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Detection of sentinel bacteria in mangrove sediments contaminated with heavy metals.

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ABSTRACT

Mangroves in the Northwest Coast of South America are contaminated with heavy metals due to wastewater discharges from industries, affecting the biota from this environment. However, bacteria proliferate in these harsh environmental conditions becoming possible sentinel of these contaminations. In this study, bacterial community composition was analyzed by throughput sequencing of the 16S rRNA gene from polluted and pristine mangrove sediments affected by marked differences in heavy metal concentrations. Core bacteria were dominated by Proteobacteria, Firmicutes, and Bacteroidetes phyla, with strong differences between sites at class and genus levels, correlated with metal levels. Increment of abundance on specific OTUs were associated with either elevated or decreased concentrations of metals and with the sulfur cycle. The abundance of *Sulfurovum lithotrophicum, Leptolinea tardivitalis, Desulfococcus multivorans* and *Aminobacterium colombiense* increases when metals rise. On contrary, *Bacillus stamsii, Nioella nitrareducens and Clostridiisalibacter paucivorans* abundance increases when metal levels are reduced. We propose these OTUs as bacterial sentinels, whose abundance can help monitor the restoration programs of contaminated mangrove sediments in the future.

1. Introduction

Mangrove forests are complex ecosystems located in the coastal zones of tropical and subtropical regions (de Souza Queiroz et al., 2017). Their complexity sources on the interaction between terrestrial, estuarine and marine systems create a unique environment for a diverse biota (Himes-Cornell et al., 2018). Mangrove forests provide constraints ecosystem services, including protection from tidal events and erosion (Sandilyan and Kathiseran, 2014), tourism and fisheries (Dias et al., 2012), as well as carbon accumulation and storage (Vo et al., 2012), and maintenance of biogeochemical cycles (Vo et al., 2012). Despite their importance, mangroves have become one of the most threatened ecosystems due to industrial wastewater discharges and high concentrations of heavy metals (Brady et al., 2015).

Mangroves harbor diverse bacterial communities, which represent the largest biodiversity pool on Earth (Flemming and Wuertz, 2019). In here, bacteria perform redox reactions in various biogeochemical cycles, such as carbon, nitrogen, sulfur, and phosphorus (Thatoi et al., 2013; Mendes and Tsai, 2018). Heavy metals, e.g., Ag, Cd, Cu, Pb, Sn, Zn, produce oxidative stress either through disrupting the antioxidant defense system or depleting the thiol status in bacteria-inhabiting sediments (Roosa et al., 2014; Gillan et al., 2015; Wu et al., 2016). This oxidative stress results in changes in bacterial abundance and diversity on polluted mangroves, which will then affect mangrove functioning (Paingankar and Deobagkar, 2018).

Studies in polluted mangroves have mainly focused on determining how a pollutant affects the general assemblage and abundance of the bacterial community and the presence of essential genes related to pollutant resistance (Cabral et al., 2016; Balakrishnan et al., 2017; Guo et al., 2018). Nonetheless, research is still scarce in the identification of specific bacterial OTUs which can change according to variations in contaminated environments. These changes may be fluctuations in pollutants levels that affect physiological processes of mangrove bacteria (Saha, 2018). The ability to contrast the composition of bacterial community with pollutants will allow protecting health status on mangroves sediments, as well as facilitate the characterization,

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production, and application of bacteria with biotechnological potential (Tan et al., 2015; Marquez, 2018; Dikit et al., 2019).

Therefore, this study assesses that concentrations and fluctuations in heavy metal levels affect the composition of the overall bacterial community, emphasizing on the most abundant Operational Taxonomic Unit – OTUs, to identify possible sentinel bacteria. We take as study case a mangrove swamps area surrounded by approximately 573 industries (Ministerio del Ambiente, 2012; Mariscal-Santi et al., 2018) within Guayaquil, Ecuador, in the north-west coast of South America. At present, sources of clandestine discharges continue in this region, which constantly contaminates the estuary (Mariscal-Santi et al., 2018). It is worth mentioning that, before sampling during 2013, the Ministry for the Environment of Ecuador carried out a biological remediation program based on the addition of bacterial cultures to the estuary (Expreso, 2013). The bioremediation program lasted 6 months approximately and resulted in a transient decrease of heavy metal levels (Expreso, 2013). Unfortunately, there is no publicly available information about this bioremediation program (type and numbers of bacteria added), which prevents a more detailed interpretation.

In summary, the estuary inside Guayaquil allows us this unique scenario for the identification of sentinel bacteria: an ecosystem where the decrease and subsequent increase in levels of heavy metals, in a previously polluted ecosystem, can be evaluated over three consecutive years. The contribution of our results relies on the identification and potential use of specific sentinel bacteria to inform mangrove conservation and management programs.

2. Materials and methods

2.1. Sampling sites and sample collection

Ecuador has approximately 105,230 Ha of mangroves on its coastal areas, where 81% of the ecosystem is found in the Gulf of Guayaquil (Calle et al., 2011). This site was selected because of the history of industrial water discharges in mangrove branches within the city of Guayaquil (Fernández-Cadena et al., 2014) but also by the presence of more pristine areas. Two sampling areas were selected: One within the city of Guayaquil (hereinafter, GYE; 2°10′13.80″S, 79°54′50.27″W), and a second one in the same estuary but inside a protected wildlife reserve in Puerto Hondo (hereinafter, PH; 2°11′55.66″S, 80° 0°26.28″W) (Fig. 1). The selected polluted site (GYE), located within Guayaquil city, has suffered direct discharge of industrial wastewater in the last 25 years and it has been reported as one of the world's most polluted mangrove areas (Fernández-Cadena et al., 2014). The unpolluted site (PH), located inside a protected wildlife reserve, has no reports of high levels of heavy metals. In GYE, sediment samples were taken in three different sampling stations, during three consecutive years (2012–2014). In PH, samples were collected in five sampling stations, during the year 2013 due to logistics constraints (Fig. 1). Surface sediment samples were named according to the year and site of sampling, as 12-GYE (December 7th- 2012), 13-GYE (December 6th - 2013), and 14-GYE (December 6th - 2014), and 13-PH (December 7th - 2013).

