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ENVIRONMENTAL MICROBIOLOGY

Diversity and Distribution of Heavy Metal-Resistant Bacteria in Polluted Sediments of the Araça Bay, São Sebastião (SP), and the Relationship Between Heavy Metals and Organic Matter Concentrations

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Abstract Heavy metals influence the population size, diversity, and metabolic activity of bacteria. In turn, bacteria can develop heavy metal resistance mechanisms, and this can be used in bioremediation of contaminated areas. The purpose of the present study was to understand how heavy metals concentration influence on diversity and distribution of heavy metal-resistant bacteria in Araça Bay, São Sebastião, on the São Paulo coast of Brazil. The hypothesis is that activities that contribute for heavy metal disposal and the increase of metals concentrations in environment can influence in density, diversity, and distribution of heavy metal-resistant bacteria. Only 12 % of the isolated bacteria were sensitive to all of the metals tested. We observed that the highest percentage of resistant strains were in areas closest to the São Sebastião channel, where port activity occurs and have bigger heavy metals concentrations. Bacterial isolated were most resistant to Cr, followed by Zn, Cd, and Cu. Few strains resisted to Cd levels greater than 200 mg L^{-1}. In respect to Cr, 36 % of the strains were able to grow in the presence of as much as 3200 mg L^{-1}. Few strains were able to grow at concentrations of Zn and Cu as high as 1600 mg L^{-1}, and none grew at the highest concentration of 3200 mg L^{-1}. *Bacillus* sp. was most frequently isolated and may be the dominant genus in heavy metal-polluted areas. *Staphylococcus* sp., *Planococcus maritimus*, and *Vibrio aginolyticus* were also isolated, suggesting their potential in bioremediation of contaminated sites.

Keywords *Bacillus* sp. · Bacterial resistance · Contamination · Heavy metals · Port areas

Introduction

A major concern nowadays is the impact of heavy metal-release into natural environments by man [1]. This kind of pollution has ecological consequences and presents a serious risk to human health because heavy metals are ubiquitous and very persistent pollutants [2].

The marine environment is often a dump site for contaminated wastewater produced by many industries. It is also the location of oil exploration and active harbors, and it is crossed by transport routes of large ships. All of these activities can generate heavy metal contamination [3]. Losses, leaks, and spills occur during loading and unloading of transport vessels, during materials storage, during chemical transfers, upon washing of isocontainers, and during maintenance of wash bays, and during commercial leaching [4].

Thus, the interest over the impacts of heavy metals on the marine environment is a growing area of scientific research. In the sea, these contaminants largely accumulate in the sediment, and their deposition generates toxicity to aquatic biota including microorganisms, which are present in the sediment [5, 6].

Heavy metals play an important role in processes involving microorganisms in sediments. Metals such as calcium, zinc, nickel, iron, potassium, magnesium, manganese, and cobalt

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are essential nutrients at low concentrations. These elements participate in biochemical catalysis, acting as protein and nucleic acid stabilizers, and enzyme cofactors, and participating of several biological processes in the cell as osmotic balance, oxidative phosphorylation, motility, and regulation of membrane channels activity [7, 8].

High metal concentrations are toxic to microorganisms due to their abilities to affect conformational changes on the structure of nucleic acids and proteins, and also could interfere in several biological processes [7]. Abiotic stress caused by metals can affect the growth, morphology, and metabolism of bacteria [9, 10]. Consequently, heavy metals can change the size, composition, and activity of the microbial community [11], altering, as well, the bacterial densities and diversity [6], which is worrisome, once marine microorganisms are of critical importance to the health of the environment, and the life on the planet. They are integral to all major biogeochemical cycles, fluxes, and processes that occur in marine systems where elements move between oxidized and reduced forms. Microorganisms are extremely abundant and diverse, and they play a key role in the regulation of the earth's climate [12]. Marine microorganisms also provide essential goods and services to human societies through their production of oxygen, their support of a sustainable food supply, and their part in regulating the health of the marine environment [13]. Bacteria are also the first step in the transfer of toxic compounds to higher trophic levels of the food chain [14].

Bacteria have developed several defense mechanisms to combat stress caused by heavy metals. These mechanisms include metal-complex formation, reduction of certain metals to less toxic species, or efflux of the metals from the cell [15, 16].

Because these microorganisms are able to transform these toxic compounds into less toxic or non-toxic, they are potentially useful in bioremediation of contaminated soils and sediments [17]. Such application is especially useful with marine bacteria, which live in the extreme environments conditions and can be utilized in situ [18].

Studies to detect, isolate, and monitor the occurrence of bacteria that are resistant to heavy metals in the marine environment are relatively recent and scarce, particularly in Brazil, but studies on marine areas in several countries have shown the importance of the presence of these bacteria in marine environments [12, 19, 20].

Some studies show that heavy metal discharge into the environment not only causes selection of heavy metal-resistant bacteria but may also cause selection of antibiotic-resistant bacteria. This occurs because some mechanisms for heavy metal resistance function in a similar way of those for resistance to antibiotics. Thus, an environmental problem involving heavy metal contamination may become a public health issue [12].

These results reflect the importance of studies on heavy metal-resistant bacteria in coastal and estuarine areas, particularly in areas that receive domestic and industrial sewage and waste from harbor activities. Several regions of Brazilian coast are chronically contaminated by untreated sewage discharge that brings to coastal waters various substances, including heavy metals. An example of a contaminated region is the area in which the Port of São Sebastião is located [4]. Additionally, in these areas, the problem is compounded by the presence of tourist routes, high population densities. At the same time, there is the presence of rich and important biodiversity. For these reasons, studies on heavy metal-resistant bacteria in polluted areas are very important.

Therefore, this study aimed to isolate and identify heavy metal-resistant bacteria in Araça Bay and São Sebastião Channel sediments to test the hypothesis that activities that contribute for heavy metal disposal and the increase of metals concentrations in environment, like harbors and sewage discharge, can influence in density, diversity, and distribution of heavy metal-resistant bacteria.

Material and Methods

Study Area

Araça Bay is located in the city of São Sebastião, in north coast of São Paulo State, southeastern Brazil, in an area adjacent to the Port of São Sebastião. This port is of significant economic importance to the country. Its natural setting places it as the third best port in the world [21].

Araça Bay includes ecosystems such as rocky shores, sandy beaches, and mangroves. It is part of a sublittoral zone that extends into the São Sebastião Channel. It also has widespread biological diversity [22].

Because of the proximity of urban human developments, this set of different ecosystems is exposed to different types of human activity, including illegal occupation and waste dumping. The bay adjoins the Port of São Sebastião and the Petrobras Waterway Terminal, where there are frequent oil spills and other complications. In addition, there is a proposal to expand the harbor on Araça Bay, threatening all its biodiversity [22].

One of the largest oil terminals in Brazil is located in the center of this region. At the Araça Bay, a marine outfall is located, by which almost all domestic sewage from the city of São Sebastião is discharged [23].

Sample Collection and Analysis

Sediment samples were collected quarterly using a corer (10 cm of length) at 37 sites along the Araça Bay during 1 year. The Araça Bay was divided in two sample regions: the intertidal zone and the sublittoral zone (Fig. 1). The intertidal zone is dominated by a tidal regime and is exposed during low tide. The sublittoral zone is not exposed during low tide, has a greater depth, and is more dominated by currents [24].

