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ENVIRONMENTAL MICROBIOLOGY - RESEARCH PAPER

Application of extracellular polymers on soil communities exposed to oil and nickel contamination

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Abstract

The petrochemical industry is responsible for many accidental releases of pollutants in soil such as hydrocarbons and toxic metals. This co-contamination is responsible for a delay in the degradation of the organic pollution. Many successful technologies to remove these metals apply extracellular polymeric substances (EPS). In this study, we tested the application of an EPS from a *Paenibacillus* sp. to aid the bioremediation of soils contaminated with crude oil and nickel. We conducted a microcosm experiment to soils containing combinations of oil, nickel, and EPS. The final concentration of oil was evaluated with an infrared spectrometer. Also, we sequenced the metagenomes of the samples in an ion torrent sequencer. The application of EPS did not aid the removal of hydrocarbons with or without the presence of nickel. However, it led to a smaller decrease in the diversity indexes. EPS decreased the abundance of Actinobacteria and increased that of Proteobacteria. The EPS also decreased the connectivity among Actinobacteria in the network analysis. The results indicated that the addition of EPS had a higher effect on the community structure than nickel. Altogether, our results indicate that this approach did not aid the bioremediation of hydrocarbons likely due to its effect in the community structure that affected hydrocarbonoclastic microorganisms.

Keywords Metagenome · Metal contamination · Hydrocarbon remediation · Extracellular polymeric substances

Introduction

The current industrial model relies on petrochemical compounds for many applications ranging from fuels to high-performance polymers [1]. This leads to the necessity to extract, move, and refine massive quantities of petroleum and its components [2]. Given the size of this industry, despite all the efforts to improve its security, these processes are exposed to accidents which may lead to the release of compounds that are often toxic [2]. Environments affected by these components thus need to be remediated in order to decrease risks [1]. The application of biological methods to remediate the environments exposed to these accidental releases is often indicated due to its generally lower cost and environmental impact [3, 4].

One common complication to the process of bioremediation is the multi-contamination with hydrocarbons and metals [5]. This co-contamination more often than not make the degradation of the organic compounds slower due to the toxicity of the metal to most microbial species [5, 6]. In such cases, it is indicated that technology to remove hydrocarbons comes before the metals [5]. One strategy to overcome the inhibition by

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toxic metals is to use substances that bind to them, making them less available to the indigenous populations, thus allowing these populations to decompose the hydrocarbons [5, 7]. These substances, used in such applications, must have ionizable functional groups that can bind to these metals, such as many extracellular polymeric substances (EPS) produced by certain bacteria [7].

EPS is a term used to designate a wide variety of high molecular weight molecules produced by microorganisms [8]. They can be formed by proteins, polysaccharides, lipids, and lipopolysaccharides [8]. Due to the presence of ionizable groups in their surfaces, they can be used to bind electrostatically to metals [9–12]. Thus, different authors proposed technologies applying EPS to the removal of metals from the environment [13–17].

The application of bioremediation methods, despite its frequent success, often leads to unexpected results due to the complex interactions taking place in the environment [18]. Hence, it is crucial to comprehend this complexity to fully understand how different factors might influence these processes in order to improve its management.

Therefore, this study aims to test the application of an EPS from *Paenibacillus polymyxa*, capable of binding Ni^{+2} cations, in the bioremediation of a soil contaminated with crude oil and nickel in a microcosm setting. Also, we evaluated how the microbial communities behave in this experimental setting by metagenome sequencing and the application of a layered statistical analysis with multivariate, machine learning, and network analysis, to get an in-depth view of these complex interactions.

Materials and methods

Sampled material and microcosm design

In order to test the effect of the application of extracellular polymeric substance (EPS) as an adjuvant in the bioremediation process in soils contaminated with crude petroleum and nickel, we have selected uncontaminated soil samples from an area of *onshore* oil extraction in the northeast of Brazil (10° 38′ 44.13″ S, 36° 57′ 41.81″ W) and used crude oil obtained in the area (paraffin oil °API 24.1—average organic composition: 55.00% saturated, 20.46% aromatics, 24.72% asphaltenic compounds) [3]. We have applied an EPS obtained from *Paenibacillus* sp. CETEM grown in RCV medium [19] modified with the addition of 30 g L^{-1} of saccharose and 0.1 g L^{-1} of yeast extract for 24 h at 30 °C. After growth, cultures were boiled for 15 min and concentrated in a rotary evaporator. The EPS was then precipitated with the addition of 3 volumes of − 20 °C 95% ethanol and incubated overnight at − 20 °C. The medium was centrifuged for 10 min at 6.000 g, and the supernatant discarded. The pellet was purified twice by dialysis using a Medicell International MWCO (12-14,000 Da) membrane in distilled water 12 h. The EPS was then air-dried at 60 °C until its weight was constant.