For heavy metal analyses, five samples were collected at each station using 10 mL polypropylene tubes previously cleaned with 10% HNO_3. Samples were then dried at 37 °C for three days and kept at 4 °C until further analysis. For nucleic acid extractions, ~ 1g of surface sediments were collected in triplicates at each sampling site and each time and stored in 2 mL sterile polypropylene tubes at −80 °C until further community DNA extraction.

2.2. Heavy metal determination

The concentration of the heavy metals Ag, Cd, Co, Cu, Mn, Mo, Ni, Pb, Se, Sn and Zn in the surface sediment samples were determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS, Thermo Fisher Scientific Finnigan X series 2), as described previously (Fernández-Cadena et al., 2014). The analytical quality was checked against a reference material (marine sediment, MESS-3) provided by the National Research Council, Canada (NRC-CNRC).

2.3. Assessment of heavy metal contamination

The degree of heavy metal contamination in the mangrove sediments was assessed by calculating the Enrichment Factor (EF) and the Geoaccumulation Index (I_{geo}). The EF standardizes the impact of terrestrial inputs by normalizing the metals of interest against another that has no anthropogenic source, in this case, aluminum (Qingjie et al., 2008), using the following equation.

$$EF = \frac{\left(\frac{C_x}{C_{ref}}\right) Sample}{\left(\frac{C_x}{C_{ref}}\right) Background} \quad (1)$$

where C_x is the measured concentration of heavy metal in the mangrove sediment and C_{ref} is the concentration of the metal selected as naturally enriched in all sample sites (Aluminum). EF is interpreted as follows:

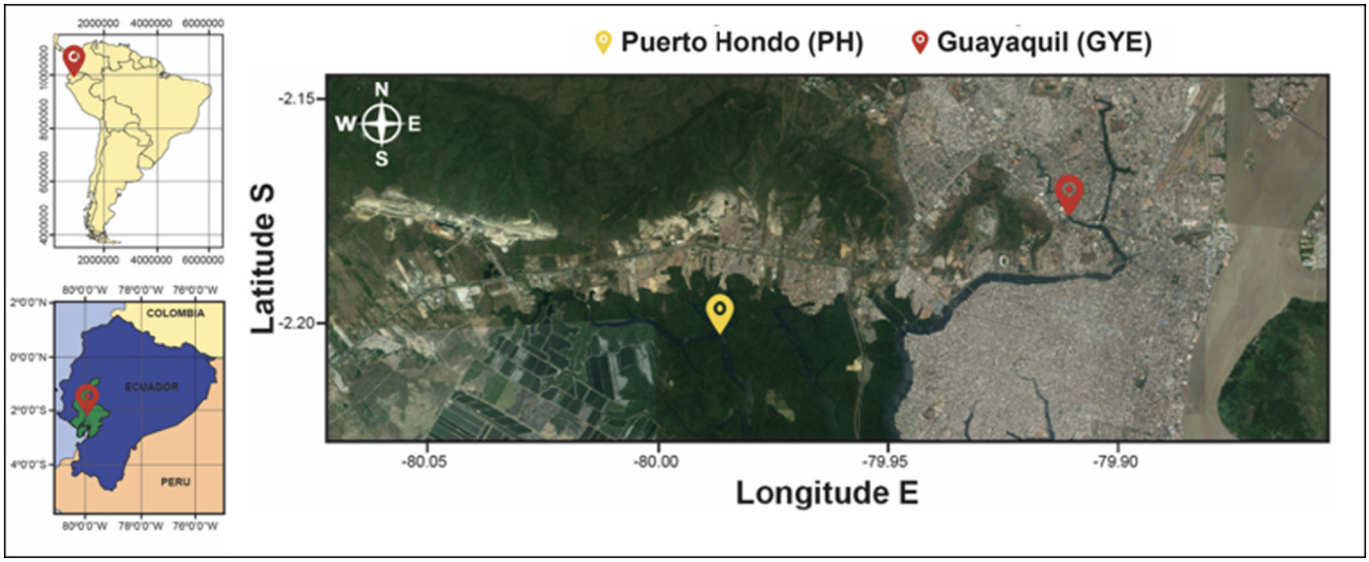

Fig. 1. Geographical location of the sampling stations in Guayaquil and Puerto Hondo mangroves.

EF < 1.5 indicates that there is no anthropogenic evidence, EF between 1.5 – 3 is interpreted as low; between 3 – 5 as moderate; between 5–10 as severe; and EF > 10 is regarded as evidence of very severe contamination due to anthropogenic influence (Qingjie et al., 2008).

The I_{geo} was first introduced by Muller (1981) and has been widely used to quantify the level of contamination in mangrove sediments (Usman et al., 2013). Values were calculated using the following equation (Eq. (2)):

$$I_{geo} = Log_2(C_n/1.5B_n) \quad (2)$$

where C_n is the measured concentration of heavy metal in the mangrove sediment, B_n is the geochemical background value in average clastic sedimentary rock (Turekian and Wedepohl, 1961), and 1.5 is the background matrix correction factor due to lithogenic effects. I_{geo} values are interpreted as background concentration if $I_{geo} \leq 0$; unpolluted if $0 \leq I_{geo} \leq 1$; moderately polluted to unpolluted if $1 \leq I_{geo} \leq 2$; moderately polluted if $2 \leq I_{geo} \leq 3$; moderately to highly polluted; if $3 \leq I_{geo} \leq 4$; highly polluted $4 \leq I_{geo} \leq 5$; and very highly polluted if $I_{geo} > 5$.

As metal concentration values did not fit a normal distribution, Box-Cox transformation (Box and Cox, 1964) was used to correct the non-normality as follows:

$$y = \begin{cases} x^{\lambda} - 1 & \lambda \neq 0 \\ \frac{\lambda}{\ln(x)} & \lambda = 0 \end{cases}$$

where y is the transformed data point and x is the value to be transformed for a specific set of data ($x1$, $x2$....xn), estimated under the assumption that the transformed values are evenly distributed. The transformation becomes logarithmic when $\lambda = 0$.