For each collection station, the sample of 10 g of sediment was added to 90 mL of saline solution, homogenized, and serially diluted to 10^{-5}. From the serial dilutions, 1 mL of the 10^{-3} and the 10^{-5} dilutions was inoculated into Marine Agar 2216 (MA, Difco) and plated using the pour plate method. All plates were prepared in triplicate. Inoculated plates were incubated at 28 °C for 48 h. The colonies were counted and the densities were expressed as colony-forming units per gram of sediment (CFU g^{-1}).

One hundred colonies were randomly selected and subcultured three successive times under the same culture conditions. Resistance testing was then applied to these isolated and purified colonies. Each colony isolated was tested for heavy metal resistance by determining the minimum inhibitory concentrations (MIC). The MICs of four different heavy metals (Cd, Cr, Cu, and Zn) were determined for each isolate using Mueller–Hinton agar (Difco), which contained each metal in concentrations ranging from 12.5 to 3200 μg mL^{-1}. The four heavy metals were tested in the following form: $CdSO_4{\cdot}8/3H_2O$; $ZnSO_4{\cdot}7H_2O$; $CuSO_4{\cdot}5H_2O$; K_2CrO_4 (Merck). The isolates were considered resistant if the MICs exceeded that of the control organism. *Escherichia coli* K-12 ATCC 25922 and *Staphylococcus aureus* ATCC 29213 strains were used as controls organisms [25, 26]. The isolates that were found to be resistant to the heavy metals were then molecularly identified.

After identification, an average of the minimum inhibitory concentration of each species was made.

Identification by Microbiological and Molecular Biology Approaches

After initial characterization by colony morphology, each isolate was then identified on the basis of 16S rRNA gene sequencing. For this purpose, each isolate was grown in Nutrient Broth prepared with sterile and filtered seawater. Its genomic DNA subsequently extracted using the PureLiink®Genomic DNA Kit (Thermo Fisher Scientific). The DNA was extracted following the recommendation of the manufacturer. The DNA integrity was checked by agarose gel electrophoresis (0.8 % in TAE 1×, containing 10 μM of ethidium bromide) and the samples were quantified spectrophotometrically. Gene amplification was carried out using previously published oligonucleotides sequences: 27S-F (5′-CAAGAGTTTGATCCTGGCTCAG-3′) and 1492-R (5′-GGTTACCTTGTTACGACTT-3′). The polymerase chains reactions consisted in: 1× PCR buffer (Invitrogen, Milan, Italy), 1.5 mM $MgCl_2$, 0.25 mM of each dNTP, 15 pmol of each oligonucleotide, 100 ng of genomic DNA, and 1 U of Taq polymerase in a final volume of 50 μL. Initial denaturation at 94 °C for 5 min was followed by 30 cycles consisting of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min. A final extension at 72 °C for 10 min was used. The amplification of was checked by agarose gel electrophoresis, as described before, and PCR products were purified using the PureLink® Quick Gel Extraction and PCR purification Combo Kit (Thermo Fisher Scientific)

DNA Sequencing and Data Analysis

Purified PCR product (50 ng) was used to cycle sequence the DNA using the ABI Prism BigDye terminator cycle sequencing kit (Thermo Fisher Scientific), following the conditions suggested by the manufacturer and using 5 pmol of the oligonucleotides 27S-F or 1492-R. Then the samples were injected in the ABI 310 automated sequencer (Thermo Fisher Scientific). The chromatograms were evaluated using the Chromas software (twww.technelysium.com.au) and analyzed using BioEdit software [27]. The 16S rRNA gene sequences (~1360 nucleotides) were assembled from multiple readouts (using the forward and reverse primers) and compared to nucleotide sequences of the NCBI GenBank using the Blastn (http://blast.ncbi.nlm.nih.gov) to the organisms' identification. The multiple sequence alignments were performed using Clustal Omega [28] Columns and the gaps were removed. The final tree was obtained using MEGA 6 [29]. The sequences were assigned to species using the highest-scoring sequence for which species information was available when the sequence similarity was greater than 97 %.

Evaluation of the Heavy Metal Concentrations and Organic Matter Content

To determine the organic matter content of the sediment samples, 100 g of each sample was dried at 105 °C and then heated to 250 °C for 5 h. Next, the dried sample was weighed, and the difference between the initial and final weight was determined. The weight difference was used as a measure of the organic matter content of the sediment.

Four heavy metal for analyses, chromium (Cr), copper (Cu), cadmium (Cd), and zinc (Zn), were chosen. These metals were chosen because they present significant concentrations in Brazilian coastal waters near harbors and industrial areas [30]. To analyze the concentration of these metals, sediment samples were frozen and lyophilized according to the SW 846 method 3051 from the EPA [31]. Metals were quantified using inductively coupled plasma atomic emission spectroscopy (ICP-AES). Certified reference materials (CRMs) EnviroMAT SS-1 and EnviroMAT SS-2 (SCP Science) were used as stoichiometry standards in elemental analysis.