The microcosms consisted in sealed, sterile 250-mL Erlenmeyer flasks containing 50 g of soil with humidity adjusted and kept to 70% of the field capacity [20]. No adjustments were necessary in the nitrogen and phosphorus concentrations as the C:N:P ratio was close to 100:10:1. With this material, the different treatments were set in triplicates, as described in Table 1. The flasks were kept at 30 °C (the average temperature at the sampled location) for 42 days. Humidity and aeration were maintained with sterile distilled water and filtered compressed air (respectively) with mixing using a sterile glass bar. Hydrocarbon volatilization was quantified based on sterile soils under the same conditions mentioned before.

Analysis of total petroleum hydrocarbons from the soil

The total concentrations of hydrocarbons in soil were quantified using infrared spectrometry (Infracal®, model HART-T, Wilks Enterprise, Inc., CT, USA) using hexane as the extractor as described previously [3]. Loss due to volatilization was quantified from sterile soils and was not significant ($p > 0.05$) and is not shown here.

DNA extraction and metagenome sequencing

DNA was extracted using the FastDNA spin kit for soil (MP Bio) following the manufacturers' protocol. DNA quantity and quality were accessed on a NanoDrop 1000 spectrometer. The DNA was sequenced in an Ion Torrent Personal Genome Machine following the steps described previously [21].

Metagenome sequence annotation and data analysis

The sequencing data was then uploaded to the MG-RAST web server [22], trimmed for quality and annotated using the standard protocol and are publicly available under metagenome project id mgp13704. The sequences were

Table 1 Concentrations of nickel, crude oil, and extracellular polymeric substances (EPS) in the different soil microcosms

Treatment	Crude oil (% w/w)	$NiCl_2$ (g kg^{-1})	EPS (g kg^{-1})
Oil-Ni-EPS	5%	1.3	18.6
Oil-Ni	5%	1.3	-
Oil-EPS	5%	-	18.6
Oil	5%	-	-
Ni-EPS	-	1.3	18.6
Ni	-	1.3	-

taxonomically assigned using Best Hit Classification against the RefSeq database with an *E* value cut-off of 10^{-5}, the minimum identity of 60%, and a minimum alignment of 50 bp and the functional annotation was performed by Hierarchical Classification against the KEGG database using an *E* value cut-off of 10^{-5}, the minimum identity of 60%, and a minimum alignment of 15 amino acids [23]. The data was exported in biom format and analyzed within the R environment using RStudio. The biom tables were imported as a Phyloseq [24] object, samples with counts lower than 15.000 removed, and the remaining samples were rarefied to an even depth. After the rarefaction, all samples remained with coverage above 90% according to the coverage estimator of the microbiome package [25]. Plots were produced with ggplot2 [26] and edited with Inkscape. NMDS plots were produced based on Bray-Curtis distance matrices using Vegan [27]. Alpha-diversity analysis and plots were produced with Phyloseq. Indexes were compared with ANOVA and Tukey's HSD post hoc test with R. The R package Boruta [28] was used to select informant feature using the Random Forest algorithm. Heatmaps were constructed using gplots package [29]. The 500 most abundant features (taxa or function) were used for network construction using Spiec-Easi [30] within R. The resultant network properties were analyzed and exported with igraph [31], qgraph [32], and visualization generated with Gephi [33].

Results

Quantification of total petroleum hydrocarbons

The addition of EPS reduced the removal of total petroleum hydrocarbons (TPHs) in these soils (Fig. 1). The lowest TPH concentration was in the presence of only oil (32.27 ± 4.83 mg g^{-1} of soil) with the removal of 37.7% of TPHs. The addition of EPS and nickel had the lowest removal, 23.7% (39.52 ± 2.56 mg g^{-1} of soil). However, alone, nickel had a higher inhibition than EPS (2% lower removal) which indicates that it has a higher effect on the hydrocarbonoclastic populations. This indicates that EPS does not aid the removal of hydrocarbons if added to the soil by itself.

Communities of alpha-diversity patterns

The alpha-diversity analysis of these metagenomes indicates that there were only minor, not significant, differences between the samples when we looked at the functional annotation against the KEGG database (Fig. 2a). Neither the observed diversity (S_{obs}, Fig. 2a) nor Shannon's indexes (H', Fig. 2a) displayed significant differences. However, the three-way ANOVA, considering nickel, EPS, and oil as individual factors for the model, indicated that only nickel had a

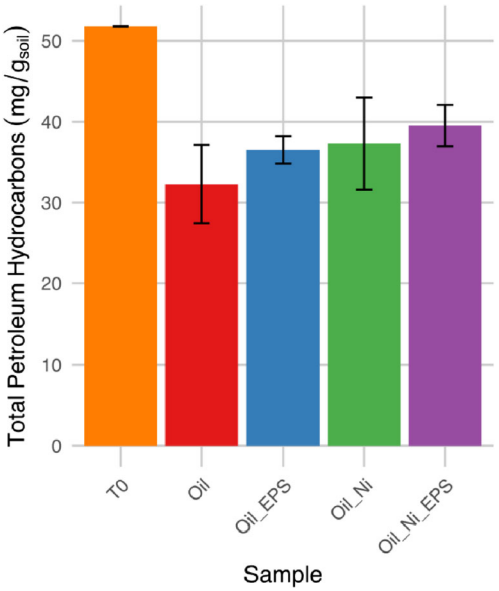


Fig. 1 Total petroleum hydrocarbon quantification of samples contaminated with hydrocarbons (oil) and nickel (Ni) treated with extracellular polymeric substances (EPS). Hydrocarbons in soil were quantified using infrared spectrometry

significant effect ($p < 0.05$) on both diversity indexes; i.e., samples containing nickel had lower values of both indexes.

