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Effect of nickel in the degradation of oil in soils contaminated with petroleum and nickel

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Abstract *— The pollution originated from the petrochemical industry is currently one of the main environmental concerns. Most of this is due to the high volume of toxic hydrocarbons that are produced and transported around the globe. However, this industry is also associated with toxic metals that are minor components of petroleum and used during refining processes. Here we have evaluated the impact of nickel in the biodegradation of crude oil in natural soils. We have assessed bacterial community profiles in these samples using ion torrent 16S rRNA gene sequencing and Real-Time PCR quantification and shotgun metagenome sequencing. We have found that the contamination with oil and nickel reduced the bacterial abundance compared with only soil. There was also an increase in the abundance of Actinobacteria. This group substituted the Proteobacteria as the dominant hydrocarbonoclastic bacteria as shown by metagenome sequencing. The presence of both contaminants also increased the removal of hydrocarbons. Thus, this indicates that the shift between Proteobacteria and Actinobacteria was beneficial to the removal of the organic pollutant.*

Keywords *— **Metagenome, metal contamination, hydrocarbon remediation, Actinobacteria.***

I. INTRODUCTION

Petroleum is one of the biggest source of environmental contamination with organic and inorganic pollutants. Besides the organic compounds, elements such as vanadium, arsenic and nickel, are found between oil constituents(Hamme, Singh, & Ward, 2003). Thus, the Petrochemical Industry is considered not only a major source of organic pollutants but also metals (Nadal, Schuhmacher, & Domingo, 2007).These waste generated by the oil industry not only offers great risks to the environment but also represent a great risk to public health (Knox & Gilman, 1997).Data provided by the United States Environmental ProtectionAgency(USEPA, 1996, 2004)has shown an increase in metal contamination associated with organic compounds of nearly 300% between 1994 and 2003. Besides the environmental and health hazards, the toxic and inhibitory effects of heavy metals on microbial growth require attention (Vivas, Biro, Nemeth, Barea, & Azcon, 2006). These metal ions, depending on the nature, concentration and availability will interfere with essential activities of micro-organisms in the soil and in the degradation process of organic compounds (Amor, Kennes, & Veiga, 2001).

Hydrocarbons and heavy metals are naturally found in soils in low concentrations as constituents of organic matter and minerals (Atlas, 1991). Therefore, the presence of organisms capable of metabolizing these components is widespread (Atlas, 1991). So, the exposure to

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hydrocarbons,either by accidental spillage of oil, or by natural phenomena, is important for determining the time of the hydrocarbon removal process on the environment(R. G. Taketani, Franco, Rosado, van Elsas, & Elsas, 2010). The contact with hydrocarbons results in an increase in the oxidizing potential of the community of hydrocarbonoclastic microorganisms(Atlas, 1991; R. G. Taketani et al., 2010). There are three inter-related mechanisms which lead to adaptationphenomenon are: (a) induction of specific enzymes; (B) genetic changes that results in new metabolic pathways; (C) selective enrichment of microorganisms. With respect to selective enrichment, the number of different microbial populationscapable of using hydrocarbons in an environment, as well as its proportion in the community increases with exposure to oil and oil products, generally, reflecting the degree of contamination (Korda, Santas, Tenente, & Santas, 1997).

Heavy metals and polycyclic aromatic hydrocarbons (PAH) have an adverse effecton the microbial community of different soils using dehydrogenase activity in relation to varying quantities of organic matter found in each soil type(Irha, Slet, & Petersell, 2003). Other studies also found a negative effect of metals in the hydrocarbon biodegradation in soil (AL-Saleh & Obuekwe, 2005; J J Kelly & Tate, 1998; John J Kelly, Ha, & Tate, 1999; Shen, Lu, Zhou, & Hong, 2005; N. F. Taketani et al., 2015).