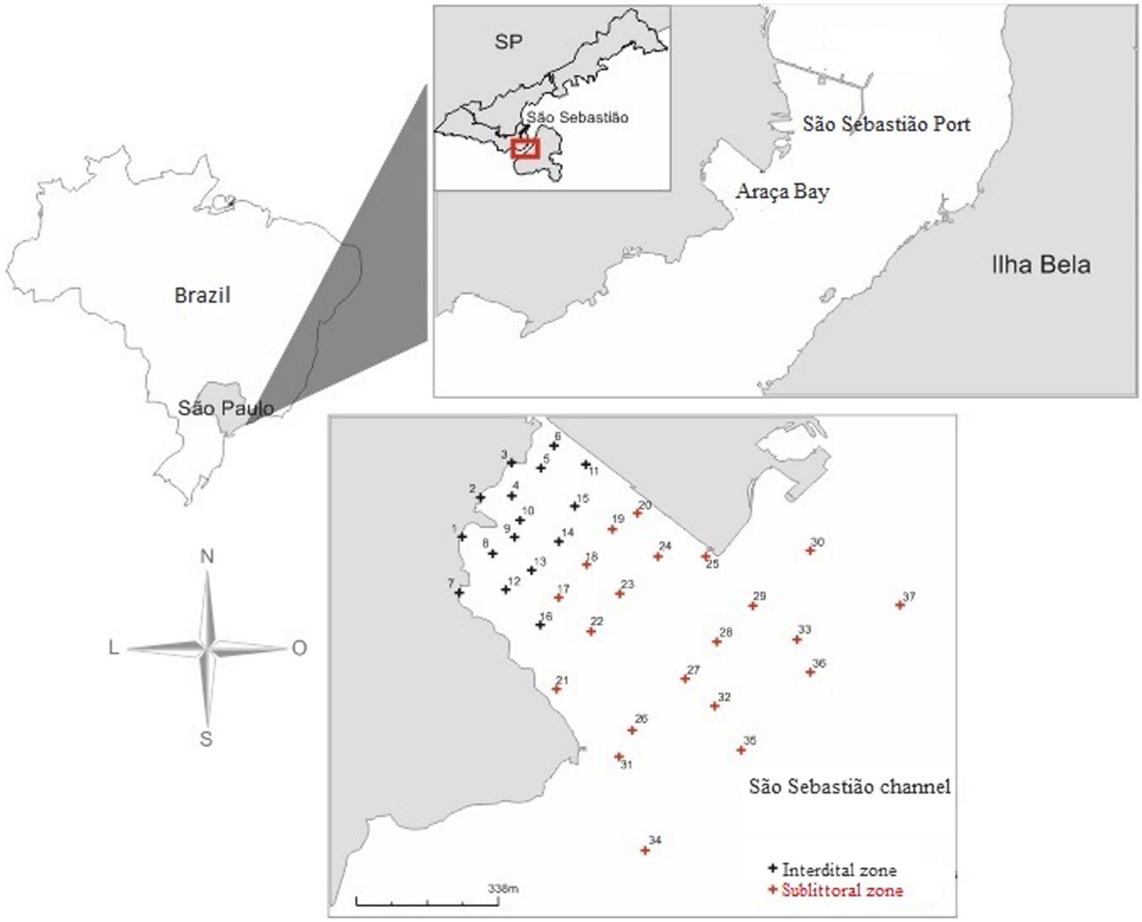


Fig. 1 Map of São Sebastião region showing the location of the São Sebastião Channel and the Araça Bay in detail with the sampling sites located in the sublittoral zone [1–16] and in the intertidal zone [17–37]

Statistical Analyses

Statistical analyses were conducted using "R" version 2.5.0. All the environmental and biological parameters were analyzed in order to evaluate normality through a Shapiro–Wilk test (SW) and equality of variances through a Levene test (L). Data with not normal distribution and heteroscedasticity was compare using Mann–Whitney non-parametric test for independent samples and correlation was made using Spearman correlation. To analyze frequency of heavy metal resistance strains, the Chi square test was used. Finally, a multivariate analysis was made using Principal Components Analysis (PCA) (Past3).

Results

Bacterial Abundance, Percentage of Organic Matter, and Metal Concentration in Sublittoral and Intertidal Zones

The bacterial concentration varied among the stations, especially among those from the sublittoral zone. Maximum values reached 85 10^4 CFU g^{-1}. The lowest values were between 7 and 10^4 CFU g^{-1}. Cell concentrations were significantly higher in the sublittoral zone ($p = 0.000025$) (Fig. 2).

The percentage of organic matter in the sediment ranged from 4.9 to 14.8 %. The highest peaks were recorded at points within the sublittoral zone, which had an average percentage of 13.7 %. Meanwhile, the average among the points within the intertidal zone was 6.5 % ($p = 0.002$). The metal with the highest concentration in the sediment was Zn: its average concentration was 38.84 mg kg^{-1}. It was followed by Cr at 19.8 mg kg^{-1}, Cu at 5.47 mg kg^{-1}, and Cd at 0.25 mg kg^{-1} (Fig. 3). All metal concentrations tested were significantly higher in the sublittoral zone(Cd: $p = 0.0088$; Cr: $p = 0.002$; Cu: $p = 0.037$; and Zn: $p = 0.049$).

Since Brazil has no legislation concerning the heavy metal concentrations in sediment that are able to produce harmful effects on the biota and in the surrounding ecosystem in this study, we used the reference values of Canadian Sediment Quality Guidelines (SQG) for marine sediments [32] (Table 1).

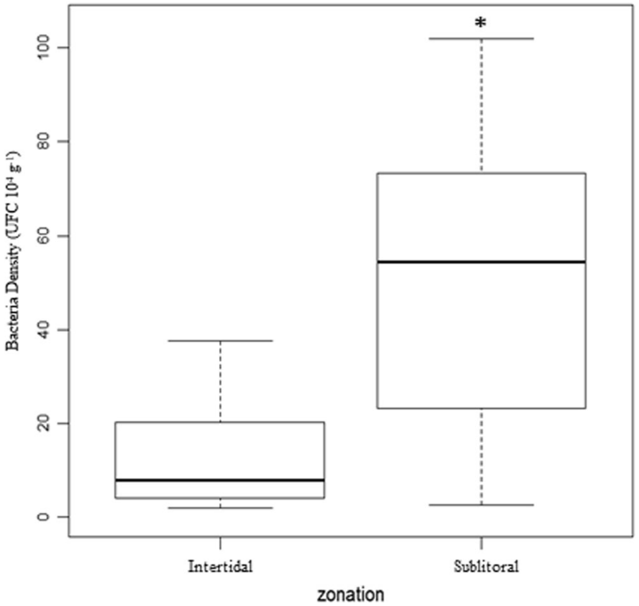


Fig. 2 Average bacterial density in the sublittoral zones and intertidal zones. Where *black line* = mean; *box* = mean ± standard error; *whiskers* = mean ± 5 % confidence interval; and *symbol* (*asterisk*) = represents statistical difference ($p < 0.05$)

Evaluation of the Resistance from Isolated Bacterial Strains to Heavy Metals

Out of 100 isolated strains (50 from intertidal zone and 50 from sublittoral zone), 53 % were resistant to chromium, 52 % to zinc, 38 % to cadmium, and 10 % to copper (Fig. 4). The bacteria were more resistant to the metals that were present at higher concentrations (Cr and Zn) in the study region. However, despite the low concentration of Cd in the sediment, a significant percentage of resistant strains were found.

The tolerance of the isolated bacterial strains for each heavy metal tested is shown in Table 2. Few strains tolerated Cd concentration levels greater than 200 μg mL^{-1}. In the case of Cr, 36 % of the strains even grew at a concentration of 3200 μg mL^{-1}. In respect to Zn and Cu, few strains (4 and 2 %, respectively) tolerated 1600 μg mL^{-1}, and none were able to tolerate the highest concentration (3200 μg mL^{-1}).

A multi-resistance profile of each strain was also evaluated. Our results revealed that only 23 % of the strains were susceptible to any heavy metal tested, 49 % were resistant to more than two heavy metals simultaneously, and 7 % of the bacteria were resistant to all four metals (Cd, Cu, Cr, and Zn). A higher percentage of metal-resistant bacteria were found in the sublittoral zone (note that this is also the zone in which the highest concentrations of metals were found). Only the correlation with cadmium was reverse. In the intertidal zone, both a lower concentration of metals and a smaller percentage of resistant strains were observed (Fig. 4).

In the sublittoral zone, bacteria were more resistant to Zn, Cu, and Cr, whereas in the intertidal zone, the strains were more resistant to Cd and Cr (Fig. 4). Note that all higher heavy metal concentrations occur in the sublittoral zone when resistance to Zn and Cu was considered (Zn: $p = 0.\ 0077$, X2 = 7.1, df = 1; Cu: $p = 0.0003$, X2 = 13.31; df = 1).

Spatial Distribution of the Heavy Metals and Resistant Strains

We also investigated the correlation between the metal-resistant bacteria and the heavy metal concentration within the sampling areas. Figure 5 shows the maps of metal concentration distribution in the sampled areas within the São Sebastião Channel and the Araça Bay. The sampling sites in the sublittoral zone are numbered 1–16, and those in the intertidal zone are numbered 17–37. Notice that all higher heavy metal concentrations were in the sublittoral zone, which is most influenced by the harbor.

