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RESEARCH ARTICLE

Effects of metals on activity and community of sulfate-reducing bacterial enrichments and the discovery of a new heavy metal-resistant SRB from Santos Port sediment (São Paulo, Brazil)

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Abstract

Sulfate-reducing bacteria (SRB) can be used to remove metals from wastewater, sewage, and contaminated areas. However, metals can be toxic to this group of bacteria. Sediments from port areas present abundance of SRB and also metal contamination. Their microbial community has been exposed to metals and can be a good inoculum for isolation of metal-resistant SRB. The objective of the study was to analyze how metals influence activity and composition of sulfate-reducing bacteria. Enrichment cultures were prepared with a different metal (Zn, Cr, Cu, and Cd) range concentration tracking activity of SRB and 16S rRNA sequencing in order to access the community. The SRB activity decreased when there was an increase in the concentration of the metals tested. The highest concentration of metals precipitated were 0.2 mM of Cd, 5.4 mM of Zn, 4.5 mM of Cu, and 9.6 mM of Cr. The more toxic metals were Cd and Cu and had a greater community similarity with less SRB and more fermenters (e.g., *Citrobacter* and *Clostridium*). Meanwhile, the enrichments with less toxic metals (Cr and Zn) had more sequences affiliated to SRB genera (mainly *Desulfovibrio*). A new *Desulfovibrio* species was isolated. This type of study can be useful to understand the effects of metals in SRB communities and help to optimize wastewater treatment processes contaminated by metals. The new *Desulfovibrio* species may be important in future studies on bioremediation of neutral pH effluents contaminated by metals.

Keywords Metals · *Desulfovibrio* · Sulfate-reducing bacteria · Metal resistance · Bioremediation · Metal removal

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Introduction

Metals are persistent and dangerous contaminants in the environment. Chronic exposure to metals in the environment is a real threat to living organisms, for example metal concentrations above threshold levels (e.g., Cr 37.3 μg mg^{-1}, Cd 0.6 μg mg^{-1}, Zn 123 μg mg^{-1}) affect the microbiological balance of soils and can reduce their fertility (Wieczorek-Dąbrowska et al. 2013; CCME 2002). Another issue is that bioaccumulation of toxic metals in biota of different ecosystems may have adverse effects on animals. Higher levels of metals in biota can have negative effects on the ecological health of aquatic animal species and may contribute to declines in their populations. In the same way, the microbial community is influenced by metal concentrations; high metal levels in the environment can decrease the microbial diversity and their degradation capacity (Ali et al. 2019).

The increase in metal concentrations is most often found near industries and other human activities. The presence of metals in effluents and industries and the lack of proper treatment and disposal contribute to this fact (Masindi and Muedi 2018). Various industries such as mining, chemicals, paper, pesticides, glass, and ceramics contribute to the increase in the concentration of metals found in wastewater and sewage sludge (Sarioglu et al. 2010; Fu and Wang 2011).

Biological sulfate reduction can remove metals from wastewaters, and immobilize metals from soils (e.g., compost). This application is possible due to the difference in the chemical properties of sulfate and sulfite metals. Metals such as cadmium, cobalt, copper, iron, zinc, chromium, and nickel are soluble when in the form of sulfate, but insoluble when in the form of metal sulfide (Paulo et al. 2015). Therefore, sulfide production by sulfate-reducing bacteria (SRB) causes precipitation of metals present in the environment, facilitating their removal (Ayangbenro et al. 2018; Azabou et al. 2007).

SRBs are divided into 7 phylogenetic groups: 5 belong to the *Bacteria* domain (*Deltraproteobacteria, Nitrospirae, Thermodesulfobiaceae, Thermodesulfobacteria, Clostridia*); and 2 to the Archaea domain (*Euryarchaeota* (genus *Archeoglobus*) and *Crenarchaeota* (genus *Caldivirga* and *Thermocladium*)) (Muyzer and Stams 2008).

This group of anaerobic microorganisms can be found in many anoxic environments, where they use sulfate as the final electron acceptor for the degradation of organic compounds or hydrogen, resulting in sulfide production. The sulfide produced can bind metal ions dissolved, forming metal sulfide (Hu et al. 2020; Muyzer and Stams 2008).

Beside this SRB application, there is a challenge to find an efficient treatment for effluents contaminated with metals using this microbial group (Paulo et al. 2015; Zhang et al. 2016). SRB activity decreases due to the presence of metals in higher concentrations (Bhattacharya et al. 1995). For example, some industrial effluents can have a concentration of 4.6–17.6 mM of Zn and 5.8–22.1 mM of Cr (Sancey et al. 2011). However, some Zn and Cr toxic concentrations, reported in the literature, during the effluent treatment are as follows: 0.85 mM of Zn and 0.65 mM of Cr in methanogenic granules (Paulo et al. 2015); 0.22 mM of Zn and 0.16 mM of Cr in a sewage sludge digester (Lin 1992); 0.57 mM of Zn for anaerobic mixed culture (Zayed and Winter 2000); 8.4 mM of Cr and 12.6 mM of Zn in acetogenic granules (Paulo et al. 2015). This demonstrates that the presence of metals in sewage can affect the performance of SRB during treatment, since the concentrations found in some effluents are much higher than the concentration which represents toxicity to microorganisms.

However, bacteria, including SRB, can develop resistance through a large diversity of metal resistance genes via both vertical evolution and horizontal gene transfer in order to adapt to the exposure of metal toxicity (Ture et al. 2018; Argudín et al. 2019). Many studies indicate that in contaminated sites, where bacteria were exposed to metal toxicity, the number of resistance genes increases. Moreover, the community in contaminated sites can adapt to metal contamination (Imchen et al. 2018; Guo et al. 2017a; Gillan et al. 2015). Thus, the knowledge about how metals can influence the activity and composition of sulfate-reducing bacteria and which of these SRB can survive in high concentrations of metals can be crucial for the development of bioremediation strategies.

Usually, port areas have metal concentrations above levels considered acceptable by environmental companies (Buruaem et al. 2012). Therefore, in these areas, microbial communities are usually exposed to metal contamination over long periods of time. Allied to this fact, in marine environments, there is a significant abundance and diversity of sulfate-reducing bacteria, since this group is very important in carbon cycling (Jørgensen 1982; Muyzer and Stams 2008).

In this way, in a long-term metal contaminated sediment, microorganisms would present higher resistance to metal toxicity. Thus, there would be greater chances of isolating strains resistant to metals. Selection-resistant SRB is important for enhancing the performance of bioreactors treating metal-contaminated wastes. Thus, the objective of the present work was to analyze, using a long-term metal contaminated sediment from a port area as inoculum, how metals influence the microbial community composition and activity of sulfate-reducing bacteria. Besides that, the study also aimed at performing isolation of SRB-resistant strains.

Material and methods

Study area and sample collection

The Port of Santos is located in the municipality of Santos, central region of the state of São Paulo coast (Brazil).

