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Microbiological profile of urban mangrove sediments exposed to effluents from the pharmaceutical industry

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ABSTRACT

This study aimed to evaluate the impact of the release of effluents from the pharmaceutical industry, for a long period, on the microbiota of a mangrove in São Luís, MA. Six equidistant points were randomly sampled and pH was analyzed through the KCl solution. Furthermore, the microbiological profile of the sediment of the area was studied through isolation by the serial dilution technique using Muller Hinton (MH), Bushnell Hass (BH) plus antibiotic, Sabouraud Dextrose Agar (SAB), and Eosin Methylene Blue (EMB). After isolation and counting of the plaques, a larger number of colonies were observed in the MH medium (30000 CFU/mL), followed by the BH medium (3000 UF/mL), SAB medium (2500 CFU/mL), and EMB (400 UFC/mL). The pH of the six samples were in the Neutral pH range, ranging from 7.10 to 7.40. The results demonstrate that the microbiota of the mangrove sediments adapted to the effluent contamination of the pharmaceutical industry because there was an expressive growth of microorganisms in all the media, including the culture medium BH plus antibiotic, which is an indication of bacterial resistance to the antibiotic used.

Keywords: Environment; Resistance; Microorganisms

1 INTRODUCTION

Population growth and urbanization have aggravated the phenomenon of environmental pollution, especially in large urban centers. In particular, some ecosystems have been more affected due to their proximity to factories and industries that produce and release effluents without prior treatment. One of the most affected ecosystems are the mangroves because they occupy an extensive urban area that suffers constant anthropic influence.

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The soil of mangroves is composed basically of organic matter mixed with sediment, being anaerobic with the exception of the surface sediments (PEREIRA FILHO *et al*., 1999). Moreover, mangroves have great biodiversity of vegetal species and microorganisms endowed with several adaptive characteristics due to the high salinity, strong winds, tidal fluctuation, high temperatures, muddy soil, and low oxygen content (POLONI, 2014).

The Brazilian coastline stretches ~7,408 km. It is composed of a large diversity of ecosystems, such as dunes, islands, bays, estuaries, swamps, cliffs, and rocky shores, that include a large number of species that possess characteristics to adapt to such localities (CORREIA; SOVIERZOSKI, 2009).

The biodiversity that exists today is the result of 3.5 million years of evolution, shaped by natural processes and, increasingly, by human action. In an inopportune way, due to the high exploitation of natural resources, the unsustainable development of human societies, and the consequent degradation of the environment, the planet is undergoing a crisis of biodiversity and degradation of natural ecosystems (ABDEL, 2003).

Located in a transition environment between land and sea, mangroves contain organisms well adapted to natural stress conditions such as high temperatures, salinity, anoxia, and ultraviolet radiation. However, this ecosystem has shown tolerance limits, being sensitive to several changes created by anthropic action (BADOLA *et al*., 2008). Furthermore, its proximity to urban environments further contributes to its exploitation. Industrial effluents have contributed to the contamination of sediments with heavy metals, and several mangroves have already been contaminated with oils due to oil spills. These events have several negative effects on the mangrove environment (BADOLA *et al*., 2008).

The dynamics of human occupation on the Maranhão coast favors the premise that mangroves are also actors in the construction of the history of Maranhão, because globally, natural resources provided by mangroves have been used by humans prehistorically, and in pre-Columbian America there are extensive records of the extraction of compounds, resins, wood, and proteins of animal origin (LACERDA *et al*., 1993). Studies in the coastal states of Pará and Maranhão revealed that mangrove resources had been extracted for more than 4,000 years (BANDEIRA, 2006; BARBOSA; CASCABULHO, 1996; GASPAR, 1996). In addition, in large urban centers, mangrove areas have suffered from the most diverse types of environmental impacts, such as waste disposal, effluents, and domestic sewage.

On the island of São Luís, that served as study area of this research, a pharmaceutical company responsible for discarding its effluents in the mangrove was functional for thirty years. According to Neto Ferreira (2019), the pharmaceutical company began its activities in Maranhão in 1968, extracting products of biotechnological origin for the manufacture of medicines and supplying them to the pharmaceutical industry. In April 2010, there was a change in management and acquisition of the company's Natural Products Division, becoming the main producer of flavonoid quercetin in the state for the combat of free radicals (cell-damaging molecules). Because of these factors, it is believed that the area houses a specific microbiota that may have acquired resistance to drugs such as antibiotics.