The taxonomic annotation against the NCBI's RefSeq database has shown a different pattern (Fig. 2b). The co-contamination with oil and nickel decreased the S_{obs} and H' around 10% which is significant ($p < 0.05$). The addition of EPS to the co-contamination helped maintain the diversity in values closer to those without the two contaminants, which indicates that the EPS helped maintain the size of the diversity of these communities higher than if it is co-contaminated. The ANOVA, followed by Tukey's post hoc test, has shown that this pattern is significant. In three-way ANOVA, the model was significant for nickel, EPS, and the interaction between oil and EPS.

Beta-diversity analysis

The variation between samples has shown an apparent clustering of samples from the same treatment (Fig. 3a and c). Despite the annotation type (i.e., KO or RefSeq), both plots indicated a separation between samples that received EPS and those that did not. Samples that received oil also have shown some separation, but not so clear. The PERMANOVA indicated that the models presented in Fig. 3a and c were significant ($p < 0.001$). However, if a separate model was constructed for each substance (oil, nickel, and EPS), only EPS was significant.

The plotting of annotated groups (KOs or taxa) side-by-side with samples indicated a different profile between annotation types (Fig. 3b and d). The functional annotation has shown no clear grouping between functional groups and

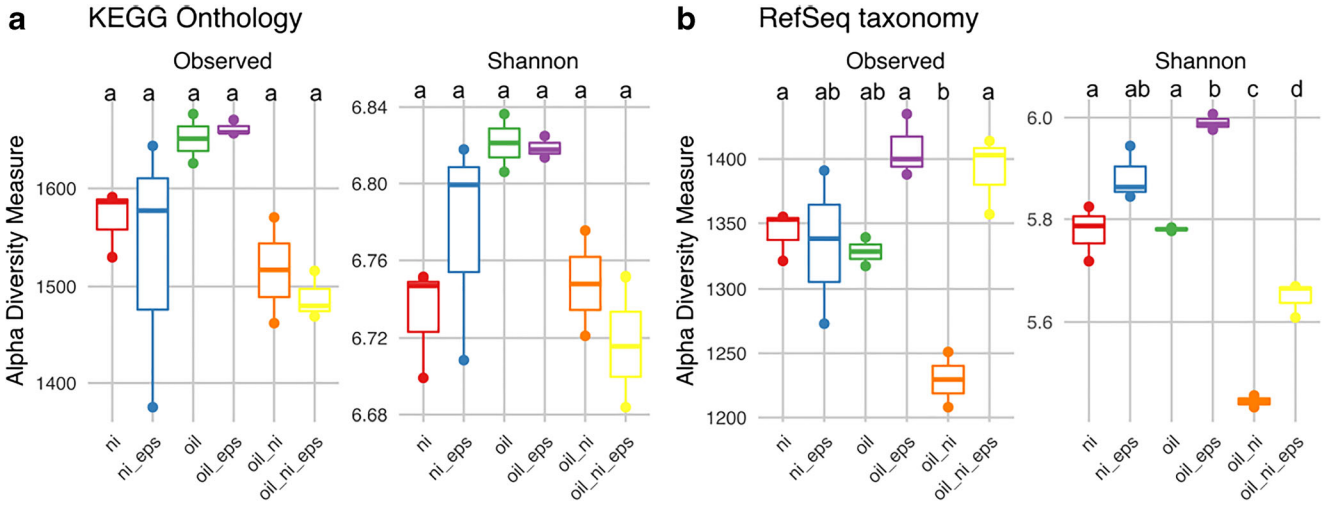


Fig. 2 Alpha-diversity indexes obtained from the functional (**a**) and taxonomic (**b**) annotation of the metagenomes obtained from soil samples contaminated with hydrocarbons (oil) and nickel (Ni) treated with extracellular polymeric substances (EPS). Different letters correspond to significantly different values according to Tukey's test ($p < 0.05$)

samples (Fig. 3b); i.e., all different functional groups had a similar dispersion between samples. However, the taxonomic annotation indicated a clear distinction between samples that received EPS and those that did not (Fig. 3d). Samples that did not receive EPS clustered with Actinomycetales taxons while those that had EPS correlated with different Proteobacteria and Acidobacteria.

Group abundance analysis

The taxonomic annotation of the samples indicated that all samples contained a high abundance of Actinobacteria, Alpha, Beta, Gamma, and Deltaproteobacteria (Fig. 4b). However, as observed in Fig. 3d, the samples that received EPS had a larger proportion of Proteobacteria (mostly Alpha and Gammaproteobacteria) than those that did not have more Actinobacteria. In samples without EPS, those that received nickel had a larger proportion of Actinobacteria.

