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The use of effective microorganisms changed the soil microbiota of canals in Maricá city, Brazil

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ABSTRACT. The objective of this study was to evaluate the effect of bioremediation, through the application of two different strategies of effective microorganisms as treatment of effluents in the parameters of water quality and in the bacteriological profile of the soil of 'Canal da Cidade' - Maricá, Rio de Janeiro, Brazil. The metagenomic analyzes indicated that the abundance and richness of microorganisms in the substrate increased significantly after the bioremediation strategy used in this study. Because of bioremediation, 31 bacterial species disappeared from the environment when comparing the initial and final bacterial profiles of the soil, where 94% of these 31 species were anaerobic. Furthermore, 61 new aerobic or facultative aerobic species appeared in the channel substrate after bioremediation. As a consequence of bioremediation, the dissolved oxygen concentration and pH values increased significantly throughout the bioremediation process, indicating that the changes found in the microbiological profile of the soil contributed to the improvement of water quality parameters, helping the environment to move from anaerobic to aerobic characteristics. Thus, it is possible to state that the use of effective microorganisms directly affected the physical-chemical parameters of water quality and the microbial profile of the soil community of 'Canal da Cidade'.

Keywords: bioremediation; metagenomics; effective microorganisms; water, microbiology.

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Introduction

Contamination of aquatic ecosystems with untreated or insufficiently purified wastewater is one of the most dangerous environmental problems of today. A large population in developing countries could use surface water for irrigation, recreation, consumption, and other domestic purposes (Garcia-Armisen, Prats, & Servais, 2007).

In developing countries, wastewater treatment is not effective, and urban rivers receive large amounts of untreated sewage, industrial pollutants, and domestic sewage. The pollution of natural waters by biological agents and toxic chemical compounds reduces the self-purification capacity of the receiving ecosystems, resulting in the accumulation of contaminants at high and harmful levels. The increasing deterioration of this natural resource is a serious global problem that the international community must face because of the high demand for water (Li et al., 2022).

Wastewater can include biodegradable organic, non-biodegradable organic and inorganic substances, heavy metals, and potential inhibitors that end up in watersheds or groundwater, ponds, and rivers. Its presence and concentration can be evaluated through the analysis of samples that determine the water quality index through several physicochemical parameters such as pH, electrical conductivity, BOD, COD, and total suspended solids (TSS) among others (Shah & Xá, 2020).

The implementation of wastewater treatment plants for pollution and recovery of contaminated water contributes to environmental protection; however, this solution presents several difficulties in terms of its implementation and high costs, making it unfeasible for most cities, especially those with a reduced number of inhabitants (Vidali, 2001). A possible alternative solution to this environmental problem could be the use of microorganisms with potential capacity for bioremediation of this ecosystem as a more ecological and economical means (Guo et al., 2010).

Bioremediation, which involves using microbes to detoxify and degrade environmental contaminants, is receiving increasing attention as an effective biotechnological approach to clean polluted environments. In general, bioremediation approaches involve environmental modifications such as nutrient application and aeration and addition of suitable degraders by seeding. Bioremediation offers several advantages over conventional chemical and physical treatment technologies, especially for dilute and generalized contaminants. *In situ* treatment is one of the most attractive advantages of this technology (Li et al., 2022).

The bioremediation process is based on the action of fungal, bacterial, or plant species (phytoremediation) or the use of aerobic, anaerobic, or membrane bioreactors to modify and maintain a stable state of chemicals in the aquatic ecosystem. The bioremediation technique focuses on treating xenobiotics and plastics due to microbial transformation that makes water less toxic and more stable than its original polluted state (Luca et al., 2018).

The objective of this work was to evaluate the effect of the use of effective microorganisms as treatment of effluents on the parameters of water quality and on the bacteriological profile of the soil of the 'Canal da Cidade'.

Material and methods

Study site

This study was conducted in a water channel called the 'Canal da Cidade', located in the municipality of Maricá, state of Rio de Janeiro – Brazil. In total, three collection points were demarcated for monitoring along the channel (P1, P2 and P3). The points are spaced 125 m apart. Point 1 is the point upstream of the channel and point 3 is the point further downstream of the channel (Figure 1).