The development of antimicrobial resistance in microorganisms is no longer a novelty for the scientific community. However, this phenomenon has reached large scales, and is becoming a crisis for global public health as drug resistance has been increasingly accumulating over time (CHOFFNES *et al.*, 2010).

Bacterial resistance to antibiotics is considered one of the major public health problems since many microorganisms that were previously susceptible to commonly used antibiotics no longer respond to these agents (WHO, 2005).

Considering all the above, this work aims to know the impact caused by pharmaceutical industry effluents on the mangrove sediment microbiota.

2 MATERIALS AND METHODS

2.1 Sediment Collection and Study Area

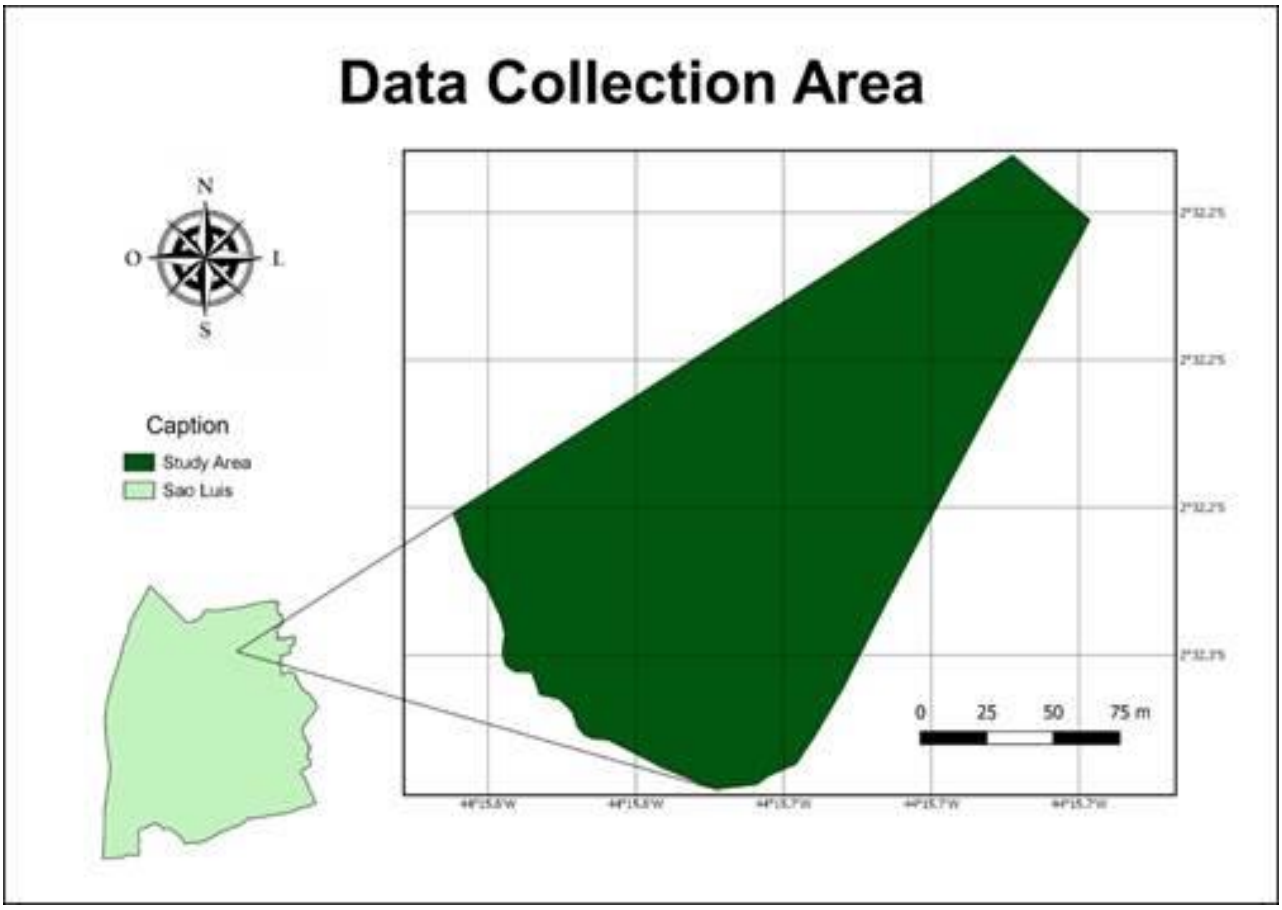
The study area is located in the city of São Luís - MA obeying the following coordinates 2º24'10" and 2º46'37" south latitude and 44º22'39" and 44º22'39" west longitude, and concerns to an urban mangrove area subjected for approximately 30 years to a pharmaceutical effluent discharge. Nowadays, the area is home to a higher-level institution and continues to undergo constant degradation serving as a deposit of solid waste and sewage as shown in Figures 1 (a) and 1 (b).