2.4. Heavy metal statistical analysis

The R framework was used for all the statistical analysis. To group samples based on metal level profiles, cluster analysis was conducted using group average based on Euclidian distances of the metal concentration. Clustering results are shown in a dendrogram as a visual representation of the steps of the hierarchical clustering solution (Le and Zidek, 1992). To test whether the metal distribution within the clusters has a non-random structure, SIMPROF was used with hierarchical clustering using *simprof* and *simprof.plot* from the *clustsig* package (Whitaker and Christman, 2014). SIMPROF routine is a hypothesis test with no a *priori* groupings, against the null hypothesis of absence of structure (Clarke et al., 2008). To test the significant differences between sampling locations, ANOSIM test was executed with *anosim* function from Vegan library (Oksanen et al., 2013), which required *a priori* grouping and is a hypothesis-based test for differences between groups.

2.5. DNA extraction and 16S rRNA gene tag-sequencing

Community DNA was extracted using the Power-Soil DNA Isolation Kit (Qiagen), and following manufacturer recommendations, with ~0.3 g of sediment. DNA integrity was evaluated by 0.8% agarose gel electrophoresis at 70 V, quantified using Quant-iT Picogreen (Invitrogen), and stored at −20 °C until further analysis.

Community DNA samples from 42 surface sediment samples collected from the two mangrove areas (27 from GYE and 15 from PH) were used for bacterial 16S rRNA gene Tag-sequencing analysis. The V4 hypervariable region of the 16S rRNA gene was amplified by PCR using 515F and 806R primer pair, as previously described in the Earth Microbiome Project (Caporaso et al., 2011). The reaction contained 1X reaction buffer, 2 mM of $MgCl^{+2}$, 0.3 mM of each dNTPs, 0.3 μM of each primer, 2.5 units of Kapa *Taq* DNA Polymerase (Kapa Biosystems) and 1 to 5 ng of template DNA with sterile water to 35 μL final volume. The PCR condition was as follows: 3 min of initial denaturation at 94 °C, 28 cycles at 94 °C for 30 s, at 57 °C for 1 min and at 72 °C for 1.5 min, followed by a final extension at 72 °C for 10 min.

After confirmation of the product by agarose gel electrophoresis (2% at 70 V), triplicate amplicons were pooled, then quantified using a standard *q*PCR assay using a Library Quant Kit Illumina (Kapa) according to manufacturer instructions, equimolarly pooled and sequenced using Illumina 300 cycles Miseq kit, following Caporaso et al., (2011) protocol. The sequences obtained in the present study are publicly available in the Sequence Read Archive database under the accession number PRJNA439352. The amplification of the 16S region in 12GYE1 and 13GYE2 samples was complex, due to low amount of DNA and nonspecific bands in the PCR. This was reflected in the sequence analysis showing low quality results and, for these reasons, were removed for further analysis.

2.6. Bacterial 16S rRNA gene sequences pre-processing

16S rRNA gene sequences were processed using Mothur (Schloss et al., 2009). Sequences were first demultiplexed, then assembled and assigned to samples by matching to barcode sequences using *make.contigs* script, and primers were removed using *cutadapt* (Martin, 2011). Sequences with undesired length (< 200–300 > bp), ambiguous nucleotides and homopolymers longer than 8 bp were removed from further analysis. After that, sequences were first aligned using the re-created Silva SEED v128 (Quast et al., 2013) as a reference. Chloroplast, Eukaryote, Archaea and mitochondria sequences were removed. Sequences were also checked for PCR chimeras using UCHIME version 4.2.40 (Edgar et al., 2011). High-quality sequences were clustered into operational taxonomic units (OTUs) with the furthest neighbor algorithm, with a minimum sequence identity cut-off of 97%. Taxonomic assignations were performed against Silva v128 (Quast et al., 2013).

In order to determine if the sequencing effort was representative, rarefaction curves were calculated per sample using *rarefaction.single*. For each sample, OTU richness (Chao) and Alpha-diversity measures (Shannon, Inverse Simpson) were computed with *summary.single* script using Mothur (Schloss et al., 2009). Differences in richness and diversity values of impacted and non-impacted groups were evaluated by one-way ANOVA followed by a post-hoc Tukey test (Box et al., 1978).

2.7. Taxonomy ordination of bacterial microbiome

The taxonomic and its statistical analysis were performed with R framework. To determine the components of the core microbiome from the mangrove sediments, a Venn diagram by OTU occurrences across communities was computed using LIMMA package (Ritchie et al., 2015).

Non-metric multidimensional scaling (NMDS) ordination based on Bray-Curtis distance with the five most abundant phyla was utilized to place the sample sites in two-dimensional spaces (*ordinate* script, Phyloseq software; McMurdie and Holmes, 2015). Beta-diversity analysis was performed to see how often the same taxon was observed in the mangrove microbiome (Lozupone et al., 2010). To determine its phylogenetic diversity, a Principal Coordinates Analysis (PCoA) using *ade4* R-library (Dray and Dufour, 2007) was conducted based on Weighted UniFrac (WUniFrac) (GUniFrac R-library; Chen, 2012) scores to visualize it graphically. Heatmaps were created using *gplots* R-package (Warnes et al., 2009), with relative abundance data of the 50 more-abundant OTUs.

2.8. Heavy metal and top abundant OTUs correlations

To identify possible sentinel bacteria, both Canonical Correspondence Analysis (CCA) and Redundancy Analysis (RDA) using *cca* function from *vegan* R-package (Oksanen, 2013) were conducted with the 10 more-abundant bacterial OTUs in all the sequences

analyzed. Besides, the taxonomic assignation of these OTUs was manually checked and corroborated by BLASTn algorithm (Altshul et al., 1990) against the 16S ribosomal RNA sequences [(Bacteria and Archaea) – NCBI database (last updated on March 6, 2017)]. The abundance of the 10 OTUs that show a more marked influence of heavy metal concentration based on CCA analysis were correlated with heavy metal levels using Pearson methodology.