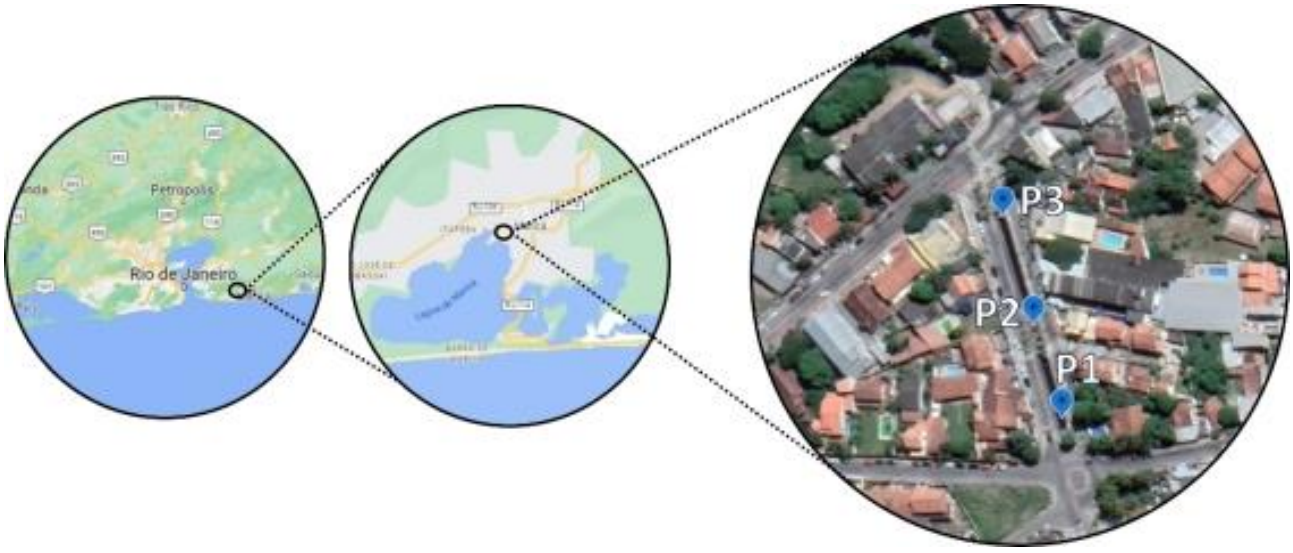

Figure 1. The location of the 'Canal da Cidade' – Maricá, Rio de Janeiro-Brazil (Images collected from Google Earth).

In addition to collecting rainwater, the channel receives sewage directly, coming only from family treatment units or even lacking any previous treatment, making it a highly impacted water body. The geographic coordinates of the Stud Area are -22.9214192 and -42.8209768 of latitude and longitude, respectively.

Effective microorganisms activation

Two preparations of 'effective microorganisms' (EM) were used. Both were produced from the basic EM concentrate EM-1® by the manufacturer (Emro, Japan). According to the manufacturer's specifications, they contained 1.3×10^4 colony forming units (CFU) of *Saccharomyces cerevisiae* ml^{-1}, and 3.3×10^4 CFU of Bacilli mL^{-1}. EMA (activated EM) was applied as a liquid agent and mudballs and was processed from EM-1 by fermentation under anaerobic conditions in water with sugar cane molasses for 7 days until pH was below 3.5. The final concentrations of microorganisms after 7 days of fermentation were 2.6×10^6 CFU mL^{-1} of *S. cerevisiae*, and 3.3×10^8 CFU mL^{-1} of Bacilli.

Microorganisms application protocol

The microorganisms were applied directly to the water and substrate of the canal for 7 days. Daily, at 9:00 am, 1,000 L of the active microorganism solution was applied at point 1. The liquid application was carried out at a flow rate of 50 L $min.^{-1}$ to avoid turning the substrate. Additionally, 10 kg of mudbricks, containing the active microorganisms, were deposited on the substrate of each of the 3 monitoring points.

The placement of the bricks was performed along the channel, to avoid errors in the positioning of the clay bricks and resuspension of the soil in the channel. The mudbricks were manufactured using the mixture according to Table 1. Clay blocks containing the fermented microorganisms were made in pottery and later cut into mudbricks of approximately 1 kg each, with approximate dimensions of 15 cm x 10 cm x 5 cm (length x width x height).

