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Effects of organic matter addition on chronically hydrocarbon-contaminated soil

Rocío Medina · Pedro M. David Gara · Janina A. Rosso · María T. Del Panno

Abstract Soil is the recipient of organic pollutants as a consequence of anthropogenic activities. Hydrocarbons are contaminants that pose a risk to human and environmental health. Bioremediation of aging contaminated soils is a challenge due to the low biodegradability of contaminants as a result of their interaction with the soil matrix. The aim of this work was to evaluate the effect of both composting and the addition of mature compost on a soil chronically contaminated with hydrocarbons, focusing mainly on the recovery of soil functions and transformations of the soil matrix as well as microbial community shifts. The initial pollution level was 214 ppm of polycyclic aromatic hydrocarbons (PAHs) and 2500 ppm of aliphatic hydrocarbons (AHs). Composting and compost addition produced changes on soil matrix that promoted the release of PAHs (5.7 and 15 % respectively) but not the net PAH elimination. Interestingly, composting stimulated AHs elimination (about 24 %). The lack of PAHs elimination could be attributed to the insufficient PAHs content to stimulate the microbial degrading capacity, and the preferential consumption of easily absorbed C sources by the bacterial community. Despite the low PAH catabolic potential of the aging soil, metabolic shift was driven by the addition of organic matter, which could be monitored by the ratio of Proteobacteria to Actinobacteria combined with E_4/E_6 ratio. Regarding the quality of the soil, the nutrients provided by the exogenous organic matter contributed to the recovery of the

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global functions and species diversity of the soil along with the reduction of phytotoxicity.

Keywords Bioremediation · Chronically hydrocarbon-polluted soil · Composting · Microbial community · Phytotoxicity assays · Stimulation with compost

Introduction

Organic pollutants are continuously released to the environment as a product of anthropogenic actions such as industrial, petrochemical and agricultural activities. They affect soil, water, and air modifying their chemical properties and consequently, their microbial community structure as well as their functionality (Sayara and Sánchez 2020). Some organic pollutants are more recalcitrant than others due to their chemical structure and the interactions with the environment (Gaur et al. 2018).

Polycyclic aromatic hydrocarbons (PAHs) are organic contaminants that are released to the soil by petrochemical activities and represent a risk to human, animal, and soil health due to their toxic, genotoxic, mutagenic, and potentially carcinogenic properties (Tsibart and Gennadiev 2013). PAHs are considered priority pollutants by the United States Environmental Protection Agency (USEPA). They are ubiquitous recalcitrant pollutants due to their chemical structure (which contains more than one benzene ring (Varjani 2017)). The interaction of PAHs with soil particles limits the biological access to them (and subsequent biodegradation): a process known as "aging".

Soil, as a complex and dynamic system, is a vital component of the terrestrial ecosystem (Sayara and Sánchez 2020). Soil microbiota plays a key role in cycling of elements such as C, N, S, P, as well as in the stabilization of soil structure. A large community of microorganisms are involved in a wide range of metabolic processes (Nannipieri et al. 2002) and then, a decrease in microbial diversity could negatively impact on soil functionality. Biodiversity guarantees a functional biosphere, conferring to an ecosystem the capacity to withstand serious disturbances, probably as a result of the redundancy of functions in the soil (Nannipieri et al. 2017). The exposure over long periods of time and a subsequent re-exposure to a pollutant enhance the metabolic potential of autochthonous microorganisms of soil, a phenomenon known as "soil memory" (Maitra 2018).

Studies on the degradation of pollutants in soil have shown that microorganisms and some organisms are extremely versatile at catabolizing recalcitrant molecules (Semple et al. 2001). The development of bioremediation strategies based on microbial communities (bacterial and fungal strains) as well as superior organisms such as plants could be used to remove the pollutants from a contaminated area (Macchi et al. 2019; Petruzzelli et al. 2016). These technologies can be applied both in situ and ex situ, and are economical and environmentally friendly (Varjani 2017). In bioremediation processes organic pollutants act as C source to environmentally adapted organisms, which through co-metabolism and synergism with others are able to remove the contaminants (Megharaj et al. 2011).

Composting bioremediation is an aerobic process that requires oxygen, optimal moisture content, and porosity to stabilize the organic waste and contaminant (Haug 2018). During composting, changes in humic and fulvic compounds occur and can be monitored through spectroscopic methods (Antízar-Ladislao et al. 2006; Vázquez et al. 2015). Exogenous organic matter addition (by composting and/or compost addition) provides porosity and aeration to soil improving the soil properties. The stimulation of the autochthonous population is due to the addition of nutrients and a new non-indigenous population that competes with the first one (Gandolfi et al. 2010).

Several researchers described composting of PAH-contaminated soil (Oleszczuk 2007; Plaza et al. 2009; Ren et al. 2018). The first step of the composting process of a PAH-contaminated soil is the stabilization phase, where the bioavailable PAH fraction is consumed. During the second step, the maturation phase, PAH sequestration occurs as consequence of the high content of organic matter (Oleszczuk 2007). In addition, sorption, desorption, and degradation of PAHs vary with soil type (Ren et al. 2018). The success of the process depends on several factors such as soil type, amendment origin, temperature, moisture content, as well as concentration of hydrocarbon and aging (Wu et al. 2013; Leech et al. 2020). These factors affect the adsorption/desorption equilibrium, the bioavailability and mobilization of the PAH and

the microbial activity. It is noteworthy that, despite no PAH elimination, the soil could recover its functionality and the toxicity could be reduced, thanks to a composting treatment (Cipullo et al. 2019).

Bioremediation processes are generally monitored by determining contaminant concentrations, microbial degrading populations, the recovery of the functions of the soil (estimators of microbial diversity and enzymatic activities), among other variables (Tabak et al. 2003; Huesemann et al. 2004; Nannipieri et al. 2017). Remediation process conduces to reduction of toxicity (Sayara and Sánchez 2020), and the recovery of the global functions of the initial contaminated material (Bastida et al. 2008).

In a previous work, the impact of remediation combining chemical oxidation followed by biological treatments of a chronically hydrocarbon contaminated soil was studied. In this study, the spectroscopic analysis became a useful tool for following and comparing those treatments (Medina et al. 2018).

More recently, Medina et al. (2020) have used composting technologies after oxidation of a chronically hydrocarbon-contaminated soil. In this work the authors demonstrated that persulfate addition to chronically contaminated soil removed PAHs and increased the bioavailable PAH fraction. Despite the PAH-degrader stimulation and high concentration of bioavailable PAH fraction, a poor PAH elimination was found as a consequence of derivative metabolism to more easily degradable compounds than hydrocarbons (Medina et al. 2020). Due to the fluorescence properties of PAHs and humic and fulvic compounds, the spectroscopic analysis of these systems could markedly improve the understanding of the time evolution of these processes.

In this context, the aim of the work was to study the effect of composting and compost addition on a chronically hydrocarbon-contaminated soil, focusing on organic matter changes and the recovery of functionality. For this purpose, we evaluated the treatments through chemical (total hydrocarbons and available PAH concentrations) and physical properties (including a spectroscopic study), microbiological counts, enzymatic activities, toxicity tests, as well as genetic diversity analysis in order to know the dynamics of the processes.