Sediment was collected from 5 stations in the Port of Santos (1- 23°56′06.97″S, 46°23′09.30″O; 2- 23°54′01.26″S, 46°22′40.10″O; 3- 23°55′08.48″S, 46°21′11.57″O; 4- 23°55′35.99″S, 46°18′38.69″O; 5- 23°58′11.78″S, 46°17′38.63″O): near industrial effluents or areas proven to be critically contaminated by metals (Pinto et al. 2015; Abessa et al. 2008; Buruaem et al. 2012) (Fig. 1).

Sediment characterization

Each sampling station was characterized physiochemically using a multi-parameter probe (Horiba U-52G). The parameters measured were pH, salinity, temperature, depth, dissolved oxygen, and conductivity. In the laboratory, sediments were characterized. The percentage of organic matter (OM) content was analyzed through muffle: 20g of oven-dried sediment sample (105 °C; 24 h) was placed in a high-form porcelain crucible and set in a muffle furnace for combustion at 600 °C for 6 h. The organic matter content was determined through the mass difference in relation to the original soil sample (Jiménez and Garcia 1992). The percentage of total organic carbon (TOC) was carried out from oxidation with potassium dichromate and sulfuric acid, and subsequent titration of dichromate over oxidation with ammoniacal ferrous sulfate, as described in Gaudette et al. (1974).

The metal concentration was determined by the SW 846 US EPA 3051 methodology (USEPA 1994), based on an acidic digestion (Navarro et al. 2011). Metals were quantified by inductively coupled plasma atomic emission spectrometry (ICP-AES) (Spectro Arcos, Germany). Analysis was performed by using the operational parameters recommended by the manufacturer: incident power of 1.2 kW; plasma, nebulizer, and auxiliary argon flow rates of 20, 1.0, and 1 L min^{-1}, respectively, and observation plasma height of 15 mm above the induction coil (Navarro et al. 2011). Target metals for analysis were Cr, Cu, Zn, and Cd due to their environmental relevance in the area (Pinto et al. 2015; Abessa et al. 2008; Buruaem et al. 2012).

Enrichment set-up and activity track

First, 45 mL of Postgate C medium (Supplementary material) was dispersed in 150 mL anaerobic bottles and different concentrations of metals were added (Cabrera et al. 2006; Gardner and Stewart 2002; Luptakova and Kusnierova 2005). Lactate was used as electron donor (60 mM) and sulfate was used as electron acceptor (30 mM). Cadmium was used in the form of $CdSO_4xH_2O$ at different concentrations of 0.2, 0.3, 0.4, and 0.5 mM. Copper was used in the form of $CuSO_4$ at concentrations of 0.3, 1.3, 3.0, and 5 mM. Chromium was used in the

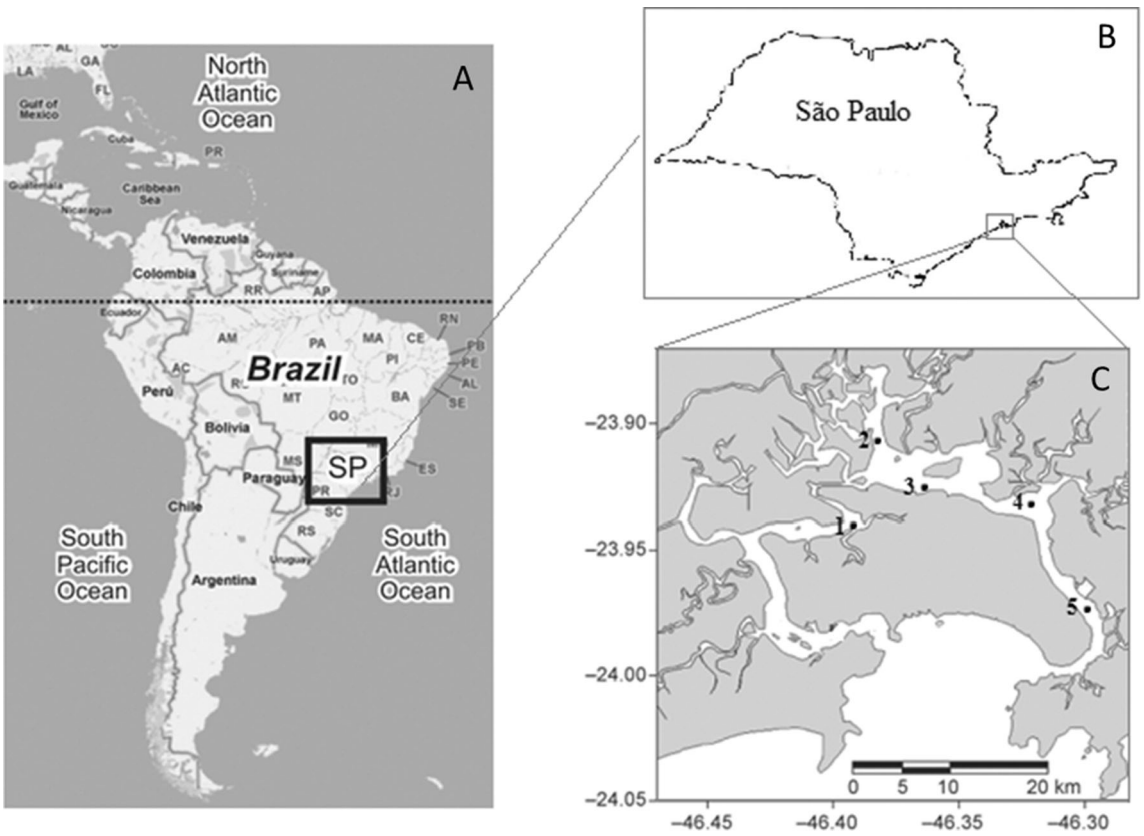

Fig. 1 Map of the study area located in Brazil (**A**). Port of Santos located at São Paulo State (**B**). In detail, location of the 5 stations where the sediment samples were collected (**C**)

form of $Cr_2(SO_4)_3$ at concentrations of 0.4, 4.0, 7.0, and 13 mM. Zinc was used in the form of $ZnSO_4$ at concentrations of 0.3, 1.0, 6.0, and 10 mM. Each metal was tested separately.

The inoculum was prepared using the three sediment samples (~10 mL each) mixed and diluted in 40 mL of a NaCl anoxic solution (0.9 % (w/v)). A 10% v/v of sediment slurry (5 mL) was added to the bottles with 45 mL of Postgate C medium, to a final volume of 50 mL. A positive control with medium without metal addition and negative control (medium and metals without inoculum addition — the highest concentration used in each enrichment was used in the negative control, also to check spontaneous precipitation) was prepared. Enrichments were incubated statically in the dark at 30 °C for 35 days. All the experiments were performed in triplicates.

