Isolation and evaluation of nickel-resistant bacteria from estuary sediment of Itajaí River.

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Abstract. This study focused on the Saco da Fazenda estuary in Brazil, an area affected by nickel contamination, and aimed to identify nickel-resistant bacteria with bioremediation potential. Sampling took place at two locations, with point 2 showing a higher degree of microbial adaptation to nickel. Nine isolates capable of thriving in a 1M nickel concentration were selected for further characterization, and they predominantly exhibited morphological and physiological traits associated with the *Bacillus* genus, potentially *Bacillus cereus*. Two standout isolates, namely LAMA 1507 and LAMA 1508, retained their nickel resistance under laboratory conditions. These isolates showed promise in nickel removal, likely employing biosorption or bioaccumulation mechanisms. However, both isolates displayed antimicrobial activity against *Vibrio fisheri*, even without nickel, a common characteristic in their taxonomic group. This research underscores the importance of exploring microbial communities in contaminated environments for potential bioremediation candidates. While LAMA 1507 and LAMA 1508 exhibit strong nickel removal potential, their antimicrobial activity highlights the need for comprehensive studies on these isolates for effective environmental remediation strategies.

Keywords. Estuarine environments**,** Nickel-resistant bacteria, Bioremediation.

1. Introduction

Estuarine waters, where freshwater meets the ocean, play a vital role in nutrient transport in the hydrosphere. However, they face significant challenges due to release of chemical contaminants from various sources, including industry and urban areas [1][2]. These contaminants, including heavy metals from industrial activities, accumulate in water and sediment, impacting bacterial communities [3]. One such affected estuarine system is the Itajaí-Açu estuary in southern Brazil, known for its economic significance and environmental concerns due to pollution [4]. Elevated nickel levels in sediment samples from the Itajaí River, exceeding regulatory limits, have raised concerns [5].

High nickel concentrations are known for their toxicity [6], driving the adaptation of microorganisms that retain resistance to this metal in contaminated environments [7]. This selective pressure has been observed in various microorganisms, including *Ralstonia eutropha*, *Alcaligenes denitrificans, Alcaligenes xylosoxydans, Klebsiella oxytoca, Hafnia alvei*, and *Escherichia coli*[8]. Nickel-resistant microorganisms have also been isolated from polluted soils[9][10], including halophilic microorganisms found in estuarine environments [11].

Addressing nickel contamination challenges, bioremediation emerges as a cost-effective strategy. It involves using microorganisms to reduce metal toxicity in contaminated sites [12]. Metals, unlike hydrocarbons, are not degraded into CO2 and H2O but are often precipitated or transformed into less toxic species [13][14]. Microorganisms can alter metal chemical forms, decreasing their toxicity or bioavailability through mechanisms like metal accumulation linked to resistance. Studies have ranked metals accumulation by heterotrophic marine bacteria, with zinc most accumulated, followed by nickel and iron [15].

The potential of a bacterium for bioremediation can be demonstrated through various approaches. Bioassays or toxicity tests involve culturing a microorganism with bioremediation potential in the presence of the toxic substance and assessing the medium's toxicity using a sensitive organism. Toxicity testing with bioluminescent bacteria like *Vibrio fisheri* is standart for such evaluations [16]. Thus, this study aims to isolate and identify resistant bacteria from sediment samples in the Saco da Fazenda estuary and explore their bioremediation

potential.

2. Research Methods

2.1 Location an sample collection

The sediment samples were collected from the Saco da Fazenda estuary, which, according to Branco [17]:

"It is located between coordinates (26°53'33" - 26°55'06" S; and 48°38'30" - 48°39'14" W), at the mouth of the Itajaí-Açú River, Itajaí, SC. It is a semi-closed body of water with an area of approximately 0.7 km2, the result of anthropic action that modified the original river mouth with the construction of containment jetties. It has silty-clayey substrate, a maximum depth of 2 meters, except in the connecting channels with the river, which reaches up to 9 meters, and a tidal range of less than 1.4 meters. The estuary receives freshwater input and domestic effluents from the Schineider Creek and Saco da Fazenda neighborhood."