Methods

Soil

The soil (S_0) used in this work was sampled from a chronically hydrocarbon- contaminated site, belonging to a petrochemical industry in La Plata city where about twenty years ago a landfarming treatment had been completed. It contains about 214 ppm of PAHs and 2500 ppm of AHs (Medina et al. 2018, 2020). A soil from the same region as S_0, without hydrocarbon contamination, was taken as control soil to compare the physical, chemical, and biological properties. It was named PS.

Treatments

All the microcosms were made in triplicate, maintained at 25 °C, and mixed manually twice a week. Sayara et al. (2009) recommended an optimal moisture content value at 40–60% for composting treatments, which corresponds to 80% of water holding capacity (WHC) for our composting treatment (CS). Then, all the microcosms were adjusted to 80 % WHC. Due to the differences between the systems, the same WHC value corresponds to different moisture content values. Moisture content was maintained by spraying with neat water.

Bioremediation treatment (BS). A Microcosm of 500 g of contaminated soil (S_0) at moisture content 22% for 12 months was taken as control system.

Stimulation treatment with compost (SS). A subsample of S_0 was stimulated with mature compost (MC) at a ratio of 7 g dry S_0: 3 g dry compost, as described by Sayara et al. (2010a). Microcosm was made using 500 g of the resulting mixture and was incubated at controlled moisture content (40%) for 45 days.

Composting treatment (CS). A subsample of S_0 was composted for 12 months in glass reactors (32 cm height), thermally isolated, and aerated. For this purpose, S_0 was amended with goat manure (GM) at a ratio of 7 g dry S_0: 3 g dry GM. A bulking agent (oat straw) was added to the resulting mixture at a ratio of 1:1 v/v, to provide a proper porosity to maintain aerobic conditions, as described by Sayara et al. (2009). The moisture content was maintained at 50%.

Determinations

Hydrocarbon content

In order to know the efficiency of the treatments applied, the PAHs and AHs concentrations were quantified. Total hydrocarbon was extracted using an ultrasonicator (400 W, 40 kHz) by an extraction process according to USEPA´s method 3550b. Triplicate samples were extracted with acetone/dichloromethane (1:1 v/v) as solvent, as described by Medina et al. (2018). The solvent was evaporated, then the residue was dissolved in dichloromethane, filtered, and injected in a gas chromatograph (GC Clarus 500-Perkin Elmer) equipped with flame ionization detector (FID) and 5HT PE column (Perkin Elmer, length: 30 m, internal diameter: 0.25 mm). The polycyclic aromatic hydrocarbon (PAH) and aliphatic hydrocarbon (AH) determinations were described in previous work (Medina et al. 2018). The available PAH concentration was estimated as described by Medina et al. (2018) to know the proportion of PAHs that could be degraded by microorganisms or that affect the toxicity of each system.

Spectroscopic analysis of dissolved of organic matter from soils

The extractions of organic matter from contaminated and treated soils were made as described by Medina et al. (2018). The water-soluble fraction was used for absorption spectra analysis (E_4/E_6 ratio), dissolved total carbon (DTC) determinations, and fluorescence excitation-emission matrices (FEEMs).

The E_4/E_6 ratios were determined by a spectrophotometer Shimadzu UV 1800. DTC was determined using a Total Organic Carbon Analyser, Shimadzu TOC5000 (Mora et al. 2009). The FEEMs were generated using a computer-interfaced Near-IR Fluorolog-3 Research Spectrofluorometer by recording successive emission spectra from 260 to 650 nm at excitation wavelengths ranging from 240 to 580 nm, with a 5-nm scan step. Excitation and emission slits were set to 5 nm.

The FEEM analysis was conducted with MATLAB 7.7 (MathWorks, Natick, MA, USA) using PLS_Toolbox version 4.0 (Eigenvector Research, Manson, WA, USA). Several pre-processing steps were used to minimize the influence of scatter lines and other attributes of the FEEMs that are due to the background solution matrix prior to modelling. A non-negativity constraint was applied to the parameters to allow only chemically relevant results. The determination of the correct number of components in the data set was assessed by the core consistency diagnostic score and validated by visual inspection of the estimated parameters and additional model diagnostics (Medina et al. 2018).

This analysis allows determining the consumption and/or mobilization of organic matter as well as of PAHs during the processes.

Microbiological counts

Enumerations of generalist and specialist cultivable populations along the assays were done to know the dynamic of populations that are stimulated by composting technologies, which reflects the soil functionality and diversity and its possibility to degrade hydrocarbons as well as them recovery. Cultivable heterotrophic bacterial (HB) count was carried out for each treatment. Samples (0.1 ml) of 10-fold dilution were spread on plates containing R2A agar (Reasoner and Geldreich 1985). Inorganic phosphorus solubilizing bacteria (PSB) were counted in phosphorus inorganic medium (PIM), (Goldstein 1986). Agar plates were incubated at 24 °C for 10 days. The most probable number (MPN) of PAH, AH and cellulose-degrading bacteria (MPN-PAH-DB, MPN-AH-DB, and MPN-C-DB) were determined in 96-well microtiter plates, using mineral salt medium supplemented with the corresponding source of carbon (Vecchioli et al. 1990; Wrenn and Venosa 1996). The inoculated microtiter plates were incubated at 24 °C for 21 days.

Enzymatic assays

Enzymatic estimators were measured to evaluate the changes of soil functionality in each treatment. Dehydrogenase activity was determined before, during, and after the treatments. Dehydrogenase assays were performed using soluble triphenyltetrazolium chloride (TTC) as described by Del Panno et al. (2005). Lipase (Margesin et al. 2002), urease

(Kandeler and Gerber 1988), arylsulphatase (Whalen and Warman 1996), proteinase (Ladd and Butler 1972), and phosphatase (Verchot and Borelli 2005) activities were determined before and after each treatment.

Toxicity tests

Soil toxicity was evaluated by testing seed germination and root elongation in accordance with the USEPA (1989) protocols. The germination index (GI%) and the elongation inhibition rate (EI%) were calculated as described by Visioli et al. (2014) using the following equation:

$$EI\% = (Lc - Ln)/Lc * 100$$

$$GI\% = (Gn * Ln/Gc * Lc) * 100$$

where G_n and L_n were the mean values of germinated seeds and root length, respectively, in the microcosms, and G_c and L_c were the mean values of these parameters in water.

DNA extraction from soil, 16S PCR-DGGE and pyrosequencing

In order to know the soil recovery after composting technologies diversity was estimated. This study also allows inferring about the microbial potential metabolic at the end of each treatment. Total DNA was extracted from 1 g soil aliquots from each microcosm after treatment, using the E.Z.N.A.™ Soil DNA Isolation Kit (Omega Bio-tek, Inc., Norcross, GA, USA) according to the manufacturer's instructions. The DNA was stored at −20 °C prior to amplification.