The SRB activity in each enrichment was monitored through weekly sulfate and sulfide measurements. Sulfate was measured using turbidimetric method (APHA 2012).

The total dissolved sulfide concentration was determined using the methylene blue method (APHA 2012) with the USEPA Methylene Blue Method kit (HACK®, USA) following the manufacturer's instructions. Sulfide was measured immediately after removing the sample from the enrichments.

The lactate and other organic acids produced in each bottle at the end of the experiment were measured through gas chromatography GC-2010 (Shimadzu Scientific Instruments Columbia, MD, USA) equipped with HP-INNOWAX column (30 m length × 0.25 mm inner diameter × 0.25 mm film thickness) and flame ionization detector (FID), using hydrogen as a carrier gas, according to the methodology described by Adorno et al. (2014). The concentration of dissolved metals was measured at the beginning and at the end of the experiment using a High-Resolution Continuum Source Atomic Absorption Spectrometer (model 300, Analytik Jena, HR-CS AAS).

Microbial community analysis

The sediment used as inoculum and 1 mL of each enrichment after incubation were taken for DNA extraction. DNA extraction was performed using a PowerSoil® DNA Extraction Kit (MOBIO, USA), according to the manufacturer's instructions. A DNA purification kit Wizard® DNA Clean-Up System (Promega, USA) was used to reduce PCR inhibition.

DNA concentration was measured with a fluorimeter (Quibit® 2.0). PCR was performed in triplicate at a total volume of 50 μL containing 10–20 ng μL^{-1}, 1X HF PCR buffer, 0.2 mM dNTPs, 2 U μL^{-1} of Phusion Hot start II DNA polymerase (Promega, Madison, WI), 10 μM of primer 515F-806R (Caporaso et al. 2011) with different barcodes for each sample, 0.2–0.4 ng μL^{-1} of DNA and nuclease-free water to the final volume. The amplification program consisted of one step of denaturation at 98 °C for 30 s, 25 cycles of denaturation at 98 °C for 10 s, annealing at 56 °C for 10 s, and elongation at 72 °C for 10 s; and a final extension step at 72 °C for 7 min.

The size of the PCR products was checked by gel electrophoresis on a 1% (w/v) agarose gel containing 1x SYBR® Safe (Invitrogen, Carlsbad, CA). PCR products were purified using a HighPrep™ PCR Clean-up System (MagBio Genomics Inc., USA). The purified PCR products were mixed in equimolar amounts with a DNA concentration of 100 ng μL^{-1}. The clustered amplicons were sequenced using FLX Genome Sequencing in combination with titanium chemistry (GATC-Biotech, Konstanz, Germany).

All sequence readings were processed by the NGS SILVA rRNA analysis pipeline (SILVAngs 1.0) (Pruesse et al. 2012). Identical readings were identified, and the exclusive readings were grouped into operational taxonomic units (OTUs), applying identity criteria of 0.98. To obtain the taxonomic profiles, the generated sequences were submitted to computational analysis using the MOTHUR bioinformatics program (Schloss et al. 2009). The cluster analysis of bacterial communities was done using PAST4.01.

All data were submitted to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) (https://submit.ncbi.nlm.nih.gov/subs/sra/) through BioProject number PRJNA531183.

Isolation of metal-resistant SRB from the enrichments

The enrichments with the highest metal concentrations that presented sulfate reduction activity were chosen as a source for the isolation process.

A solid media was made with the same composition of the media described before adding 13% of Nobel Agar (Sigma-Aldrich), and distributed in petri dishes, using the anaerobic tent to preserve the anoxic conditions. One hundred microliter of the chosen enrichments was inoculated using the Spread plate technique. The plates were disposed in anaerobic jars pressurized with N_2/CO_2 (80:20, v/v) that were incubated for a maximum of 28 days at 30 °C, or until colony growth was observed.

The colonies observed in solid media were inoculated in 15 mL liquid cultures with lactate, sulfate, and metals (with the same concentrations used in the previous enrichments), and without yeast extract to avoid fermenter contamination improving the purity and certifying that the colonies reduced sulfate in the presence of metals. At the end of the experiment, nine pure cultures were identified and analyzed.

Identification and phylogenetic reconstruction

Strain identifications were made through 16S rRNA gene sequencing using the Sanger sequencing technique. First, 1.0 mL of liquid culture was centrifuged for 5 min at 14,000 *g* and the supernatant was removed. After this process, the pellet

was resuspended in 100 μL of Tris-HCl buffer (T.E.). From this cell suspension, 2 μL was used as template material for PCR using the 27F and 1492R primers (Timmers et al. 2015). The PCR product was examined by gel electrophoresis and cleaned using the MasterPure® DNA Purification Kit according to the manufacturer's instructions. DNA was sent to GATC Biotech (Konstanz, Germany) for Sanger sequencing using the same primers as sequencing primers. The partial sequences were quality trimmed, checked for vector contamination, and merged into full-length sequences with DNA Baser version 4.20.0. The resulting sequences were aligned and classified with SINA v1.2.11 (Pruesse et al. 2012) using the SILVA Ref NR SSU r128 database (Yilmaz et al. 2014).

Genome sequencing

For genome sequencing, the strain was grown in 100 mL cultures described previously for the isolation process. Biomass was collected by centrifugation at 4,700 *g* and 4 °C for 20 min, after which the supernatant was discarded. To avoid contaminant that could interfere in the DNA extraction, two wash steps were carried out. The pellet was resuspended in sterile phosphate buffer, centrifuged at 13,400 *g* and 4 °C for 10 min, and the supernatant was discarded again. This step was repeated twice. For a high quality and quantity DNA for a good genome sequence, the protocol described by Salvà-Serra et al. (2018) was used. The quality and concentration of genomic DNA were observed through electrophoresis gel, nanodrop, and with a fluorimeter (Quibit® 2.0). Genomic DNA was frozen and sent to BaseClear BV (Leiden, The Netherlands) where sequencing was performed using the Illumina HiSeq2500 platform. The quality and duration of Illumina readings was inspected with FastQC, version 0.10.12.

The draft genomes were checked for completeness, contamination and strain heterogeneity with CheckM (Parks et al. 2015).

An analysis of the average nucleotide identity (ANI) between the genome dataset pairs was performed using the online ANI calculator tool, available at http://enve-omics.ce.gatech.edu/ani/index. DNA–DNA in silico (DDH) hybridization values were also determined using the Genome-to-Genome Distance Calculator (GGDC), version 2.0 web server (Meier-Kolthoff et al. 2013).

Results

Sediment characteristics

The results of sediment characteristics are shown in Table 1. The salinity ranges from 16.0 to 23.3, the DO ranges from 37.7 to 69.7% and the percentage of OM changed in each station, being the lowest value of 1.6% at P4 and the highest 5.1% at P5. The lowest value of TOC percentage was 2.6% at P2 and the highest was 4.8% at P5.