The functional annotation was very homogeneous among all samples (Fig. 4a). As expected, most of the functional groups found are related to central metabolism, while the degradation of xenobiotics was only minor (Figure S1). Only genes related to the degradation of benzoate, xylene, and polycyclic aromatic hydrocarbons (PAH) were slightly higher in samples that received oil. However, samples that received oil and EPS had a lower proportion of sequences related to these metabolisms.

Feature selection

In order to identify features that vary between treatments and are features that are uncorrelated and non-redundant, we have applied the Boruta method [28] of feature selection (Figs. 5 and 6). Applying this method to the functional annotation has shown that there are more functional groups that aid the differentiation of the samples with and without EPS than with nickel or oil (Fig. 5). Selected features involved in energy, carbohydrates, and amino acid metabolism had lower abundances while those involved in membrane transport and translation had higher abundances. Despite the features related to the metabolism of carbohydrates in the samples with EPS, none of the features had a direct link with the substance added to the microcosms.

The features selected from the taxonomic annotation table had a similar profile to that identified in the results presented before (Fig. 6). The EPS was the factor that allowed the selection of more features, and most of these features were related to Actinobacteria. Oil had a strong effect on Proteobacteria, mostly Alphaproteobacteria, which indicates that these groups explain most of the differences observed between samples that received the addition of these substances.

Network analysis

The network analysis showed that the communities found in these soils were affected by the different treatments (Fig. 7 and Figure S2). In general, the networks constructed based on the functional annotation of the metagenomes decreased their overall betweenness centrality if we analyzed each factor in separate. Also, the presence of oil increased the number of negative edges in the network; it also decreased the number of nodes related to genetic information processing and environmental information processing and decreased the number of nodes with high betweenness centrality. These networks were also considered small-world (small-worldness ≥ 3); i.e., all nodes can be accessed by a small number of steps.