This pattern is very similar to that of Zn- and Cr-resistant bacteria, which are present in higher numbers in the areas where the concentrations of these metals are more abundant. In the case of Cd-resistant bacteria, this relationship is reversed (Fig. 6).

Multivariate Analysis

Now it is possible to see in Fig. 7 how all variables influence in each other. To confirm which factor influenced the most in explaining the data, a PCA was performed. It was possible to notice that heavy metals concentration had a clear influence in heavy metal-resistant bacteria in sediment. Only for Cd that this relationship was inverse. The components 1 and 2 demonstrated higher strength explaining together 76 % of the variance (56.8 and 19.5, respectively).

Evaluation of the Bacterial Strains Abundance by 16S rRNA Subunit Gene Analysis

The genetic molecular analysis from the gene of 16S rRNA subunit allowed revealed the prevalence of *Bacillus* sp. out of the 50 strains isolated in this study. Quantitatively, only four species account to ~75 % of the bacterial identified in this work. *Bacillus pumilus* occurred most frequently in the samples (38 %), followed by the *Bacillus cereus* (18 %), *Vibrio alginolyticus* (10 %), and *Planococcus maritimus* (8 %). The abundance of other bacteria varied from 2 % (*Bacillus thuringiensis*, *Bacillus safensis*, and *Bacillus boroniphilus*) to 3 % (*Bacillus aerophilus*, *Enterobacter asburiae*, *Exiquobacterium* sp, *Micrococcus luteus*, *S. aureus*, *Staphylococcus* sp, *Staphylococcus epidermidis*, *Staphylococcus warneri*) (Fig. 8).

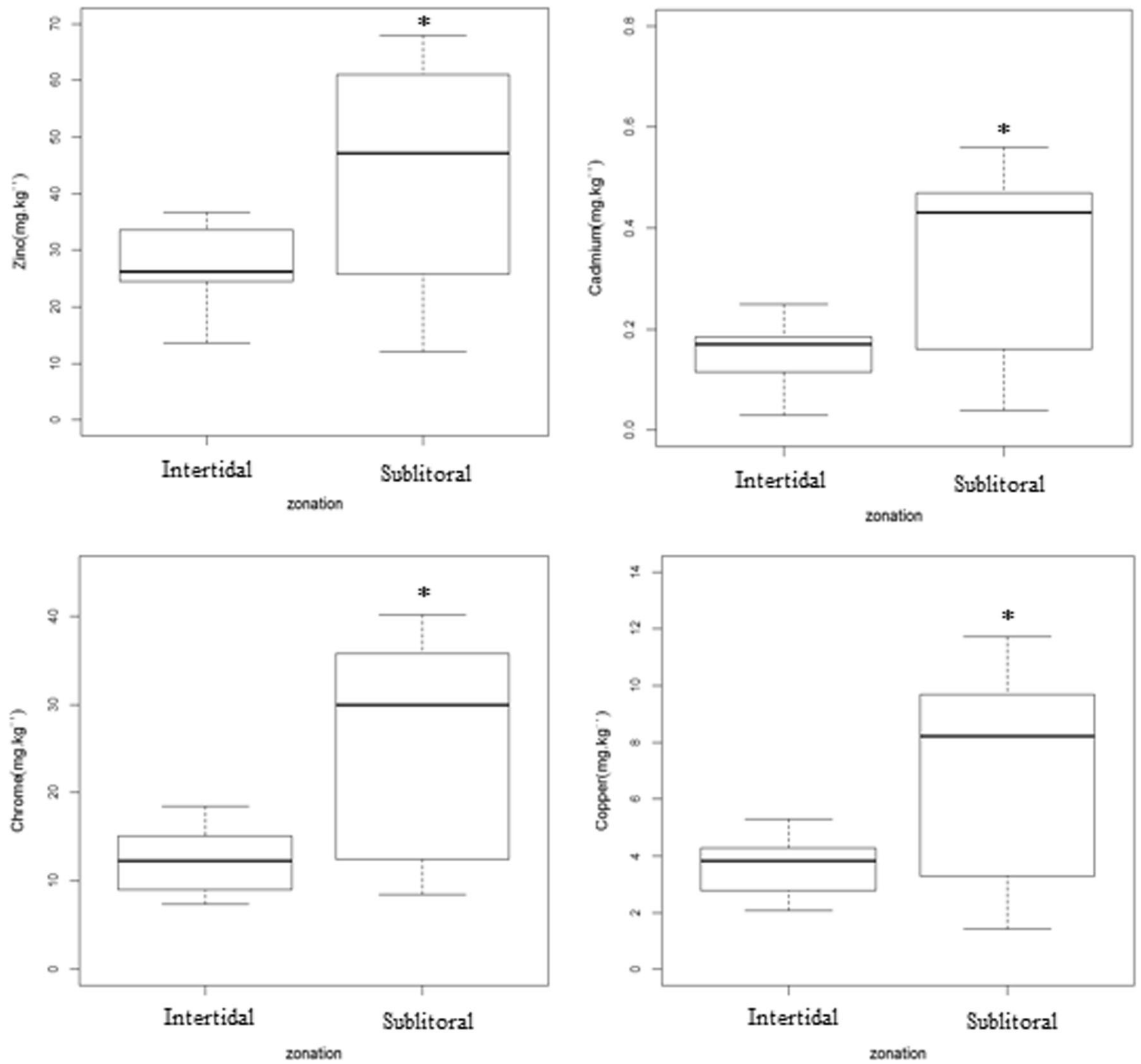


Fig. 3 Heavy metal concentrations (mg kg^{-1}): zinc (**a**), cadmium (**b**), copper (**c**), and chromium (**d**) from two sampling zones (intertidal and sublittoral). Where *black line* = mean; *box* = mean ± standard error; *whiskers* = mean ± 5 % confidence interval; and *symbol* (*asterisk*) = represents statistical difference ($p < 0.05$)

Minimum Inhibitory Concentration Analysis

Each genus responded differently to the heavy metals tested in this study when MIC was considered. In the case of zinc, *Enterobacter* sp. and *Exiquobacterium* sp. exhibited the highest average MIC (1600 μg mL^{-1}). The lowest MIC (12.5 μg mL^{-1}) was exhibited by *Micrococcus* sp. (Fig. 9a).

Table 1 References values for heavy metal concentrations in estuarine sediments as per the Canadian Sediment Quality Guidelines (SQG)

Metal	TEL (mg kg^{-1})	PEL (mg kg^{-1})
Zn	124.0	271.0
Cr	52.3	160.0
Cu	18.7	108.0
Cd	0.7	42

TEL threshold effect level, *PEL* probable effect level

All genera presented high MICs of chromium. *Exiquobacterium* sp. reached 3200 μg mL^{-1}, followed by *Bacillus* sp., *Vibrio* sp., and *Staphylococcus* sp., whose MICs were similar and ranged from 2400 to 2614 μg mL^{-1} (Fig. 9b). *Enterobacter* sp. had the highest MIC of copper (1600 μg mL^{-1}). The other genera exhibited values ranging from 250 to 568 μg mL^{-1} (Fig. 9c).

Cadmium was the metal with the lowest MIC among the strains. The highest peak was obtained by *Micrococcus* sp. (400 μg mL^{-1}), while the lowest peak was obtained by *Exiquobacterium* sp. (12.5 μg mL^{-1}). Concentrations among the other genera ranged from 200 to 249 μg mL^{-1} (Fig. 9d).

