different
386 (Permanova analysis) when comparing: (i) internal and external sectors ($p = 0.001$) and (ii) the QR
387 index classifications ($p = 0.02$). It is important to note that microorganisms involved in the sulfur
388 cycle were found more abundant in the internal sector (Figure 4b), especially at the stations close to
389 the mouth of the main tributary rivers (SB06 to SB11) with high organic load from domestic
390 effluent, which represents a reduced environment (Carvalho et al., 2021, 2020, n.d.; Paissé et al.,
391 2008).

392 Following the reducing nature of sediments from the internal sector, Thermoplasmata class,
393 related to methanogenic activities (Ma et al., 2016) exclusively detected in this sector (absent in
394 SB06, SB08, SB10, and SB11), presents positive correlations with all metal CF ($r > 0.44$, $p < 0.05$;

395 Figure 4c). Similarly, ASVs affiliated to Thermococci were found only at SB06 and SB07, with the
396 latter presenting 19 times greater abundance (Figure 4b). These microorganisms are
397 hyperthermophilic heterotrophs, strictly anaerobic, and related to reducing environments rich in
398 organic matter with high concentrations of sulfur, which is a limiting factor for their growth (Teske,
399 2009; Zillig and Reysenbach, 2015). In the present study, Thermococci proves to be resistant to
400 metallic contaminants due to its significant ($p < 0.05$) positive correlation with Cr, Cu, and Hg
401 (Spearman; $r = 0.42, 0.42, \text{ and } 0.47$, respectively - Figure 4c).

402 Also, ASVs affiliated to *Thermodesulfovibrionia* and *Dehalococcoidia* presented higher
403 abundances in the internal sector (Figure 4b) and significant ($p < 0.05$) positive correlation with
404 some metal CF (Spearman; $r > 0.39$ - Figure 4c). *Thermodesulfovibrionia* is known for the
405 reduction of sulfate and other sulfur compounds (Tamburini et al., 2020) that have been found
406 abundant in coastal sediments with a high contamination degree by metals and polycyclic aromatic
407 hydrocarbons (PAHs) (Tangherlini et al., 2020). *Dehalococcoidia* is an organohalide respiring
408 bacteria, playing a role in detoxifying anoxic contaminated sediments with polychlorinated
409 biphenyls (PCBs) through reductive dechlorination activities (Maturro et al., 2016; Nuzzo et al.,
410 2017). In the present study, *Dehalococcoidia* is present in 9 stations from the internal sector (except
411 SB01, SB06, and SB10 – Figure 4b), with average relative abundance an order of magnitude higher
412 than that found in the external sector, where they were found in only 5 stations (SB21 > SB22 >
413 SB15 > SB16 > SB13). Such observation suggests that *Dehalococcoidia* present selective
414 advantages for living in environments chronically impacted by multiple contaminants such as SB,
415 where metallic contaminants are indicators of the pollutants' mixture (Carvalho et al., 2021, 2020;
416 Moreira et al., 2021).

417 In decreasing order, the stations SB06, SB11, and SB07 also present the highest relative
418 abundances of ASVs affiliated to *Anaerolinea* (Figure 4b), which was consistent with the results
419 shown in Figure 4c – presenting strong correlations with metals (Spearman; $r > 0.39, p < 0.05$,
420 except for Cr) – and with previous reports showing that members of *Anaerolinea* were found
421 abundant in river sediments, soils, and coastal sediments contaminated with metals (Meng et al.,
422 2019; Sun et al., 2013; Yin et al., 2015). In contrast, *Nitrososphaeria* was present in all the stations
423 of the external sector (except SB15 and SB18) and at the SB10 station in the internal sector (Figure
424 4b). This observation was in accordance with reports showing that members of the *Nitrososphaeria*
425 have been found more abundant in less contaminated sites (Tangherlini et al. 2020). The negative
426 correlation between *Nitrososphaeria* and the metals (Spearman; $r < -0.39, p < 0.05$) in the present
427 study (Figure 4c) indicates their sensitivity to metallic contamination.