For this study, the samples were collected at two distinct points located on the riverbank, accessed by existing piers, on two occasions. The samples were placed in sterile plastic bags using previously sterilized spatulas. They were then refrigerated until processing in the laboratory, which occurred in less than six hours.

Fig.1- Collection sites. Point 1 highlighted in red and point 2 highlighted in yellow.

2.2 Enumeration and isolation

To enumerate microorganisms, 10 grams of sediment from each sampling point were diluted in 90 milliliters of filtered and autoclaved estuarine water. After 30 minutes of homogenization using a magnetic stirrer, two consecutive decimal dilutions were prepared. Then, 100 microliters of each dilution were inoculated onto plates containing R2A Agar. These plates were made with filtered estuarine water and supplemented with NiCl2 at concentrations of 5 millimolar (5mM) and 10 millimolar (10mM) to facilitate the enumeration of cultivable nickel-resistant bacteria. The choice of these nickel concentrations was based on prior studies involving nickel-resistant microorganisms [11][18]. This inoculation process was performed in triplicate. The cultures were incubated at 30°C for seven days, and microorganism counts were conducted on replicates with colony counts ranging from 10 to 200. The colony count per gram of sediment was determined by calculating the average colony count and multiplying it by the dilution factor.

For the isolation of resistant microorganisms, colonies with distinct morphologies were collected from plates supplemented with 10mM NiCl2. These selected colonies were then inoculated onto new plates containing R2A Agar supplemented with nickel and prepared using sterilized estuarine water. These plates were incubated at 30°C for two days. After incubation, subcultures were performed to obtain pure cultures. The obtained pure cultures were preserved in Marine Broth supplemented with 20% glycerol and stored in an ultrafreezer at -80°C.

2.3 Maximum tolerated nickel concentrations

To determine the maximum tolerated nickel concentrations, isolated organisms were streaked at four different points on R2A agar plates containing various concentrations of the metallic salt $NiCl2{\cdot}6H2O$, including 100 millimolar (100 mM), 500 mM, 1000 mM, 5000 mM, and 1 molar (1 M). A control plate without the metal was also prepared. Duplicates were inoculated for each concentration level, along with a parallel control. Inoculation began with a pre-culture cultivated in R2A medium, which had been incubated at 30ºC for 48 hours. Following inoculation, the plates were incubated at 30ºC for seven days. After incubation, a thorough examination of the plates was conducted to determine the presence or absence of microbial growth. Colonies showing growth on both the control plate and the plates with the highest nickel concentrations were selected for further analysis. This rigorous evaluation was carried out for each microorganism, with five organisms from each sampling point and collection date being tested, resulting in a total of 30 organisms.

2.4 Phenotypical characterization

Following the nickel resistance tests, the top 10 microorganisms demonstrating the highest nickel tolerance were selected. Their morphological characteristics were examined through optical microscopy, focusing on attributes like arrangement, shape, motility, and the presence of endospores. Based on the observed arrangement patterns, it was deduced that all these microorganisms likely belonged to the *Bacillus* genus [19]. Consequently, a simplified taxonomic key technique, as presented by Reva, Sorokulova, and Smirnov [20], was used for the preliminary identification of species. This preliminary description and characterization laid the groundwork for subsequent analyses. To determine the precise taxonomic grouping, a series of biochemical tests assessing primary metabolic traits

was conducted. These tests included Gram staining, the presence of cytochrome C, the existence of amylase, anaerobic growth capacity, and the presence of catalase. The selection of these specific tests was based on metabolic characteristics described in earlier studies for groups within the *Bacillus* genus [21][22].

