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Effectiveness of biochar application and bioaugmentation techniques for the remediation of freshly and aged diesel-polluted soils

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ABSTRACT

In order to contribute to a minimum impact on soil biocenosis during the application of in-situ bioremediation techniques, this work assessed the efficiency of a scarcely used combination of biochar and a bioaugmentation based on an autochthonous bacterial consortium. Bioaugmentation-biochar combination was assessed by using soil samples from a polluted site with two pollution scenarios: a) soil with aged diesel, and b) clean soil to which fresh diesel was later added simulating a recent pollution event. The autochthonous consortium, isolated from the aged-diesel soil, was genetically, taxonomically and functionally characterized by these authors in a previous work. The biochar used was obtained from tree pruning residues. In both scenarios, four treatments were carried out under short-term test conditions: i) natural attenuation, ii) biochar, iii) bioaugmentation, and iv) biochar-bioaugmentation combination. Our results show that the bioaugmentation-biochar combination was significantly more effective than the simple treatments. This combination produced more than 20% diesel degradation in both scenarios over twelve weeks. Simultaneously, an increase in bacterial diversity was observed in that period. Therefore, using biochar combined with bioaugmentation suggests synergies that lead to a highly efficient and environmentally friendly bioremediation processes.

1. Introduction

Petroleum hydrocarbons constitute the largest group of environmental pollutants worldwide. Petroleum products are found in approximately 60% of contaminated soils within Europe (Panagos et al., 2013). Soil pollution by petroleum hydrocarbons can have different sources, among which spills and leaks from storage tanks or pipelines are the most common (Das and Chandran, 2011). At sites exposed to diesel contamination, soil biota in general and microbiota in particular are severely affected, limiting soil ecosystem services and even transforming previously pristine soils into a secondary source of contamination (Koshlaf and Ball, 2017). Diesel fuel is a complex mixture of aliphatic, cyclic, and aromatic saturated and unsaturated hydrocarbons that pollute the ecosystems, changing key soil physicochemical properties. When a hydrocarbon spreads through the soil, the lightest fractions volatilize, leaving the longest ones, as well as the aromatic chains with a greater number of rings, decreasing their solubility in water (Truskewycz et al., 2019). The recalcitrance of hydrocarbons in contaminated soils increases with the time of contact between the soil and the contaminant. This may be the effect of the incorporation of part of the pollutant into the soil fraction of organic matter, as well as the penetration of the hydrocarbon into small pores, which hinder the access of microorganisms, resulting in low bioaccessibility to the hydrocarbon (Hatzinger and Alexander, 1995; Khan et al., 2012; Koshlaf and Ball, 2017; Semple et al., 2003). This process is known as aging, and it results in lower rates of biodegradation due, in general, to a low bioavailability of the hydrocarbon.

Many petroleum hydrocarbon constituents are toxic and have mutagenic effects (Gallego et al., 2001; Gong et al., 2003; van Dorst et al., 2014). The potential toxicity and mutagenicity of hydrocarbons in soils leads to the implementation of effective soil remediation techniques. Physical and chemical soil remediation methods are usually expensive and often harmful to the environment, due to the aggressive use of potent oxidative agents combined with high temperature and

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pressure (Adipah, 2018; Usman et al., 2016). Biological techniques, however, include the use of microorganisms that, by forming communities, can metabolically cooperate in the use of hydrocarbon as a source of energy and carbon (Faust and Raes, 2012; Meulenberg et al., 1997; Sutherland et al., 1995). On the other hand, there may be exclusion interactions in the same ecological niche, where two or more species can compete for shared resources, and where the predominance of any of them will depend on a large number of environmental variables (Faust and Raes, 2012; Freilich et al., 2011; Ghoul and Mitri, 2016).

Bioremediation techniques enhance or accelerate the natural biodegradation capacity of native biota in contaminated soil. The degradation of hydrocarbons can be increased either by biostimulation of these populations, with the addition of nutrients, or by bioaugmentation, increasing the biomass of certain functional microorganisms in the degradation of the pollutant (Brzeszcz et al., 2020; Margesin et al., 2007; Yu et al., 2005). The added microorganisms can be autochthonous, including microbial consortia isolated by enrichment culture (Garrido-Sanz et al., 2019), or allochthonous, isolated in a different environment (Brzeszcz et al., 2020). The lack of specific biodegradation pathways for the target pollutants might require the addition of exogenous populations; however, the use of native populations is preferred as they are adapted to soil conditions. Similarly, different authors (Chen et al., 2012; Partovinia and Rasekh, 2018; Zhang et al., 2019) have tested the immobilization of bacteria as a bioaugmentation technique in contaminated soils, using different supports, including biochar.