Figures 1 (a) and (b) – Urban mangrove area studied in this work serving as a deposit of waste and household materials

(a) (b)

In this area a sediment collection was carried out randomly at six different equidistant points as seen in Figure 2. The samples were then stored in hermetically sealed bags and transported for their use in later stages.

Figure 2 – Map of the area of collection of the sediment samples for the isolation of the microorganisms



For the isolation of the microorganisms from the sediment, a composite sample was elaborated mixing 10 g of the samples from each point. The isolation of the microorganisms was performed according to Clark (1965), in which 25 g of the composite sample was added to 225 mL of sterilized distilled water. Serial dilutions of 1:10 were taken to the 1:100 dilution. Then, 0.1 mL of the last dilution was inoculated in Mueller-Hinton (MH), Sabouraud Agar (SAB), Eosin Methylene Blue (EMB), and Bushnell Hass (BH) agar medium supplemented with carbapenem antibiotic class. The experiment was carried out in triplicate. The plates were incubated at 30 °C for a maximum of 10 days. Subsequently, the colonies was screened on each plate, where the macromorphological characteristics of the colonies were observed. All reagents used in this study were 95% pure, and the culture media were all Himedia™ brand.

2.3 pH analysis

The hydrogenation potential of the six samples was determined according to the ionic activity of hydrogen in suspension, which is based on the use of water or a solution of potassium chloride (KCl) or calcium acetate ($CaCl_2$). For the experiment, 10 g of soil was used, and the samples were measured in volume instead of weight because this is not relevant in the process. The samples were placed on the agitator table for 10 min and a reading was taken with a pH meter through electrodes dipped in the sample according to methodology recommended by Alef (1995).