2.5 Evaluation of nickel bioremediation potential

To evaluate the potential of the isolated bacteria for nickel bioremediation, a modified method inspired by Ruiz et al.[16] was employed. In this experimental approach, nickel-resistant strains were cultured in Marine Broth enriched with 5 mM of nickel for 48 hours. After incubation, cells were separated via centrifugation at 11,000 rpm for two minutes, and the resulting supernatant was sterilized by filtration through 0.22 µm pore size membranes to remove any remaining microorganism cells from the medium.

As a control, organisms were cultured in Marine Broth without nickel under the same conditions, and cell removal was carried out in the same manner. These control experiments were conducted to account for any potential influence of other molecules produced by the bioremediating organism and the culture medium on *V. fischeri* exclusively. Both control media, one with nickel and one without, were prepared but left uninoculated. They underwent the same filtration and centrifugation procedures as previously described. All control samples and the treated medium were then assessed for their impact on the inhibition of bioluminescence in *Vibrio fischeri*[23]. This assessment followed established protocols and utilized the Lumistox equipment. Microorganisms with bioremediation potential demonstrate a noticeable reduction in the toxicity of nickel towards *V. fischeri*.

3. Results and discussion

Following sample collection and processing, we conducted inoculation using triplicates from the second and third serial dilutions. These were introduced into mediums with 5mM and 10mM nickel supplementation, alongside a control medium.

Substantial growth was seen in the control medium, while no noticeable growth occurred in the supplemented media. To expand our collection of resistant isolates, we inoculated 10 new plates with 100 µl from the first dilution series, specifically supplemented with 5mM and 10mM nickel. This modified approach resulted in growth at both metal concentrations (Fig. 2). Sample 2 displayed a more diverse range of morphologically distinct resistant colonies, especially when supplemented with 10mM nickel, achieving the highest CFU count per gram of sediment. This suggests that the microbial community at point 2 is more adapted to the presence of nickel. This adaptation could be attributed to the different collection locations, with point 1 having a higher water current flow, while point 2 was near an outlet with lower water current flow.

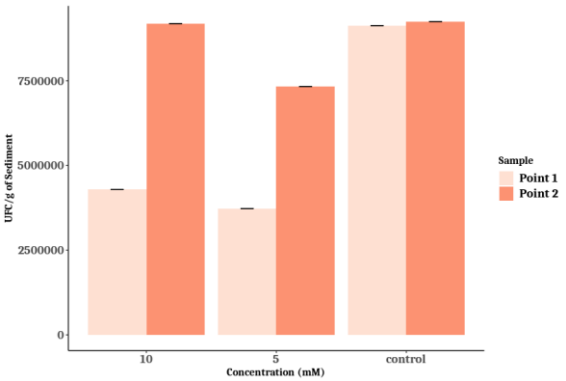


Fig.2- Number of colony-forming units (CFUs) per gram of sediment found in the treatment without nickel supplementation (control) and with nickel supplementation at 5mM and 10mM.

In contrast, in sample 1, the CFU count per gram of sediment was higher in the non-supplemented medium, with only a slight increase at 10mM in the supplemented media. This is consistent with metal toxicity, indicating a community with fewer resistant organisms. In this case, compromised growth was observed due to the stress imposed by the heavy metal on cells [3][7].

Another interesting observation was the increase in CFU/g of sediment at the higher nickel concentration (10mM), consistent with a study by Guzman et al. [24], which found increased growth of nickel-resistant organisms in a medium supplemented with nickel. Heavy metals, even in small quantities, can significantly alter soil community structure. Higher metal concentrations create a selective environment favoring microorganisms with greater resistance. This phenomenon of pollution-induced tolerance has been observed in ecological systems, where prolonged exposure to pollution leads to the development of greater tolerance in resident communities[25][26]. Soils chronically polluted with trace metals like nickel show induction of tolerance in their microbial communities due to the selection of more resistant organisms, physiological acclimatization, and genetic adaptation[27][28][29]. A total of 10 isolates were chosen from sample 1, and 20 isolates from sample 2. Priority was given to colonies grown in the 10mM supplemented medium of sample 2 to preserve the most resistant organisms.