The concentration found for each metal in each station is presented in Table 2. The cadmium concentrations were under detection limits in the sediments of all stations. Station P1 was the station with the highest metal concentration. Zinc was the metal found at the highest concentration, an average of 108 μg mg^{-1}.

Enrichment analysis

Sulfate removal

Tracking activity of the enrichments showed the effect of metal concentration on SRB activity (Fig. 2). Increasing metal concentrations had a progressive effect on both total sulfate removal and sulfate reduction rates. In the positive control without metals and in enrichments with 0.3 mM of Zn and 0.4 mM of Cr, sulfate was completely consumed in an average of 28 days. The conditions with Cr 0.4 mM and Zn 0.3 mM presented higher sulfate removal rate than the positive control.

In the Zn enrichments, the microbial community was able to reduce 30 mM and 21.9 mM of sulfate at Zn concentrations of 0.3 and 1.0 mM, respectively. Remarkably, at the highest Zn concentration used in our enrichments (10 mM), 5 mM of sulfate was reduced. In Cr enrichments, sulfate removal occurred in almost all of the Cr concentrations. In the lowest Cr concentration (0.4 mM), 30 mM of sulfate was removed, and in the initial Cr concentrations of 4.0, 8, and 12 mM, 12 mM of sulfate was reduced.

The enrichments with different concentrations of Cu and Cd had a lower sulfate reduction, 10 mM of sulfate was reduced in initial Cd concentration of 0.7 mM and the amount reduced was progressively lower with an increase in Cd concentration. All enrichments with Cu reduced an average amount of 10 mM of sulfate.

Organic acids produced

The amount of lactate and sulfate consumed, sulfide and acids produced, and metal removal in liquid phase at the end of the experiment are shown in Table 3.

SRB consume lactate, reduce sulfate, and produce sulfide that can precipitate metals according to Eqs. 1 and 2:

$$2C3H5O3- + SO4-2 \rightarrow 2CH3COO- + 2HCO3- + HS- + H + (lactate)\ (acetate) \quad (1)$$

$$HS + M \rightarrow MS(S) + H+ \quad (2)$$

Thus, having consumed the concentrations of lactate and sulfate, the number of organic acids produced through

Table 1 Physicochemical data at 5 sampling stations during sample collection (dissolved oxygen (D.O.); organic matter (O.M.); total organic carbon (T.O.C))

	Depth (m)	Temperature (°C)	Salinity	D.O. (%)	pH	O.M. (%)	T.O.C. (%)
P1	1.0	26.3	16.0	46.5	5.7	4.1	3.2
P2	1.7	26.6	14.8	38.0	5.8	2.1	2.6
P3	1.7	27.0	24.4	69.7	5.8	2.0	4.2
P4	2.0	27.0	23.2	45.9	6.0	1.6	4.3
P5	1.8	27.8	23.3	37.7	6.1	5.1	4.8

sulfidogenesis could be calculated. In the positive control, the acetic and isolaveric acids detected were produced due to sulfidogenesis (55.5 mM). In Zn enrichments, the acids produced due to sulfidogenesis were significantly lower in the bottles with Zn initial concentration of 6.0 and 10.0 mM.

The highest levels of dissolved sulfide detected were in the enrichments without metals (positive control), with initial Zn concentration of 0.3 mM and 1.0 mM, Cr 0.4 mM, Cd 0.2 mM, and Cu 0.3 mM. Low concentrations of dissolved sulfide were detected in the other metal enrichments (Table 3).

At the end of the experiment, the highest metal concentration removal in liquid phase was 4.5 mM of Cu, 5.4 mM of Zn, 9.6 mM of Cr, and 0.2 mM of Cd.

Acetic acid and propionic acid were the main organic acids produced in the enrichments (Table 4). In the positive control, acetate represents almost all the organic acids produced (54.5 mM). Cu enrichments (0.3, 1.3, and 3.3 mM) showed similar propionic acid production (32.3–35 mM), higher than acetate concentration (16.3–22.2 mM). In Cd enrichments, higher concentrations of propionic acid as average (29.0 mM) were found when compared to the average amount of acetic acid detected (13.5 mM). Zn and Cr enrichments had similar proportions of organic acids produced. For the lowest metal concentrations tested, higher acetate productions were found in average (30.3–38.8 mM), whereas higher concentrations of propionate were found in the bottles with higher metal concentrations

Microbial community analysis

The microbial community composition of the sediment slurry used as inoculum had approximately 9.3% of the sequences that belong to SRB genera. *Desulfobulbus*-related sequences presented the highest abundance ranging from 4.3 to 7% of the total sequence count. The other SRB sequences were assigned to *Desulfatiglans* (1.2%) *Desulfococcus* (0.5%), *Desulfovibrio* (0.3%), and *Desulfosarcina* (0.1%).

The microbial community found in the metal enrichments is shown in Fig. 3, highlighting the genus *Desulfovibrio*, the most abundant SRB. The positive control also showed other SRB as *Dethiosulfovibrio* and *Thermodesulfovibrio*. *Desulfovibrio* appeared in higher concentrations in the enrichments: Cu 0.3; Cu 1.3; Cu 3.0; Cd 0.2; Zn 0.3; Zn 1.0. *Citrobacter* was an important genus that appeared in enrichments with the highest metal concentration of Cu, Cr, and Cd.

Up to 6 mM zinc initial concentration, a significant number of sequences affiliated to the genera *Desulfovibrio* and *Thermodesulfovibrio* was not observed. At the highest concentration (Zn 10 mM), the presence of sulfate-reducing bacteria was not observed. *Clostridium*, *Alkaliphilus*, and *Lactococcus* were more abundant.

Sulfate-reducing bacteria were only found at 0.4 mM concentration of Cr, with sequences belonging to *Desulfovibrio* sp. and *Desulfobulbus*, and at the 4.0 mM concentration where only the *Desulfovibrio* genus was found. At higher concentrations, there was dominance of *Clostridium* and *Citrobacter*.

In the cadmium enrichments, sequences belonging to *Desulfovibrio* were observed only in concentrations of 0.2 mM and 0.3 mM. In the two highest concentrations, *Clostridium, Citrobacter*, and *Vibrio* dominance were predominant.

In copper enrichments, the only genus of sulfate-reducing bacteria found was *Desulfovibrio*, and the abundance ranges from 0.25 to 0.30.

The similarity analysis (Fig. 4) among the microbial communities of the enrichments showed clustering by metal concentrations more than metal types. Within metal types, the communities present in the Cd and Cu enrichments were closer while the Cr and Zn enrichment communities were more similar.