Since biochar is a particularly heterogeneous material, several authors have found that its physicochemical properties, as well as the optimal charge density of the bacteria associated with biochar, are determining factors of the effectiveness of bioremediation processes (Chen et al., 2012; Galitskaya et al., 2016; Partovinia and Rasekh, 2018; Ren et al., 2020). The origin of the biochar and the pyrolysis temperature, condition different physical-chemical properties of the biochar, such as pH, water retention capacity or specific surface, among others; which determine the retention of nutrients and pollutants, contributing to the bioremediation process (Beesley et al., 2011, 2013; Xiao et al., 2018; Zhang et al., 2013). Specifically, biochar produced at high temperatures, as a by-product of gasification, exhibits higher sorption efficiency for the remediation of organic soil pollutants (Ahmad et al., 2014). Furthermore, biochar particles have an important role in the structure of microbial populations (McBeath et al., 2014; Obia et al., 2016; Peake et al., 2014), as well as in the formation of microhabitats, whose characteristics are conditioned by the structure and chemical properties of biochar (Noyce et al., 2016; Schnee et al., 2016).

Some bioremediation treatments, although they have proven their effectiveness, may show a negative impact on the soil biota or groundwater. On this matter, since biostimulation is based on the addition of nutrients such as N, the amount added to the soil can inhibit or promote the nitrification process, with the risk of increasing nitrogenous pollutants in leachates (Peltola et al., 2006; Simpanen et al., 2016; Thomé et al., 2017). In combination with bioaugmentation, the addition of surfactants increases the bioavailability of the hydrocarbon, but at the same time, surfactants modify the microbial structure of the soil with negative effects on some microbial populations (Wolf et al., 2019; Xu et al., 2018).

In this work we used a previously isolated and characterized bacterial consortium that was obtained from the enrichment culture of an aged diesel-polluted soil, which bioremediation efficiency was previously tested in short-term microcosm conditions (Garrido-Sanz et al., 2019). We hypothesize that the effectiveness of this autochthonous consortium can be significantly improved when biochar is part of the treatment. For this purpose, we compared the bioremediation ability of this consortium, like free cells, in combination with other remediation treatments, including natural attenuation and the addition of biochar, alone and combined with the autochthonous bacterial consortium. These treatments were evaluated in soils with two pollution scenarios: (1) an unpolluted soil on which fresh diesel was added, simulating a recent pollution event, and (2) a soil polluted by aged diesel. Also, the structure and diversity of bacterial communities were analyzed in order to study their evolution under the different bioremediation treatments and their relationships to the specific hydrocarbon fractions in response to such treatments. The application to contaminated soil of an autochthonous bacterial consortium and a biochar obtained from a gasification process of local pruning residues, makes this approach an environmentally friendly strategy for bioremediation of soils polluted with petroleum hydrocarbons.

2. Materials and methods

In order to achieve the objectives described above, the following tasks have been carried out, including tests under short-term microcosm conditions.

2.1. Site description, soil sampling, pre-treatment, and analysis

The polluted site is located in a fuel storage and loading facility in San Fernando de Cádiz, Spain (Garrido-Sanz et al., 2019) where restricted oil spills (currently inactive) have occurred during previous decades. Soil sampling was carried out according to pollutant distribution patterns based on the presence of old tanks and pipes, which has led to define two scenarios for the same soil type: soil A, in pollution-free sites, and soil B, in directly polluted sites by old spills. In both cases, soil samples correspond to slightly developed soils in fine-textured recent marine deposits and were obtained by means of pits at variable depths between 0 and 30 cm (del Reino et al., 2014). Directly from the above-mentioned site, approximately 3 kg of each type of soil (A and B) were dried, manually homogenized and sieved at 4 mm size. Average field concentration of total petroleum hydrocarbons (TPH) in both samples was 80 $\pm$ 4 mg kg^{-1} for unpolluted soil A and 2974 $\pm$ 143 mg kg^{-1} for aged-diesel polluted soil B. Subsequently, 200 g of each sieved soil were again homogenized in a mechanical rotary mixer for 24 h. Then, soil A was artificially polluted with 10,000 mg kg^{-1} of diesel obtained from current storage tanks, simulating a recent pollution event. Later, both soils were again subjected to manual and mechanical homogenization (rotary mixer) for 24 h. A quantity of 200 g of each soil was placed in pots for one month, prior to remediation treatments. After this month, initial average TPHs concentration (T0, included in Table 1) of the pot samples were 4816 $\pm$ 300 mg kg^{-1} for soil A (fresh diesel) and 3032 $\pm$ 97 mg kg^{-1} for soil B (aged diesel). TPHs variability between pots, within the same soil, were 10.8% and 5.5%, respectively. Prior to artificial pollution in soil A, average values for total carbon (TOC Analyzer) were as follows. Soil A: 0.30% total organic C, and 2.67% total inorganic C; Soil B: 6.17% total organic C and 2.62% total inorganic C. The easily oxidizable C (Walkley-Black wet oxidation method) was 0.1% and 2.9% in soils A and B respectively. When soil A was artificially polluted, the oxidizable C increased up to 0.5%. Each of the pots containing polluted soil were watered with 10% (v/w) of sterile Fåhraeus plant solution (Fahraeus, 1957), prior to the weekly bioaugmentation treatments. It is a generic mineral medium that allows an initial conditioning of the soil, whose contribution of nutrients is lower than that of any biostimulation.