To determine their maximum tolerated concentrations, the triplicates with supplementation were divided into intervals of 100mM, 500mM, 1000mM, 5000mM, and 1M (10000mM). As a result, 3 isolates from sample 1 exhibited tolerance up to 1M, and 5 isolates from sample 2 showed the same capability(Table 1). Variability in resistance test results can be attributed to different responses exhibited by various microorganism

species[30][31][32].

Isolate – Point 1	Max. [Ni] tolerated	Isolate – Point 2	Max. [Ni] tolerated
1	100mM	1	100mM
2	100mM	2	100mM
3	100mM	3	100mM
4	500mM	4	100mM
5	1000mM	5	500mM
6	1000mM	6	500mM
7	5000mM	7	500mM
8	10000mM	8	500mM
9	10000mM	9	500mM
10	10000mM	10	500mM
-	-	11	1000mM
-	-	12	1000mM
-	-	13	1000mM
-	-	14	5000mM
-	-	15	5000mM
-	-	16	10000mM
-	-	17	10000mM
-	-	18	10000mM
-	-	19	10000mM
-	-	20	10000mM

Tab.1 - Nickel resistance tests with microorganisms isolated from point 1 and point 2, indicating the maximum tolerated nickel concentrations.

Among the resistant microorganisms, those that showed growth in 1M (10000mM) of nickel were selected for further characterization. The selected microorganisms from the resistance tests were cultured in nutrient broth and observed under an optical microscope at 1000x magnification. Various characteristics were documented, including arrangement, shape, motility, and the presence of endospores. The predominant arrangement observed was streptobacilli, most of these isolates showed motility. Endospores were present in the majority of cases as well. All isolates exhibited a rod shape (Table 2).

Point - Isolate	Arrange	Shape	Motility	Endospores
1 - 7	Streptobacilli	Rod	+	+
1 - 8	Streptobacilli	Rod	+	+
1 - 9	Diplobacilli	Rod	+	+
1 - 10	Streptobacilli	Rod	+	+
2 - 16	Streptobacilli	Rod	-	+
2 - 17	Diplobacilli	Rod	-	-
2 - 18	Diplobacilli	Rod	+	-
2 - 19	Diplobacilli	Rod	+	-
2 - 20	Diplobacilli	Rod	+	+

Tab.2 - Characteristics of each isolate obtained, observed under an optical microscope at a magnification of 1000x.

Based on this general characterization of microorganisms, it was determined that their likely taxonomic origin is within the genus *Bacillus*, as the observed features are common characteristics within this group[19]. Following a simplified taxonomic key for aerobic bacteria[20], selective media were employed to elucidate their physiological characteristics.

Gram staining revealed a positive result for the cell wall type in all strains. Furthermore, cytochrome C oxidase was detected in all strains, and they exhibited anaerobic growth capability. Notably, none of the strains demonstrated starch degradation (amylase absence). In the catalase test, only strains 1-8, 2-17, and 2-19 tested positive, while all strains exhibited nitrate reduction(Table 3).

Point. ID	1.7	1.8	1.9	1.10	2.16	2.17	2.18	2.19	2.20
G (+)	+	+	+	+	+	+	+	+	+
Cyt. C	+	+	+	+	+	+	+	+	+
(-)O^2	+	+	+	+	+	+	+	+	+
N.R.	+	+	+	+	+	+	+	+	+
Catal.	-	+	-	-	-	+	-	+	-
Amy.	-	-	-	-	-	-	-	-	-

Tab.3 - Biochemical characterization of isolates, indicating cell wall type through Gram staining (G(+)), presence of cytochrome C (Cit. C), starch hydrolysis capability (Amy.), growth under anaerobic conditions((-)O^2), presence of catalase (Catal.), and nitrate reduction capacity (N.R.).