In order to explain the inhibition of biodegradation of monoaromatic hydrocarbons by the presence of heavy metals, Amor et al., (2001) tested many heavy metals (zinc, nickel and cadmium) for inhibiting the degradation of toluene in a culture medium and obtained the result that nickel is the metal with the greater negative influence, followed by cadmium and finally zinc. This metal also promotes a delay in the biodegradation of crude oil, but changes on microbial populations suggest an adaptation to the presence of the metal in the environment(N. F. Taketani et al., 2015).

The change in populations of microorganisms by pollution or natural factors in the soil can generate changes in different levels. The degree of influence on microbial populations of the soil depends on several factors, including the ability of microorganisms to survive the disturbance and perform functions that restore ecological balance, for such a genetic and metabolic framework is necessary. When there is more than one change to the environment, the chances of survival and execution of metabolic functions by microbial populations are even more at risk(Griffiths & Philippot, 2013).However, if different taxonomic groups of microorganisms have the same metabolic potential, this metabolic redundancy becamea very important tool for environmental resilience to the different changes(Taketani et al. 2014).

The application of metagenomic tools to correlate community structure and function allows a deeper look into the functional redundancy and the response to different environmental conditions. Therefore, this study aims to unveil the relationship between microbial community structure and function and influence of nickel on the biodegradation of crude oil in the bioremediation.

II. MATERIALS AND METHODS

Environmental samples and microcosm design

In order to mimic the conditions found in an accidental spill of oil on soil samples we have used soil samples from an area of *on shore* oil extraction in the city of Carmópolis(state of Sergipe) 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)(N. F. Taketani et al., 2015). We have chosen to use nickel as our model heavy metal because it is common constituent of oil and is also widely used during the process of refining as a catalyst.. Hence, 5.0% of crude oil and 260mg/kg of nickel chlorite were used in the microcosms. Four different treatments were applied, a control soil without oil and nickel, one containing only oil, only nickel and one containing both oil and nickel. Sterile controls containing the oil and oil+nickel were added to account for the volatilization of hydrocarbons which were not significant and are not shown.The microcosms were destructive and were set in duplicated 250ml autoclaved Erlenmeyer flasks closed with sterile hydrophobic cotton plugs. Each contained 50g of soil that was sieved in a 5mm mesh prior to utilization. Soil humidity was kept constant at 70% with addition of sterile distillated water and carbon, nitrogen and phosphorus ration (C:N:P) of the soil was 100:10:1 in the microcosms containing oil. Flasks were randomly distributed and a bench top and clustered and rearranged Microcosms were kept at room temperature for 42 days after which samples were taken and kept at -20°C.

Analysis of total petroleum hydrocarbons from soil

The total concentration of hydrocarbons in soil was quantified using infrared spectrometry (Infracal®, model HART-T, Wilks Enterprise, Inc, Connecticut, USA) using hexane as the extractor as described previously (N. F. Taketani et al., 2015).A standard curve was constructed with a serial dilution of the same oil used in the microcosm and was measured each time the analysis was performed.

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Controls using sterile soil were used to evaluate the loss of hydrocarbons due to chemical and physical processes which was below the limits of detection of the technique and was considered negligible.

Polymerase chain reaction of bacterial 16S rRNA gene.

DNA was extracted using Fast DNA spin kit for soil (MP Bio) following the manufactures' protocol. DNA quantity and quality was accessed on a NanoDrop 1000 spectrometer. The 16S rRNA gene present in the samples obtained from these microcosms was quantified by Real-time PCR as described previously (R. G. Taketani, dos Santos, van Elsas, Rosado, & Elsas, 2009) using primers P1 e P3 (Muyzer, Dewaal, & Uitterlinden, 1993). The qPCR standard curve had an R^2 of 0.99–0.96 and an efficiency of 95–105 %.

The amplification of 16S rRNA gene V6 region was amplified using the fusion primers using primer sequences described previously (Sogin et al., 2006)with the addition of barcodes and PCR conditions and purification were performed according to the protocol described by Taketani et al. (2016).