Table 1. Raw materials and their concentration for the manufacture of the mudbricks applied in the 'Canal da Cidade' soil.

Raw Materia	g Kg^{-1}
Clay Soil	700
Wheat Bran	35
Cane Molasses	35
Effective Microorganisms Fermented	175
Charcoal Powder	50
NaCl	5

Sample collection

A soil sample from each point was collected (in triplicate) before the beginning of the use of effective microorganisms (time zero) and 24 hours after the last release of effective microorganisms in liquid form, totaling 18 samples (3 points in 2 different times in triplicate). Soil collection was performed at 10 am, without rain, using a Van-Veen and Petersen dredge.

The collected material was homogenized, and a 50 g sample was collected and stored in a sterilized Falcon tube. To avoid contamination, the dredger was sterilized with sodium hypochlorite and deionized water between collections from different points. During the entire collection period, the samples were kept on ice. Finally, the samples were sent to the laboratory and stored at -80ºC.

Channel water parameters were measured daily in P3 at 9:00 am for 7 days before the addition of EMA using a multiparameter sensor (Hanna HI98194, Portugal). In addition, water parameters were measured at P3 at 9 am during the 7 days of bioremediation, daily, and on day 8, before the soil collection. The water parameters collected before bioremediation are showed in the Table 2.

Table 2. Water parameters of the Canal da Cidade collected daily for 7 days before the start of the treatment at 9:00 am in the P3. The values are shown as mean ± SD.

Water Parameter	Mean ± SD
Temperature, ºC	27.44 ± 0.22
pH	6.17 ± 0.04
Dissolved oxygen, mg L^{-1}	0.05 ± 0.01
Salinity	0.43 ± 0.01
Transparency, cm	5.13 ± 0.07

Metagenomic

Soil samples were subjected to metagenomic analysis. To extract deoxyribonucleic acid (DNA) from the bacteria present in the collected material, 200 mg of the sample were used, using the QuickDNA ™ Fecal / Soil Microbe Miniprep kit (Zymo Research), following the supplier's specifications. The extracted DNA was then quantified in a NanoDrop™ 1000 spectrophotometer (Thermo Scientifc DE, US) and maintained at a concentration above 100 µg μL^{-1}. After DNA extraction, the amplification of the region of the 16S gene of ribosomal ribonucleic acid (rRNA) of the preserved regions V3 and V4 was carried out. The V3-V4 region of the 16S rRNA gene was amplified by PCR using primers 314F / 806R.

In the sequencing of the samples, Illumina SBS technology was used, which marks the nucleotides by fluorescence when they bind to the complementary strand in each cycle. Noisy sequences were removed, and the remaining representative reads from the clusters were grouped using complex algorithms in Operational

Taxonomy Units (OTUs), through fast length adjustment of short reads (FLASH). Reads were grouped with 100 % identity (ID) using CD-HIT-DUP in a single file. OTUs will be collected using a quality filter to ensure 97% ID at the species level. For sequencing, a minimum alignment of 300 pb and 100 k of readings per sample were used. Sequences were analyzed using Quantitative Insights Into Microbial Ecology (QIIME).

Statistical analysis

To assess the homogeneity of the bacterial community at each collection time, a one-way ANOVA was performed using the data of the 3 monitored points at each monitoring time (T0 and T8). The results among the bacterial communities before and after bioremediation were submitted to Student's t test, comparing the data before and after the use of effective microorganisms. All statistical analyzes were performed using GraphPrism 8.0 software (GraphPad Software). Ecological indices were calculated using PAST 4.11 software. All statistical analyzes were performed with 5% significance.

Results and discussion

At T_0, there was no significant difference between the 3 monitored points in the bacterial community profile ($p>0.05$). Samples collected before bioremediation showed a very similar soil bacterial community among the 3 monitored points, indicating that the microbiological community in this channel is very restricted and specific. These results are corroborated by the water quality parameters, since the results indicated that the channel has a low concentration of dissolved oxygen and low pH. These characteristics act as a selection system for species able to live in these environmental conditions (Lorenzo, 2011), which helps to explain the low variability of the species found in the three different monitoring points. Table 3 shows the results of the bacterial community of the 3 monitored points before the bioremediation.