Genetic diversity analysis of the soil microcosm bacterial community was performed by PCR amplification of bacterial 16S ribosomal DNA (rDNA) fragments followed by denaturing gradient gel electrophoresis (DGGE) as described by Medina et al. (2018). Additional details are presented in Supporting Material S1.

Also, DNA samples were used for PCR amplification using the 16S rDNA universal bacterial primers, 341Fbac (Muyzer et al. 1993) and 909R (Tamaki et al. 2011) to amplify a 568-bp fragment of the 16S rDNA flanking the V3 and V5 regions. PCR and pyrosequencing procedures were carried out as described in a

previous work (Medina et al. 2018). Experimental procedure details are listed in Supporting material S2.

Analysis of the pyrosequencing data set

Pyrosequencing raw data were converted to sequence reads using Mothur software (version v.1.34.0) (Schloss et al. 2009) as detailed in Supporting material S2. The sff files were submitted to the NCBI Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra) and are available at the accession number PRJNA507470. For statistical analysis of the data, Good´s coverage index and Hill's numbers (species richness [^{0}D], the exponential of Shannon entropy [^{1}D], and the inverse Simpson index [^{2}D]; Hill 1973) were used as diversity measures in accordance with current consensus criteria (Jost 2006; Chao et al. 2012) and were calculated using Mothur software (version v.1.34.0, (Liao et al. 2015)).

Statistical analyses

The effect of different operational parameters on the evaluated indicators during treatments was investigated using a two-way multivariable ANOVA analysis and post hoc Tukey test with XLStat.

Results

Effect of exogenous organic matter addition on hydrocarbon content and on the hydrocarbon-contaminated soil matrix

The different treatments applied to S_0 modified its properties, as described in Table 1. As described for other composted systems, the amendment microcosms incremented organic carbon, nitrogen, and phosphorus content as well as electrical conductivity (Sayara et al. 2010a, b, 2011; Medina et al. 2020).

All the treatments showed a fall in DTC concentration that could be assigned to microbial metabolic activity associated with pH drop. Through the year of bioremediation in the BS microcosm, a 51 % decrease in DTC was observed. SS and CS had an estimated initial DTC value of 176 mg C/l, based on the soil:amendment ratio of 7:3 used for making them. In SS microcosm, approximately a 29 % decrease in DTC

Table 1 Physical and chemical properties of the chronically contaminated soil (S$_0$) and final microcosms. PS and MC determinations were included as reference values for different measurements

		Bioremediation treatments			Controls	
	S$_0$	BS	SS	CS	MC	PS
pH	8.8 ± 0.1	7.57 ± 0.04	7.62 ± 0.05	7.16 ± 0.02	7.6 ± 0.5	7.3 ± 0.2
EC [mS/cm]	0.63 ± 0.01	0.54 ± 0.04	6.54 ± 0.03	7 ± 1	–	0.34 ± 0.01
OC [%]	2.2 ± 0.9	2 ± 1	9.3 ± 0.5	10.7 ± 0.5	–	2.4 ± 0.3
N [%]	0.2 ± 0.1	0.20 ± 0.03	0.7 ± 0.1	0.9 ± 0.1	–	0.24 ± 0.03
P [mg/kg]	8.3 ± 0.6	6.0 ± 0.3	159.0 ± 0.7	153.0 ± 0.1	–	3.7 ± 0.6
DTC [mgC/l]	96 ± 3(a)	46.5 ± 0.8(c)	125.9 ± 0.8(b)	97 ± 1(a)	363 ± 3	151 ± 2
E$_4$/E$_6$	15.0	3.3	23.1	32.7	4.6	13.7
AH [g/kg]	2.5 ± 0.3(a)	3.3 ± 0.3(a)	5.0 ± 0.9(b)	1.9 ± 0.7a)	2.4 ± 0.2	< 0.001
PAH [g/kg]	0.21 ± 0.02(a)	0.28 ± 0.05(a)	0.21 ± 0.01(a)	0.27 ± 0.08(a)	*udl*	< 0.0002
PAH* [%]	1.0 ± 0.7(a)	1 ± 1(a)	15 ± 2(c)	5.7 ± 0.3(b)	–	–

OC Organic Carbon, Walkley – Black method. *N* Nitrogen, Microkjeldahl method. *P* Phosphorous, Bray-Kurtz No 1 method. *DTC* Dissolved total carbon. *AH* Total aliphatic hydrocarbon. *PAH* Total polyciclyc aromatic hydrocarbon. *PAH** bioavailable PAH *udl* under detection limit. **–** : not determined

For the same parameter, values followed by the same letter are not significantly different (p < 0.05)

was observed after 45 days of treatment, while in CS microcosm after a year, the reduction of DTC was of 45 %, reaching a remaining value similar to the S$_0$ one.

At the end of the treatments, the AHs, PAHs, and bioavailable PAHs fraction concentrations were measured (Table 1). Results indicated that the effectiveness of each treatment on hydrocarbon elimination as well as on the PAH bioavailability was different.

Figure 1 shows the relative abundance of each fraction of AHs (low, medium, and high molecular weight, named as C9-C19, C19-C29, and C29-C35, respectively) and in Supporting Material, Table S1 lists the average values and standard deviation of each fraction.

The BS microcosm showed no change in the AHs concentration or in the relative abundance of the AHs fractions (compared to S$_0$). The addition of organic matter to the SS and CS (4 months) microcosms increased the AHs content, reaching 5.0 and 10.4 g/kg, respectively. It is noteworthy that CS microcosm achieved a significant elimination of AHs (about 24 %) after one year (Table 1).

In CS microcosm at the end of the treatment (12 months) an increment in the relative abundance of the C29-C35 fraction was observed. The final AH profile
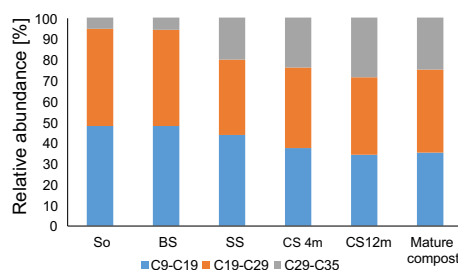

Fig. 1 Aliphatic hydrocarbon (AH) profiles of S$_0$ and bioremediation treatments. Mature compost was included as comparative system. Composting soil in vitro was measured at 4 months and at the end of the treatment for monitoring the process

was similar to that observed for MC, suggesting a typical metabolic activity of a composting treatment.

No treatment produced the net PAH removal. However, changes in the relative PAH composition were detected in the amendment microcosms. Fluorene, phenanthrene, and acenaphthene were significantly reduced in SS and CS (12 months) microcosms. Particularly, an increase in benzo[g,h,i]perylene was observed in CS microcosm, suggesting that during the composting treatment this high molecular weight PAH was released from the soil matrix (see Supporting Material, Table S2).

A 2.1 ppm of bioavailable PAHs (1 % of the total PAH, Table 1) was determined in S_0, represented by pyrene, benzo[a]anthracene and benzo[a]-pyrene (Figure S1). Only the amendment treatments (SS and CS) affected the bioavailability of PAHs, suggesting a great transformation of the soil matrix (Figure S1). A significant increase in bioavailable benzo[a]pyrene was detected after a year of composting (CS), while acenaphthylene, acenaphthene, fluoranthene, pyrene, benzo[a]anthracene, benzo[b]fluoranthene and benzo[a]pyrene were the bioavailable PAHs detected in SS microcosm. In both treatments, the increased bioavailability did not contribute to PAH elimination.