Ion torrent sequencing, sequence processing and analysis

The 16S rRNA amplicon obtained as described above was used as libraries for barcoded amplicon sequencing using ion torrent technology as described in Taketani et al. (2016). Sequences obtained were processed according to a modified version of the 454 tutorial from que Quantitative Insights Into Microbial Ecology toolkit (QIIME) (Caporaso et al., 2010)as described in Taketani et al. (2016).

Total DNA extracted from soil was sequenced using a Ion Torrent Personal Genome Machine (Thermofisher Scientific) using a 316 chip using according to the manufacturers' protocol as described previously (R. G. Taketani, Kavamura, Mendes, & Melo, 2014). The obtained sequences were uploaded to MG-RAST (Meyer et al., 2008) for quality control and annotation. Sequences were taxonomically assigned using Best Hit Classification against the M5NR database using an E-value cut-off of 10^{-5}, minimum identity of 60% and a minimum alignment of 50 bp and the functional annotation was performed by Hierarchical Classification against the Subsystems database using an E-value cut-off of 10^{-5}, minimum identity of 60% and a minimum alignment of 15 amino acids (Delmont et al., 2011). All sequences are deposited in the MG-RAST database under project number mgp857 and mgp89055.

Data Analysis

Plots were produced in MG-RAST, Excel, RStudio, using ggplot2 (Wickham, 2009) and cowplot(Wilkes, 2017) packages. 16S rRNA sequencing biom file obtained in QIIME was analyzedin phyloseq(Mcmurdie & Holmes, 2013), principal coordinate analysis(PCoA) and adoniswas performed using the vegan package (Oksanen, 2010). Differences were tested using analysis of variance (ANOVA) followed by Tukey HSD *posthoc*test in RStudio.

III. RESULTS

Quantification of total petroleum hydrocarbons (TPH)

The quantification of TPHs present in the samples that received only oil was 42.14 ± 1.71 mg/kg which corresponds to a removal of 30.53% of the total hydrocarbons added to these samples. The treatment that also received nickel (Oil + Nickel) had concentration of 28.05 ± 2.85 mg/kg corresponding to 53.76% of removal. These indicated that the addition of nickel improved the removal of oil in these microcosms. The loss hydrocarbons due to volatilization was not statistically significant ($p>0.05$) and is not shown.

Quantification of bacterial 16S rRNA genes

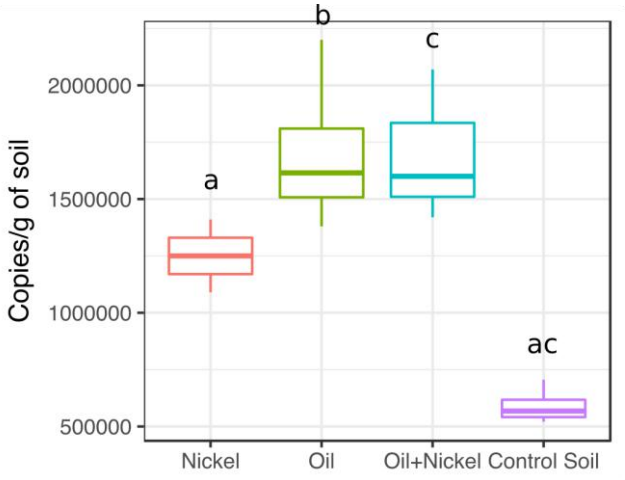

Fig.1: Real-time PCR quantification of the bacterial 16S rRNA gene of the samples contaminated with petroleum and nickel. Letters above the indicates significant differences in Tukey's HSD posthoc test.

The Real-time PCR quantification of bacterial 16S rRNA genes indicated that the addition of oil increased the number of gene copies despite the co-contamination with nickel (figure 1). However, although in smaller proportion, the addition of nickel alone also increased the number of gene copies if compared to the control soil. This indicates

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that the contamination with oil and hydrocarbons led to an increase in the bacterial numbers.