The profile of the microbiological community of 'Canal da Cidade' in T_0 (MCP-T_0) and T_F (MCP-T_F) are the absolute observations considering the 3 replicates simultaneously (P1, P2, and P3). In other words, MCP-T_0 and MCP-T_F represent the sum of all the different information found for each bacterium in each of the 3 points of its collection. The values of mean-T_0 and Mean-T_F are the average of the values of the 3 points (P1, P2, and P3) in 'zero time' and 'final time', respectively.

Table 3. Summary of the information about the soil bacteriological community of the 3 different monitored points in the 'Canal da Cidade' before the bioremediation. The values are presented as the mean. Mean-T_0 is the mean considering all 3 points simultaneously in 'time zero'.

Monitored Point	Phyla[1]	Class[1]	Order[1]	Family[1]	Genus[1]	Species[1]
P1	9	17	29	55	81	115
P2	9	20	34	55	83	159
P3	9	17	28	48	61	128
[2]ANOVA p -value	0.9999	0.7589	0.3514	0.6412	0.4963	0.2058
Mean-T_0	9.0	18.0	30.3	52.7	75.0	134
SD-T_0	0.0	1.7	3.2	4.0	12.2	22.60
[3]MCP-T_0	9	24	41	77	131	236

[1]The values presented for P1, P2, and P3 are the mean of the three replicates collected for each point. [2]The p value corresponds to the comparison among the 3 monitored points. [3]MCP-T_0 indicates the absolute observations of T_0. This means that MCP-T_0 is the sum of all different observation for each parameter.

The MPC-T_0 was greater than the mean-T_0 observed at the 3 points. This occurred because some species were not observed at the 3 points simultaneously. However, despite the richness having approximately 82 species more than the average of the 3 points, the bacterial communities in the 3 points are very similar to each other, since these 82 species were found in very low concentrations in each point. This causes the richness index to be directly influenced even though it does not represent a structural difference between the communities in the 3 points.

At T_0, the most abundant phyla were Firmicutes with approximately 54.67 % of the bacteria found in 'Canal da Cidade' soil. The most abundant species of this phylum were *Clostridium ruminantium* and *Clostridia bacterium*. *C. ruminantium* is one of the main species of bacteria that cause contamination of bovine carcasses during and after processing. Those species can survive and find good growing conditions even in vacuum packaging (Reid et al., 2017). 'Canal da Cidade' is in an urban space and near of one slaughterhouse and this can be the reason of the high concentration of this bacterium in the 'Canal da Cidade' soil.

Each species observed and their respective absolute observations at the three points form the bacterial community. Only after determining the MCP-T_0 were the ecological indexes calculated for T_0.

Samples collected after bioremediation showed a very similar soil bacterial community among the 3 monitored points, indicating that the microbiological community in this channel is very restricted and specific, as previously found for bioremediation. At T_F, there was no significant difference between the 3 monitored points in the bacterial community profile ($p>0.05$). Table 4 shows the results of the bacterial community of the 3 monitored points after the bioremediation. At T_F, there was no significant difference between the 3 monitored points in the bacterial community profile ($p>0.05$).

Table 4. Summary of the information about the soil bacteriological community of the three different monitored points in 'Canal da Cidade' before the bioremediation. Mean-T_F is the mean considering all 3 points simultaneously in the 'time final'.

Monitored Point	Phyla[1]	Class[1]	Order[1]	Family[1]	Genus[1]	Species[1]
P1	10	19	31	54	78	200
P2	10	20	34	66	100	157
P3	10	20	37	70	113	164
[2]ANOVA p -value	0.9999	0.7686	0.3514	0.6412	0.4963	0.2255
Mean-T_F	10.00	19.67	34.00	63.33	97.00	173.67
SD-T_F	0.00	0.58	3.00	8.33	17.69	23.07
[3]MCP-T_F	10	22	38	77	135	269

[1]The values presented for P1, P2, and P3 are the mean of the three replicates collected for each point. [2]The p value corresponds to the comparison among the 3 monitored points. [3]MCP-T_F indicates the absolute observations of T_F. This means that the MCP-T_F is the sum of all different observations for each parameter.

