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

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## Abstract

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

## Environment implication

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

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

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

## 1 Introduction:

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

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

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

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

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

## 2. Materials and Methods

### 2.1 Site description

Sepetiba Bay is located between latitudes 22°55'00" S and 23°03'60" S and longitudes 43°56'30" W and 43°36'20" W, 60 km distant from Rio de Janeiro city (Brazil). It is highly influenced by the tidal cycle (Signorini, 1980), ranging from 419 to 447 Km<sup>2</sup> with an average depth of 6 m, 6-day water renewal time, and a volume of  $2.56 \times 10^9$  m<sup>3</sup>, approximately (Barcellos et al. 1997; Molisani et al. 2004; Smoak et al. 2012).

The São Francisco Channel is the main tributary of the 10 rivers that make up the drainage basin, being responsible for 86% of the freshwater inputs (Barcellos et al. 1997; Molisani et al. 2004; Smoak et al. 2012). According to IBGE (2010), the 10 municipalities occupying the SB basin release sewage produced by more than 7.611.000 inhabitants into the SB tributary rivers with no proper treatment. In addition, the bay presents a history of chronic metal contamination (mainly Cd and Zn) from the activity of the former Campania Siderúrgica Mercantil Ingá SA. Since the beginning of its operation, more than 2 million tons of tailings were dumped in the Bay. The 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 ( $\Sigma CF$ ) 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 
$$\text{CF} = \frac{C}{C_0} \quad (1)$$

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

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

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

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

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

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

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

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

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

## 2.4 DNA extraction, PCR amplification and sequencing

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

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

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

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

## 2.5 Data analysis

The community data (ASVs table) was transformed into relative abundances, and those greater than 1% were kept for the analysis. Statistical and community composition/diversity 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 -  $\Sigma$ 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  $376.6 \pm 3.5$  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 ( $\Sigma CF_{\text{mean}}$  SB  
 319 external sector stations =  $49 \pm 6.8$ ; Table S1), had significantly higher (Mann Whitney's U test;  $w =$   
 320 143,  $p = 0.002$ ) microbial ASV richness ( $S_{\text{mean}}$  external sector stations =  $159 \pm 10$ ; Table 1) and  
 321 significantly higher (Mann Whitney's U test;  $w = 134$ ,  $p = 0.01$ ) Shannon's diversity index ( $H_{\text{mean}}$   
 322 external sector stations =  $4.7 \pm 0.3$ ; Table 1) than the stations of the SB internal sector, exhibiting  
 323 high contamination degree ( $\Sigma CF_{\text{mean}}$  SB internal sector stations =  $536 \pm 246$ ; 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 ( $\Sigma CF_{\text{mean}}$  SB internal sector stations =  $536 \pm 246$ ; Table S1)  
 336 and significantly (Mann Whitney's U test;  $w = 16$ ,  $p = 0.0005$ ) higher geochemical term ( $GT_{\text{mean}}$  SB  
 337 internal sector =  $9989 \pm 3442$ ; Table S2). Despite these differences, both sectors showed similar  
 338 (Mann Whitney's U test;  $w = 52$ ,  $p = 0.1$ ) microbial terms ( $MT_{\text{mean}}$  SB internal sector =  $3.78 \pm 1.3$ ;  
 339  $MT_{\text{mean}}$  SB external sector =  $5.2 \pm 1.7$ ; 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

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

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

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

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

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

Following the reducing nature of sediments from the internal sector, Thermoplasmata class, related to methanogenic activities (Ma et al., 2016) exclusively detected in this sector (absent in 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 (Matturro 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 CO<sub>2</sub> 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

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

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

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

### 3.2.3 Influence of metallic contaminants on microbial assemblages

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

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



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

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

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

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

The ASVs linked to ASVs directly correlated to metals (Figure 7a, green edges) are likely to constitute a transformation network involving cooperation, probably using metal compound transformation intermediates (Nogales et al., 2011). Bacteria resistant to metallic contamination have homeostatic mechanisms that make them able to eliminate the metals that have been excessively absorbed by cells, through efflux pumps (Nies, 1995). They also produce sulfur-rich 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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952

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

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

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

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