Figure 2 shows the FEEMs of the extracts from the S_0, BS, SS, and CS microcosms. An aqueous solution with a mix of 16 PAHs, alkaline extract of MC and pristine soil (PS) were included as controls to interpret the FEEMs. The matrix for the PAH mix (Fig. 2e) showed an intense emission in the region [λ^{em} = 280–440 nm, λ^{exc} = 250–325 nm], named as *region 1* and assigned to the emission of PAHs. The alkaline extract from PS (Fig. 2f) showed a dominant peak in the region [λ^{em} = 360–460 nm, λ^{exc} = 290–350 nm]. MC (Fig. 2g) showed a similar emission region to that of PS but with lower intensity of fluorescence. This region was named as *region 2* and assigned to the emission of organic matter.

The FEEMs of S_0 (Fig. 2a) showed two major contributions, corresponding to *regions 1* and *2*. BS (Fig. 2b) showed a major peak in *region 1*, and a minor contribution in *region 2*. The amendment microcosms, SS and CS (Fig. 2c, d, respectively) showed only one peak, assigned to the organic matter fluorescence (*region 2*). It is noteworthy that the excitation wavelength range of *region 2* presented a shift to lower values from S_0 to CS (Fig. 2a, d), reaching the MC profile. A similar behaviour was observed during a domestic waste composting process by Vieyra et al. (2009), which was related to the humification degree of the composting process.

In order to obtain more information about the effect of soil treatments on the FEEMs a PARAFAC analysis was performed. This is a mathematical analysis employed to study complex systems, which allows determining the number of fluorescent components (fluorophores) that contribute to the matrices, as well as to convolve their spectra. The experimental data

could be adequately fitted to a model consisting of 13 fluorophores. The emission and excitation spectra of each one can be seen as Supporting Material (Fig. S2A and S2B). This analysis revealed significant differences in the behaviour of each system (Supporting Material, Fig. S3).

From the analysis of the control systems (PAHs, MC and PS), the components could be bunched into 3 groups: 5 components for the fluorophores from PAHs, 4 components for the fluorophores from MC, and 3 components for the fluorophores from PS. One component (yellow in Supporting Material, Fig. S3) had a low contribution in the control systems but an important one in S_0 and BS. The relative contribution of these 4 groups to the systems is shown in Fig. 3. Microcosms S_0 and BS presented similar profiles, with contributions of the 4 groups. Microcosms SS and CS showed an increased contribution of groups from MC and PS, with a profile similar to MC. In both microcosms the higher E_4/E_6 value (Table 1) suggested the presence of low molecular weight compounds with a minor condensation degree and aromaticity. The changes observed in SS and CS microcosms suggested that the addition of organic matter allowed the mobilization of contaminants through the transformation of the soil matrix.

Effect of exogenous organic matter addition on soil biological activity

Among all enzymes in the soil environment, dehydrogenases are one of the most important due to their intracellular presence in all living microbial cells (Moeskops et al. 2010). Dehydrogenase activity was used to monitor the biological activity in the microcosms during all the incubation period. To test the effect of the bioremediation strategies on the microbial activities, arylsulphatase, urease, lipase, and phosphatase activities were determined at the end of the treatments and were compared with S_0 values.

Only the amended microcosms (SS and CS) developed dehydrogenase activity (Supporting Material Fig. S4). The activity period lasted approximately one month in SS microcosm and then, a decrease to the initial value was observed until the end of the treatment, 45 days (132 ± 14 µg TPF g^{-1} dry soil). Although with high variability attributed to the lack of homogeneity, the highest dehydrogenase activity was
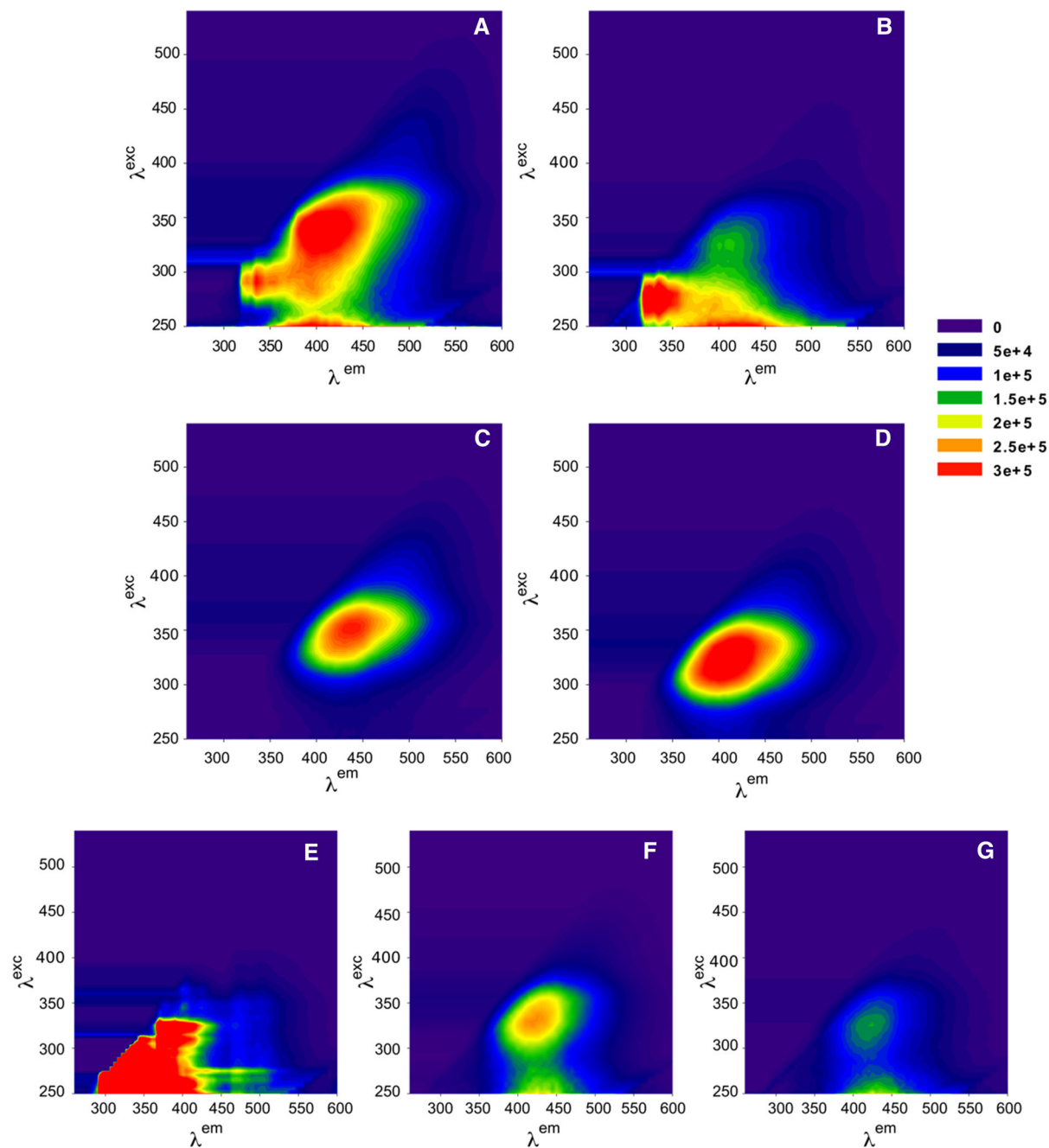
Fig. 2 Fluorescence emission–excitation matrices (FEEMs) of the dissolved organic matter (DOM) from **a** S_0; **b** BS microcosms; **c** SS microcosms; **d** CS microcosms. Controls: **e** Aqueous solution of 16 PAHs; **f** Alkaline extract of pristine soil (PS); **g** Alkaline extract of mature compost