3. Results and discussion

3.1. Heavy metals concentration is higher in polluted surface mangrove sediments compared to pristine mangroves

Metal concentration values were significantly higher at GYE in comparison to that at PH (Table S1). Maximum Cu concentration in GYE was up to 5 times higher than in PH (177.5 $\mu g\,kg^{-1}$ and 21.1 $\mu g\,g^{-1}$, respectively) and Zn was up to 10 times higher in GYE than in PH (431.4 $\mu g\,kg^{-1}$ in GYE and 37.4 $\mu g\,kg^{-1}$ in PH). The elevated concentrations of Ag, Cd, Cu, Pb, and Zn detected in Guayaquil are similar to levels detected in other mangroves polluted by industrial water discharges in Brazil and India (Cabral et al., 2016; Balakrishnan et al., 2017). Se, due to its natural presence in the sediment (Suzuki et al., 2012), was the only metal that was present in higher concentrations in PH in comparison to GYE (11.1 $\mu g\,kg^{-1}$ and 3.4 $\mu g\,kg^{-1}$ respectively). Besides, metal levels from 2012 to 2014 in GYE, shown a decrease in the concentrations during 2013 and a subsequent increase during 2014. For example, Zn levels in 2012 ranged from 327.1 - 527.2 $\mu g\,kg^{-1}$, which decreased to a range of 194.2–291 $\mu g\,kg^{-1}$ in 2013, while in 2014 was between the levels of 210.6–637.3 $\mu g\,kg^{-1}$ (Table S1).

To detect if the levels of metals found here are due to anthropogenic influence, we estimate the EF and I_{geo} indexes. Except for Se, the metal signature of 12-GYE, 13-GYE and 14-GYE in comparison to 13-PH were between 2 to 7 times higher in EF and I_{geo} indexes (Table S1). EF and I_{geo} are indicators used to assess the presence and intensity of anthropogenic contaminant deposition on surface soil (Barbieri, 2016). The increase of these indexes in GYE and the proximity between sampling sites and industrial water discharge pipes, strongly support the anthropogenic origin of heavy metals in GYE. The relationship between industrial wastewater discharges and increases in metal levels has also been reported in other mangrove areas (Machado et al., 2002; Chakraborty et al., 2014; Almasoud et al., 2015; Kumar et al., 2016).

Considering the stability of heavy metal levels detected in the mangrove sediments, GYE samples shown changes on heavy metal concentration between years. Unexpectedly, the concentration of metals decreased between 2012 and 2013, and then increased in 2014. Samples taken at GYE in 2013 clustered apart from those taken in GYE in 2012 and 2014, with a non-random grouping (SIMPROF $p = 0.01$ at 999 iterations; Fig. 2).

3.2. Bacterial community changes among polluted and non-polluted mangrove sediments

After the removal of undesired data, pre-processing and taxonomic classification, the initial dataset with 4,608,839 sequences decreased to 2,316,159 sequences, with a total of 8648 Operational Taxonomic Units (OTUs). The summary of the dataset is shown in Table S2. The maximum number of OTUs was observed in 12GYE, with an average of 5757 ± 137 OTUs, whereas 13PH reached a minimum at 4163 ± 275 OTUs (Table S2). Rarefaction curves showed good coverage, indicating that it was possible to recover most of the OTUs for all GYE and PH samples (Fig. S1).

A core microbiome analysis (Fig. 3a) indicated that of all the bacterial OTUs detected, 3406 (39.38%) were common to all sample sites and years (912,215 sequences), indicating that almost 40% of the bacterial communities seems to be not affected by the pollution. In addition to this, it was observed that in GYE, more OTUs are shared among them (21.8%) compared to PH (up to 3.4%), indicating that the presence of pollution decrees the heterogeneity of the area. Nogueira et al. (2015) determined that the anthropic homogenization of microbial communities is a potential risk of microbial diversity on mangroves (Nogueira et al., 2015). Members of the core microbiome have critical functions such as health and nutrient cycling, as previously described in mangroves and coraline environments (Cabral et al., 2016; Hernández-Agreda et al., 2017).

From 58 phyla detected in the core microbiome, Proteobacteria, Firmicutes, and Bacteroidetes were the most abundant (33.8%, 25.14% and 17.59% of relative abundance, respectively; Fig. 3b). Other phyla detected were Chloroflexi (5.57% ± 2.21) Planctomycetes (1.63% ± 1.05) and Acidobacteria [0.21% ± 1.1] (Fig. 3b). This phyla distribution is similar to that described in other mangroves, such as in Brazil and Hong Kong (Lin et al., 2018; Jiang et al., 2013). NMDS and Beta-diversity analysis by WUniFrac metric distances showed dissimilarity between GYE and PH. However, within GYE the sample sites are closer to each other in each sampling year (Figs. S2 and S3).

In GYE, 48.39% of the Proteobacteria abundance was associated with Delta-proteobacteria, a bacterial class related to sulfate reduction metabolism (Varon-Lopez et al., 2014). The high abundance of Delta-proteobacteria has been recently observed in ecosystems affected by heavy metals (Cabral et al., 2016) and other pollutants such as hydrocarbons (Dos Santos et al., 2011; Andreote et al., 2012; Varon-Lopez et al., 2014), which stimulate the proliferation of these taxa. On the other hand, sequences associated with Gamma-proteobacteria in sediments contaminated by heavy metals were decreased by 1.6 X in 13-GYE (31.04%) in comparison with 13-PH (49.36%). It has been described that sulfur-oxidizer Gamma-proteobacteria in sediment particles are responsible for up to 70% of CO_2 fixation (Wang et al., 2016). Its presence is related to unpolluted mangrove estuaries (Cabral et al., 2016).

In contrast with GYE, in Puerto Hondo mangrove bacterial sequences associated to Firmicutes represented a 40% of the relative abundance (Fig. 3b), a high abundance even in comparison to other unpolluted mangroves from Brazil, China, and India (Behera et al., 2013; Cabral et al., 2016; Chen et al., 2017). Most abundant Firmicutes classes were Clostridia (Fig. 3e), with a high number of sequences associated with SOB bacteria.

Finally, Bacteroidetes were distributed evenly both over the years and geographically between GYE y PH (Fig. 3b). Nonetheless, the abundance of sequences associated with Flavobacteria and VC2.1_Bac22 classes dramatically change between PH and GYE (Fig. 3c). Previous studies have shown that the abundance of Bacteroidetes is favored in pristine mangroves compared to those polluted by hydrocarbons (Andreote et al., 2012; Basak et al., 2015). Here, it was observed that VC2.1_Bac22 is also favored in an environment with high concentration of heavy metals. If this variation could be directly associated with changes in heavy metal concentrations, then it would make this OTU a good candidate to sense pollution.