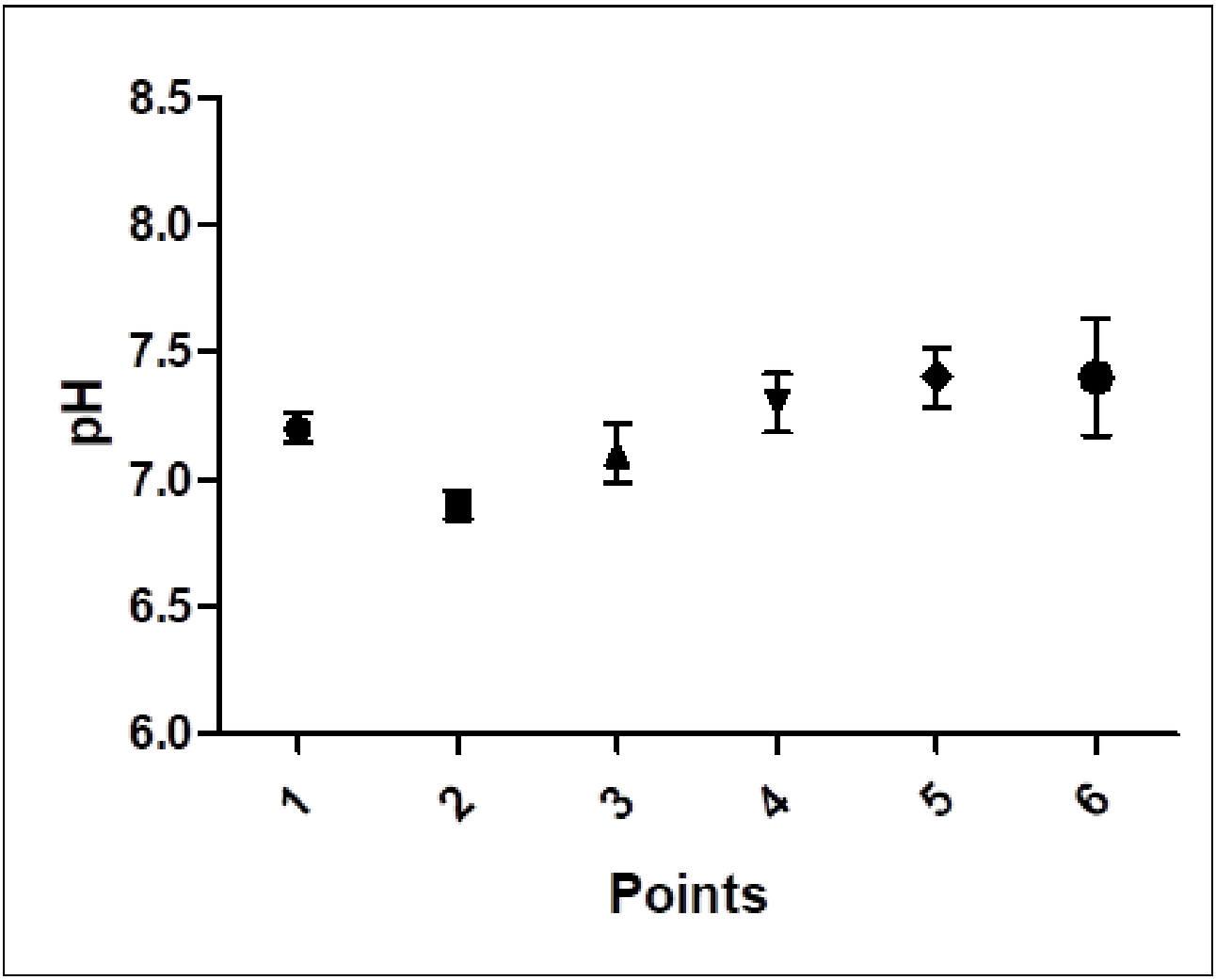
2.4 Statistical analysis

The results obtained during the study were evaluated comparatively through the Student's T test because data were nonparametric. The analyses were performed using the Statistic 8.0 software, considering $p < 0.05$ as statistically significant. Data on pH and the number of isolated colonies were compared using Spearman's correlation analysis.

3 RESULTS

The area under study often suffers from the disposal of solid waste and domestic sewage. In addition, for a long time, it was an effluent site of the pharmaceutical industry. These impacts cause a direct change in the local microbiota and physical and chemical factors that contribute to the balance of the area. Figure 3 shows the pH variation of the six collected samples. In relation to this factor, a slight variation was observed, with point 1 presenting a pH of 7.2, similar to points 2 and 3, which presented pHs of 7 and 7.1, respectively. Point 4 presented a pH of 7.3, while points 5 and 6 presented the highest pHs with a value of 7.4. These results show that the mangrove sediments remained with pHs around neutrality with an alkaline tendency and may favor the adaptation of some microbial groups in relation to others.

Figura 3 – Variation of sediment pH at the six points collected in the mangrove



The mangrove sediments are an excellent habitat for microorganism because they represent an environment rich in organic matter, favoring the metabolism of decomposing organisms. The results of the isolation can be seen in Figure 4. All the culture media presented a growth in colonies; however, among the different culture media used, the MH, showed the greatest colony growth of 30,000 CFU/mL, followed by BH plus antibiotic with 3000 UFC/mL. Furthermore, the SAB medium showed a total of 2500 CFU/mL while the EMB culture medium showed 400 UFC/mL.