detected in CS microcosm during the first month of treatment. After that, the activity slowly declined over the 120 incubation days and remained detectable after the year of treatment (24 ± 13 μg TPF g^{-1} dry soil).

However, it could be assumed that composting generated a more stable material than stimulation.

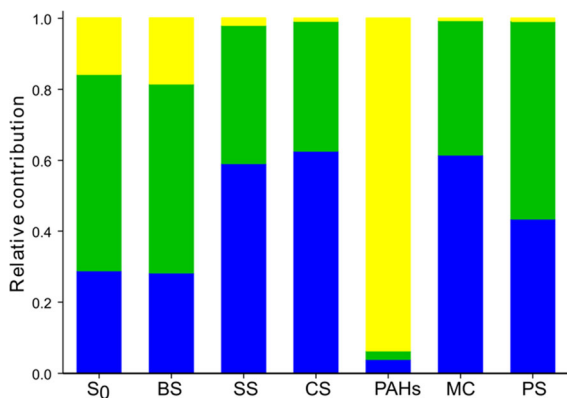Table 2 shows the enzymatic activity values from the microcosms at the end of the treatments. All the

Fig. 3 Contribution of different types of fluorophores determined from the PARAFAC analysis of the FEEMs of the alkaline extract for each microcosm, including the pristine soil (PS), the aqueous extract from the mature compost (MC), and an aqueous solution of 16 PAHs (PAHs). Organic matter contribution is shown in blue and green, and PAH contribution in yellow

enzymes remained at a high level in the amendment microcosms (SS and CS).

The significant increase of the lipase activity can be associated with the aliphatic hydrocarbon mobilization detected in SS and CS microcosms, as reported (Brzeszcz et al. 2016). The relatively lower lipase value in CS microcosm could be attributed to the advanced process of transformation of organic matter (one year in CS vs. 45 days in SS). Taking into account that lipase activity can remain at elevated values for a long period of time in hydrocarbon-contaminated soils (Margesin et al. 2000), our results

suggested the contribution to the degradation of aliphatic hydrocarbons by a microbial community be lately established in the CS microcosms.

Urease, arylsulphatase, and phosphatase activities in SS and CS were higher than in S_0 demonstrating the potential of the microbial community for the degradation of organic matter.

Effect of exogenous organic matter addition on the acute toxicity

The GI% was determined by testing the aqueous extract (100 %) and four dilutions (5 %, 10 %, 25 %, and 50 %) of the microcosms at the end of the treatments. Only the aqueous extract showed significant differences between treatments. The values of the index were 28 ± 15, 5 ± 13, 42 ± 23, and 98 ± 4 for S_0, BS, SS, and CS, respectively. Even though the GI% showed high deviation, it is interesting to note that this index increased along time in CS reaching a higher value than S_0 (data no shown).

Morphometric parameters of *Lactuca sativa* L. germination are a sensitive toxicity test for the phytotoxicity of long-term petroleum hydrocarbon-contaminated soils (Masakorala et al. 2013). Then, particular aspects of the morphometric variables, such as the radicle and hypocotyl elongation, were recorded (Fig. 4). The seeds exposed to the aqueous extract (100 %) of S_0 and BS microcosms produced seedlings without secondary radicles (Fig. 4b, c, respectively). The primary root was thinner than the control roots (water, Fig. 4a). In addition, the elongation of the

Table 2 Enzymatic activities determined from microcosms at the end of the treatments

| | S_0 | Bioremediation treatments | | |
		BS	SS	CS
Lipase [µg pNP g_{ds}^{-1}]	0.19 ± 0.01(a)	0.17 ± 0.01(a)	0.32 ± 0.03(c)	0.26 ± 0.04(b)
Urease [µg N-NH4 g_{ds}^{-1}]	23 ± 5(a)	17 ± 2(a)	64 ± 6(b)	76 ± 4(b)
Arylsulphatase [ngpNP g_{ds}^{-1}]	33 ± 1(a)	*udl*	59 ± 1(c)	47 ± 2(b)
Protease [mg Tyr g_{ds}^{-1}]	*udl*	*udl*	0.20 ± 0.01(a)	0.09 ± 0.05(a, b)
Ac. Phosphatase [µg pNP g_{ds}^{-1}]	0.10 ± 0.02(a)	0.04 ± 0.03(a)	0.6 ± 0.1(b)	1.4 ± 0.4(c)
Alk. Phosphatase	0.18 ± 0.02(a)	0.30 ± 0.02(a)	1.34 ± 0.07(c)	0.8 ± 0.1(b)

uld under detection limit

For the same parameter, values followed by the same letter are not significantly different (p < 0.05)

hypocotyl was observed. On the other hand, seeds exposed to the aqueous extract of SS and CS microcosms produced smaller seedlings with a thickened primary root with secondary roots and a shorter hypocotyl (Fig. 4d).

Effect of exogenous organic matter addition on soil microbial populations

Figure 5 shows the time evolution of PAH degrading bacteria (PAH-DB), AH degrading bacteria (AH-DB), and cellulose degrading bacteria (C-DB). No changes in PAH-DB were observed, in agreement with the lack of effect of the treatments on PAH concentration. SS and CS presented an increase in AH-DB and C-DB with respect to S_0 at the beginning of the treatments, according to the amendment addition. On the other hand, AH-DB and C-DB in BS did not present significant changes compared to S_0.

The heterotrophic bacterial community decreased at least one order at the end of the bioremediation treatments (Fig. S5, Supporting Material). Compost addition (in SS) and composting in situ (in CS) produced an increase in phosphorous solubilizing bacteria (Figure S5). Interestingly, this community was stimulated at the start of the composting process, probably as a result of the P provided by the amendment.

The effects of different remediation treatments on the bacterial community structure were analysed by PCR-DGGE. Banding patterns of two independent replicates at the end of each treatment were analysed (Supporting Material, Fig. S6). The dendrogram obtained with primers 341F-GC and 907R showed changes in bacterial community structure in the microcosms. The proximity of samples indicated a higher degree of community similarity.