Table 2 Metal concentrations (Zn, Cr, Cd, and Cu) in μg mg^{-1} of sediment for each of the sampling stations

	Zinc	Chromium	Copper	Cadmium
P1	230 ± 0.010	24 ± 0.000	29 ± 0.001	< 0.004
P2	144 ± 0.003	12 ± 0.001	8.7 ± 0.000	< 0.004
P3	79 ± 0.003	22 ± 0.001	23 ± 0.000	< 0.004
P4	60 ± 0.001	15 ± 0.000	13 ± 0.001	< 0.004
P5	28 ± 0.000	13 ± 0.001	6.0 ±0.005	< 0.004

Isolation of metal-resistant SRB from the enrichments

Seven different strains were isolated. The phylogenetic tree based on 16S rDNA is presented in Fig. 5. All the isolated strains were assigned as the same species. The closest relative

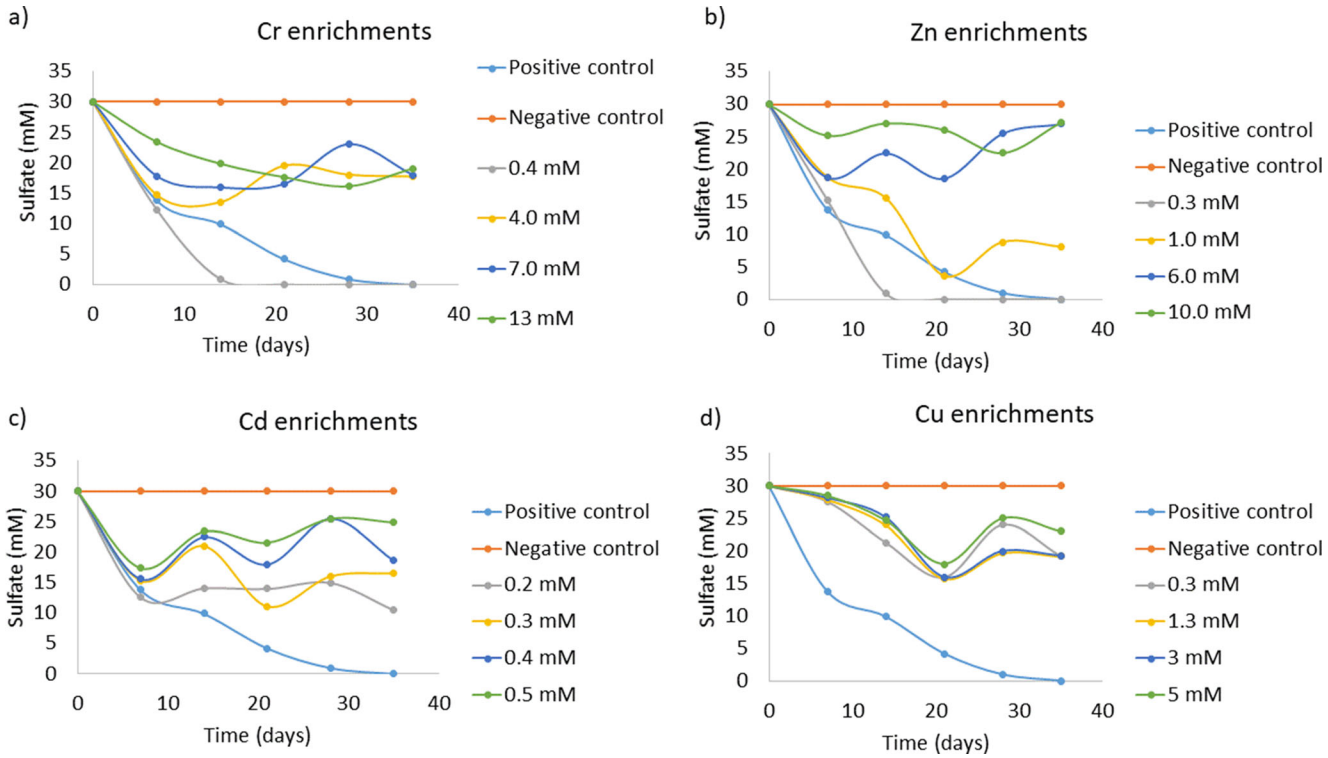

Fig. 2 Sulfate concentrations in enrichments with different concentrations of Cr (**a**); Zn (**b**); Cd (**c**); Cu (**d**) for 35 days

is *Desulfovibrio desulfuricans*. The similarity level indicates that the isolated strains were not the same as *D. desulfuricans*.

The percentage similarity between the isolates and the closest relatives can be seen in Table 5.

Table 3 Values of lactate (mM) and sulfate (mM) consumed, dissolved sulfide (mM), and organic acids produced in total and through sulfidogenesis (mM), metal removal in liquid phase (mM) in each enrichment after 35 days

	Lactate consumed (mM)	Sulfate consumed (mM)	Organic acids produced (mM)	Organic acids produced through sulfidogenesis (mM)	Dissolved sulfide produced (mM)	Metal removal in liquid phase (mM)
Negative control	0	0	0	0	0	0
Positive control	56.7	30	55.5	55.5	15	–
Cu 0.3	56.0	10.8	54.4	21.6	3.1	0.2
Cu 1.3	51.8	8.8	51.0	17.6	0.1	1.25
Cu 3.0	50.7	7.7	49.9	15.4	0.1	2.5
Cu 5.0	42.3	5	38.5	10	0.1	4.5
Zn 0.3	45.1	28.0	41	41	15	0.25
Zn 1.0	61.2	21.9	59.2	43.8	13	0.8
Zn 6.0	42.3	3	39.5	6	1.6	5.4
Zn 10.0	55.0	2.8	51.7	5.6	0.1	4
Cr 0.4	45.1	30	32.9	32.9	13	0.32
Cr 4.0	30.2	12.3	24.8	24.8	0.65	3.2
Cr 7.0	28.1	10	27.2	27.2	0.1	6.5
Cr 13.0	27.1	9	22.9	22.9	0.4	9.6
Cd 0.2	41.0	19.5	40.4	40.4	3	0.16
Cd 0.3	59.8	13.5	58.8	27	1.9	0.2
Cd 0.4	43.0	11.4	41.9	22.8	0.4	0.3
Cd 0.5	47.8	5.1	46.5	10.2	0.6	0.1

Table 4 Acids produced (mM) in enrichments with different concentrations of Zn, Cr, Cu, and Cd

Enrichments	n-Buthanol (mM)	Acetic ac. (mM)	Propionic ac. (mM)	Butyric ac. (mM)	Isovaleric ac. (mM)	Sum of produced acids (mM)
Negative control	0.0	0.0	0.0	0.0	0.0	0.0
Positive control	0.0	54.5	0.0	0.0	1.0	55.5
Cu 0.3	0.0	22.2	32.3	0.0	0.0	54.5
Cu 1.3	0.0	16.8	35.0	0.0	0.0	51.8
Cu 3.0	0.0	16.3	33.6	0.0	0.0	49.9
Cu 5.0	0.0	4.1	34.4	0.0	0.0	38.5
Zn 0.3	0.0	38.8	1.6	0.0	0.6	41.0
Zn 1.0	0.0	38.8	15.9	0.0	6.5	61.2
Zn 6.0	0.0	15.9	23.5	0.1	0.0	39.5
Zn 10.0	0.0	17.2	34.4	0.0	0.0	51.7
Cr 0.4	0.0	40.3	2.6	0.0	0.0	42.9
Cr 4.0	0.0	32.4	2.4	0.0	0.0	34.8
Cr 7.0	0.0	3.9	13.4	10.9	0.0	28.2
Cr 13.0	0.0	2.3	13.2	7.4	0.0	22.9
Cd 0.2	0.0	17.1	23.3	0.0	0.0	40.4
Cd 0.3	0.0	17.3	41.0	0.0	0.5	58.8
Cd 0.4	0.0	9.5	26.1	6.3	0.0	41.9
Cd 0.5	2.3	10.0	25.9	8.3	0.0	46.5

Percentages lower than 98% can indicate new species (Yarza et al. 2014).