In the T_F, the most abundant phylum was Firmicutes, with approximately 54% of the total bacteria found in the soil of Canal da Cidade. However, these 54% represent an absolute observation (abundance) substantially greater than those found at the beginning of the experiment. The firmicute population after bioremediation was 1.63 times greater than that found naturally in the channel soil. The most abundant species were *C. bacterium, C. ruminantium, Eubacteriales bacterium, Clostridium cellulovorans,* and *Caldisericaceae bacterium*.

After bioremediation, the presence of a new phylum was verified in the soil of 'Canal da Cidade'. The phylum Nitrospirae was found at all monitored points and in all 9 collected samples. The species of the phylum Nitrospirae found was the *Nitrospiraceae bacterium*. The globally distributed phylum Nitrospirae represents the most diverse known group of nitrite-oxidizing bacteria (Daims et al., 2015).

The ecological success of Nitrospirae has been associated with substantial metabolic versatility (Lücker et al., 2010), which includes utilization of various organic compounds in addition to nitrite and CO_2, cyanate (Palatinszky et al., 2015) or urea degradation and nitrification by reciprocal feeding with ammonia-oxidizing microbes, and chemolithoautotrophic aerobic hydrogen oxidation. This versatility allows these bacteria to be found even in degraded and/or contaminated environments if it offers minimum conditions in relation to their physicochemical parameters and the presence of compounds used in the growth of these bacteria. In this sense, the presence of these bacteria can be an indication of the improvement of the water and soil of 'Canal da Cidade' after bioremediation.

When we compared the bacterial communities before and after bioremediation, at all 3 monitoring points, we can observe a substantial change in the species found before and after bioremediation. In all, 300 species were found in the soil of 'Canal da Cidade'. Of these, 205 were found in both collections. That is, there was a variation of approximately 32% in the bacterial community during the treatment period.

Of the 236 species that were identified in the soil before bioremediation, 31 species were not found in the channel soil after the use of effective microorganisms. In contrast, of the 269 species identified in the soil after bioremediation, 64 species were not present in the initial community. The Venn Diagram illustrates these characteristics of the communities found in the two monitoring moments (Figure 2). The bacterial communities before and after bioremediation were different ($p<0.05$) considering all groups: phyla, class, order, family, genus, and species. A summary of the comparison of the microbiological community before and after bioremediation is showed in Table 5.

These alterations in the community of bacteria in the soil of 'Canal da Cidade' may be associated with changes in water quality during bioremediation. Considering the bacterial species that disappeared from the environment after bioremediation (T_0 exclusive species), approximately 94% of them were anaerobic bacteria. Likewise, of the new species found at the end of the treatment (T_F), 100% were aerobic or facultative anaerobic bacteria. Regarding the modification of water quality parameters, a significant (even if small) increase in both dissolved oxygen and water pH ($p<0.05$). No differences were found in the other monitored parameters. Figure 3 shows the behavior of pH and dissolved oxygen over 15 days of monitoring.

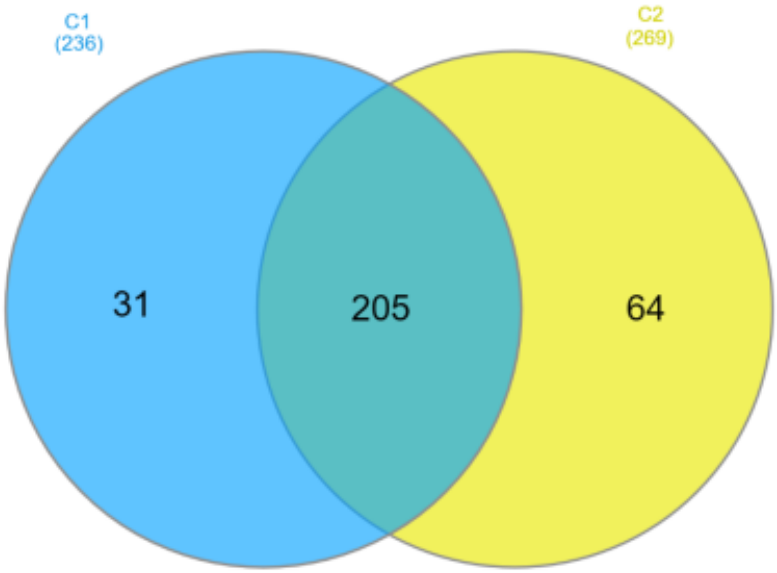

Figure 2. Bacterial microbiota species level at the two moments of soil sample collection. The intersection of the two ellipses (green area) indicates the number of shared OTUs in the bacterial communities of C1 and C2. Numbers outside the intersection area correspond to the OTU that were uniquely found at each time of sample collection. Community C1 is represented in blue and C2 in yellow (collected from the author's collection).