2.2. Bacterial consortium growth

The bacterial consortium previously obtained (Garrido-Sanz et al., 2019) was routinely grown in a liquid culture of minimal salt medium (MM) (Brazil et al., 1995) supplemented with 1 ml l^{-1} of phosphate buffered mineral medium salts (PAS) (Bedard et al., 1986) and 0.005% of yeast extract. Diesel oil was supplemented at 1 ml l^{-1} as the sole carbon and energy source. The specific composition of the diesel oil has been previously described (Garrido-Sanz et al., 2019). The culture was grown at 28 °C in a rotary shaker at 140 rpm for 7 days (Garrido-Sanz

Table 1
Evolution of mean concentrations (mg·kg^{-1}) of aromatic and aliphatic fractions for soils A and B, treatments NT (Natural attenuation), BC (Biochar), BA (Bioaugmentation), SN (Biochar + Bioaugmentation) and times T0, T1 and T2. Data in brackets indicate the percentage of degradation of each treatment with respect to T0.

SOIL	FRACTION	T0	T1 = 6 weeks (mg·kg^{-1})				T2 = 12 weeks (mg·kg^{-1})			
			NT (%)	BC (%)	BA (%)	SN (%)	NT2 (%)	BC2 (%)	BA2 (%)	SN2 (%)
A	>C10–C12	354	32 (91)	21 (94)	24 (93)	13 (96)	18 (95)	20 (94)	20 (94)	16 (95)
	>C12–C16	1064	781 (27)	716 (33)	713 (33)	602 (43)	563 (47)	553 (48)	652 (39)	533 (50)
	>C16–C21	1340	1230 (8)	1188 (11)	1225 (9)	1120(16)	1116 (17)	1099 (18)	1296 (3)	1074(20)
	>C21–C35	439	582 (−33)	739 (−68)	576 (−31)	550 (−25)	407 (7)	414 (6)	465 (−6)	402 (8)
	>C35	28	9 (67)	14 (52)	7 (77)	6 (77)	10 (63)	9 (67)	7 (74)	8 (72)
	>EC10-EC12	296	108 (64)	42 (86)	58 (81)	36 (88)	50 (83)	37 (87)	48 (84)	20 (93)
	>EC12-EC16	153	65 (58)	66 (57)	58 (62)	47 (69)	47 (69)	47 (69)	42 (72)	33 (78)
	>EC16-EC21	992	566 (43)	553 (44)	603 (39)	481 (52)	553 (44)	529 (47)	492 (50)	401 (60)
	>EC21-EC35	134	100 (25)	80 (40)	97 (27)	83 (38)	69 (49)	69 (48)	61 (54)	55 (59)
	>EC35	18	10 (44)	29 (−62)	12 (31)	11 (38)	7 (61)	10 (45)	10 (43)	6 (66)
	Aliphatic	3225	2634(18)	2672 (17)	2544 (21)	2292 (29)	2114 (34)	2095 (35)	2440 (24)	2033 (37)
	Aromatic	1592	848 (47)	769 (52)	826 (48)	658 (59)	725 (54)	692 (57)	654 (59)	515 (68)
	TPH	4816	3482(28)	3442 (29)	3370 (30)	2950 (39)	2840 (41)	2787 (42)	3094 (36)	2548 (47)
B	**FRACTION**	**T0**	**NT (%)**	**BC (%)**	**BA (%)**	**SN (%)**	**NT2 (%)**	**BC2 (%)**	**BA2 (%)**	**SN2 (%)**
	>C10–C12	4	3 (38)	1 (75)	1 (67)	2 (58)	4 (0)	5 (−25)	5 (−25)	2 (44)
	>C12–C16	156	164 (−5)	138 (12)	146 (6)	134 (14)	152 (3)	147 (6)	153 (2)	103 (34)
	>C16–C21	580	548 (5)	542 (7)	550 (5)	477 (18)	571 (1)	572(1)	600 (−4)	489 (16)
	>C21–C35	1061	920 (13)	1382 (−30)	1159 (−9)	1051 (1)	973 (8)	1044 (2)	699 (34)	944 (11)
	>C35	113	184 (−63)	56 (51)	51 (55)	57 (49)	82 (28)	80 (29)	21 (82)	54 (52)
	>EC10-EC12	12	6 (48)	4 (66)	3 (75)	28 (−136)	8 (31)	12 (1)	9 (21)	5 (56)
	>EC12-EC16	9	7 (19)	6 (27)	5 (45)	11 (−21)	7 (16)	8 (4)	4 (49)	5 (47)
	>EC16-EC21	520	470 (9)	440 (15)	408 (22)	427 (18)	551 (−6)	548 (−5)	514 (1)	354 (32)
	>EC21-EC35	551	565 (−3)	661 (−20)	528 (4)	500 (9)	517 (6)	533 (3)	387 (30)	354 (36)
	>EC35	28	104(-276)	130 (−371)	132 (−377)	79 (−186)	66 (−140)	74 (−166)	52 (−88)	54 (−94)
	Aliphatic	1914	1816 (5)	2118 (−11)	1907 (0)	1720 (10)	1783 (7)	1848 (3)	1477 (23)	1592 (17)
	Aromatic	1119	1153 (−3)	1368 (−22)	1198 (−7)	1202 (−7)	1150 (−3)	1174 (−5)	966 (14)	772 (31)
	TPH	3032	2969 (2)	3486 (−15)	3104 (−2)	2922 (4)	2932 (3)	3022 (0)	2443 (19)	2364 (22)

et al., 2019), achieving an optical density (OD) at 600 nm between 0.25 and 0.3, which corresponds to 1–1.5 10^7 CFU ml^{-1}. The autochthonous bacterial consortium used in this study is mainly composed of the following genera: *Pseudomonas* (27.0%), *Aquabacterium* (22.4%), *Chryseobacterium* (15.3%), *Sphingobium* (5.2%), *Novosphingobium* (3.7%), *Dokdonella* (3.3%), *Parvibaculum* (3.2%) and *Achromobacter* (2.5%), as previously described (Garrido-Sanz et al., 2019).