The genome sequence was used to confirm the presence of a new *Desulfovibrio* species. Average nucleotide identity and in silico DDH values obtained from pairwise comparison of the available genome sequences of *Desulfovibrio* genus members are shown in Table 6. The values confirm *Desulfovibrio* sp. FE33 as a new species.

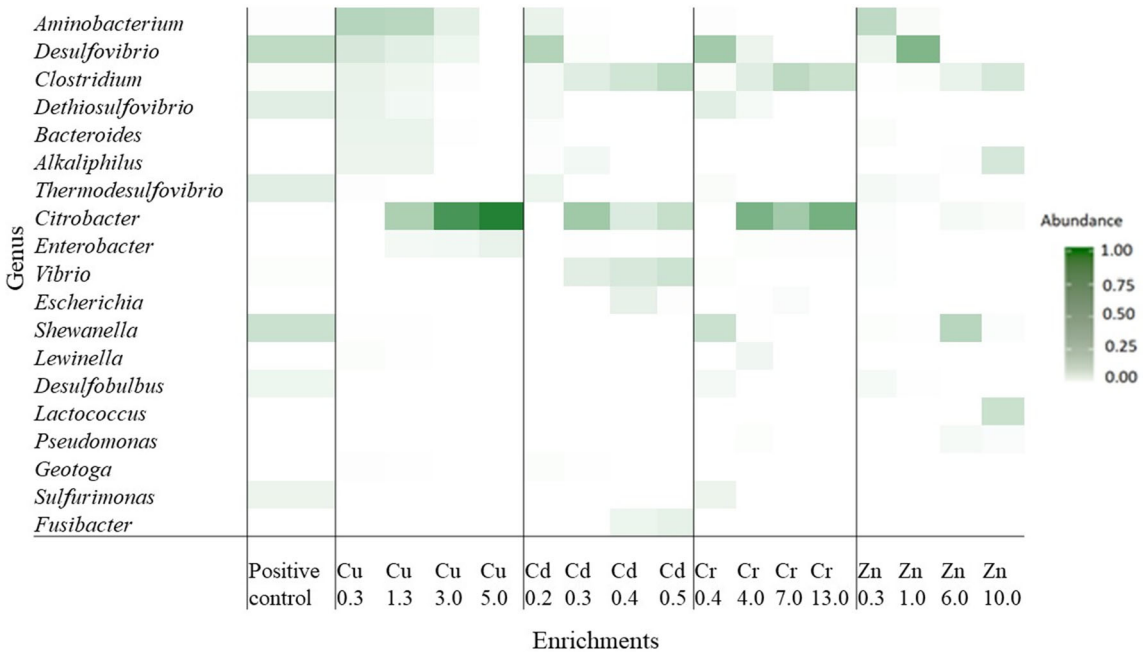

Fig. 3 Heat map reflecting the abundance of different genera identified in the samples of each enrichment. Dark green colors indicate higher abundance as reflected in the legend

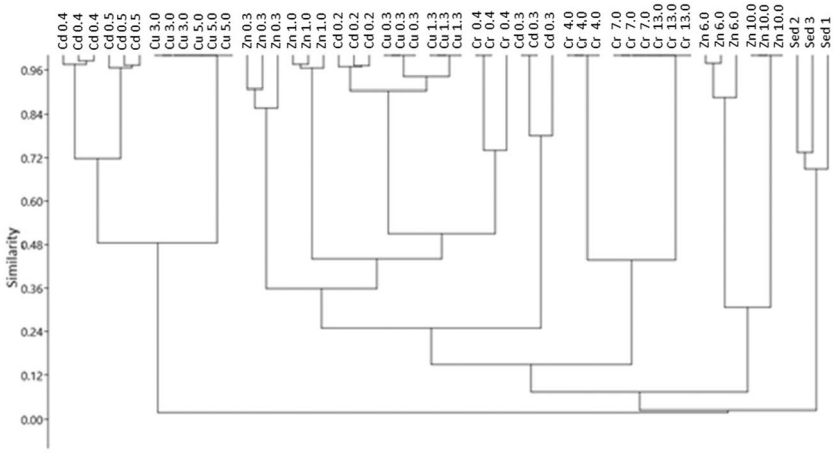

Fig. 4 Cluster analysis of bacterial communities found in different enrichments (in each triplicate) and in the sediment triplicates used as inoculum (sed 1, sed 2, and sed3) based on the "Bray-Curtis" dissimilarity matrix.

Discussion

Several studies have shown metal contamination in the port of Santos. According to Kim et al. (2015), the port of Santos is a good example of an environment that suffers from the continuous introduction of various contaminants.

Metal concentrations found in the sediment at the stations in the study area are considered safe for the biota according to the environmental agencies, which is 35.7 μg g^{-1} for Cu, 37.3 μg g^{-1} for Cr, 0.6 μg g^{-1} for Cd, and 123.1 μg g^{-1} for Zn (CCME 2002). However, there are many studies and reports that found metal concentrations above these limits at the Port of Santos (CETESB 2018; Kim et al. 2015; Buruaem et al. 2012). According to Buruaem et al. (2012), the proximity to the industrial sources is responsible for increasing the metal concentration; in some regions, the Zn concentration reached 1077 μg g^{-1} (Choueri et al. 2009) and Cr reached 97.5 μg g^{-1} (Hortellani et al. 2008). Thus, metal concentration can vary along the port area in different stations and periods of time. This long-term exposure causes these microorganisms to tolerate high levels of metals as indicated by the enrichments. In addition, in the port sediment used as an inoculum, there was a large abundance of SRB that were exposed to metal contamination. The community present in the inoculum was able to reduce sulfate in very high metal concentrations.