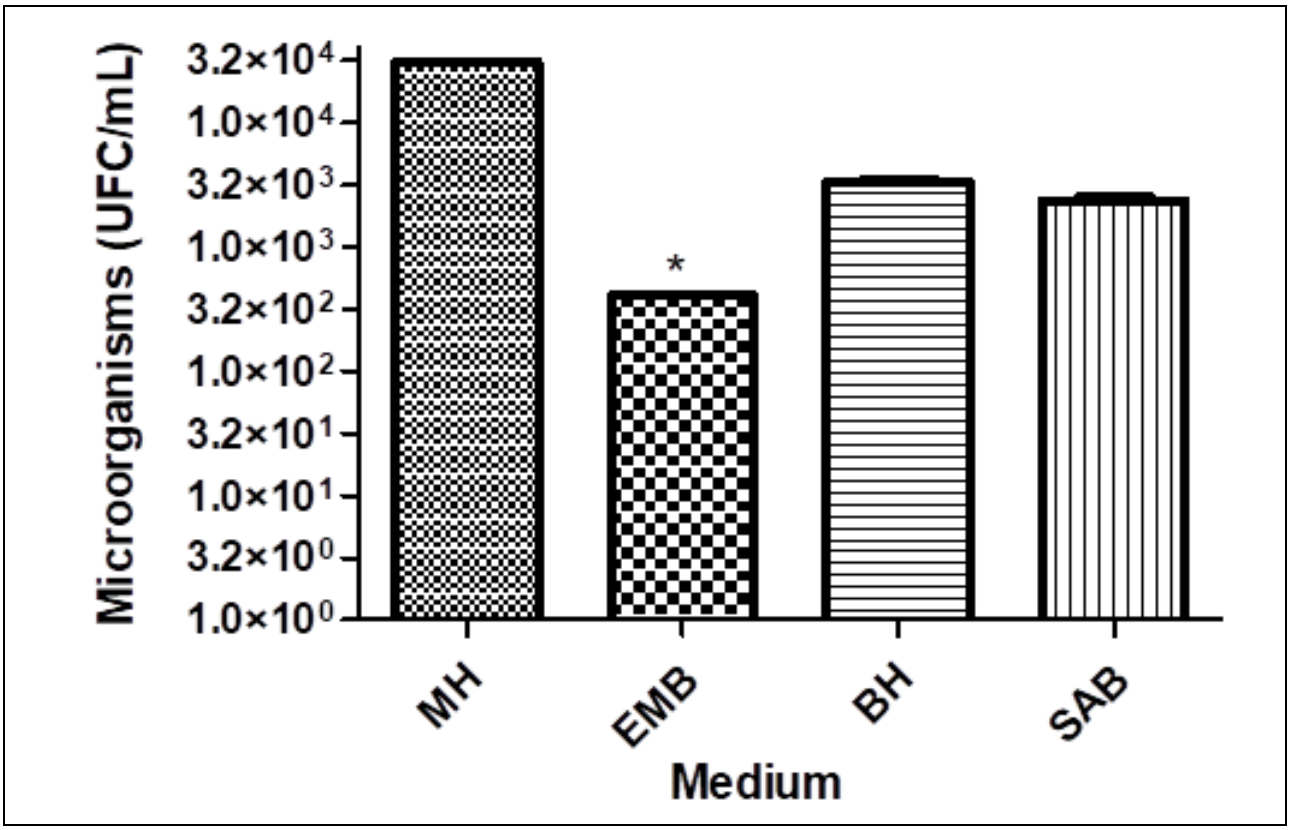
Among the tested media, MH is the richest in relation to macro and micronutrients. It consists of a defined micronutrient composition with known concentrations of $CaCl_2$, $MgCl_2$, and $ZnCl_2$, as well as peptone and casein that transmit the macronutrients to the medium, favoring the growth of bacteria. This explains the number of colonies present in the medium (COSTA *et al*., 2018).

The BH presented the second highest quantification and the highest colonies diversity, with macromorphologically different colonies, mainly due to the composition of the medium. BH is a least chemically defined medium and the antibiotic of the carbapenem class was used as the only carbon source, thus proving that bacterial colonies adapted most to soil contaminated by effluents from the pharmaceutical industry (COSTA *et al*., 2018).

The SAB culture medium is a growth medium for yeast and filamentous fungi. It is composed of a mixture of dextrose and peptone, which makes it rich in macronutrients such as carbon and nitrogen, favoring the growth of heterotrophic organisms (COSTA *et al*., 2018).