428 The enrichment of methanogenic and sulfate-reducing organisms - as detected in the SB
429 anoxic polluted sediments from the internal sector stations, Figure 4b - could represent the taxa

430 exhibiting function involved in the natural attenuation of contaminants, as suggested by Li et al.
431 (2019). Their high abundances reflect the impact of contamination on the structure of microbial
432 communities. Also, energy metabolism specifically linked to methane metabolism is pointed out in
433 the literature as typical in metal-contaminated environments (Bergsveinson et al., 2020; Coclet et
434 al., 2021; Guo et al., 2018). Recently, predicted metabolic pathways from 16S rRNA gene revealed
435 methane metabolism as an indicator of high metallic pollution in the stations of SB internal sector
436 (Moreira et al., 2022). Studies considering sedimentary microbial community functional data show
437 an increase in the relative proportion of genes involved in methanogenesis, which are correlated
438 with eutrophic, anoxic, and metal-polluted sediments (Coclet et al., 2021; Li et al., 2019). Research
439 conducted in the study area shows that the content and quality of organic matter are not the main
440 factors that influence methanogenesis (Marinho et al., 2012). Recently, it has been shown that
441 microbial communities inhabiting pristine (non-contaminated) sediments exhibit metabolic potential
442 involved in greenhouse gases consumption (Li et al., 2019). In contrast, it has been shown that the
443 production of greenhouse gases in marine coastal areas is correlated with urban waste load (Burgos
444 et al., 2015), particularly including metals (Brown et al., 2022). The presence of adapted
445 microorganisms to highly polluted anoxic sediments (as those mainly inhabiting the SB internal
446 sector) have the capacity to increase the greenhouse gases production (Wang et al., 2022). This is
447 due to the activation of adaptive mechanisms for coordinating intracellular toxic-metal homeostasis
448 (Coclet et al., 2021; Prabhakaran et al., 2016) such as metal immobilization. Thus, sulfate reduction
449 is reported as one of the anaerobic metabolism that promotes metal immobilization, resulting in
450 CO₂ emission (Ayangbenro et al., 2018). Additionally, the comparison of metagenomes from
451 contaminated sediment to that of pristine sediment revealed that the presence of contaminants,
452 particularly heavy metals, increased methanogenesis and sulfate reduction capabilities while
453 reducing the capacity of nitrogen fixation (Li et al., 2019). In contrast, the pristine site harbored
454 higher consumption of methane limiting the amount of greenhouse gases (Li et al., 2019).

455

456 3.2.2 Microbial taxa specific to QR risk level and habitat specific

457

458 Linear discriminant analysis effect size (LEfSe), performed with the 50 most abundant
459 genera, revealed the ASVs showing significant different abundance according to the habitat
460 (internal vs. external sectors, Figure 5a) and to the QR risk level of sediments (high vs. moderate vs.
461 low risks, Figure 5b). This observation was consistent with previous reports showing habitat
462 (Jeanbille et al., 2016a,b; Ben Salem et al., 2019), and/or contamination specificities (Bourhane et
463 al., 2022; Hafez et al., 2022) for microbial taxa. Such ASVs differentially abundant to a specific

464 condition are considered as bioindicators (Segata et al., 2011), which can be useful to develop tools
465 for monitoring contamination (Aylagas et al., 2017; Bourhane et al., 2022; Hamza-Chaffai, 2014).

466 Among the genera found specifically more abundant in the metal contaminated internal
467 sector by LEfSe, the Uncultured *Thiotrichaceae* and *SCGC-AB-539-J10* genera, known for their
468 anaerobic metabolism (Sharrar et al., 2017; Wasmund et al., 2014), were also found specifically
469 more abundant in stations classified as high QR risk (Figure 5b). Such observation further supports
470 the role of anaerobic microorganisms in metal contaminated sites (Liu et al., 2019; Zárata et al.,
471 2021). Consistently, the specific presence of the *Desulfatiglans*, *SEEP-SRB1*, *Candidatus Thiobos*,
472 *Spirochaeta 2*, uncultured (Class: *Anaerolineae*), and Uncultured *Calditrichaceae* genera known for
473 their anaerobic metabolisms (Kublanov et al., 2017; Miyazaki et al., 2014; Petro et al., 2019; Suzuki
474 et al., 2014; Yamada et al., 2006) also characterizes the internal sector.