16S rRNA gene tags taxonomic affiliation

The taxonomic classification of 16S rRNA gene libraries obtained from the treatments indicated a distinction between them (figure 2). All samples that received the petroleum addition had a clear increase in the abundance of Actinobateria (figure 2A) while decreasing Acidobacteria and Proteobacteria. Samples containing nickel and oil had an even larger growth in Actinobacteria. The addition of nickel stimulated the abundance of Bacteroidetes and Firmicutes with a decline in the relative abundance of Actinobacteria.

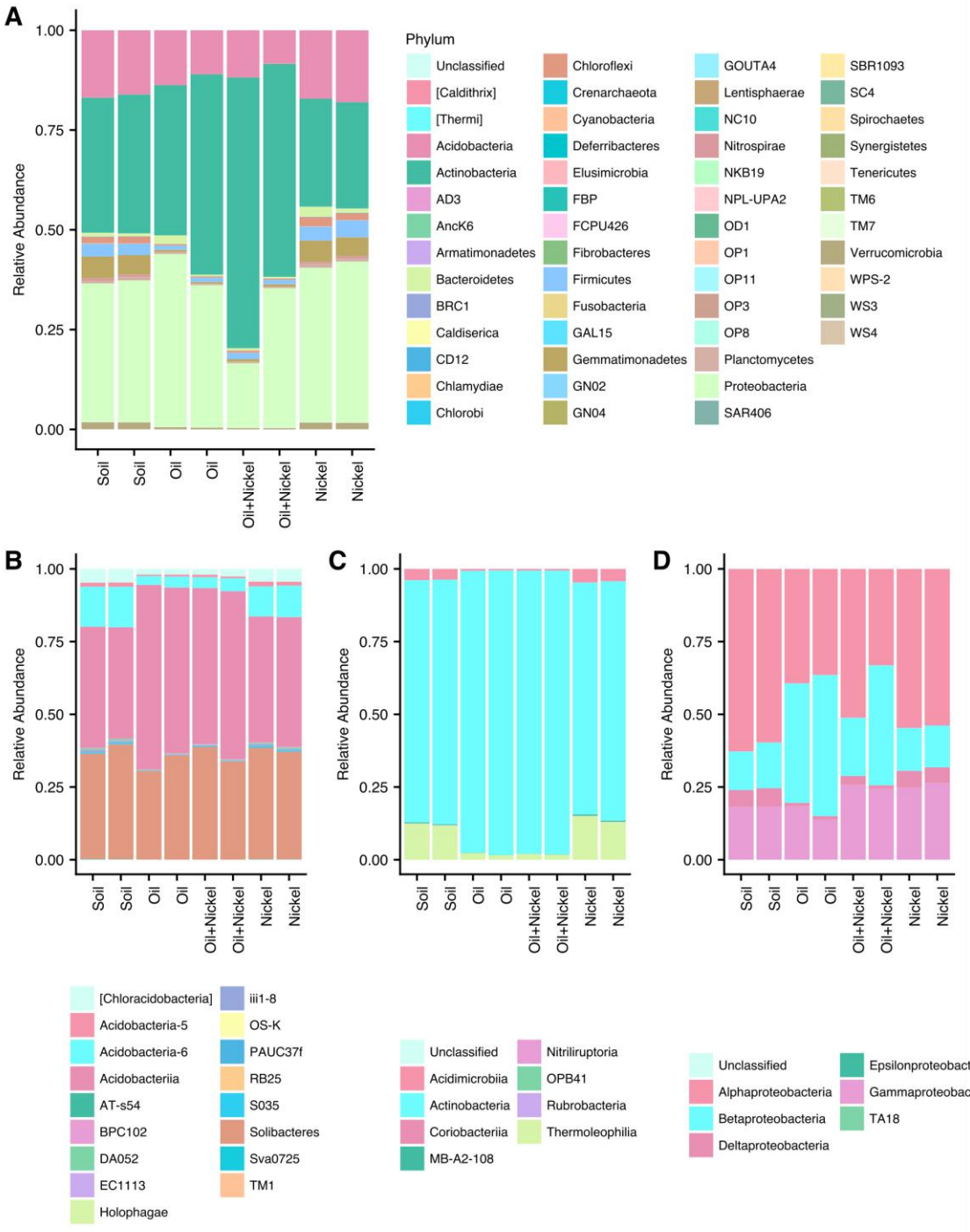

Fig.2: Bar chart of the classification of 16S rRNA gene sequences from soils. Classification was obtained in Qiime 1.9 using the Greengenes database. A. Phylum level classification. B – Class level classification of the Acidobacteria. C - Class level classification of the Actinobacteria. D. Class level classification of the Proteobacteria.

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Within the Acidobacteria phylum the class Acidobacteriia was positively influenced by the addition of oil while Solibacteres and Acidobacteria-6 (group 6) were negatively affected. The addition of nickel led only to minor changes in the class affiliation of the sequences due to a decrease in the relative abundance of group 6 with an increase in Acidobacteriia.