Table 5. Summary of the comparison about the soil bacteriological community of the Canal da Cidade before and after the bioremediation. The values are presented as the mean. To and T_F denote 'time zero' and 'time final', respectively.

Monitored Point	Phyla	Class	Order	Family	Genus	Species
[1]MCP-T_0	9	24	41	77	131	236
[2]MCP-T_F	10	22	38	77	135	269
[3]Mean-T_0	9.0	18.0	30.3	52.7	75.0	134
[4]Mean-T_F	10.00	19.67	34.00	63.33	97.00	173.67
[5]t-Student p Value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Significant difference	Yes	Yes	Yes	Yes	Yes	Yes

[1]MCP-T_0 indicates the absolute observations of T_0. This means that MCP-T_0 is the sum of all different observation for each parameter. [2]MCP-T_F indicates the absolute observations of T_F. This means that the MCP-T_F is the sum of all different observations for each parameter. [3]Represents the results considering all 3 points simultaneously in T_0 (time zero). [4]Represents the results considering all 3 points simultaneously in T_F (time final). [5]The p value corresponds to the comparison between the mean-T_0 and the mean-T_F values.

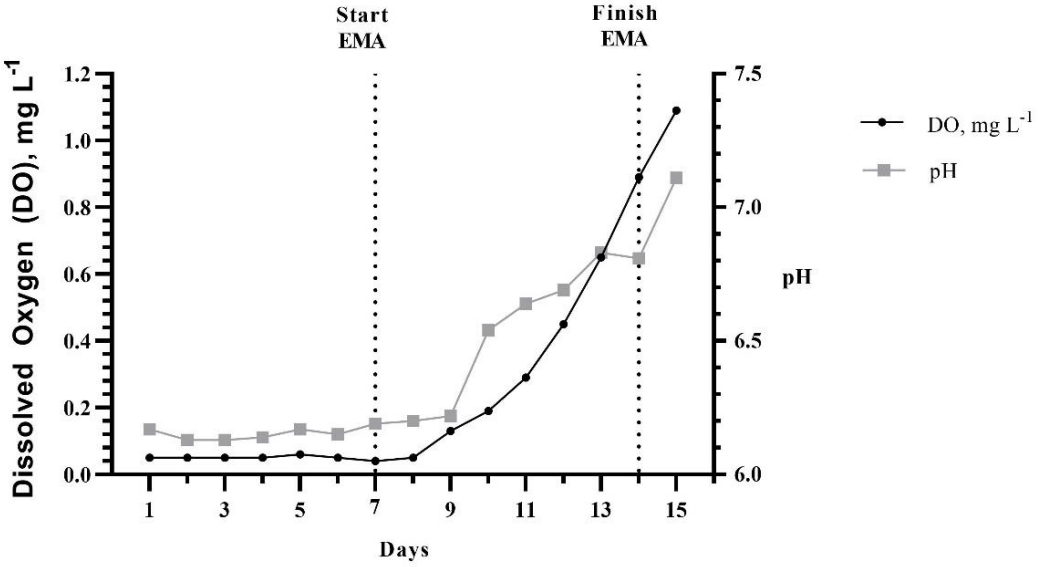

Figure 3. Dissolved oxygen (mg L^{-1}) and pH of Canal da Cidade beyond 15 days of monitoring. The vertical dotted lines represent the beginning and end of EMA use (collected from the author's collection).

The increase in the dissolved oxygen levels appears to be the main cause of this alteration. Strictly aerobic bacteria have low tolerance for the presence of oxygen in the environment, even at a low concentration. In

addition, aerobic and, mainly, facultative anaerobic, bacteria are able to carry out their metabolic activities, even if not optimally, in environments with low oxygen concentrations (Lücker et al., 2010).