475 In contrast, the genera found specifically more abundant in the external sector (low metal
476 content) by LEfSe are known for their aerobic metabolism, such as Subgroup 10 (*Acidobacteria*),
477 *Actibacter*, *Exiguobacterium*, *Blastopirellula*, *Paeniclostridium*, *Romboustia* genera (Kim et al.,
478 2008; Kristensen et al., 2021; Tedesco et al., 2021). Consistently, these genera were also found
479 specifically more abundant in stations classified as low and moderate QR risks (Figure 5b).

480

481 3.2.3 Influence of metallic contaminants on microbial assemblages

482

483 A cluster correlation analysis was carried out to determine the interactions between the 50
484 most abundant genera and metallic contaminants (Figure 6). Following the LEfSe analysis, the
485 identified bioindicators for the internal sector/high ecological risk - such as *SCGC-AB-539-J10* and
486 the uncultured *Thiotrichaceae* - presented a significant positive correlation (Spearman; $r > 0.39$; $p <$
487 0.05) with five or more metals. Thus, the microbial community in the internal sector presents a high
488 abundance of taxa resistant to contaminants (Figure 4b). In contrast, bioindicators for the external
489 sector/low and moderate ecological risk - such as *Actibacter*, *Candidatus Nitrosopumilus*,
490 *Thermoanaerobaculaceae Subgroup 10*, uncultured *Cyclobacteriaceae*, and uncultured
491 *Sandaracinaceae* - presented significant negative correlation (Spearman; $r < -0.39$; $p < 0.05$) with at
492 least 4 metals. Thus, the microbial community in the external sector presents a high abundance of
493 taxa sensitive to contaminants (Figure 4b).

494 The co-occurrence network analysis shows four sub-networks (Figure 7). But the
495 interactions between ASVs and metals were evidenced only in the main network (Figure 7a).
496 Among the 97 ASVs that showed significant non-random interactions (Spearman; $r > 0.6$, $p <$

497 0.003) in the network, 39 ASVs were directly linked with metals (Figure 7b), while the other 58
498 ASVs showed indirect correlations (Figure 7a).

499 Among the ASVs directly correlated with metal contamination (Figure 7b – connected by
500 green edges), 8 ASVs (6 gray ellipses representing the Proteobacteria phylum, belonging to
501 Gammaproteobacteria class; and 2 white hexagons representing the Bacteroidetes phylum,
502 belonging to Bacteroidia class) are directly positively linked (Spearman; $r > 0.6$, $p < 0.003$) to metal
503 contamination, which indicates that they are probably resistant to metallic contamination. Among
504 them, the abundance of 3 ASVs was specifically correlated with Hg contamination level, (ASV-3
505 and ASV-609, both Gammaproteobacteria class, and ASV-1659, Bacteroidia class). The abundance
506 of the other 5 ASVs presented a significant positive correlation with the contamination level of at
507 least 2 metals.

508 From these 8 ASVs which abundance was found to be positively and directly linked to metal
509 content, 3 belong to the genera that were identified as bioindicators for the metallic contamination
510 of the internal sector, and for the high ecological risk stations (Figure 5a): ASV-483 (uncultured
511 Thiotrichaceae) and ASV-1321 (Robiginitalea) (Figure 6).

512 The 31 ASVs, presenting abundances with significant negative correlations (exclusions -
513 Spearman; $r < -0.6$, $p < 0.003$) with metal contents (Figure 7b – connected by red edges), represent
514 taxa probably sensitive to contaminants influencing other ASVs by mutual exclusion (Figure 7a –
515 red edges). These 31 ASVs belong to the Proteobacteria (grey ellipses; Gammaproteobacteria,
516 Deltaproteobacteria, and Alphaproteobacteria classes), Bacteroidetes (white hexagons; Bacteroidia
517 and Ignavibacteria classes), Firmicutes (white rectangle; Bacilli class), Actinobacteria (blue
518 hexagon; Acidimicrobia class), Acidobacteria (white ellipse; Blastocatellia class - Subgroup 4), and
519 Planctomycetes (blue ellipse; Phycisphaerae class) phyla. Among them, 7 ASVs – ASV-755
520 (Ulvibacter), ASV-1398 (uncultured Cyclobacteriaceae), ASV-2097 (Methyloceanibacter), ASV-
521 2191 (Eudoraea), ASV-2949 (Desulfobulbus), ASV-3252 (Exiguobacterium), and ASV-3419
522 (Actibacter) – were among the top 50 genera. Except for ASV-755, all the other 6 ASVs were
523 revealed, by LEfSe analysis, as bioindicators for the SB external sector and for low ecological risk
524 (ASVs 1398, 3252, and 3419) (Figure 5). The 7 ASVs were affiliated to genera showing
525 abundances with negative or no correlation with metallic contaminants (Figure 6).