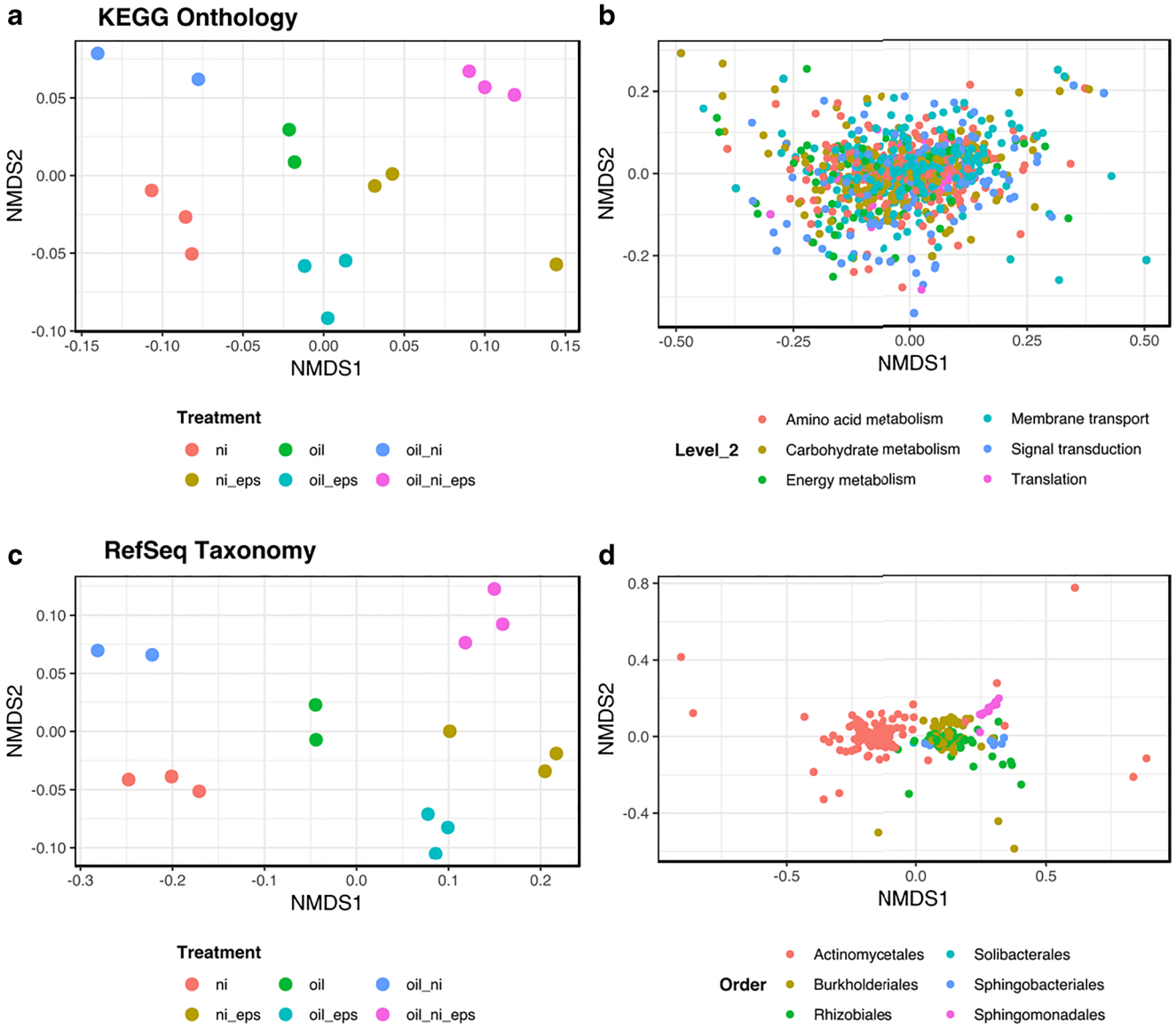


Fig. 3 Non-metric multidimensional scaling (NMDS) plots of metagenomic data based on MG-RAST classification of sequences obtained from soil samples contaminated with hydrocarbons (oil) and nickel (Ni) treated with extracellular polymeric substances (EPS). **a**-**b** NMDS of the functional classification of the metagenomic data (**a**—dots represent the samples, **b**—dots represent functions within the five most abundant functional groups). **c**-**d** NMDS of the taxonomic classification of metagenomic data (**a**—dots represent the samples, **b**—dots represent the taxa within the five most abundant taxonomic groups). Samples are colored as displayed on the legend

The taxonomic networks for the different treatments also varied according to the presence of each contaminant (Fig. 7 and Figure S2). Most of the networks have shown a high connection between nodes from actinobacteria (Fig. 7) as indicated by the proximity of nodes and the number of edges between them. However, samples containing EPS did not present this clustering of nodes. We can also note that the betweenness centrality was also more homogeneous in samples with EPS with a decrease in the betweenness of node from Proteobacteria. Also, this network had a higher proportion of negative edges.

Discussion

The application of bioremediation technologies in situ is often challenged by unanticipated outcomes and impaired performance [18]. Differences in environmental parameters (e.g., pH and temperature), indigenous populations, and the complex interactions between their differences may lead to this uncontrolled behavior [34]. Here, we have presented the first result of the application of an extracellular polymeric substance (EPS) obtained from *P. polymyxa* CETEM capable of binding the metal [35] in a soil contaminated with petroleum and nickel.