3.3. Identification of sentinel bacteria in polluted mangrove sediments in the Gulf of Guayaquil

To identify candidate sentinel bacteria that are related to heavy metal concentrations, we consider those OTUs that showed a significant difference in relative abundance between both mangrove areas. Fig. 4 shows the 50 more-abundant OTUs, that account of 65% of the total number of sequences (1,504,160 sequences). Cluster analysis separates all the samples in two main groups: one formed by GYE samples and other by PH samples, with a strong taxonomic differentiation between sites and years, in the case of GYE. The taxonomic assignation of the 50 most abundant OTUs described by mothur, was also check manually using the BLAST algorithm at NCBI (Table S3). This allowed us, in some cases, to identify bacterial sequences at the species level. This analysis

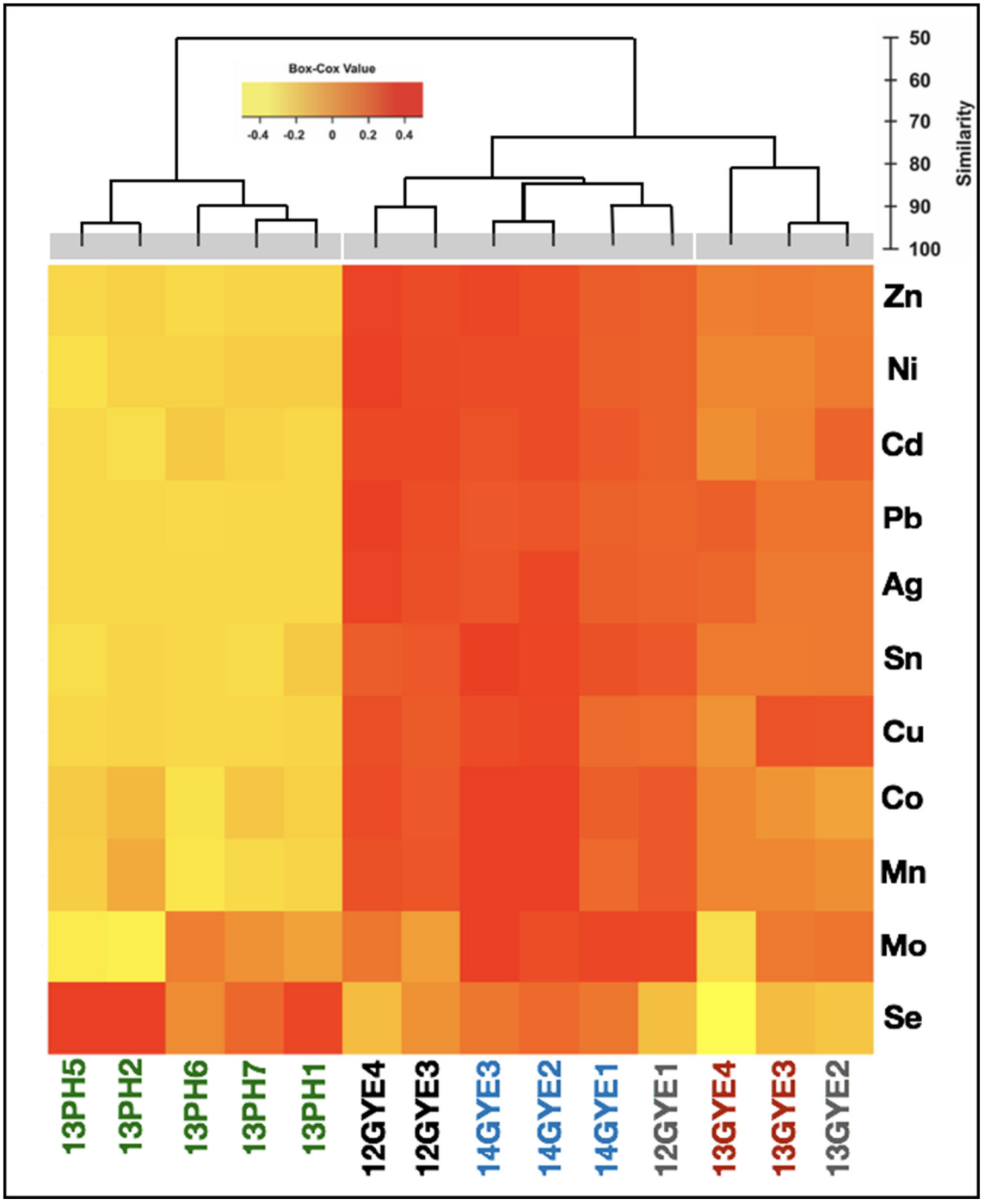

Fig. 2. Hierarchical cluster analysis based on heavy metal concentrations at Guayaquil (GYE) and Puerto Hondo (PH) mangroves. Heatmap represent metal levels (Box-Cox transformation) distributed over superficial mangrove sediment from GYE and PH. Cluster analysis was conducted on Euclidean distance between metal levels at each sample; gray boxes represent real groups (P = 0.01 at 999 iterations, SIMPROF).

was also corroborated using the same databases, but with the DADA2 workflow instead (Callahan et al., 2916). While DADA2 has proven to be a powerful tool for identifying species, mothur results show similar results in terms of bacterial community composition and identifying the most abundant sequences. In the case of mothur for the 50 most abundant, the BLAST manual search was performed, giving similar results with DADA2. Since mothur was unable in some cases to identify some of the OTUs at species level, manual identification was performed in NCBI (Table S3) giving similar results with DADA2.