Despite being an open environment, with constant capture of rainwater and untreated sewage, the changes found in the parameters seem to be strictly linked to the use of the EMA. As previously presented, the water parameters did not show significant variation over the 7 days of monitoring before bioremediation, which demonstrates the stability of these parameters in this impacted environment. Furthermore, it is important to point out that during the 15 days of monitoring (7 days before bioremediation and 8 days after the start of treatment), no atypical releases of sewage were observed and there was no record of rain in the entire city and neighboring cities.

However, considering the 15 days of water quality monitoring, these parameters (dissolved oxygen and pH) showed statistically significant changes and increases from the 2nd day of treatment (first water quality monitoring after the first EMA release), which corroborates that the use of EMA was the responsible for changing the water parameters. Thus, the alteration in the bacterial community seems to be strictly related to the use of EMA in the water and soil of 'Canal da Cidade'. Some bacteria that were found just before bioremediation are *Syntrophales bacterium* and *Chloroflexi bacterium*. Both species have strictly anaerobic (Kjeldsen, Joulian, & Ingvorsen, 2004; Muyzer & Stams, 2008) and sulfate-reducing (Delgado-Baquerizo et al., 2018) metabolism.

Analyzing the ecological indices, the bacterial community in T_0 showed high values of diversity (Shannon and Margalef) and low values of dominance (Table 6). Although the Firmicutes phylum is the most abundant in the community, the diversity at the site is high because several species contribute (in richness and specific specie abundance) to the formation of the bacterial community.

Table 6. Ecological indices of the soil bacteriological community at the three different monitoring points in 'Canal da Cidade' before the bioremediation. The values are presented as mean. T_0 means 'time zero' and represents the results considering all 3 points simultaneously.

Ecological Index	P1	P2	P3	[1]MCP-T_0	[2]ANOVA p-value
Richness	115	159	128	236	0.2058
Abundance	3310	8323	5279	16912	0.0739
Dominance	0.072	0.062	0.067	0.055	0.8069
Shannon	3.499	3.246	3.197	3.487	0.3408
Margalef	14.07	17.27	15.23	3.487	0.0622

[1]MCP-T_0 indicates the absolute observations of T_0. This means that MCP-T_0 is the sum of all different observation for each parameter.[2]The p value corresponds to comparisons among the three monitored points.

Although it is not possible to statistically compare the values of MCP-T_0 and MCP-T_F, since they do not have replicates, it is worth mentioning the substantial increase in the richness and abundance of bacterial species found in the soil of 'Canal da Cidade' after treatment with EMA. For richness, the number of species found was almost 9% higher at the end of treatment. Although the difference in richness values before and after treatment was 21 species, it is important to emphasize that 64 new species were found exclusively in TF. Regarding abundance, the number of cells found in the T_F samples was almost 79% higher than those found in T_0. Table 7 presents the values for the ecological indices in TF.

The combination of these factors, mainly, collaborated so that the dominance index in T_F decreased 8 times in relation to that found in T_0. Nevertheless, both diversity indices were higher at the end of the experiment. These scenarios corroborate the hypothesis that the use of EMA for 7 days was responsible for the improvement in water quality parameters and the increase in bacterial biodiversity in the soil of 'Canal da Cidade'.

Table 7. Ecological indices of the soil bacteriological community at the three different monitoring points in 'Canal da Cidade' before the bioremediation. The values are presented as mean. T_F means 'time final' and represents the results considering all 3 points simultaneously.

Ecological Index	P1	P2	P3	[1]MCP-T_F	[2]ANOVA p-value
Richness	200	157	164	257	0.4468
Abundance	8638	7436	13083	30238	0.0623
Dominance	0.062	0.05935	0.07607	0.06261	0.1002
Shannon	3.41	3.301	3.142	3.359	0.4302
Margalef	21.7	17.5	17.2	24.82	0.0737

[1]MCP-T_F denotes the absolute observations of T_F. This means that the MCP-T_F is the sum of all different observations for each parameter. [2]The p value corresponds to comparison among the 3 monitored points.

Conclusion

The use of EMA, even if only for 7 days, was responsible for the improvement in water quality parameters, increase in bacterial biodiversity, and the disappearance and emergence of aerobic species in the soil of 'Canal da Cidade', proving to be an interesting tool and effective as an alternative effluent treatment method.

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