In order to know the bacterial community structural changes, by species richness [^0D], diversity [^1D], and equality [^2D], pyrosequencing analysis was done. The pyrosequencing process provided 24970 sequences, and pyrosequencing-based analysis and subsequent statistical inference provided 20928 prokaryotic sequences (average length of 389 bp) after the trimming process (Table 3). The Good's coverage in all samples was above 76 %, and the rarefaction curves did not approach saturation in any of the samples, indicating that there might be some undetermined microbes (Supporting Material, Fig. S7). Sequences were clustered in operational taxonomic units (OTUs), on the basis of a distance of 3 %. Rarefaction curves estimating OTU richness confirmed the difference between S_0 and the samples after different treatments.

Regarding the stimulated microcosms, the diversity index only showed difference in SS microcosm, with similar ^1D and ^2D values compared to S_0, indicating that the mature compost addition increased the species richness in the soil but kept a similar number of typical species with a similar number of dominants. Contrariwise, the composting in situ, CS microcosms, preserved the species richness but reduced the number of typical species yielding a more uneven community. The low ^0D, ^1D, and ^2D values determined in BS microcosm suggested that the bioremediation treatment produced the selection of a community reduced in species richness and diversity after one year.

Figure 6 shows the proportion of dominant orders that define each microbial community in each microcosm. The common dominant orders (with relative abundance > 2 %) were Actinomycetales (> 13.6 %), Rhizobiales (> 8.3 %), Acidimicrobiales (> 4.8 %), Sphingomonadales (> 3.8 %), and
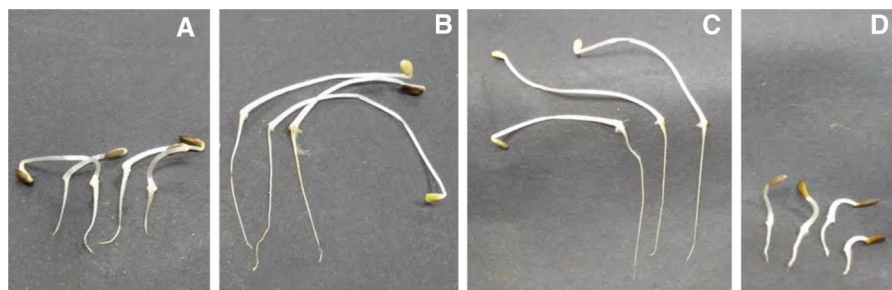

Fig. 4 Macroscopic aspect of *Lactuca sativa* L. seedling after 5 days with water (**a**), and pure extract of S_0 (**b**), BS (**c**), and SS (**d**)
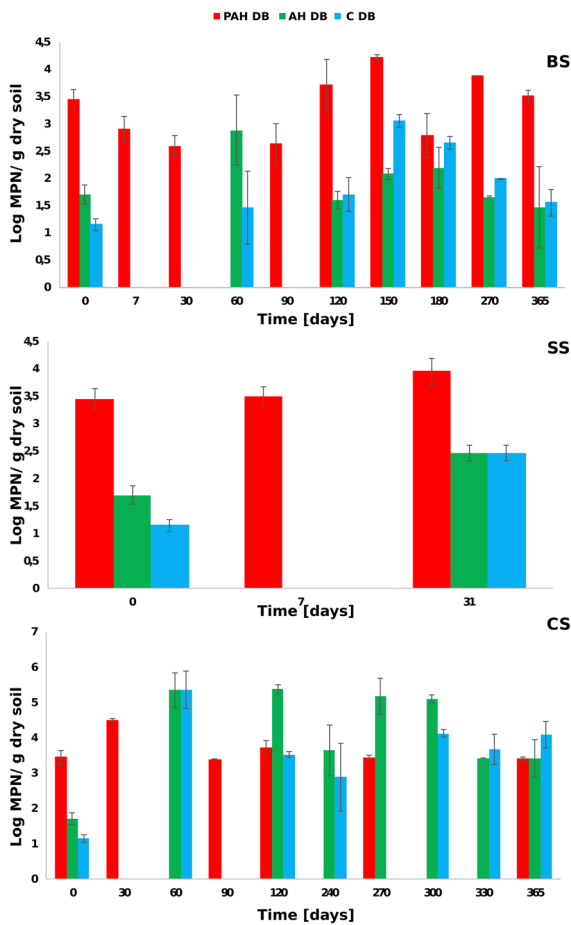
Fig. 5 Dynamics of PAH degrading bacteria, AH degrading bacteria, and cellulose degrading bacteria throughout the incubation period by MPN for bioremediation (BS), stimulation with mature compost (SS) and composting (CS). The results are the means of triplicate independent microcosms with the brackets representing the standard deviation

Xanthomonadales (> 2.0 %). Abbasian et al. (2016a) found that soil contaminated with crude oil had a microbial community where dominant members were Actinomycetales (Gram-positive bacteria) as well as Rhizobiales and Sphingomonadales (both belonging to Gram-negative bacteria). Abbasian et al. (2016b) found *Acidimicrobiales* as dominant member, a known genus belonging to Actinobacteria, which is able to use iron after aeration treatment of aliphatic hydrocarbon-contaminated soil. Stefani et al. (2015) also found Xanthomonadales as dominant bacterial member in soils collected from a petrochemical plant. This bacterial order includes hydrocarbon degraders, generalist and obligate hydrocarbonoclastic bacteria (Gutierrez 2019). Interestingly, all biological treatments produced the positive selection of Ohtaekwangia (Gram-negative bacteria) along with the decrease of Acidobacteria Gp3, Acidobacteria Gp7, and Acidimicrobiales. Although the Ohtaekwangia's ecological role in the soil is still unclear, its abundance in hydrocarbon-contaminated soils subjected to remediation treatments was reported by other authors (Hou et al. 2015; Wang et al. 2016; Medina et al. 2018, 2020).

The relative order frequency that defines the taxonomic profiles of the bacterial community at order level (relative abundance > 2 %) is shown in Supporting Material (Fig. S8), and its complete phylogeny is listed in Table S3. The most abundant classified orders found in S_0 were Actinomycetales (38.8 %) and Sphingomonadales (12.0 %). Among Actinomycetales, *Nocardioides* was the dominant genus in S_0, while the Gram-negative

Table 3 Diversity parameters for the different communities obtained by analysis of pyrosequencing data at the end of each remediation treatment. The results correspond to the original contaminated soil (S_0) and after the bioremediation treatments (BS, SS, and CS microcosms)

Microcosms	Initial total sequences	Final total sequences	Observed OTUs	Good´s coverage	^0D	^1D	^2D
S_0	10666	6950	230	85	473	62	43
BS	5078	3306	169	91	236	47	27
SS	5848	3830	306	76	704	68	37
CS	3378	1808	262	84	418	66	33