Furthermore, the class Actinobacteria (phylum Actinobacteria) was the dominant class in all samples (figure 2A), however, the addition of oil has led to a reduction in the incidence of Thermoleophilia that was substituted by Actinobacteria.

The changes observed within the Proteobacteria were more pronounced between samples that received only oil and oil and nickel (figure 2B). The addition of oil and nickel led to agreat increase in Gammaproteobacteria and a smaller one in Betaproteobacteria. The addition of only oil had a positive effect in the relative abundance of Betaproteobacteria in spite of Alphaproteobacteria. Additionally, the expansion in Gammaproteobacteria was also observed in samples that received only nickel.

Alpha diversity patterns

The abundance distribution of OTUs found in each sample was used to evaluate the effects of each treatment in the diversity patterns by means of diversity indices (figure 3). The number of OTUs observed in the treatments containing nickel was similar to the control soil while both treatments that received the addition of oil had a lower number of OTUs. However, these differences were not significant according to ANOVA test. The Shannon's H' index showed the same pattern of lower diversity in the samples that received oil. This effect was confirmed by ANOVA with *posthoc* test TukeyHSD. This disagreement between both indexes indicate that besides lowering the amount of OTUs observed, the addition of oil must have changed the evenness of this OTUs leading to a higher dominance of the most frequent OTUs.