Among the most abundant and frequent OTUs in GYE were sequence affiliated to sulfate-reducing bacteria (SRB) members from the Delta-proteobacteria, e.g. Desulfobulbaceae, *Desulfatiglans* sp., *Desulfonatronobacter* sp., *Desulfococcus* sp., Desulfarculales, and Desulfobacteraceae. In PH, there is a marked presence of OTUs associated with autotrophic facultative SOB such as the Gamma-proteobacteria *Shewanella* sp. and *Microbulbifer* sp. and the Alpha-proteobacteria *Thioclava* sp. All these SOB genera are proven to be beneficial for the development of mangrove rhizosphere (Jiang et al., 2013). This difference in SRB and SOB abundances between polluted and pristine mangrove sediments was also reported on mangrove sediments from Brazil, which changes were suggested to be due to the ability of SRB to cope with the contaminants during the sulfate reduction process

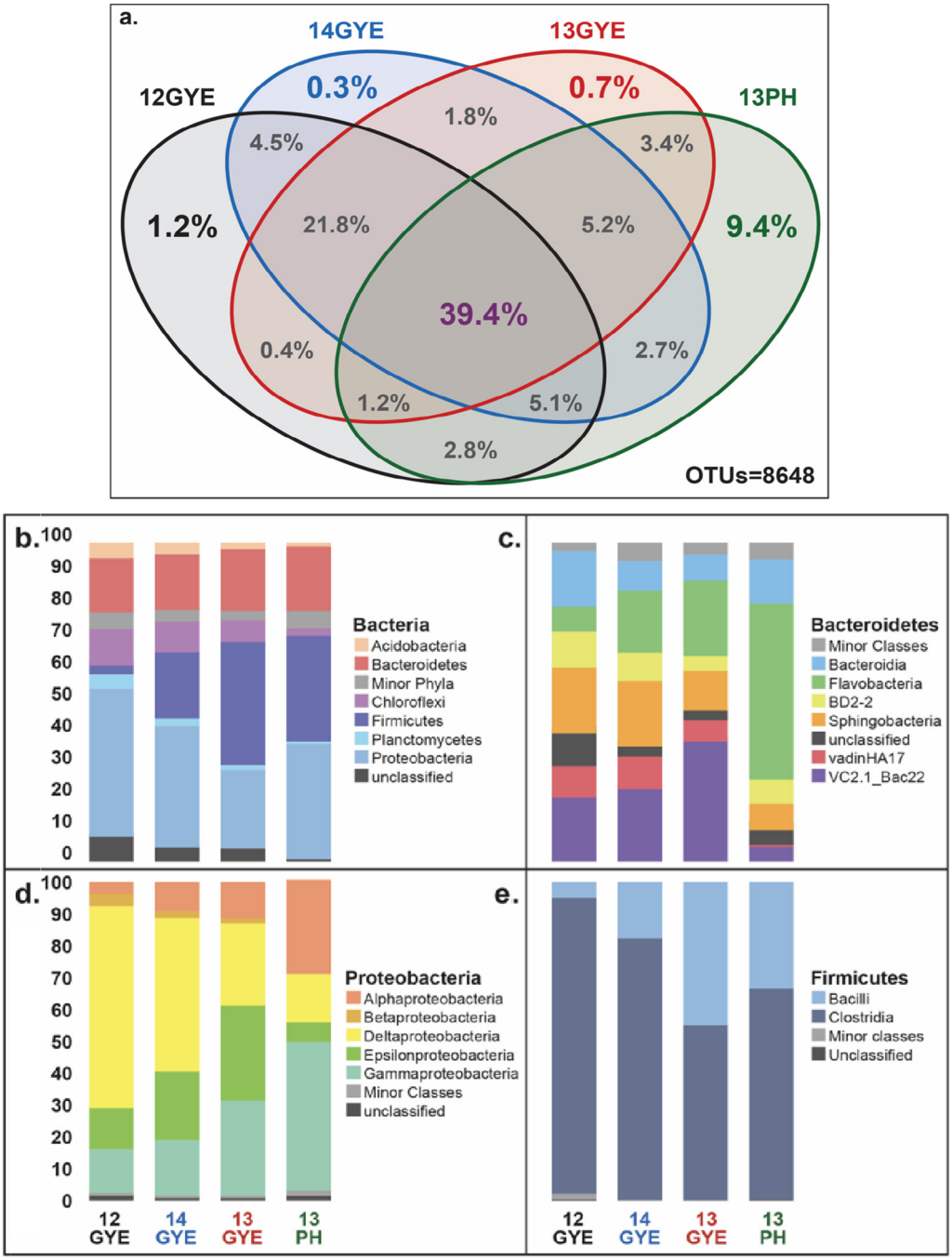

Fig. 3. Core bacterial microbiome and taxonomic composition of Bacteria from Ecuadorian mangrove sediments. (a) Venn diagram showing shared OTUs between sample sites. (b–e) Taxonomic composition based on partial 16S rRNA sequences. (b) corresponds to Phylum level, (c), (d) and (e) correspond to class level.

(Varon-Lopez et al., 2014).

There are taxa that, in addition to being favored by anaerobic conditions, are also capable of succeeding in ecosystems with high heavy metal concentrations. An example is *Sulfurovum lithotrophicum* (OTU00004 in Fig. 4) that was abundant in all the sampling sites and during all years, including when heavy metals increased (12GYE and 14GYE). *Sulfurovum lithotrophicum* has been identified in environments dominated by oxidation and reduction of sulfurous compounds (Zouch et al., 2017), since its metabolism allows, in addition to oxidizing sulfide, reduce thiosulfate (Jeon et a., 2017).

Here, associated to the bioremediation program developed in the Gulf of Guayaquil between 2013-2014, we demonstrate that heavy metal levels decrease and, consequently, bacterial community composition and structure changed, suggesting that the pilot remediation plan used was effective and point the presence of potential sentinel bacteria of pollution. For example, there was a decrease in the abundance of members of the Deltaproteobacteria class (Fig. 3d) between 12GYE and 13GYE, with a subsequent increase in 14GYE (e.g. *Desulfatibacillum alkenivorans* – OTU000125; Fig. 4). Moreover, when all metals were tested (Table S1) and in order to detect if any of the OTUs are related to any of the environmental variables, CCA indicates a strong correlation between bacterial community changes at GYE and the concentration of Ag, Cd, Cu, Mn, Pb, Se, Sn, and Zn, that explain a 67.8% of the total sample variance (Fig. 5). Similar results are shown with Redundancy Analysis (RDA), when considering linear relationship between variables instead unimodal (Fig. S4). OTUs assigned as