2.3. Biochar characteristics

Biochar raw materials were composed of tree pruning residue (pine and olive trees). Prior to the start of the pyrolysis, raw materials were subjected to a drying process at 95 °C for 2 h, and large pieces were fragmented to a maximum diameter of 1–2 cm. Since the aim of pyrolysis was specifically energetic, the process was carried out at temperatures up to 700 °C, yielding approximately 10% biochar (w/w). The nature of raw materials and pyrolysis parameters determined the physicochemical properties of the obtained biochar; among them: TPHs = 0.507 mg g^{-1}; surface area = 7.8 m^2 g^{-1} (BET method); pH = 8.4 (1:10 ratio); easily oxidizable organic carbon = 5.1% (w/w; Walkley-Black method) and total organic C (TOC) = 66.0%.

2.4. Experimental design and remediation treatments

A short-term microcosm experiment has been developed in order to test the hypothesis cited above. Four different treatments, three replicates each, were applied to each pot containing 200 g of both A and B soils. These treatments consisted in the following:

1. Natural attenuation (NT): No biochar or bacterial consortium was applied to the soils.
2. Biochar (BC): Soils were amended with 10% of the biochar described above, manually homogenized and then in an automatic tumbler for 2 h and three weeks prior the start of the experiment (see below). The use of this proportion of biochar is supported by previous studies that show that certain increases in the addition of biochar imply a higher sorption capacity on organic pollutants (Cheng et al., 2017; Denyes et al., 2012).
3. Bioaugmentation (BA): 5 mL of the bacterial consortium described above were surface inoculated to the soils each week. The bacterial load was of 1–1.5·10^7 CFU ml^{-1} (Colony-Forming Units, calculated by serial dilutions platted in agar medium).
4. Application of biochar and bioaugmentation (SN): A combination of the biochar amendment and the addition of the bacterial consortium, were applied to both soils as described above in points 2 and 3.

Triplicates of each of the four treatments in the two different soils (24 pots in total) were maintained for a period of twelve weeks in a culture chamber with a 16/8 h light/dark photoperiod, with temperatures of 25 °C (light) and 18 °C (dark) to simulate natural conditions. 40–60% soil moisture (w/w) was maintained with sterile water. Each time (weekly) that the bacterial consortium was added to the corresponding pots, all the pots were homogenized by manual mixing (1 min each), in order that all the pots maintained the same aeration conditions. Samples of each pot were collected at week 6th (T1) and week 12th (T2). Samples at T1 were designated NT, BC, BA and SN, while at T2 these were named NT2, BC2, BA2 and SN2, for natural attenuation, biochar, bioaugmentation and combination of biochar and bioaugmentation treatments, respectively.

2.5. TPH quantification on soils and statistical analysis

Four TPH determinations were assessed in each triplicate of treatment to reduce measurement variability. In the case of the initial soil prior treatments (T0), only one sample per pot was analysed. TPHs and hydrocarbon fractions were analysed by gas chromatography with a flame ionization detector (GC-FID) following the procedure previously described by (Garrido-Sanz et al., 2019; Pindado Jiménez et al., 2014). Degradation of the different petroleum hydrocarbon fractions was calculated as percentages (CC_{T0}-$CC_{T1/T2}$/CC_{T0})*100, where CC_{T0} is the initial hydrocarbon concentration at T0 (prior to treatments) and $CC_{T1/T2}$ is the hydrocarbon concentration at T1 or T2, respectively. The

SPSS 14.0 statistical program was used to evaluate the effects of the treatments on the sampling times T1 and T2, through an error diagram with means and confidence intervals (0.95), and its significance ($p < 0.05$) was evaluated by ANOVA repeated measurements and Bonferroni *post hoc* comparisons, with residual normality and heteroscedasticity tests. The differences between the two polluted soils in the three sampling times (T0, T1 and T2) were assessed by Principal Component Analysis (PCA), using R 6.3.1 FactoMiner package.