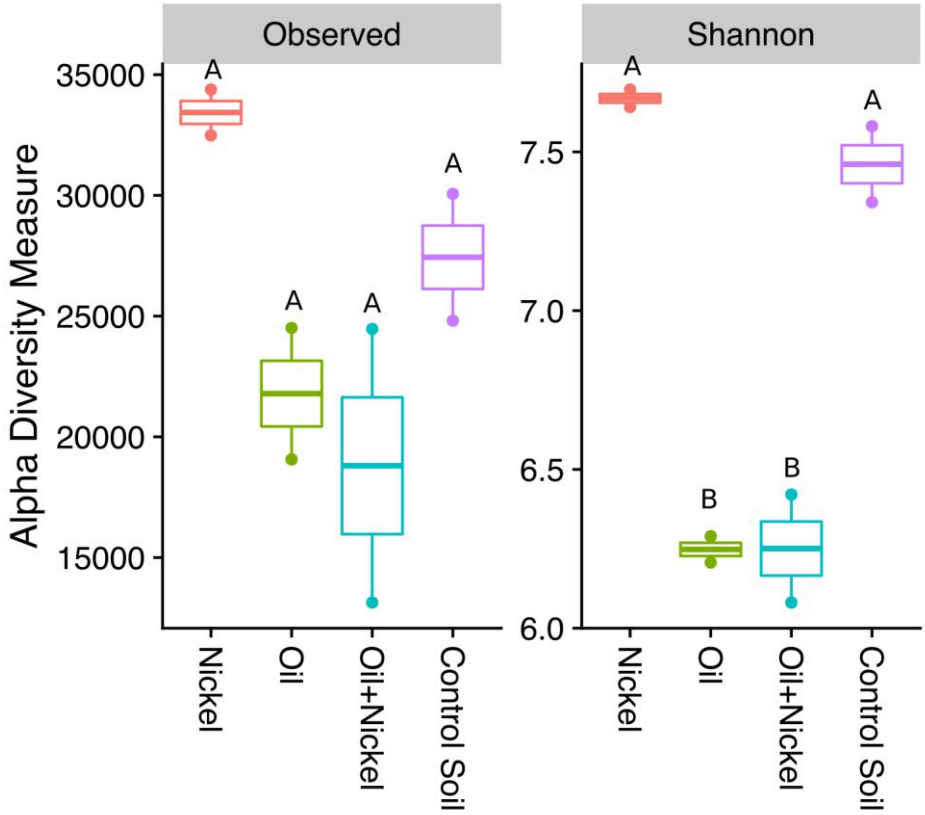

Fig.3: Alphadiversity patterns of 16S rRNA gene sequences from soils

Beta diversity patterns

To investigate the relationship between this samples and the most abundant OTUs we have applied a principal coordinate analysis (PCoA) based on a Bray-Curtis similarity index (figure 4). As observed on the previous analysis the PCoA indicated a clear clustering of samples that did not receive oil (figure 4A). On the other hand, although the samples that had oil added did not present a tight clustering on a two-dimensional scale, they were tightly clustered on the first dimension (figure 4A, Axis.1)

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which represents 71.5% of the variance observed on this samples.

Plotting the OTUs from the five most abundant orders on top of the samples (figure 4B) indicate that some of OTUs were more correlated with samples that contained oil than the ones that did not. Most of the OTUs belonging to the Burkholderiales order were highly correlated with oil samples. On the other hand, the Sphingomonadales and Xantomonadales were better correlated with samples without oil. Nevertheless, we could not observe any taxonomy-based trend for the Acidobacteria and Actinobacteria.

The Adonis test indicated that the different treatments were significantly different (p=0.012, table S1) and that the addition of oil also had a significant effect over the bacterial community (p=0.04). The addition of nickel however did not have a significant effect over the bacterial community (p=0.535).

Table 1: Quantification of total petroleum hydrocarbons (TPHs) by infrared spectrometry

Treatment	Initial concentration (mg/kg)*	Final Concentration (mg/kg)	Removal (%)
Oil	60.66±1.42	42.14±1.71	30.53
Oil+Nickel	60.66±1.42	28.05±2.85	53.76

* - Initial concentration was considered the same for both treatments since it was evaluated before the addition of Nickel