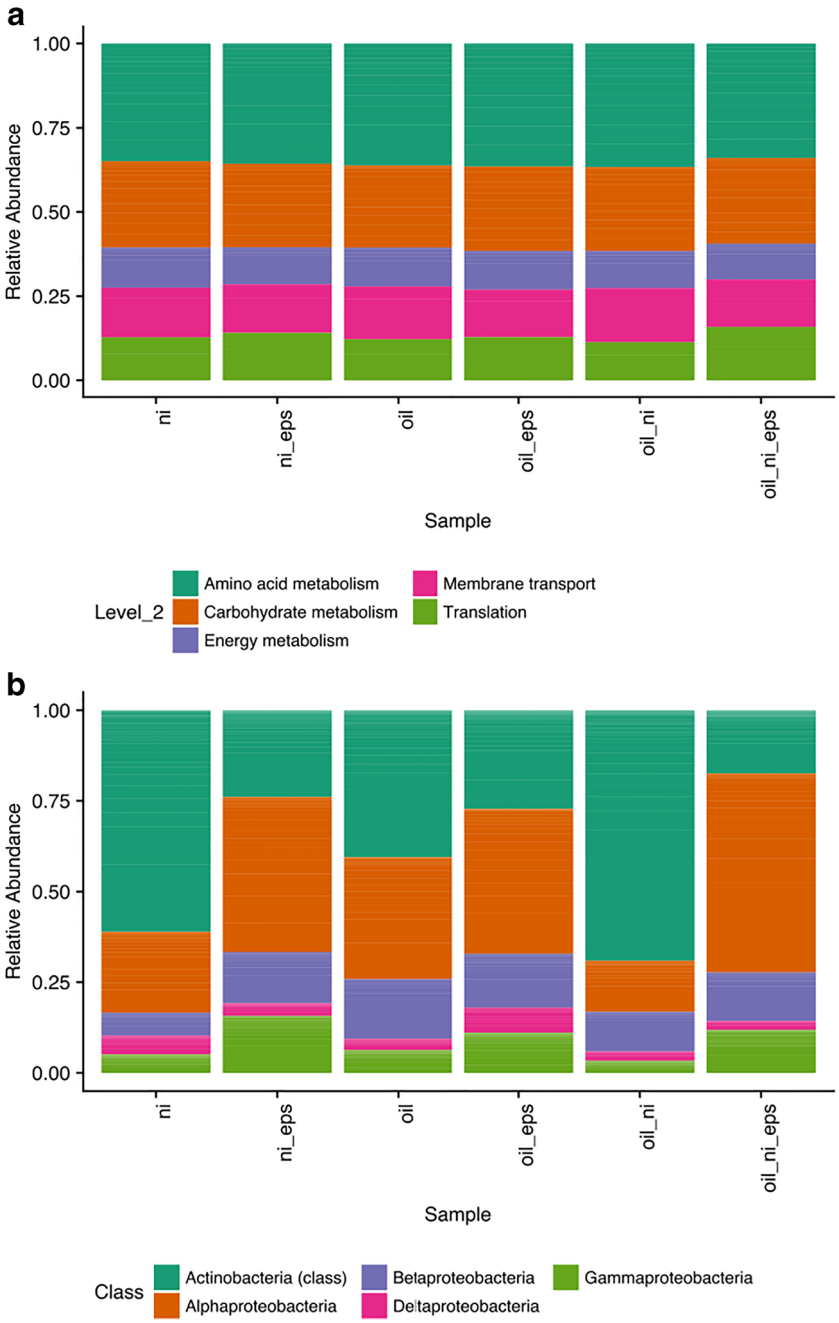


Fig. 4 Classification of metagenomic sequences based on the MG-RAST classification of sequences obtained from soil samples contaminated with hydrocarbons (oil) and nickel (Ni) treated with extracellular polymeric substances (EPS). **a** Classification of sequences in functional KEGG ontology groups. **b** Classification of sequences to the level of class

The current literature indicates the addition of EPS to soils for the remediation of metals and hydrocarbons separately [36]. The EPS would increase the solubility of the hydrocarbon and adsorb the metal [36]; however, its effect in co-contaminated environments is unknown. Binding of the EPS to the metal was expected to aid the biodegradation of the organic pollutant by reducing its toxic potential [7]. However, our results indicated that this addition did not aid in the removal of the hydrocarbons, and it reduced it. The soils from this region are prone to natural attenuation due to its characteristics [3], and the co-contamination with lower amounts of nickel improved the biodegradation of these compounds [20]. However, the concentration of nickel added to the soils had an inhibitory effect on the bioremediation process. Thus, if EPS was reducing the metal availability, we expected to see an improvement in the mineralization of hydrocarbons. Thus, the EPS applied might not adsorb the nickel in the soil either by the metal being unavailable or by some conformational changes in the polymer's functional groups [7, 37] or if it bound to it must have increased the toxicity of the metal to the populations that break down the petroleum.

Nonetheless, our results indicate that the addition of the EPS led to directed changes in the metagenome

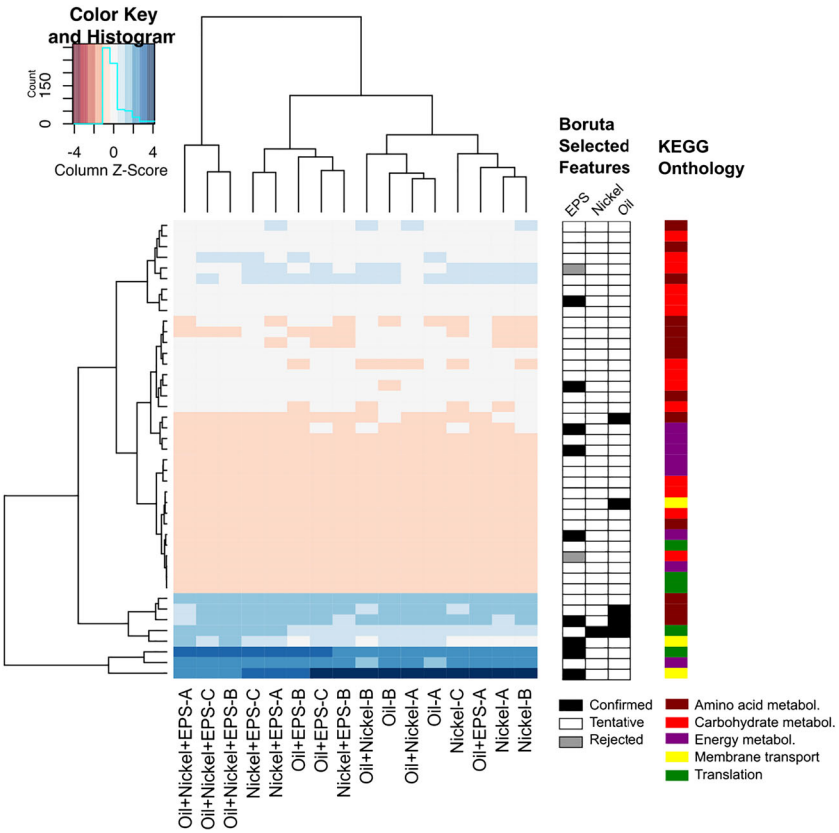


Fig. 5 Heatmap showing the relationship between KO pathways (horizontal) and samples (vertical) using complete linkage method with Euclidean distance measure. The heatmap displays the relative abundance (row *z* scores) of KOs within samples (vertically). KOs that differentially segregated across soil successional stages were identified by random forest analysis with Boruta feature selection and are displayed in the center boxes according to the legend. Classification of the pathway is shown in the right boxes according to the legend