2.6. DNA extraction, 16S rRNA amplicon sequencing and profiling

For total DNA extraction, at T0, two random soil samples (two pots) per soil A and soil B were obtained. At T1 and T2, a sample from every pot (triplicate per treatment) and soil type was obtained, except SN2 treatment (in soil B) where only DNA from a duplicate was extracted. Total DNA of soil samples was extracted using the MO BIO PowerSoil™ DNA Isolation kit according to the manufacturer's instructions. Amplicon of the 16S ribosomal RNA gene (16S rRNA) and sequencing of amplicons were performed by the *Parque Científico de Madrid* (Spain). Briefly, the V3–V4 16S rRNA region was amplified using the primers 341F (5′-CCT ACG GGN GGC WGC AG-3′) and 805R (5′-GAC TAC HVG GGT ATC TAA TCC-3′) prior to libraries preparation using the Miseq reagent kit v3 600 cycles. Amplicons were sequenced using Illumina Miseq 2 × 300. Reads were uploaded to the BaseSpace (Illumina) server and analysed by the sequencing company with the standardized 16S Metagenomic App workflow v1.1.0 for taxonomic classification of reads, using the Ribosomal Database Project (RDP) classifier (Wang et al., 2007) and the RefSeq + RDP 16S v3 database (Alishum, 2019). Reads classified to the Class taxonomic level were further used for diversity and structure analyses.

2.7. Bacterial diversity analyses

The composition of the microbial communities was studied at the Class taxonomic level. For each sample, a total of 95 different classes were analysed. Alpha diversity was calculated using Hill numbers (N) that relate richness to abundance of species (effective or equivalent number of species with the same abundance), with different orders of diversity ($q = 0, 0.5, 1, 2$), and Hill ratio (E10) was calculated for uniformity or evenness (Chao et al., 2014; Hill, 1973). Based on these q values we obtain: N0 = species richness, N0.5 = number of rare species, N1 = number of abundant species, N2 = number of dominant species, E_{10} (N1/N0) = evenness index. These diversity indices were calculated as Hill means of the three replicas and the uncertainty by bootstrapping using "Vegan Package" in the statistical environment R 6.3.1. The SPSS 14.0 software was used to compare the most common diversity indices N0, N1 and N2, between the two times (T1 and T2) with non-parametric statistical tests (Wilcoxon signed rank test) and the effect between treatments in the three samplings (T0, T1 and T2), was observed with the error diagram (mean and standard error).

The 20 most abundant classes were selected for both soils and the following statistical tests were carried out with the R 6.3.1 software. The changes in diversity between T1 and T2, that indicate the gain or loss of individuals per class, were calculated using the paired t tests with the "tpaired.krandtest" function of the "adespatial" package with a significance $p < 0.05$ (Legendre, 2019). The differences between the microbial communities between T1 and T2 were observed in the Principal Coordinate Analysis (PCoA); the class abundance matrix was performed transforming the data (Hellinger) and using the Euclidean distance; the "pcoa" and "biplot.pcoa" functions were used from the "ape" package (Borcard et al., 2018). The relationship between the TPH chains and the classes of bacteria was achieved by means of restricted redundancy analysis (RDA); data were previously transformed by "Hellinger" and were used in the "rda" function of the "vegan" package; high correlations were avoided with "VIF" (variance inflation factors); the graphic representation and the selection of the represented classes were obtained with the "Triplot.rda" and "goodness" functions (Borcard et al., 2018).

2.8. Sequence deposition

16S rRNA gene amplicons sequences of the diesel-degrading consortium have been deposited in the NCBI Sequence Read Archive (SRA). Data are available under the BioProject accession PRJNA657971, and SRAs SRR12476958-SRR12477008.

3. Results

3.1. Comparison of the effectiveness of the remediation procedure between the different treatments

3.1.1. Differences between freshly polluted soil and aged soil hydrocarbon fractions

The characterization of the aliphatic and aromatic hydrocarbon fractions of the diesel fuel from the currently operating storage tanks has been previously reported (Garrido-Sanz et al., 2019). The initial TPH composition in the freshly polluted soil A, and the aged-polluted soil B prior treatments, shows a different composition, as shown in Table S2. In general, a prevalence of the aliphatic C_{12}–C_{21} and aromatic EC_{16}-EC_{21} fractions may be noted. The fresh-polluted soil A shows a higher concentration of most hydrocarbon fractions, particularly of short to middle-chain length aliphatic and aromatic hydrocarbons (C_{10}–C_{12} to C_{16}–C_{21} and EC_{10}-EC_{12} to EC_{16}-EC_{21}). On the contrary, the aged-polluted soil B shows higher concentrations of long-chain hydrocarbons (C_{21}–C_{35} to $> C_{35}$ and EC_{21}-EC_{35} to $> EC_{35}$) compared to soil A.

The distribution of the different TPHs fractions can be observed in the principal component analysis (PCA) of both soils A and B (Fig. 1A), which shows that the first dimension explains the 62.7% of the total observed variance and clearly differentiate both soils. Most short and middle-chain hydrocarbons (C_{10} to C_{16}) are closer to the freshly polluted soil A where the aliphatic fraction is also dominant. On the contrary, long-chain hydrocarbons (C_{21} to C_{35}) and the aromatic fraction are predominant in the aged-polluted soil B. Comparing the different sampling times; at T0, the soil A shows a high dispersion (Fig. 1B).