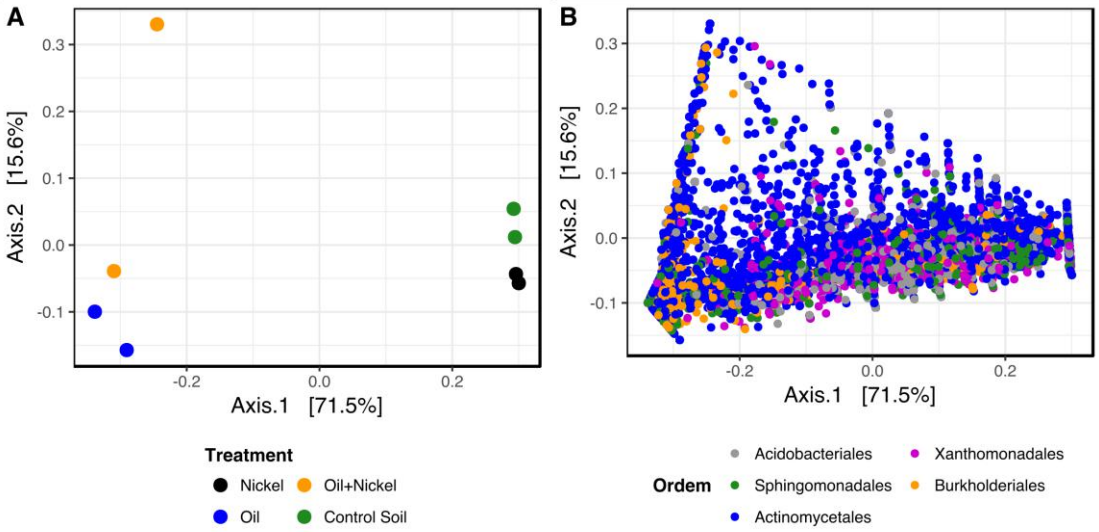

Fig.4: Principal Coordinates Analysis of 16S rRNA gene sequences from soils. A – Dots represent each sample. B – Dots represent OTUs from the five most abundant Orders.

Metagenomic profile of co-contaminated samples

The metagenomic profile of the communities was evaluated using the MG-RAST pipeline (figure S1 and S2). The taxonomic composition differed from the observed for the 16S rRNA gene, the shotgun libraries had a larger proportion of sequences derived from Proteobacteria and Actinobacteria than the 16S libraries (figure S1). The functional assignment indicated a predominance of genes related to the metabolism of Amino acids, Carbohydrates and Protein (figure S2). Comparing the taxonomic and functional affiliation of the genes indicates that there is more functional congruence between metagenomes than taxonomic.

Functions related to the metabolism of hydrocarbons were more frequent in the metagenomes that received oil than the control soil (figure 5). Additionally, the soil co-contaminated with nickel had the highest incidence of genes related to these metabolisms. Besides the highest frequency of genes related to this metabolism, the taxonomic affiliation of these were related to sequences from Actinobacteria. Conversely, the sequences obtained from the sample contaminated only with oil were affiliated to the Proteobacteria. The only exceptions were of genes

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related to benzoate degradation and salicylate and gentisate metabolism.

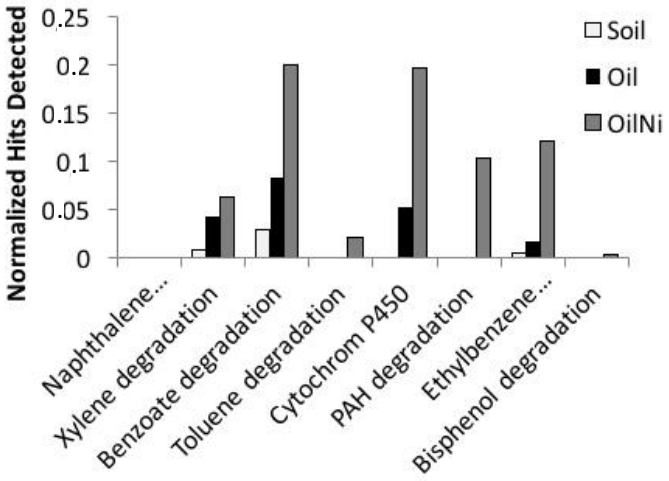

Fig.5: Number of metagenomic sequences that match genes involved in the degradation of hydrocarbons.

Fig.6: Taxonomic classification of metagenomic sequences involved in the degradation of hydrocarbons.

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IV. DISCUSSION

The contamination of the environment with a heavy metal such as nickel is considered harmful both to the environment and health (USEPA, 2004). The presence of these elements together with hydrocarbons has been pointed as a disturbing factor which slow down the degradation of the organic material (Dermont, Bergeron, Mercier, & Richer-Laflèche, 2008; Muniz, 2004; Pérez-de-Mora, Engel, & Schloter, 2011; Santos-Echeandía, Prego, & Cobelo-García, 2008; Tang et al., 2010). This disturbance however as does not impede the degradation of this hydrocarbons, it slows down the initial process of degradation (N. F. Taketani et al., 2015). Yet, the participation of different populations in this process probably led to different outcomes.