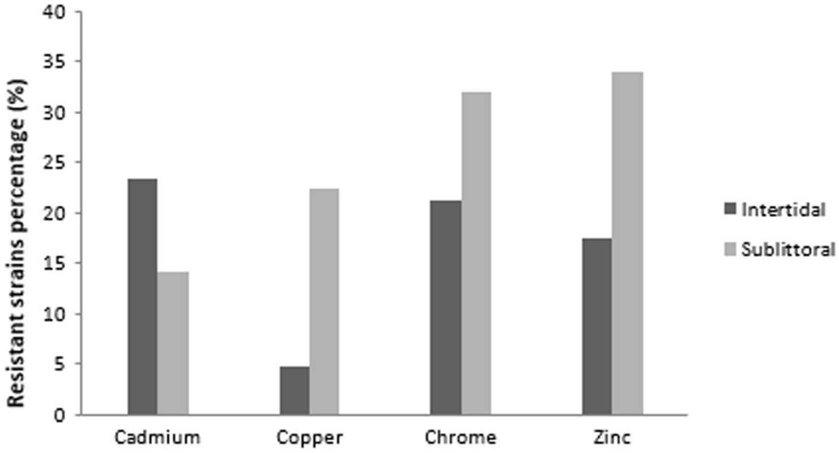


Fig. 4 Percentage of strains found to be resistant to the heavy metals tested (cadmium, copper, chromium, and zinc) in the intertidal zone and the sublittoral zone. The *symbol* (*asterisk*) represents statistical differences

Discussion

The sediment has been considered to be a layer of the water column in which pollutants accumulate because of its high adsorption and storage capacities. In the sediment, pollutant concentrations are found to be several orders of magnitude higher than those found in water. Sediments are therefore good indicators both of acute and chronic pollution [33].

The heavy metal concentrations determined in most of the samples were below the values established by Canadian law. However, in some parts of the sublittoral zone, the levels obtained were above the limits. These levels are likely because of the nearby refineries, fossil fuel burning, and industrial and domestic effluent discharge, all of which end up influencing Zn, Cr, Cd, and Cu concentrations found in these sediments [34].

In the present study, the highest metal concentrations were observed in the sublittoral zone. The collection stations in the sublittoral zone are near or even within the São Sebastião channel, where pollution originating mostly from the harbor, domestic, and industrial effluents occurs. The local hydrodynamics also allow the high accumulation of metals and organic matter in the sublittoral zone. Castro-Filho [35] and Fontes [36] showed that the circulation of the São Sebastião channel is characterized by northerly and southerly movements, with day-long intervals and little influence from tidal currents. This indicates that part of the pollution coming from the northern part of the channel, where the harbor and the Almirante Barroso Waterway Terminal (TEBAR) are located, may have an influence on the deposition of heavy metals into the sediments of the Araça Bay.

Already in 1977, it was found that bacteria in marine environments undergo selection or adjustment when heavy metals are present [37]. It has also been found that heavy metals can change the size, composition, and activity of the microbial community [11]. Abiotic stress caused by metals can affect the growth, morphology, and metabolism of bacteria [9, 10].

In the current study, however, the highest density of bacteria was found in the sublittoral zone, in which the metal concentrations are higher. This fact can be explained by the presence of wastewater discharge close to the sublittoral zone, where there is a high organic matter concentration that favors bacterial growth. Another likely factor is that the highest densities of bacteria occur in areas with higher concentrations of metals because most of the bacteria present there are already resistant as a result of chronic contamination [20].

Generally, metal contaminations in the environment cause an increase in the level of resistance among the local bacterial community [38] and reduce the bacterial diversity as a consequence of bottleneck events [39]. Therefore, the higher the heavy metal concentration, the higher will be the occurrence of heavy metal-resistant bacteria. This finding was confirmed in this study for in the case of Zn and Cr. The higher concentration of these two metals in sediments also presented more

Table 2 Heavy metal tolerance among bacteria isolated from Araça Bay sediment

Metal	Percentage (%) of tolerant isolated strain at different metal concentrations (μg mL^{-1})										Resistant isolates (%)
	12.5	25	50	100	200	400	800	1600	3200	>3200	
Cadmium	54.0	51	49	38[a]	22	7	0	0	0	0	38
Copper	100	95	94.1	86	42	25	10[a]	2.0	0	0	10
Cadmium	100	100	100	100	100	97.0	65.8	53[a]	36.0	0	53
Zinc	100	96.0	94.0	93.0	72.0	52[a]	42	4	0	0	52