3.1.2. Differences in effectiveness between treatments on diesel degradation

Petroleum hydrocarbon removal under the different treatments compared to the initial values of both soils is shown in Table 1. Regarding the degradation of the different hydrocarbon fractions in the case of the natural attenuation treatment (NT), after 6 weeks (T1), a strong reduction of hydrocarbons in soil A compared to the initial concentrations was observed. Total aliphatic hydrocarbons reduced by 18.3% while the aromatic fraction decreased by 46.7%. On the other hand, the C_{10}–C_{12} aliphatic fraction was reduced by 91.1%, while in the aromatic fractions EC_{10}-EC_{12} and EC_{12}-EC_{16} decreased more than 50%. Conversely, in soil B, natural attenuation at 6 weeks did not produce any significant reduction of the overall aliphatic or aromatic fractions, accounting for only 5% reduction and 3% increment, respectively (Table 1). Likewise, some increases in TPHs concentrations were observed, indicating an increment in the concentration of specific fractions due, among other causes, to the breaking down of long carbon chains towards shorter ones.

The highest TPHs degradation was observed at twelve weeks in the combined biochar and bioaugmentation (SN) treatment for both soils, A and B. At this timepoint, TPHs degradation in soil A is twice the one observed in soil B (47% and 22% respectively). The other treatments show reduced hydrocarbon degradation compared to SN. In soil A (freshly polluted soil), the effect of natural attenuation was similar to other simple treatments, degrading 41% of TPH. In soil B, the most effective simple treatment was the bioaugmentation, with 19% of TPH degradation (Table 1).

In soil A, the highest degradation of aliphatic and aromatic fractions

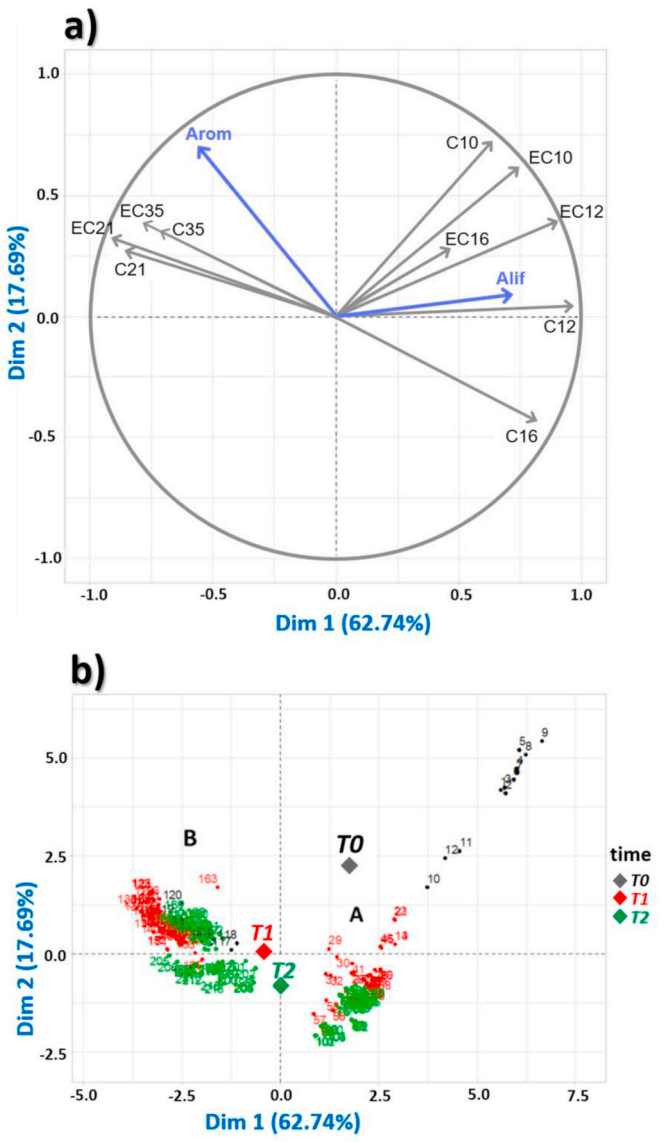

Fig. 1. Principal component analysis (PCA) of the hydrocarbon fractions in soils A and B at all sampling times (T0, T1 and T2). (A) Position of the hydrocarbon fractions (black): $>C_{10}$–C_{12} (C12), $>C_{12}$–C_{16} (C16), $>C_{16}$–C_{21} (C21), $>C_{21}$–C_{35} (C21), $>C_{35}$ (C35): $>EC_{10}$-EC_{12} (EC10), $>EC_{12}$-EC_{16} (EC12), $>EC_{16}$-EC_{21} (EC16), $>EC_{21}$-EC_{35} (EC21), $>EC_{35}$ (EC35) and total aliphatic (Alif) and aromatic (Arom) fractions in blue. Specific fractions of aromatic and aliphatic hydrocarbons are abbreviated as shown in Table 1, (B) Samples at the different time in soils freshly polluted (A) and aged (B), respectively.

was observed in the SN treatment (i.e. the combination of biochar and bioaugmentation) for both soils, with a degradation of 37% in aliphatic and 68% in aromatic fractions. In soil B, the reduction of aliphatic was greater in the BA (bioaugmentation) treatment, with a 23%. In aromatic fractions, 31% was reached with SN treatment (Table 1, Fig. 2).