[a]Values based on 1808 random sequences per sample calculated using EstimateS software version 9.1.0
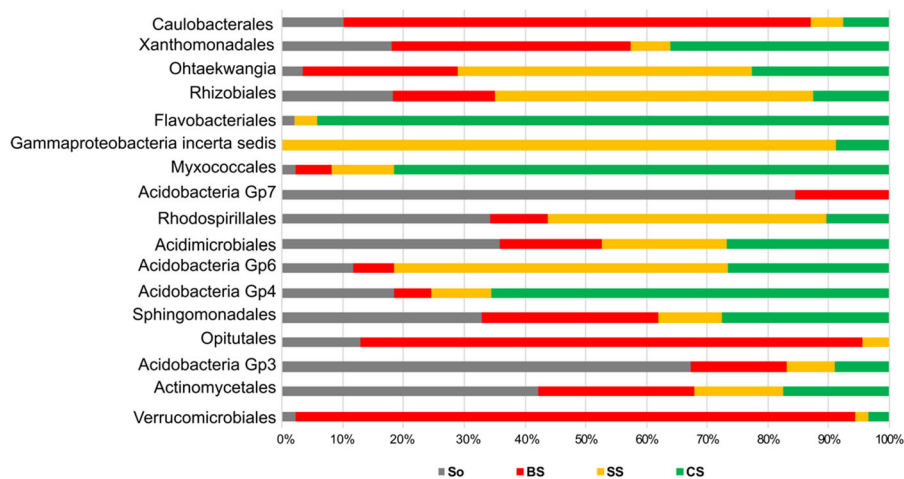
Fig. 6 Bacterial community structure. Proportion of dominant orders in each microcosm

Sphingomonadales order was composed of members of *Sphingomonas*. By bioremediation treatment, a positive selection of Verrucomicrobiales and Opitutales orders was found (Fig. 6). A decrease of Actinomycetales (23.7 %) and Sphingomonadales (10.7 %), along with the positive selection of Caulobacterales and Xanthomonadales, was found, which increased their abundance up to 14.8 % and 12.2 % respectively. However, the abundance of *Nocardioides* and *Sphingomonas* genera was not modified in regard to S_0. Moreover, among Caulobacterales order *Brevundimonas* was the dominant genus (10.1 %). Contrarily to ours results, Militon et al. (2010) found that the abundance of Gammaproteobacteria was replaced by Actinobacteria when an oxygen biostimulation process was applied to aged oil-polluted soils. Organic matter addition (SS and CS) caused the increment of Acidobacteria Gp6. On the other hand, compost addition (SS) produced the positive selection of Gammaproteobacteria with the decrease of Sphingomonadales orders, while composting of soil (CS) increased the Myxococcales, Flavobacteriales, and Acidobacteria Gp4 orders (Fig. 6a). Particularly, stimulation with mature compost (SS microcosms) resulted in a community more diverse than S_0 where the predominant orders were Rhizobiales (26.1 %), Acidobacteria Gp6 (13.9 %), and Ohtaekwangia (13.2 %). In addition, the Actinomycetales order decreased to 13.6 %. In spite of the diversity indices, composting of contaminated soil (CS microcosms) showed a bacterial community with an arrangement characterized by the presence of

Myxococcales (14.2 %) where the dominant genus was *Phaselicystis* (4.1 %). Moreover, the dominant orders in CS were Actinomycetales (16.2 %), Xanthomonadales (11.2 %), and Sphingomonadales (10.1 %) with a distinctive abundance compared to S_0.

Discussion

Effect of organic matter addition on soil matrix and on hydrocarbon elimination

Bioremediation treatments produced the microbial community stimulation, a fact that was evidenced by DTC consumption. Particularly, in both amended microcosms, CS and SS, DTC was consumed (about 29 % and 45 %), mainly from the exogenous sources, as reported in a previous work (Medina et al. 2020). As described by Shirshova et al. (2006) the E_4/E_6 ratio is indicative of the molecular size, condensation degree, and aromatization of humic acids. During composting, humic substances are modified in terms of their structure and chemical properties, releasing aliphatic compounds and carbohydrates and resulting in the increase in the degree of condensation (Plaza et al. 2009). Antízar-Ladislao et al. (2006) used FEEMs to monitor humic substance formation and showed that peak excitation wavelength shifts and peak fluorescence intensity can both be used as indicators to monitor the humification or maturation of compost.

While BS microcosms showed a lower E_4/E_6 ratio, CS and SS presented higher values than S_0 evidencing

a distinctive humic substance composition as a result of different treatments. Based on this, it is possible to assume that the BS microcosm experienced a degree of transformation of the endogenic organic matter similar to that produced in the MC, as indicated the low E_4/E_6 value associated with the aromatic and molecular weight of the humic substances determined in both substrates (Lguirati et al. 2005). On the other hand, the higher values of DTC and E_4/E_6 found in SS and CS with respect to BS suggested a low condensation degree of the organic matter, indicating that the biological process might be continued.

In a previous work we found that stimulation of soil after chemical oxidation resulted in a new soil matrix where the organic carbon content was high with a high condensation degree (DTC = 189 mg C/l, E_4/E_6 = 3.8), and as a consequence, hydrocarbon elimination by co-metabolism failed (Medina et al. 2020). However, composting after chemical oxidation resulted in a soil matrix with low organic matter content, and low condensation degree (DTC = 62.2 mg C/l, E_4/E_6 = 23). In addition, the change found in aliphatic hydrocarbon profiles was in line with the organic matter transformation described by Plaza et al. (2009).

Several factors affect PAH elimination: microbial community highly selected, aging of contamination, bioavailable PAH fraction, nature of the organic matter added as well as PAH dilution effect. In regard to organic matter interaction, some authors concluded that the quality of organic matter and the time of interaction with hydrocarbons determined the rate of release/adsorption of PAHs, modifying their elimination and extractability (Bamforth and Singleton 2005; Lladó et al. 2009; Wu et al. 2013; Godlewska et al. 2017). For example, Wu et al. (2013) described that during composting the stabilization phase was characterized by 90 % PAH losing processes at the expense of the bioavailable fraction, while many variables could interfere with the elimination processes from contaminated soil. In a previous publication (Medina et al. 2020), the authors observed that the composting after oxidative treatment produced a significant increase in the bioavailability of PAHs, reaching 56 % of total PAHs. However, in the present study, a similar composting procedure led to only 6 % of total PAHs, showing that the bioavailability of one system strongly depended on its global history.

The absence of PAH elimination in BS was probably the result of their low bioavailability and the aging of the soil. By organic matter addition, CS and SS, significant increments in bioavailable PAH fractions were observed. However, these increments did not correlate with net PAH elimination. The lack of PAH elimination could be attributed to the low PAH concentration in the soil matrix due to the soil dilution effect in SS and CS, which could be is insufficient to stimulate the microbial degrading capacity in amendment soil, or to the preference for easily available carbon sources by the microbial community. This behaviour was reported several times. For example, Zappi et al. (1996), reported that supplying growth and energy sources by itself did not improve the biodegradation rates, when studying the degradation of hydrocarbons by bioremediation of soils containing low levels of PAHs (around 0.07 g/kg). Additionally, Sayara et al. (2010b) used a sandy loam soil spiked with 6 PAHs and composted under different conditions. They observed that the lowest degradation rate (18 %) correlated with the lowest PAH concentration (0.1 g/kg) and explained this behaviour assuming that this concentration was below the levels needed to begin the degradation process in the presence of easily available materials. Moreover, in a study about the remediation of petroleum contaminated soils through composting degradation (Wang et al. 2011), the authors argued that a microorganism's selection of nutrients could delay the degradation of pollutants, as normally microorganisms prefer easily degradable materials over resistant ones. Then, it could be thought that the initial stimulation was not effective to keep the autochthonous PAH degraders from S_0 active.