Some studies demonstrate that one of the difficulties in applying SRB in reactors to treat contaminated waste is the high concentration of metals that is toxic and inhibits SRB activity (Zhang and Wang 2016; Ayangbenro et al. 2018). Other studies have demonstrated toxic metal concentrations

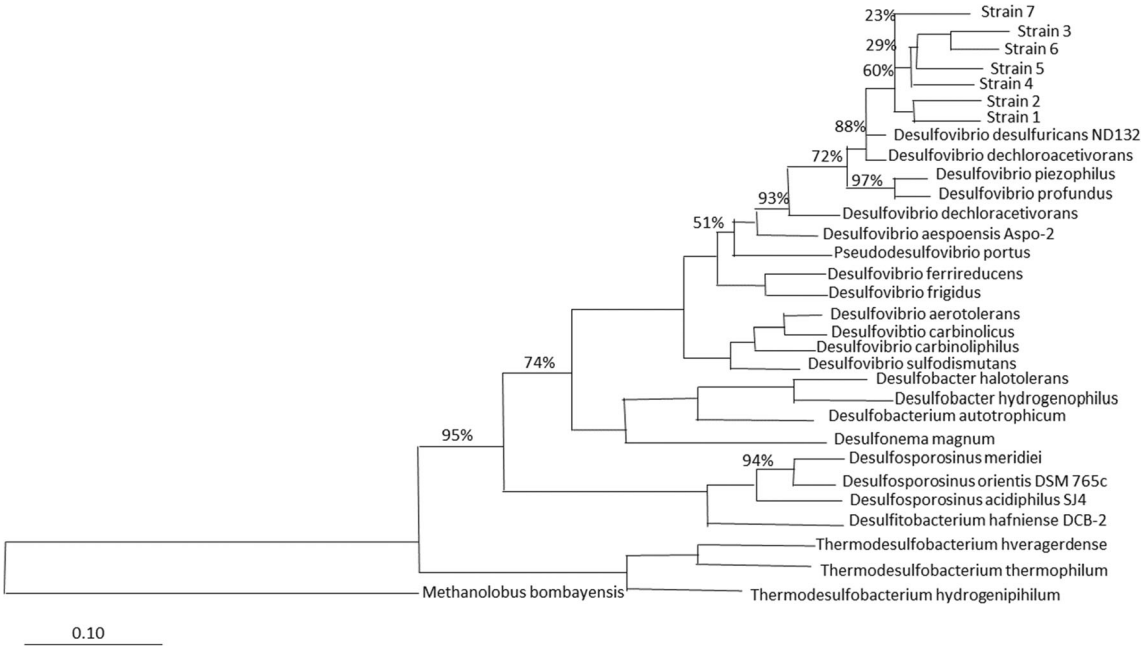

Fig. 5 Phylogenetic tree based on 16S rDNA sequence analysis, showing the phylogenetic placement of strains isolated in the present study. The tree was constructed by the neighbor-joining statistical method, and *Methanolobus bombayensis* was used as the out group

Table 5 Similarity percentage by neighbor-Joining methods between the isolates (St1, St2, St3, St4, St5, St 6, and St 7) and the two closest relatives (*Desulfovibrio desulfuricans* (DsDesu) and *Desulfovibrio dechloroacetivorans* (DsDech))

	St1	St2	St3	St4	St5	St6	St7	DsDesu	DsDech
St1	–	0.9916	0.9815	0.9863	0.9901	0.9923	0.9804	0.9760	0.9489
St2	0.9916	–	0.9875	0.9854	0.9886	0.9878	0.9791	0.9743	0.9468
St3	0.9863	0.9854	–	0.9812	0.9917	0.9939	0.9748	0.9640	0.9410
St4	0.9863	0.9854	0.9812	–	0.9894	0.9947	0.9770	0.9667	0.9416
St5	0.9901	0.9886	0.9917	0.9894	–	0.9931	0.9749	0.9750	0.9528
St6	0.9923	0.9878	0.9939	0.9947	0.9931	–	0.9762	0.9771	0.9550
St7	0.9804	0.9791	0.9748	0.9770	0.9749	0.9762	–	0.9679	0.9423
DsDesu	0.9760	0.9743	0.9640	0.9667	0.9750	0.9771	0.9679	–	0.9622
DsDech	0.9489	0.9468	0.9410	0.9416	0.9528	0.9555	0.9426	0.9622	–

for SRB. Hao et al. (1994) observed inhibition of sulfate reduction in 0.32 mM of Cu and 0.26 mM of Zn; Cabrera et al. (2006) observed inhibition of SRB activity in concentrations of 0.15 mM of Cu, 0.13 mM of Zn; Cd in concentration of 0.12 mM was toxic to SRB according to Binish et al. (2015); Guo et al. (2017b) observed inhibition of sulfate reduction in concentrations of 1.0 mM of Cr and 0.8 mM of Zn. In the present study, SRB community was able to reduce sulfate in higher metal concentration (Zn 6.0 mM, Cu 3.0 mM, Cd 0.5 mM, and Cr 8.0 mM).

However, enrichments also demonstrated the impact of metals on SRB metabolism. The higher the concentration of metals, the less sulfate was removed (Fig. 2). Sheng et al. (2011) found similar patterns, when the initial concentration of Cu was higher than 0.3 mM, the sulfate concentration barely changed during the experiment, which indicates that SRB activity was completely inhibited. In the study of Guo et al. (2017b), when the concentrations of Zn^{-2} were below 0.2 mM, the sulfate reduction efficiency was greater than 95.2%, when Zn^{-2} concentration was increased to 1.5 mM, the efficiency decreased to 10%.

In the present study, there was a removal of all the 30 mM of sulfate added to the enrichments with an initial concentration of Zn of 0.3 mM and Cr 0.4 mM. When the concentration of Zn was 1.0 mM, 21.9 mM of sulfate was reduced, which still demonstrates the community resistance. Whereas, Martins et al. (2009) showed that SRB were tolerant only at an initial concentration of 0.1 mM of Zn.

Table 6 Average nucleotide identity (ANI) and in silico DNA–DNA hybridization pairwise (DDH) comparison of *Desulfovibrio* sp. FE33 with the closest relative strains (*Desulfovibrio desulfuricans*)

		D. desulfuricans
DDH	1	12.7
	2	20.0
	3	13.1
ANI	4	83.8

Higher sulfate removal rate was achieved in the medium containing Zn and Cr. The higher removal rate was due to an intensive SRB activity, which reduces more sulfate, forming more sulfide that binds metal causing its precipitation. However, we cannot rule out other ways of removing metals, such as adsorption, especially considering their initial inoculum, or complexation with other compounds. According to Lin 1992 and Lin 1993, the sensitivity sequence of anaerobic processes to different metals is Cd> Cu> Cr> Zn> Pb> Ni. In the present study, a similar sequence was observed. The SRB in lower concentrations of Cd (0.2–0.5 mM) and Cu (0.3–5.0 mM) reduce less sulfate (5.0–10.8 mM and 5.1–19.5 mM, respectively) and precipitate less metal (0.3 and 4.5 mM, respectively) when compared to the enrichments with Zn (0.4–10 mM) and Cr (0.4–13.0 mM) that reduce more sulfate (3–30 mM and 9–30 mM, respectively) and precipitate more metals (5.6 mM and 9.9 mM, respectively).