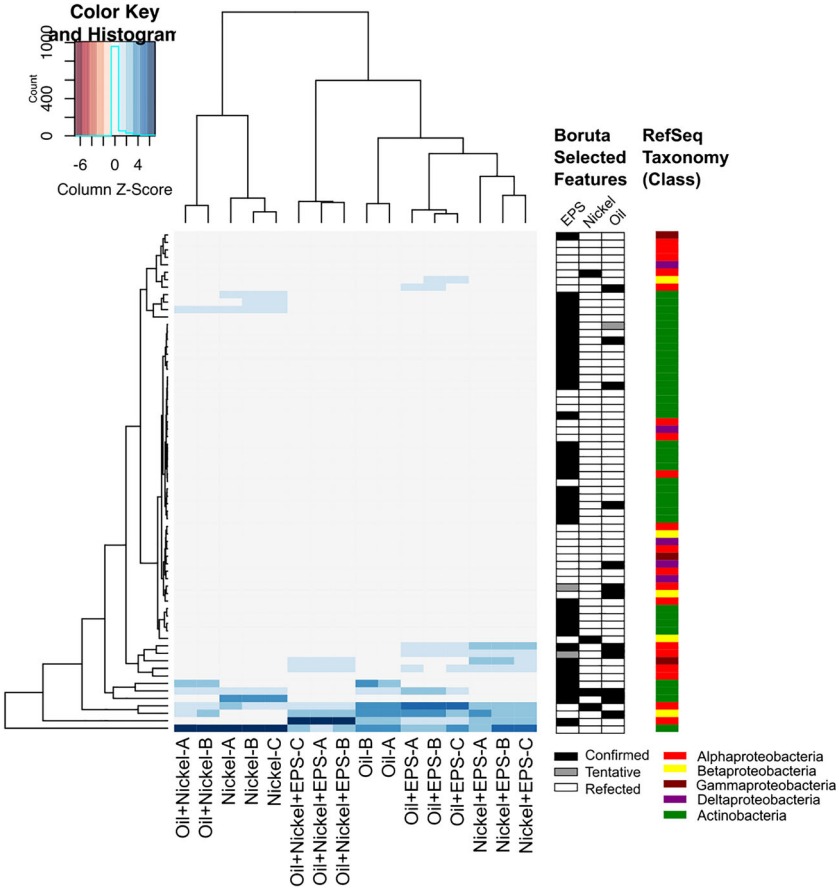
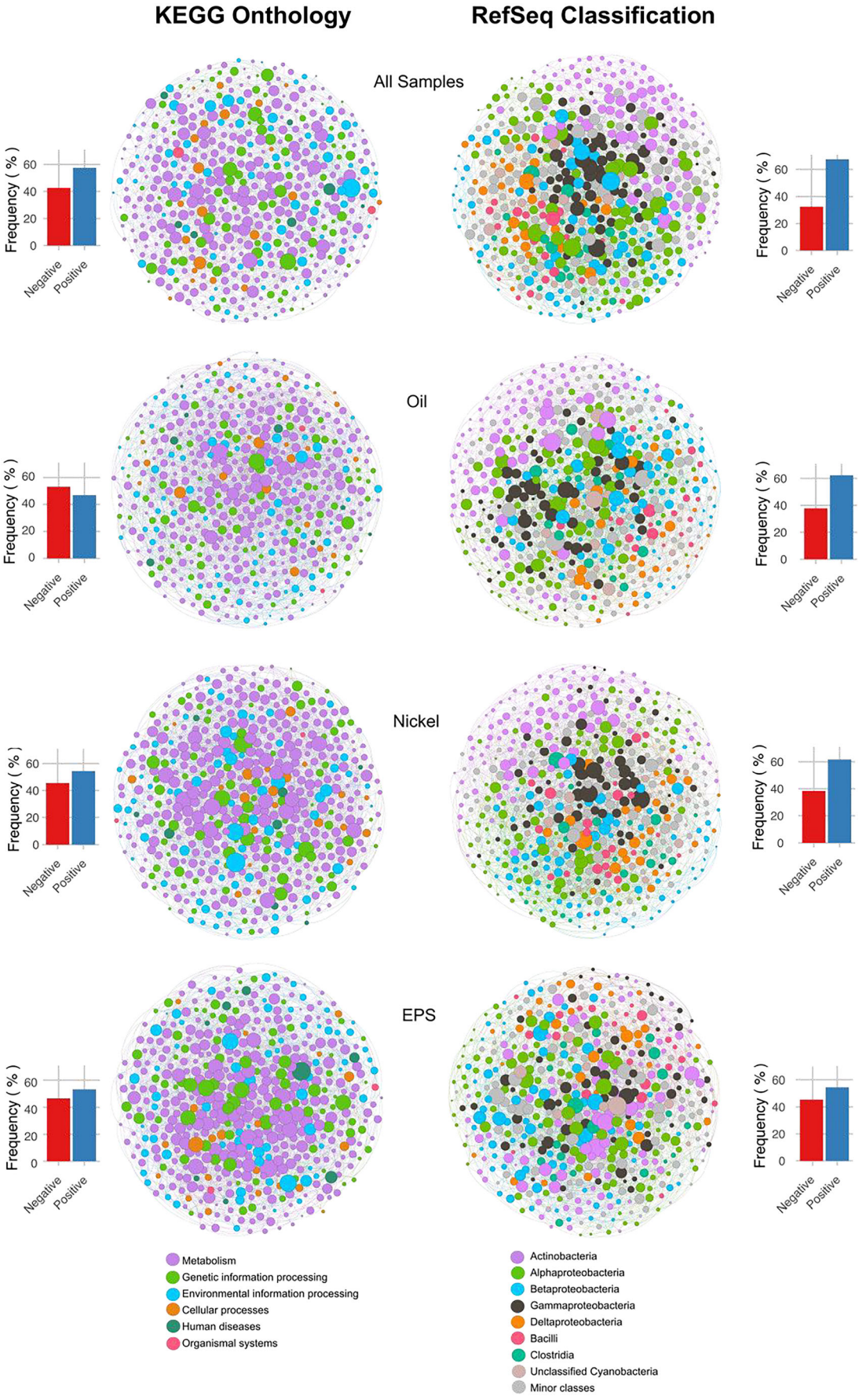