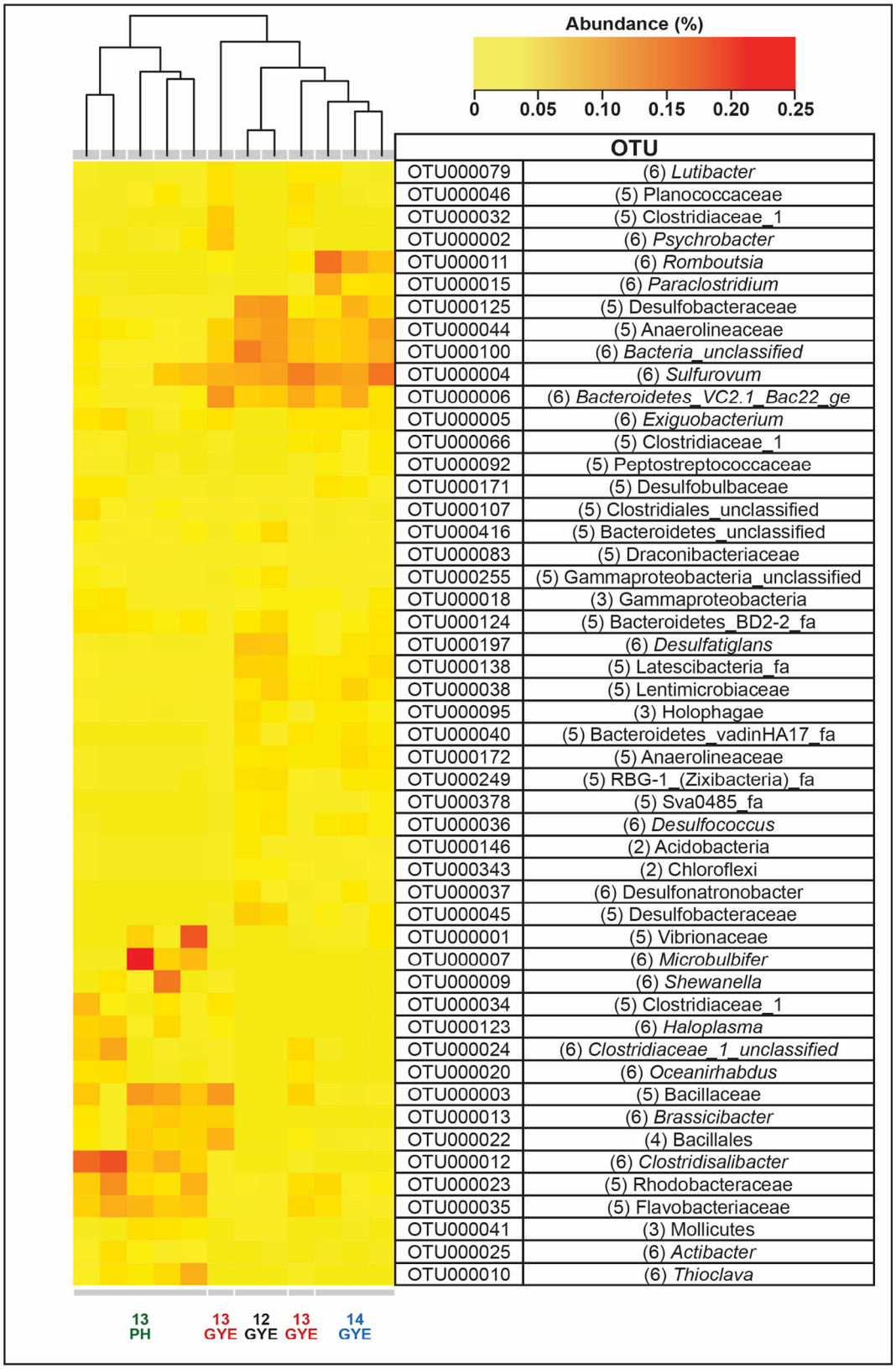

Fig. 4. OTU distribution and clustering. Heatmap represents the bacterial relative abundance (50 most abundant OTUs). Cluster above the heatmap was generated using Bray-Curtis distance. Color keys represent relative abundance in percentages. The numbers represent the taxonomic resolution level of the assignation, with (1) = Domain, (2) = Phylum, (3) = Class, (4) = Order, (5) = Family and (6) = Genus. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