Unlike other media, the EMB culture medium is a differential selective medium, which selects the Gram-negative bacteria and shows the *Escherichia coli* bacterium with a metallic green color different from the other colonies. This explains the lower number of colonies present in this culture medium (COSTA *et al*., 2018).

Figure 4 – Growth of colonies (UFC / mL) in the Muller Hinton (MH), Eosin Methylen Blue (BEM), Bushnell Hass (BH) and Sabouraud (SAB) cultures media in the isolation of microorganisms from mango sediments *P< 0.05



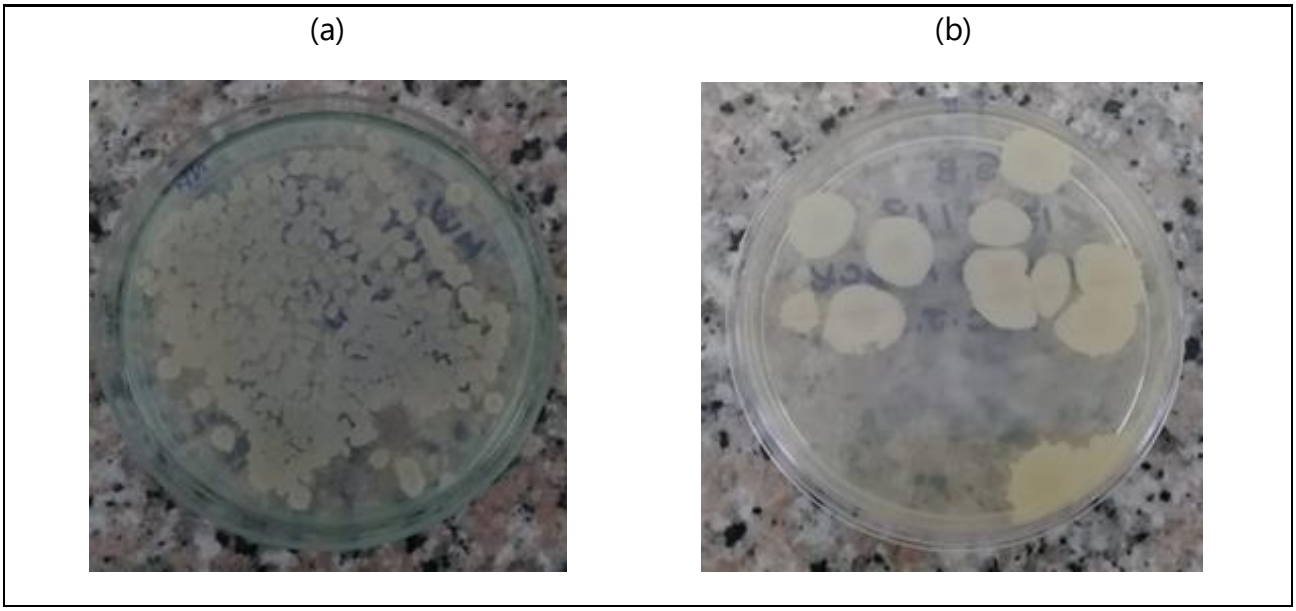
The macromorphological aspect of the colonies was also evaluated, as shown in Figure 5 (a) and (b). In the MH medium, many milky, whitish colonies were observed, forming almost a uniform layer on the Petri dish, evidencing the high number of colonies that resemble bacteria. In the middle SAB, there was a lower number of colonies, bigger, with more dense aspect, and yellowish color, evidencing morphology of colonies of yeast.

The EMB medium presented the lowest number of colonies between the four media tested; however, it showed blue and metallic-green colonies, highlighting Gram-negative bacteria like E. coli of the thermotolerant coliform group among them.

The BH medium with antibiotic was the one that took the most time for incubation; however, it showed the appearance of small, fluorescent-looking colonies, suggesting the presence of the genus Pseudomonas spp. Other characteristics could also be observed, such as colonies of differentiated colors (pink and orange) and a slightly more scattered characteristic of colonies of the genus Bacillus spp.

The macromorphological characteristics show that different microbial groups are adapted to the contaminated environment, mainly highlighting the growth in the medium BH with antibiotic as the only source of carbon.