Fig. 6 Heatmap showing the relationship between RefSeq taxonomy families (horizontal) and samples (vertical) using complete linkage method with Euclidean distance measure. The heatmap displays the relative abundance (row *z* scores) of families within samples (vertically). Families that differentially segregated across soil successional stages were identified by random forest analysis with Boruta feature selection and are displayed in the center boxes according to the legend. Classification of the families is shown in the right boxes according to the legend


KEGG Onthology
RefSeq Classification
All Samples
Oil
Nickel
EPS
Frequency (%)
0
20
40
60
Negative
Positive
Metabolism
Genetic information processing
Environmental information processing
Cellular processes
Human diseases
Organismal systems
Actinobacteria
Alphaproteobacteria
Betaproteobacteria
Gammaproteobacteria
Deltaproteobacteria
Bacilli
Clostridia
Unclassified Cyanobacteria
Minor classes

◀ **Fig. 7** Network analysis of the MG-RAST annotation of the metagenomes of sequences obtained from soil samples contaminated with hydrocarbons (oil) and nickel (Ni) treated with extracellular polymeric substances (EPS). Nodes represent different taxa or functions and its classification is color-coded according to the legend. Size of the node represents the betweenness centrality of the node, and its size is comparable only within each network. Bar plots on the side of each network indicate the frequency of negative and positive interaction between nodes

structure independent of how it acted on the metal. The shift from a community primarily dominated by Actinobacteria to one dominated by Proteobacteria may be responsible for the diminished bioremediation. However, the changes in the taxonomic profile of these metagenomes do not reflect in changes in the functional one. This functional stability is an effect of the functional redundancy; i.e., many taxa can carry genes for the same functions [38]. This redundancy allows a new taxon to explore a resource once a taxon is not present [39, 40].

This shift in the microbial community and its effect in the removal of the organic pollutant is not straightforward. Most well-known hydrocarbon degraders, such as *Pseudomonas*, belong to the Proteobacteria [41], while Actinobacteria is not commonly regarded as hydrocarbonoclastic. However, many taxa belonging to the latter present broad metabolic capabilities and can decompose complex materials such as lignin [42] and some, such as *Rhodococcus*, are recognized as versatile hydrocarbon degraders [43]. Thus, if the application of EPS is not selecting against hydrocarbonoclastic microorganisms, it is selecting those with lower fitness.

EPS are essential molecules in the process of hydrocarbon bioremediation in marine environments [7]. They act as a point of contact between the oil and the cellular biofilm formed outside the hydrophobic droplet aiding the formation of the marine oil snow (MOS) [44]. The Gamma and Alphaproteobacteria are responsible for the secretion of EPS and formation of this biofilm [7, 44] in oceanic samples. Thus, the EPS may have facilitated the access of these Proteobacteria to the oil, leading them to occupy these niches. However, despite having more fitness to occupy the EPS-oil surfaces, those organisms might not be so fast at decomposing hydrocarbons at the conditions of the experiment.

Another aspect of the EPS that may play a part in the patterns observed is that they might serve as a source of nutrients to some populations. These polymeric substances are often rich in polysaccharides and proteins [45, 46]. The addition of such nutrients may stimulate the growth of fast-growing bacteria. Fast-growing heterotrophy is a common characteristic of members of the Alpha and Gammaproteobacteria [47]. Hence, as the constituents of the EPS are more palatable sources of carbon, they might divert the community from degrading hydrocarbons.

The change in the role of Actinobacteria within the EPS-based network analysis also highlights how members of these phyla were affected by these polymers. In all other networks, most of the members from this group formed a tight and peripheral cluster, highly interconnected. However, the addition of EPS scattered the members of these phyla around the network, increasing its connection to other taxons. This indicates that EPS not only changes the way the community interacts with the pollutant but also how the populations within the community interact between themselves.

In conclusion, despite several successful applications of EPS in the bioremediation of metals and hydrocarbons in separate, our results indicate that its application in a co-contamination with both pollutants was less practical than natural attenuation. This was related to a decrease in the relative abundance of Actinobacteria and an increase in the abundance of Proteobacteria, which indicates that in these soils, these changes might have a relation. Hence, the application of EPS obtained from *P. polymyxa* CETEM was not successful because it led the microbial community away from one that was better prepared to degrade these organic pollutants.

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Compliance with ethical standards This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no conflict of interest.

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