526 The ASVs linked to ASVs directly correlated to metals (Figure 7a, green edges) are likely to
527 constitute a transformation network involving cooperation, probably using metal compound
528 transformation intermediates (Nogales et al., 2011). Bacteria resistant to metallic contamination
529 have homeostatic mechanisms that make them able to eliminate the metals that have been
530 excessively absorbed by cells, through efflux pumps (Nies, 1995). They also produce sulfur-rich
531 molecules (thiol group) capable of sequestering metal cations and immobilizing them from the

532 formation of complex molecules; also some of these organisms are capable of reducing metal ions
533 to a less toxic oxidation state (Nies, 1999).

534

535 **4 Conclusions:**

536

537 The V4-V5 hypervariable region of the 16S rRNA gene sequencing analysis revealed that
538 microbial diversity was significantly different between Sepetiba Bay sectors. Such significant
539 difference in microbial community structure between both sectors was consistent with the
540 classifications of ecological risk according to QR index, validating the QR index as a reliable tool
541 representative of the chronic contamination of the studied area.

542 Bioindicators, taxa significantly more abundant under a specific condition, identified for the
543 SB internal sector represent the selective pressure induced by metallic contaminants. They mainly
544 present anaerobic metabolism, which is consistent with the internal sector characteristics, being
545 anoxic and receiving high organic input from terrestrial drainage. Moreover, anaerobic
546 bioindicators in the most polluted sector are affiliated to taxa resistant to metals. Particularly, the
547 high abundance of sulfate-reducing taxa in the internal sector participates probably to the natural
548 attenuation function involved in mitigating the metal contamination effects by producing sulfide, a
549 metal-ligand that makes metal unavailable to the biota. But, the anaerobic processes provided by the
550 sulfate-reducing metabolisms could also contribute to greenhouse gas emissions. In contrast, the
551 identified bioindicators of the SB external sector belong to microbial taxa sensitive to multiple
552 contaminants with mainly aerobic metabolism, which is consistent with the environmental
553 conditions prevailing in the SB external sector.

554 In order to provide detailed information on the microbial communities, it is suggested that
555 future works investigate the metabolic functions affected by the chronic exposition to metallic
556 contamination of SB to gain information on the influence of contaminants on microbial metabolism.

557

558

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560

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953 **Figure legends**

954 **Figure 1 Location of sampling station in Sepetiba Bay (Rio de Janeiro – Brazil).**

955 **Figure 2 Comparison of microbial communities inhabiting SB sediments.** The comparison was
956 performed according to (a) sectors and (b) ecological risk classifications provided by the QR
957 index (Moreira et al., 2021). The Venn diagram shows the shared and the exclusive ASVs
958 between either SB sectors or QR index classification.

959 **Figure 3 Comparison of microbial community structures in SB sediments.** The canonical
960 correspondence analysis (CCA) shows the correlations between microbial communities (at
961 Phylum level - represented by white diamonds) with the physical/geochemical - fine grain
962 content ($< 63\mu\text{m}$), TOC, and metal CF – and the microbiological - EST and DHA activities -
963 parameters. Internal sector stations are represented by red triangles, while external sector
964 stations are represented by blue triangles.

965 **Figure 4 Comparison of microbial community composition in the different stations of SB.** The
966 bar plot (a) shows the phyla composition and their relative abundances (%) in the different
967 stations. The Heatmap (b) shows the class composition and their relative abundances (%) in
968 the different stations. The cluster correlation (c) shows the relationship between prokaryotic
969 classes and the metal CF, based on Spearman correlations ($r < -0.39$ or $r > 0.39$ are significant,
970 $p < 0.05$).