[a] Minimal inhibition concentrations of standard strain *E. coli* K12

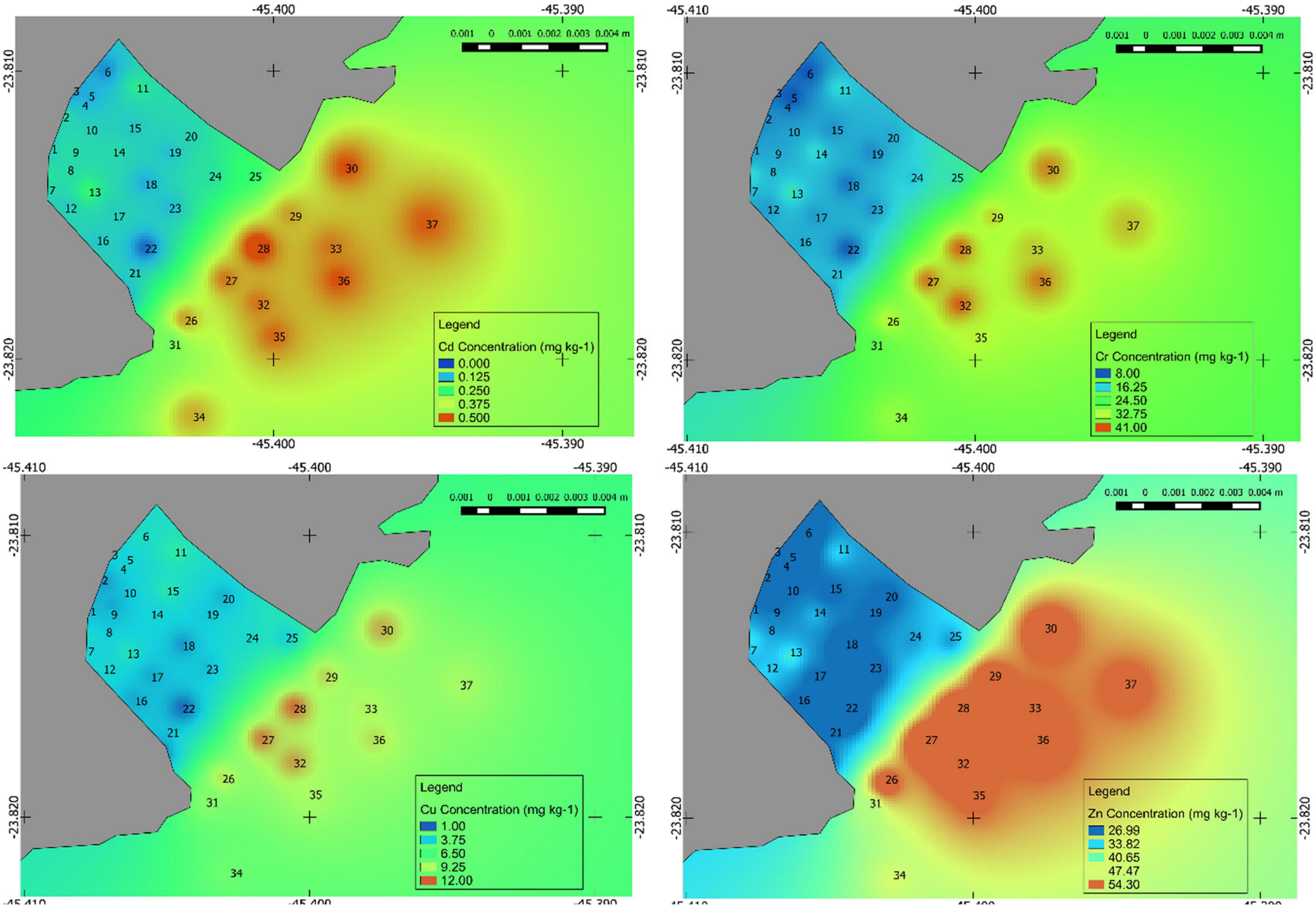


Fig. 5 Distribution of concentrations of Cd (**a**), Cr (**b**), Cu (**c**), and Zn (**d**) in sampled areas in the São Sebastião Channel and in the Araça Bay, with a sublittoral zone [1–16] and an intertidal zone [17–37]

than half of resistant strains identified in this work. Cu that was found at the lowest concentration presented the lowest percentage of resistant strains to Cu.

In this study, more bacterial strains were resistant to chromium than to the other metals tested. This difference may be explained by the relatively low toxicity of chromium compared to the toxicity of the other metals. Chromium (and particularly Cr(VI)) exhibits little biological activity because of low interaction with macromolecules and may be considered less toxic than other heavy metals [40].

It is well known that zinc is a trace element that is essential for bacterial cell growth, but it is a potent inhibitor of electron transport in cellular respiration when present at high concentrations [41]. However, its toxicity is very low compared to that of other metals such as Hg, Cd, Cu, Ni, Co, and Pb [42]. This factor may explain the results obtained in this study, in which more than half of the strains of bacteria isolated from Araça Bay were resistant to Zn.

The bacteria exhibited low resistance to Cu. This difference may occur because this metal is associated with acute and chronic toxicity in bacteria, and because it strongly affects the enzyme system and essential cellular metabolism [43].

Of the bacterial strains, 38 % were resistant to cadmium, despite the low concentrations of this metal in the sediments of the Araça Bay. In fact, several studies have reported the presence of bacteria that are tolerant of heavy metals, even at sites with very low heavy metal concentrations [44]. This difference can be explained by cadmium's toxicity. Cd is the most toxic metal for living organisms, and low doses have been found to have adverse effects on biota [32]. Low Cd concentrations are able to select resistant bacteria because higher concentrations are lethal [45].

The results of PCA analysis confirms the hypothesis proposed in this study, as well all the points discussed above. Higher bacteria resistance was found in areas with high concentration of heavy metals, except for Cd because of it is extremely toxicity. However, although the Fig. 2 has shown that intertidal zone, which had a lower heavy metal concentration in general, had a lower bacteria density, PCA showed that highest concentrations of metals reduce bacterial density in the marine environment.

Akinbowale, Peng, Grant, and Barton [25] found the same bacterial resistance to metals tested in this study. The resistance occurred in the following order: Cu > Cr > Zn > Cd.

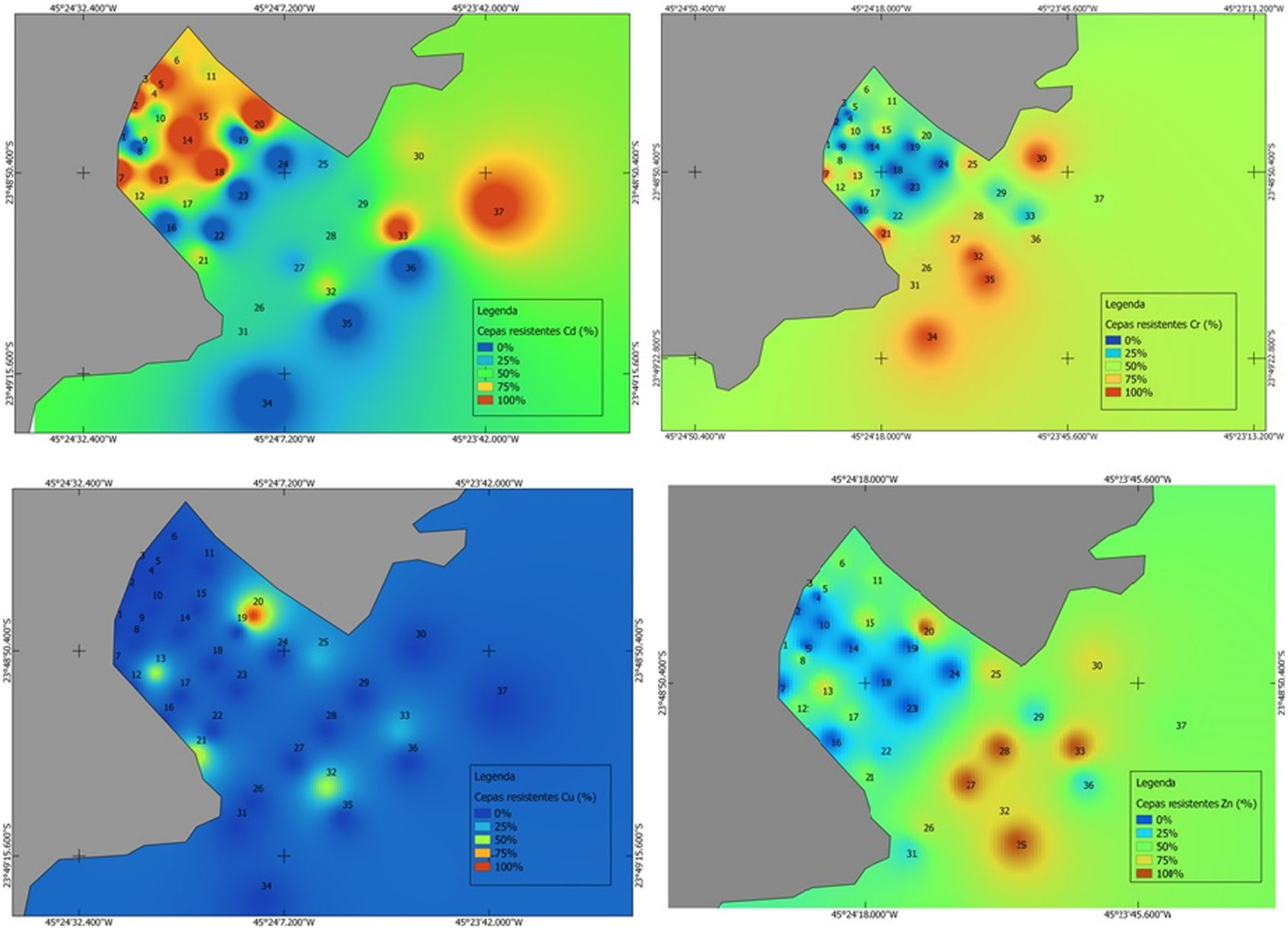


Fig. 6 Distribution of bacteria resistant to Cd (**a**), Cr (**b**), Cu (**c**), and Zn (**d**) in sampled areas in the São Sebastião Channel and in the Araça Bay, with the sublittoral zone [1–16] and the intertidal zone [17–37]

Matyar, Kaya, and Dinçer [46] studied the sediments of the Iskenderum Bay in Turkey and found bacteria to be more resistant to Cd, followed by Cu and Cr. This discrepancy suggests that there are differences in the heavy metal concentrations in sediments from different areas depending on the surrounding anthropogenic activities. These differences may be reflected in the bacterial resistance profile of the region.