Figure 5 – macromorphological aspect of the colonies in the Mueller Hinton medium (a) and in the medium Sabouraud Dextrose Agar (b)



No statistical difference was observed between the pH values at the six collection points. Regarding the isolation in the four tested media, a significant statistical difference was observed between the EMB medium and the other three media ($p = 0.0032$).

The Spearman's correlation analysis showed that when the number of isolated colonies in the culture media was correlated with the pH of the six collected points, there was a statistically significant negative correlation when the mean was the EMB ($p = 0.0025$ and $R = -0.865$).

4 DISCUSSION

One way to observe the microbial diversity of a site is to isolate them, especially in studies centered in microorganisms with some specificity as is the case of this work. The highest number of colonies found in MH medium suggests a greater bacterial diversity than fungal, which may be favored by the pH of the sediments (close to neutrality in the six points tested; DIAS *et al.*, 2017). The most conspicuous aspect in the isolation experiment was the number of colonies in the BH medium, which demonstrates the adaptation of the

microbiota to the site, acquiring resistance to the class of antibiotic used as carbon source in the isolation experiment. The small, stained colonies suggest that the adapted bacteria are pigment producers, which may be an explanation for their presence in a contaminated environment. In general, pigments are protective factors against stress situations. Among the isolated Gram-negative bacteria, it is possible to verify the presence of colonies with metallic green appearance, suggesting the presence of bacteria of the coliform group E. coli. This could be related to the discharge of domestic sewage at the site.

Due to the great importance and wide distribution of mangroves in the Brazilian regions, some studies involving combinations of independent cultivation techniques and isolation of microorganisms have been carried out (BRITO *et al*., 2006). Isolation should be as economical as possible, as well as the entire process that arises after isolation, taking into account the productivity obtained by the microbial growth and the necessary expenses for their isolation. Important factors, such as nutritional characteristics, are considered for the selection of a microorganism. In this regard, it is desired to use relatively inexpensive substrates, which can be obtained through the appropriate formulation of the medium for isolation, optimum temperature, compatibility with the fermentor used, and intended cultivation process, for their growth. Also, microorganisms should be genetically stable and, when genetic manipulation is desired, the best mechanisms for this should be considered (HASSAN; SHAIKH, 2016).

Although mangroves are scarcely studied ecosystems, some authors' results are in accordance with the results of this study. Dias *et al*. (2017) isolated bacterial quantities similar to those of this work from mangrove sediments of Maranhão. The authors used culture media similar to those used in this study and observed that the MH medium was the most efficient for the isolation of bacteria. The authors also reported the use of SAB medium to obtain isolates of yeasts and filamentous fungi. Sangkanu *et al*. (2017) isolated a bacterium of the order Actinomicetales from mangrove sediments from a historical park in Thailand and identified it as belonging to the genus *Streptomyces* sp. Saravanakumar *et al*. (2018) isolated different microbial groups from mangrove sediments in an estuarine region in India. Similar to this work, the authors reported the isolation of bacteria of the genus *Lactobacillus* spp., filamentous fungi of the genus *Trichoderma* sp., and some

Actinomycetales. Ser *et al*. (2018) isolated and sequenced the genome of *Streptomyces gilvigriseus* from a mangrove forest. The authors state that it represents a new species of Streptomyces bacteria resistant to contaminated environments.

The appearance of colonies in the BH medium shows the adaptation of bacteria to the contaminated environment. In a similar study, Costa *et al*. (2018) isolated bacteria in the BH medium using agro toxics as a carbon source in soils with a history of herbicide contamination, which evidences the efficacy of the chemically defined culture medium.

5 CONCLUSION

The isolation of microorganisms in sediments contaminated with effluents from the pharmaceutical industry shows that the local microbiota adapted to the medium and acquired resistance to the molecules to which it was exposed. This is a serious concern because this mangrove is located in an urban area, close to schools, universities, and businesses, and it still serves as a livelihood for some low-income families fishing near the place. The results of this study, although preliminary, demonstrate how much the environment is directly linked to human health and the damage that environmental impacts, such as untreated effluents spills, can cause to human societies.

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