In soil A, at twelve weeks (T2), the bioaugmentation treatment alone produced the lowest reduction of the aliphatic fraction (24% versus 34% in NT2). The comparison between T1 and T2 using ANOVA two factors, show that all comparisons are significant with a $p < 0.05$, except for BA in the aliphatic fraction for soil A (Fig. 2A). Regarding the aromatic fraction, most of the comparisons between T1 and T2 were significant except for SN, even though if this treatment achieved the highest degradation observed. Remarkably, the highest differences in the aromatic fraction between T1 and T2 were observed in BA, while this treatment showed low differences in the case of the aliphatic fractions (Fig. 2A).

In soil B, an aged hydrocarbon-polluted soil, the degradation of the aliphatic and aromatic fractions was lower than in soil A (Table 1). At T1, the aliphatic fraction slightly decreased in all treatments except for the biochar application (BC), in which the C_{21}–C_{35} fraction increased compared to the initial concentrations (Table 1). A similar behaviour is observed in BC treatment for the aromatic fraction, where an increment of EC_{21}-C_{35} and EC_{35} fractions was observed (Table 1), which could be explained by a sorption process of long hydrocarbon chains to the biochar. In both cases, these fractions decreased at 12 weeks (T2). The differences between T1 and T2 are significant at $p < 0.05$ for bioaugmentation alone (BA) and combined biochar and bioaugmentation (SN) (Fig. 2B). The highest degradation observed in soil B occurred within the combined SN treatment at T2, showing higher reduction in aromatic (31%) than aliphatic fraction (17%) while BA, at T2, produced higher reduction in aliphatic (23%) than aromatic fractions (14%; Table 1, Fig. 2B).

In the freshly polluted soil A, the least degradable fractions were the aliphatic C_{16}–C_{21} and C_{21}–C_{35}, which did not exceed 20% degradation, at 12 weeks (T2), in any of the treatments (Table 1). In soil B, a change was observed between T1-T2 in the degradation of the aliphatic and aromatic fractions, which depended to a great extent on the applied treatments. Thus, the least degradable fractions in soil B was the aromatic fraction $> EC_{35}$, which decreased between both times. Likewise, the C_{16}–C_{21} and EC_{16}-EC_{21} fractions did not exceed 1% in any of treatments at 12 weeks (T2), except for the SN treatment where there was a degradation that exceeded 15%. Furthermore, the C_{21}–C_{35} and EC_{21}-EC_{35} fractions did not exceed 9% at 12 weeks (T2), except in BA and SN, where the degradation exceeded 30% in some cases (Table 1).

3.2. *Changes in structure and diversity on each treatment and pollution scenario*

Results of the analysis of alpha-diversity at Class level are summarized in Table 2. In soil A at T0, there was a higher richness in the effective number of rare, abundant and dominant classes, with higher evenness values of the most common classes observed after six weeks (T1), where these same values decreased (Table 2, Fig. S1). With respect to the T1-T2 period, there were no significant differences ($p < 0.05$) between richness (N0) and effective number of dominant classes (N2); however, there were differences, for such p-value, between the effective number of most abundant classes (N1). The differences between the treatments in the period T1 and T2 samples are shown, in Fig. S1A, where the greatest increase in diversity (N1 and N2) is observed in the combined SN treatment, reaching values closer to the initial ones (T0). On the contrary, the BA treatment presents the least variation between T1 and T2. On soil B, T0 showed values lower than T1 for all Hill numbers. Besides, the differences between diversity indices, N0, N1 and N2, were significant $p < 0.05$, between times T1 and T2. In this period, the behaviour of the treatments showed an evolution towards a greater diversity (Fig. S1B).

Regarding the relative abundance (Fig. 5, Table S1), all microbiomes in soils and treatments were dominated by *Actinobacteria*. In the freshly polluted soil A, the Class *Actinobacteria* population increased in all treatments, from the initial 47.6% at T0, prior to artificially polluting the soil, to 53.4 %–68.9% in the case of SN at T2 and NT at T1, respectively. The most remarkable change in soil A, occurred in the Class *Bacilli*, whose relative abundance was 25% before the treatments (T0), after which its abundance decreased to less than 3.3% (Fig. 5, Table S1). It is important to note that the 20 most abundant and common classes between both soils and treatments represent more than the 95% of total relative abundance (Table S1). Members of all classes listed in this section, have been previously described as hydrocarbon degraders. In all cases, *Actinobacteria* and the different *Proteobacteria* classes (*Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria and Deltaproteobacteria)* represent more than 92% in the case of soil A and more than