From our results, the behaviour observed by composting suggested that a redistribution of the bioavailable and sequestered or unavailable PAH fractions could have occurred along the year. Although some low molecular weight PAHs were not detected at the end of the treatment in CS microcosms, the release of other PAHs probably due to the weakening of unions during the compost

maturation phase resulted in the absence of net elimination of these contaminants.

Effect of organic matter addition on biological soil properties and microbial community

Our results showed that organic matter transformations, endogenous as well as exogenous, which occurred by organic amendment addition, promoted the functional recovery of the soil and cultivable bacterial community. These processes were accompanied by the reduction of phytotoxicity in spite of high electrical conductivity and no net hydrocarbon elimination.

Dehydrogenase activity showed the recovery of oxidation-reduction biological processes after organic matter addition (Nannipieri et al. 2002). The augmented lipase values suggested the active mobilization of AHs (Brzeszcz et al. 2016; Medina et al. 2018) along with the increases of AH-DB and C-DB population. Arylsulphatase and phosphatase activities reflected the active S and P cycles, the latter evidenced by the increment in phosphorous solubilizing bacteria counts. Organic matter addition provided porosity and nutrients such as compounds with C, N, P, and S to the soil (Elfstrand et al. 2007) and then, a different microbial population was stimulated, mobilizing these elements and restoring soil functionality. In addition, S and P utilization, mediated by arylsulphatases and phosphatases, was driven by microbial demand for S and P independently of C availability (McGill and Cole 1981).

In regard to phytotoxicity assay, the toxicity of a compound may change during the bioremediation process, because toxic metabolites or transformation by-products could be produced resulting in increased soil toxicity (Morelli et al. 2005). In addition, Morelli et al. (2001) reported mutagenic persistence along the bioremediation of a petrochemical sludge and attributed this effect to the relationship between easily degradable hydrocarbons and PAH concentration. Also, the germinated seeds could show changes in root morphology due to the negative impact of hydrocarbon-contaminated soil (Masakorala et al. 2013). The increases in EC is another factor that could cause a reduction of the germination percentages, as was observed by Esechie (1994) for sorghum seeds (EC ranged from 27.2 to 37.2 dS/m; GI%<50). Therefore, the high values of EC and the bioavailable

PAH fraction in the SS and CS microcosms could contribute to the detrimental effects on germination. In the present study, roots germinated using the supernatant of amendment soil microcosms (CS and SS) showed morphology similar to the control (seeds embedded in water), suggesting the reversion of the toxicological effect despite the EC and PAH bioavailability increment.

All physical, chemical, and biological changes were accompanied by modifications of bacterial communities.

Based on the dominance of members of *Nocardioides* and *Sphingomonas* genus, both harboured of dioxygenase genes (Cébron et al. 2008), we can infer that S_0 bacterial community was highly selected with an intrinsic potential for hydrocarbon degradation.

The microbial community changes produced during bioremediation (BS microcosms) reflected substrate consumption, where members of Actinomycetales and Sphingomonadales were gradually replaced by Caulobacterales, Xanthomonadales, Verrucomicrobiales, and Opitutales. Caulobacterales members were reported as lignocellulosic polymers degraders (Wilhelm et al. 2018), Xanthomonadales included hydrocarbon degraders as well as generalist microorganisms (Gutierrez 2019), Verrucomicrobiales was suggested as aerobic organotrophic specialist (Chen et al. 2020), while Opitutales members were described as harboured of genes related to C-source utilization and N cycling (Hester et al. 2018).

The addition of organic matter (CS and SS microcosms) produced the increase of Acidobacteria Gp6. In fact, Acidobacteria members are frequently described as oligotrophic bacteria belonging to the K-strategist category and seem to be favoured under resource-limited conditions (Fierer et al. 2007). A high ratio of Proteobacteria to Actinobacteria was observed in CS (2.4), probably as the result of the nature and concentration of the available organic matter (Medina et al. 2020). In addition, Covino et al. (2016) reported that the high incidence of Actinobacteria (24.4 %) was characteristic of the thermophilic stage of the composting treatment. Then, this ratio suggested that the composting process in CS was not completed, in agreement with its E_4/E_6 value.

SS microcosms showed an increase in bacterial diversity, and this microbial community was characterized by members of Rhizobiales and Ohtaekwangia. The dominance of Rhizobiales could contribute to

functional restoration through the N cycle (Garrido-Oter et al. 2018), while members of Ohtaekwangia could degrade high molecular weight organic compounds (Drury et al. 2013). Decrease in Actinomycetales order could indicate the successional shift, probably driven by organic matter nature and content. In addition, the low abundance of Actinobacteria suggested that the process induced by compost addition (SS microcosms) was not finalized, in line with E_4/E_6 results presented in this work.

CS microcosms were characterized by the presence of members of Myxococcales, Flavobacteriales, and Acidobacteria Gp4 orders. Among Myxococcales, the dominant genus was *Phaselicystis*. Members of this genus have been isolated from soil samples and decomposing plant materials (Garcia and Müller 2014). Flavobacteriales are known Gram-negative and copiotrophic bacteria. Members of this order were isolated from soils and related to high molecular weight C-source utilization such as starch, cellulose, and chitin (Fierer et al. 2007; Eilers et al. 2010). Hence, the microbial community found in CS suggested an increase in catabolic diversity that led to the observed modifications in the organic matter and the hydrocarbon content.

Applying the same composting approaches in the same soil previously treated with persulfate Medina et al. (2020) found that organic amendments produced the increase of Gemmatimonadetes (> 8.25 %) while in this work we observed that composting of soil (CS) stimulated Bacteroidetes and compost addition (SS) stimulated Actinobacteria.

Therefore, the stimulation of the bacterial community by adding organic matter depends on the characteristics of the soil including its history: the application of a previous chemical treatment determines the populations that adapt and colonize this environment.

Conclusions

Composting and the addition of mature compost on a soil chronically contaminated with hydrocarbons stimulated the microbial populations and increased the bioavailable fraction of PAHs. However, no net PAH elimination was found. The low PAH elimination efficiency could be attributed to the lower PAH content to stimulate the microbial degrading capacity

and to the preferential consumption of easily assimilated C sources by the bacterial community.

Regarding the quality of the soil, the nutrients provided by the exogenous organic matter contributed to the recovery of the global functions and species diversity of the soil along with the reduction of phytotoxicity.

The improvement in soil condition, together with the fact that the composting process is still ongoing, allows us to suppose that the proposed strategies would reduce contamination in longer treatment times. However, the combination with other approaches such as addition of surfactants and/or inoculation with specific consortiums could be considered, taking advantage of the improvement achieved in soil quality.

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