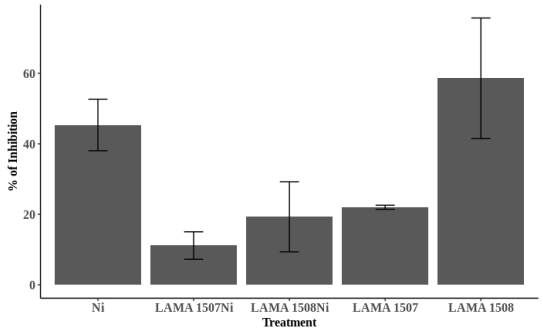
It can be concluded that all isolates are facultative anaerobic microorganisms and do not possess starch hydrolysis capability. These characteristics, along with the observed features, suggest that the majority of these microorganisms likely belong to the taxonomic group of *Bacillus cereus*, as indicated by the taxonomic key, specifically identified as potential candidates for *Bacillus cereus* [22]. This aligns with findings from other studies, where *Bacillus* was identified as a predominant genus among nickel-resistant isolates [30][31][32][34]. These Gram-positive bacteria are known for their high genetic diversity and metabolic versatility, allowing them to thrive in various environments[35]. They employ their resistance mechanisms to facilitate the absorption, transport, and efflux of metal ions out of the cell, making them efficient in removing and absorbing heavy metals [36].

During the subculturing process, two specific strains were selected for further analysis due to their consistent resistance maintenance under laboratory conditions. The first strain, originally from point 1 and formerly labeled as ID 9, was renamed LAMA 1508. The second strain, originating from point 2 and previously identified as 16, was re-designated as LAMA 1507.

When assessing the impact of each isolate on *Vibrio fischeri*, it was noted that LAMA 1507 displayed the lowest inhibition levels in supplemented media. Conversely, LAMA 1508 exhibited reduced inhibition levels compared to the sterile medium (Figure 3). Without supplementation an increase in inhibition was observed for LAMA 1508, while LAMA 1507

maintained low inhibition.

Fig.3 - Inhibition percentage measurements of *Vibrio fischeri*, divided into inocula with the metal (LAMA 1507Ni and LAMA 1508Ni), sterile medium with the metal only (Ni), and interaction in a neutral medium (LAMA 1507 and LAMA 1508). Absorbance measurements were taken at 600nm.



These results align with previous studies highlighting the genus *Bacillus* as a primary candidate for heavy metal bioremediation, employing strategies such as biosorption, mediated biosorption, bioaccumulation, or bioprecipitation [37][38]. Ayangbenro and Babalola [38] demonstrated heavy metal removal by *Bacillus cereus* NWUAB01, identifying a biosurfactant with removal efficiencies for Pb, Cd, and Cr. This further underscores the potential for heavy metal bioremediation within this taxon. However, it's essential to note that when interacting with *Vibrio fischeri* in conditions without the metal, the isolates exhibit inhibitory effects, indicating a level of toxicity. It's also worth mentioning that the *Bacillus* genus, particularly strains of *Bacillus cereus*, has documented antimicrobial activity, affecting both Gram-positive and Gram-negative bacteria [40].

4. Conclusion

This study focused on bacteria's resistance to nickel and identified promising isolates for remediating areas contaminated with this heavy metal. The results revealed the presence of bacteria highly resistant to nickel, especially in the sample from point 2, indicating a well-adapted microbial community. Initially, 9 microorganisms capable of thriving in a medium with 1M (10000mM) of nickel were selected and characterized as likely members of the *Bacillus* genus, specifically *Bacillus cereus*. This aligns with prior research emphasizing *Bacillus* as a key player in heavy metal bioremediation, including nickel, due to its genetic diversity and adaptability. The bioremediation assessment of the selected isolates, LAMA 1507 and LAMA 1508, suggests that LAMA 1507 holds more promise, displaying superior removal capabilities. Both isolates exhibited toxicity to *Vibrio fischeri* when interacting in a medium without nickel, a common trait within the potential taxonomic group *Bacillus cereus*. In summary, the isolates from this study, particularly LAMA 1507, exhibit potential for nickel bioremediation, contributing to more effective environmental remediation strategies.

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