The addition of nickel in high concentrations (>1.0g/kg) has been shown to promote decrease of the overall microbial richness and diversity(Li, Hu, Ma, & Wang, 2015; Markowicz, Cycoń, & Piotrowska-Seget, 2016; Remenár et al., 2017). However, our experiments with a smaller dose showed that Actinobacteria might be resistant to the presence of this metal (Li et al., 2015). Since the soil matrix is capable of binding the nickel ions and thus make it less available to the soil microorganisms, microbial communities from different soils respond differently to this stress (Li et al., 2015; N. F. Taketani et al., 2015). The soils used in this study presents high cation exchange capacity (CEC) and thus can bind high quantities of nickel(Ding, Hu, Wan, Wang, & Gao, 2016). The high CEC and moderately high concentration of nickel could lower the intensity of the disturbance caused by the toxicity of this metal if compared to the literature (Li et al., 2015; Markowicz et al., 2016; Remenár et al., 2017).

The incidence of Acidobacteria and Proteobacteria were affected by the addition of hydrocarbons, however the addition of nickel has led to different outcomes between these phyla. Proteobacteria are common players in hydrocarbon degradation and *Pseudomonas*(Oyetibo, Ilori, Obayori, & Amund, 2013)are commonly employed in bioremediation procedures (Evans et al., 2004). However, Actinobacteria are also important hydrocarbon degraders (Acosta-González, Martirani-von Abercron, Rosselló-Móra, Wittich, & Marqués, 2015; De Pasquale, Palazzolo, Lo Piccolo, & Quatrini, 2012; Ke, Luo, Wang, Luan, & Tam, 2010; Vila & Grifoll, 2009; Zeinali, Vossoughi, & Ardestani, 2007)and *Rhodococcus*are also important hydrocarbon degraders in soils (Acosta-González et al., 2015; Auffret, Yergeau, Labbé, Fayolle-Guichard, & Greer, 2014; Song et al., 2011). However, certain actinobacterial groups have been shown to be selected by high doses of nickel (Remenár et al., 2014). Hence, the shift between these phyla are due to the addition of this metal is likely the cause of the higher degradation observed in soils with nickel.

The shift toward the increased abundance of Actinobacteria in soils co-contaminated was also confirmed by the metagenomic data. These results shown that not only these organisms were present in the samples but reads assigned as related to the metabolism of hydrocarbons were also from actinobacterial origin. This shows that these OTUs are not only present because they can withstand both contaminants but they also have the genes to degrade components of oil.

Nonetheless, our data does not indicate that the presence of this metal stimulates the oils degradation. Given the toxic nature of this elements at the concentration used in this study (260mg/kg)(USEPA, 2004) this hypothesis is very unlikely. However, the presence of this metal might eliminate some OTUs (i.e. Proteobacteria) that are sensitive to it leaving some niches open to organisms that are resistant to it (i.e. Actinobacteria) that would in the absence of nickel lose the completion for hydrocarbons. This second hypothesis is in line with the intermediate disturbance hypothesis (Wilkinson, 1999)which predicts that after a disturbance many species may migrate to the cleared niches which maximizes the diversity and potentially also increase activity.

V. CONCLUSION

In conclusion, our data has shown that the contamination of soils with hydrocarbons and nickel leads to an increased abundance of Actinobacteria and hydrocarbonoclastic (HC) genes from organisms from this phylum. Concurrently, there is also an increase in the removal of hydrocarbons in the presence of metals thus indication that this hydrocarbonoclastic Actinobacteria are responsible for the higher rate of degradation. Hence, we propose that the presence of nickel to this soil wiped some HC Proteobacteria that are better competitors in normal soil conditions leaving this niches open to colonization by other taxa in this case Actinobacteria. These HC newcomers in the absence of other competitors outperform the original populations in the removal of oil.

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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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