This metal toxicity pattern was confirmed by analyzing the community present in the enrichments. The two more toxic metals (Cd and Cu) had a greater community similarity with less SRB and more fermenters (e.g., *Citrobacter* and *Clostridium*). While the enrichments with less toxic metals for SRB (Cr and Zn) were closer by distance analysis, where the SRB genera (specially *Desulfovibrio*) were found in higher abundance. These results show us that these metals have similar influences on the SRB community, corroborated by previous studies that also showed higher Cu and Cd toxicity when compared to Cr and Zn (Cabrera et al. 2006; Ahmadi et al. 2015; Hussain and Qazi 2016). This noticeable difference in the impact of each metal on SRB metabolism, as well as the toxic concentrations of each metal, can help predict the behavior of this community in bioreactors during treatment of metal-contaminated effluents (Utgikar et al. 2003).

It is also important to highlight that the conditions with 0.4 Cr and 0.3 Zn presented higher sulfate removal rate than the positive control. It indicates that these metals stimulate the SRB to some extent, and are toxic only in higher concentrations. This is corroborated by the heatmap that shows higher

abundance of *Desulfovibrio* at conditions 0.4 mM Cr and 0.3 mM Zn than in the positive controls. Metals such as zinc and chromium are indispensable micronutrients for the growth and development of bacteria (Nies 1999). Several studies show that essential metal supplementation in a bioreactor may stimulate bacterial activity and optimize treatment process (Gustavsson et al. 2013; Schmidt et al. 2014; Gustavsson et al. 2011; Paulo et al. 2015).

It was clear that metals influence sulfate reduction metabolism. The higher the metal concentration, the lower sulfate reduction was observed. Where there was a higher metal concentration, the lactate was consumed through fermentation and not through sulfate reduction metabolism.

The metal influence on sulfate reduction metabolism can also be confirmed when the community present in the enrichments is analyzed. Figure 3 shows a drastic decrease in the SRB community, and consequently an increase in the proportion of fermentative bacteria (e.g., *Citrobacter* and *Clostridium*) when metals increase. In the fermentation process, these bacteria oxidize lactate mainly to propionate (Gonzalez-Garcia et al. 2017; Gänzle 2015; Vollenweider and Lacroix 2004). The lactate incomplete oxidation to acetate done by SRB through the sulfate reduction process was gradually replaced by the fermentation process due to the toxicity of the metals.

Citrobacter is a genus with a very versatile physiology, having the ability to grow through both aerobic and anaerobic mechanisms. Through anaerobic metabolism, it can degrade several carbon sources through respiration or fermentative metabolism. Under strict anoxic conditions, they can make complete oxidation of organic matter generating CO_2 (Gerritse and Gottschal 1993; Brooks et al. 2015).

Several studies show the feasibility of using the genus *Desulfovibrio* for biotechnological applications (Zouch et al. 2017; Igiri et al. 2018). Kim et al. (2015) demonstrated the ability of the species *Desulfovibrio desulfuricans* to remove metals from seawater. The removal efficiency was 98.2%, 99.8%, and 90.1% for Cu^{2+}, Cr^{6+}, and Ni^{2+}, respectively, after approximately 7 days.

A new *Desulfovibrio* species was isolated at the end of the study. In general, species of this genus can be applied to several contaminated effluent treatments (Sahinkaya et al. 2015; Kousi et al. 2015; Hussain et al. 2016). However, several studies demonstrate that its activity is restricted to certain initial concentrations of metals (Sani et al. 2003; Cabrera et al. 2006; Binish et al. 2015). Nevertheless, the new strain was isolated from an inoculum that was exposed to long-term metal contamination. This genus was found in enrichments with the following metal concentrations: Zn 6.0 mM, Cu 3.0 mM, Cd 0.3 mM, and Cr 7.0 mM. This is indicative of high metal resistance.

The long-term exposure to metals can favor the development of efficient resistance mechanisms. A study by Chen et al. (2019) demonstrated that areas, which have been exposed to the contamination of different metals (Hg, Pb, Cu, As, Zn, Cd, and Cr) for a long time, presented a greater amount of resistance genes involved in the efflux of Cu, Zn, and Cd and mercury reduction.

Microorganisms are able to execute an array of metal resistance mechanisms to cope with metal toxicity by regulating intracellular metal concentrations in sublethal levels. These are (1) efflux of metals that enter cells by either specific or nonspecific transporters, (2) intracellular compartmentalization within safe sectors of cell reducing cytoplasmic availability of metals, (3) intra- or extracellular entrapment of metals by complexation with microbially generated ligands, and (4) enzymatic transformations reducing metal toxicity, etc. (Nies 1999; Sar et al. 2013).

Conclusions

Port areas are important sites for the study of metal-resistant and biotechnologically relevant sulfate-reducing bacteria. It was possible to notice that metals have a strong influence on SRB metabolism. Metals such as, zinc and chromium are indispensable micronutrients for bacterial growth, at low concentrations, stimulate the SRB, and are toxic only in higher concentrations. Essential metal supplementation in a bioreactor may stimulate bacterial activity and optimize treatment process.

Metals have different toxicities, affecting SRB activity at different ways. Cd and Cu were more toxics. The noticeable difference in the influence of each metal on SRB metabolism, as well as the toxic concentrations of each metal, can help predict the behavior of this community in bioreactors during treatment of metal contaminated effluents.

A new *Desulfovibrio* species was isolated in enrichments with high metal concentrations (Zn 6.0 mM, Cu 3.0 mM, Cd 0.3 mM, and Cr 7.0 mM). The new species was found in enrichments where higher concentrations of metals were present compared to the concentration of metals tolerated by other SRB species found in the literature. The isolation of SRB-resistant strains can be very useful in future studies to optimize the metal removal from industrial waste to avoid that this type of pollutant reaches the environment. The environmental impact caused by metals and their detrimental effect on biota makes it urgent to seek efficient bioremediation treatment. Future studies to understand the resistance mechanisms present in this new species can also be very useful.

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Author contribution Bruna Del Busso Zampieri, Gunther Brucha, and Irene Sánchez-Andrea designed the research. Oliveira AJF organized the sampling collection. Bruna Del Busso Zampieri performed sampling. Bruna Del Busso Zampieri and Elis Watanabe Nogueira performed experimental work. Bruna Del Busso Zampieri performed data analysis and wrote the manuscript with critical revision by Gunther Brucha, Irene Sánchez-Andrea, and Ana Julia Fernandes Cardoso de Oliveira with input from all authors.

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Declarations

Ethics approval and consent to participate Not applicable

Consent for publication Not applicable

Competing interests The authors declare no competing interest.

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