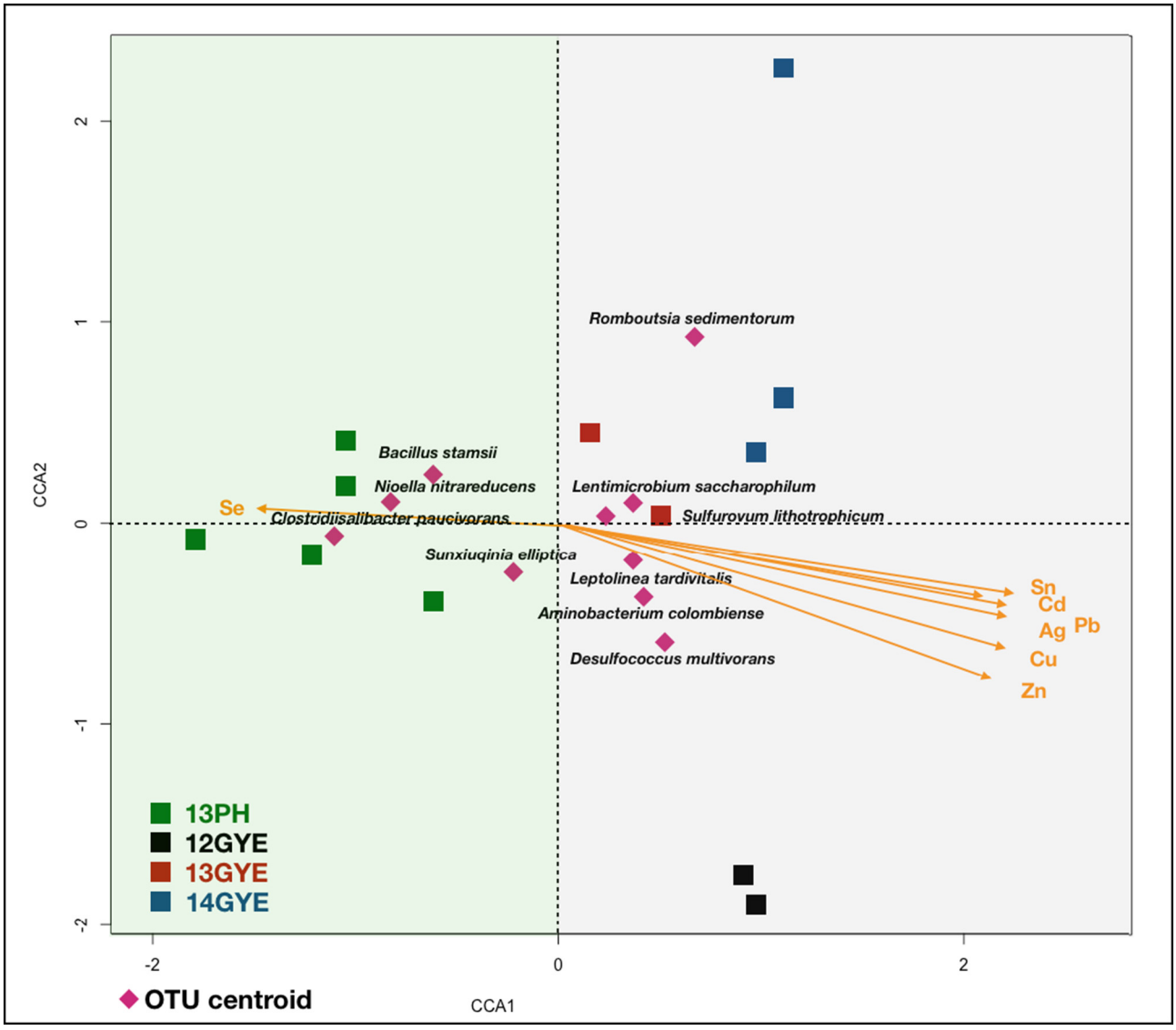

Fig. 5. Distribution of the most abundant OTUs. Canonical Correspondence Analysis (CCA) between the Top 10 most abundant OTUs in mangrove sediments and heavy metal concentration at each site and year of sampling.

Table 1
Pearson correlation coefficient matrix showing the relationship between the concentration of heavy metals and bacterial taxa that can be used as sentinel bacteria.

Taxa associated	Pb	Zn	Se	Ag	Cd	Sn
Sulfurovum litothropicum	0.76 **	0.76 **	−0.47	0.8 **	0.75 **	0.75 **
Lentimicrobium saccharophilum	0.78 *	0.46	−0.74	0.63*	0.55 *	0.47
Leptolinea tardivitalis	0.82 ***	0.8 ***	−0.3	0.8 **	0.85 ***	0.83 ***
Desulfococcus multivorans	0.68 *	0.74**	−0.17	0.7 **	0.77 **	0.7 **
Romboutsia sedimentorum	0.5	0.59	0	0.59	0.54	0.66 *
Clostridiisalibacter paucivorans	−0.9 ***	−0.81 *	0.32	−0.9 ***	−0.82 ***	−0.82 ***
Nioella nitrareducens	−0.81 **	−0.74	0.28	−0.78 *	−0.75 *	−0.74 *
Bacillus stamsii	−0.3	−0.52	−0.16	−0.39	−0.49	−0.5 *
Sunxiuqinia elliptica	0	0.13	0.17	0	0	0

*P = 0.01, **P = 0.001, ***P = 0.0001.

Sulfurovum litothropicum (OTU000004), *Lentimicrobium saccharophilum* (OTU000006), *Leptolinea tardivitalis* (OTU000172) and *Desulfococcus multivorans* (OTU000036) were the most influenced by metal levels (Fig. 5). In the case of *Romboutsia sedimentorum* and *Sunxiuqinia elliptica* it is observed that the centroid is far from vectors, additional to this and given that correlation values with metals are not significant (Table 1). its presence in the microbiome would not be significantly related to the increase or decrease of heavy metal levels (Table 1). In the case of Se, it showed a positive and significant correlation with heavy metal concentrations (Table 1). PH showed *Bacillus stamsii* (OTU000003), (OTU000035), *Nioella nitrareducens* (OTU000023) and *Clostridiisalibacter paucivorans* (OTU000012) as more representative taxa. However, Pearson test showed a non-significant correlation between these taxa and Se, the metal with highest concentration in PH (Table 1).

The increase in abundance of *Desulfococcus multivorans* (OTU00036) and *Leptolinea tardivitalis* (OTU000172) correlates with the increase of Ag, Cd, Cu, Pb, Sn and Zn in GYE. In contrast, lower metal levels were related to the increase in some SOB (e.g. *Clostridiisalibacter paucivorans* (OTU000012), *Nioella nitratireducens* (OTU000023) and *Bacillus stamsii* (OTU000003). This was previously described in mangroves from Brazil, but at that study there was not a taxonomic description of bacteria associated to that phenomenon (Varon-Lopez et al., 2014).

The results presented here support the use of bacterial community structure variation analysis, and mainly those associated to the sulfur cycle, to follow the perturbation state of mangrove sediment and propose that tool as a potential way for heavy metal monitoring programs (Sun et al., 2012; Mejias Carpio et al., 2017). This implies that some specific taxa can be easily monitored in contaminated estuaries, that is the characteristic of being a good candidate for sentinel bacteria.

Subsequently, besides to associate the presence of sentinel bacteria, it is possible to track metabolic pathways or diverse mechanisms of response to pollutants. This could be a mechanism to diagnose a pollution event and evaluate the effectiveness of the restoration programs.

4. Conclusions

- The fluctuation of heavy metal levels within Guayaquil is reflected at the same time to changes in bacterial composition.
- There are bacteria that benefit in disturbed ecosystems, such as *Desulfococcus multivorans* that increase in abundance when metal levels are higher.
- *Sulfurovum lithotrophicum* has the ability to adapt regardless of the levels of heavy metal in the sediment, while *Bacillus stamsii* have low levels of abundance when heavy metal concentrations are elevated.
- The lack of correlation of metal levels with abundant bacteria such as *Romboutsia sedimentorum* and *Sunxiuqinia elliptica* would mean that specific taxa are indifferent to metal concentrations.
- Given the anaerobic sediment condition, variation in heavy metal levels in mangrove sediments significant correlates with the variation of the abundance of several bacterial groups related to the sulfur cycle.
- This study showed that bacteria, specially sulfur-related, could be sentinels to diagnose and to execute management and remediation programs in mangrove ecosystems contaminated by heavy metals.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2019.110701.

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