MICs are important to understand the level of strains resistance and may be associated with the concentrations and toxicity of metals in the environment. Malik, Khan, and Aleem [38] isolated bacterial strains from the soil of industrial areas and reported highest MIC of 2400 μg mL^{-1} for Cu, Cd, Zn, and Cr. In this study, the MIC of almost all of the metals reached maximum values of 3200 μg mL^{-1}. The exception was Cd, the MIC of which reached a maximum value of 800 μg mL^{-1}

Malik and Jaiswal [47] also found high MICs in their isolated strains and associated this result with sampling area location. Similar to the current study, Malik and Jaiswal [47] collected their samples from locations close to points of industrial and domestic effluent discharge. In the present study, the collection points were close to domestic sewage and industrial wastewater discharge points, and were also close of the São Sebastião Port and the TEBAR.

Thus, the distribution of resistant bacteria in the Araçá Bay may be associated with effluent discharge, since both the highest concentrations of metals and the higher percentages of resistant strains were found at the points of the sublittoral zone near the São Sebastião channel. These results also support the existence of higher heavy metal contamination in the São Sebastião channel.

Though the metal concentrations found in this study were below the reference values from Canadian Sediment Quality Guidelines (SQG) overall, a high frequency of resistant bacterial strains was also found. These results suggest that the heavy metals likely have effects on the biota, particularly in the sublittoral area. The findings also suggest that these populations of microorganisms are in the process of undergoing biological and genetic changes.

This fact is very important, since bacteria are the most abundant organisms in the sediment and represent the first

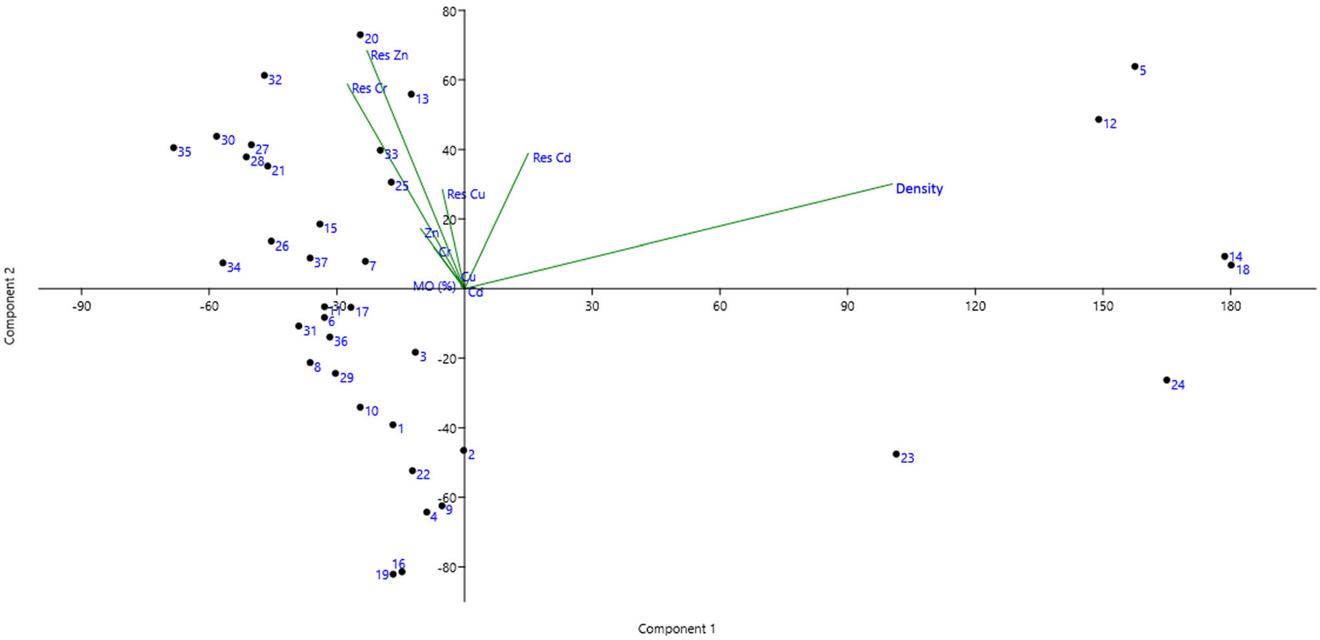


Fig. 7 Principal components analysis (PCA) of stations using variables: density; organic matter; Zn concentration (*Zn*); Cu concentration (*Cu*); Cr concentration (*Cr*); Cd concentration (*Cd*); percentage of resistant bacteria to Zn (*Res Zn*), Cu (*Res Cu*), Cr (*Res Cr*), and Cd (*Res Cd*)

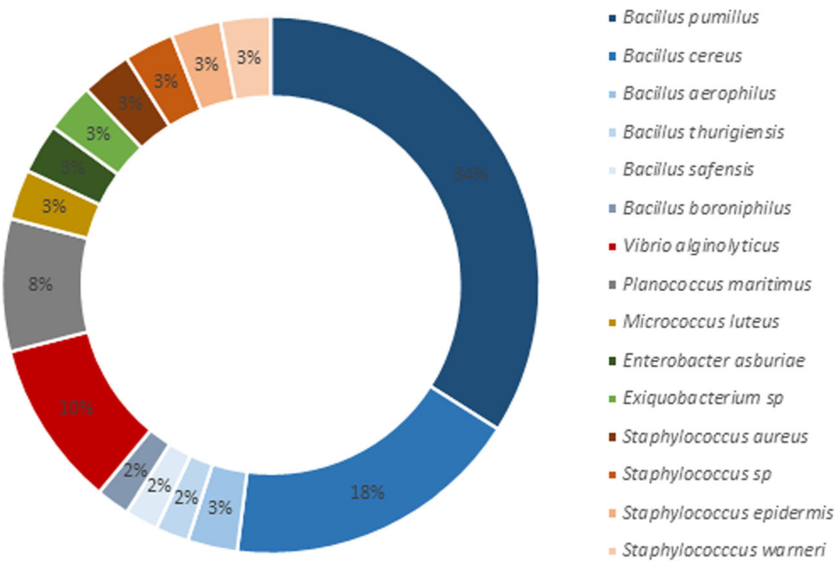
steps of toxic component transfer to higher trophic levels [14]. Because of these factors, the results suggest the need for pollution monitoring in the Araça Bay.