971 **Figure 5 Comparison of microbial communities from the SB stations.** Linear discriminant analysis
972 effect size (LEfSe) shows the significantly different abundances of prokaryotic genera
973 according to (a) SB sectors, and (b) the ecological risk classifications provided by the QR
974 index application (Moreira et al., 2021).

975 **Figure 6 Cluster correlation between microbial ASVs and pollutants.** The analysis was performed
976 with the relative abundances (%) of the most 50 abundant genera and metal CF
977 (Supplementary material - Table S1). Heatmap and clustering were based on Spearman
978 correlations, in which $r < -0.39$ or $r > 0.39$ are significant ($p < 0.05$). * Significant positive
979 correlations ($p < 0.05$).

980 **Figure 7 Co-occurrence network between ASV abundances and metal contamination factors**
981 **(CF).** The network was inferred from ASVs relative abundances and CF (Supplementary
982 material – Table S1) applying Spearman correlations (straight lines), p-value threshold 0.05.
983 The positive correlations are indicated in green lines and mutual exclusions in red lines. Metal
984 contamination factors are indicated by yellow diamonds, while the different geometric shapes
985 represent ASVs sorted by phylum.

986 **Supplementary material – Table S1** Granulometric characteristics, total organic carbon (TOC),
987 Metal concentrations and Contamination Factor (CF) for sediments at sampling sites (Moreira
988 et al., 2021).

989 **Supplementary material – Table S2** Microbial parameters and calculated geochemical term (GT),
990 microbial term (MT), Quality Ratio index (QR), threshold values and estimated sediment risk
991 (Moreira et al., 2021).

992

993 **Table 1 Sequencing and microbial community data analysis** - Number of sequences before and
 994 after demultiplexing; observed richness (S), alpha diversity (Shannon diversity index), and QR
 995 index classification for each station.

	Sample names	Input sequences	Trimmed sequences	Species Richness (S)	Shannon (H)	QR*
996						
997	SB01	24354	3701	100	4.4	4.10 ⁻⁰⁵
998	SB02	25785	4375	124	4.6	1.10 ⁻⁰⁴
999	SB03	26583	6800	156	4.8	3.10 ⁻⁰⁴
1000	SB04	25419	4921	138	4.7	1.10 ⁻⁰⁴
1001	SB05	16952	2708	82	4.2	2.10 ⁻⁰⁴
1002	SB06	22266	5117	102	4.2	1.10 ⁻⁰⁴
1003	SB07	23979	5921	80	4.1	3.10 ⁻⁰³
1004	SB08	22967	6835	111	4.3	1.10 ⁻⁰¹
1005	SB09	28696	4051	107	4.5	3.10 ⁻⁰³
1006	SB10	26469	6179	110	4.3	1.10 ⁻⁰²
1007	SB11	27844	7803	111	4.3	1.10 ⁻⁰⁴
1008	SB12	29637	4674	126	4.6	1.10 ⁻⁰⁴
1009	SB13	25209	8198	136	4.7	3.10 ⁻⁰²
1010	SB14	26544	7504	156	4.7	1.10 ⁻⁰²
1011	SB15	22612	6338	128	4.6	1.10 ⁻⁰³
1012	SB16	31310	12678	195	5.0	4.10 ⁻⁰³
1013	SB17	43498	12700	220	4.9	1.10 ⁻⁰¹
	SB18	31447	10636	210	5.0	2.10 ⁻⁰³
	SB19	30169	11350	202	5.0	1.10 ⁻⁰²
	SB20	22576	7833	126	4.6	1.10 ⁻⁰²
	SB21	22995	8142	166	4.6	4.10 ⁻⁰³
	SB22	23412	7500	176	4.9	3.10 ⁻⁰²
	SB23	30491	9464	108	4.3	4.10 ⁻⁰²
	SB24	25470	9316	92	3.9	4.10 ⁻⁰³
	SB25	26372	6432	165	4.8	1.10 ⁻⁰²
	SB26	25014	7606	144	4.7	1.10 ⁻⁰²
	Total	688070	188782			

*
 *QR are from Moreira et al. (2021)