A significant portion of the bacteria identified in this study belongs to the group of Gram-positive bacteria, which is similar to findings from other studies [20, 48, 49]. Previous studies estimated that only 5 % of the bacteria found in the ocean was gram-positive [50], but more recent studies suggest that the abundance and diversity of Gram-positive in the sediments are much larger [49, 51, 52]. The present study suggests a higher abundance and diversity of gram-positive bacteria in Brazilian marine sediments.

The isolates were also identified. The frequency of *Bacillus* genus was substantially higher relative to the other genera. Bacteria resistant to heavy metals were isolated in several other studies with results similar to ours [53–55]. This consistency may indicate that *Bacillus* sp. possesses significant potential for the bioremediation of marine and estuarine areas polluted with heavy metals.

Kamala-Kannan and Lee [56] isolated bacteria resistant to heavy metals in sediments of the Sunshon Bay in South Korea. All isolates were identified as *Bacillus* sp. This finding suggests that anthropogenic pollution ultimately selects for resistant species. In this case, Bacillus sp.

Fig. 8 Percentages of bacterial species isolated from sediment samples and identified by the analysis of the 16S rRNA subunit gene



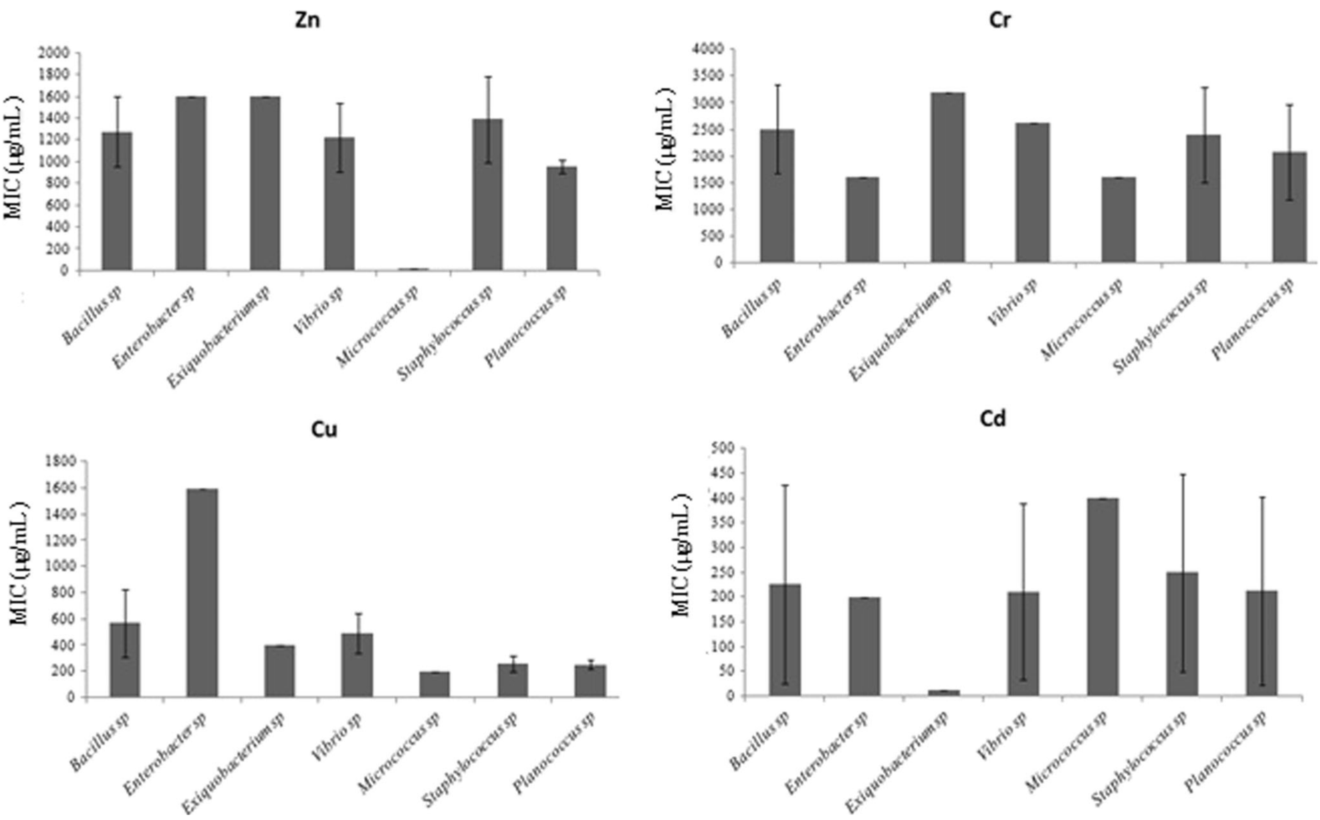


Fig. 9 Average minimum inhibitory concentration (MIC) of zinc (**a**), chromium (**b**), copper (**c**), and cadmium (**d**) for each bacterial genera isolated

was the dominant taxonomic group in the communities of heterotropic bacteria in the polluted areas.

Staphylococcus sp. is also a genus that may be used in bioremediation. *Staphylococcus* sp. may be resistant to multiple heavy metals, findings which are similar to those obtained in the present study [57]. Several species of *Vibrio* and *P. maritimus* were also isolated from marine sediments in other studies and exhibited resistance to various metals, as well as simultaneous resistance to antibiotics [20].

Pollution of the marine environment by effluents from factories, harbor activities, and other sources containing heavy metals selects for bacteria that are resistant to various metals. As a result, this pollution is a problem not only for the local biota but also for public health, since simultaneous resistance to metals and antibiotics is a reciprocal phenomenon [57].

Recently, the Scientific Committee on Emerging and Newly Identified Health Risks suggested that various biocides, including heavy metals, may contribute to the selection and maintenance of bacteria with antibiotic resistance [58]. This contribution may occur through the horizontal transfer of genetic elements carrying genes that confer resistance to antibiotics and biocides or that share the same resistance mechanisms [58–60].

This problem is exacerbated by the fact that many of these bacteria are pathogenic and can cause serious problems to humans. For example, *S. aureus* has become very relevant in recent decades in respect to nosocomial infection with high levels of morbidity and mortality; in most cases, its multidrug resistance makes treatment difficult [61]. Another example is *B. cereus*, which can cause serious food-borne illness [62].

This effect on public health is another factor that reflects the need for heavy metal contamination-monitoring in the environment. This contamination could lead to heavy metal-resistant bacteria that are more likely to be resistant to antibiotics.

The points discussed support the hypothesis of this study. In Araça Bay there is a clear relationship between heavy metal concentration and selection of heavy metal-resistant bacteria. There are groups that dominated the environment like *Bacillus* sp. Some implications are particularly important: it was possible to isolate bacteria with great biotechnological potential; the monitoring of heavy metal pollution has to be encouraging due the impact in microbial population and to the possibility of pathogenic strains also be resistant to antibiotics.

Conclusion

In this way, this study showed that the presence of harbor activities increase heavy metals concentration that influence the distribution of resistant strains, selecting a larger number of bacteria resistant to various forms of heavy metals. In addition, it may alter the microbial community and ecosystem function. We isolate several species of heavy metals-resistant bacteria with high MIC that can be used for future studies of marine bioremediation of contaminated areas in Brazil. Moreover, we observed that there is a dominance of *Bacillus* sp. in this area and it can have high resistance to metals tested that could be an indicator of heavy metal-contaminated areas.

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