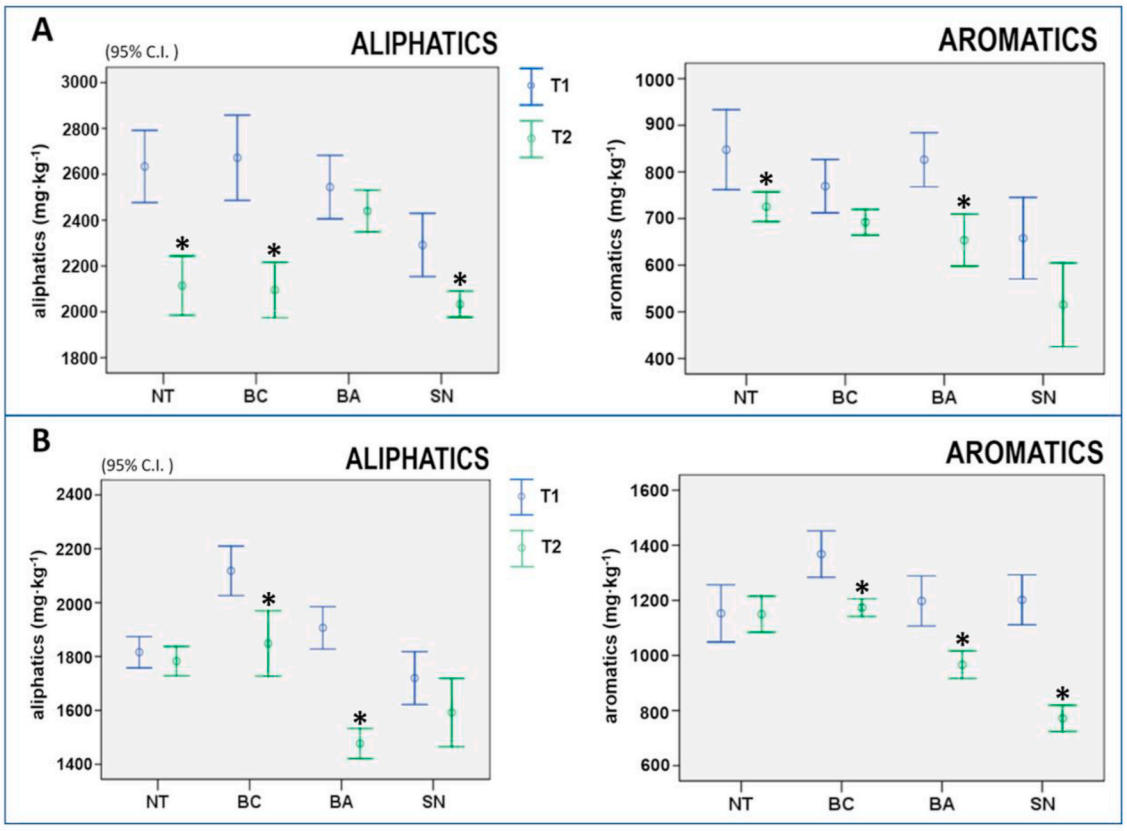

Fig. 2. Error bar diagrams representing the mean and the confidence intervals at 95% of the concentration (mg·kg^{-1}) of the aliphatic and aromatic fractions between sampling times, treatments and soils A and B. Sampling times T1 and T2 are represented in blue or green, respectively. The different treatments are: BA; bioaugmentation, BC; biochar, NT; natural attenuation and SN; combination of bioaugmentation and biochar. The asterisk added in T2 treatments corresponds to the statistically significant differences $p < 0.05$.

Table 2
Average and standard error (StdErr) of Hill numbers (N0, N0.5, N1 and N2) and Hill ratio (E10) of the diversity indexes obtained within the different treatments at T0, T1 and T2 for soils A and B.

	Time	treatment	N0	StdErr	N0.5	StdErr	N1	StdErr	N2	StdErr	E10	StdErr
SOIL A	T0	–	85	1	15.57	0.11	5.83	0.02	3.35	0.01	0.07	0.02
	T1	NT	67	1	7.80	0.06	3.11	0.01	1.99	0.01	0.05	0.01
		BC	67	1	8.60	0.06	3.53	0.01	2.23	0.01	0.05	0.01
		BA	69	1	7.93	0.06	3.31	0.01	2.12	0.01	0.05	0.01
		SN	68	1	8.92	0.07	3.83	0.01	2.45	0.01	0.06	0.01
	T2	NT2	65	1	8.31	0.06	3.35	0.01	2.05	0.01	0.05	0.01
		BC2	68	1	9.81	0.07	3.81	0.01	2.21	0.01	0.06	0.01
		BA2	64	1	8.37	0.06	3.36	0.01	2.02	0.01	0.05	0.01
		SN2	70	1	11.31	0.06	4.96	0.01	2.98	0.01	0.07	0.01
SOIL B	T0	–	79	2	15.27	0.10	5.58	0.03	2.93	0.01	0.07	0.02
	T1	NT	73	1	16.31	0.08	7.01	0.03	4.03	0.01	0.10	0.03
		BC	74	1	16.13	0.09	6.88	0.02	3.91	0.01	0.09	0.03
		BA	73	1	15.24	0.08	6.33	0.02	3.62	0.01	0.09	0.02
		SN	73	1	15.06	0.07	6.60	0.02	3.90	0.01	0.09	0.02
	T2	NT2	79	1	19.13	0.10	8.36	0.03	4.62	0.02	0.11	0.03
		BC2	79	1	19.17	0.10	8.49	0.03	4.73	0.01	0.11	0.03
		BA2	79	1	17.97	0.09	7.80	0.03	4.45	0.02	0.10	0.03
		SN2	76	1	18.87	0.12	8.67	0.05	4.97	0.02	0.11	0.04

80% in the case of soil B under the different treatments at T1 and T2. Other predominant classes include *Acidobacteria*, *Gemmatimonadetes*, *Bacilli*, *Cytophagia* and *Sphingobacteriia*, all of which represent less than 5% (except *Bacilli* at T0 in soil A, previously described).

Changes in diversity between T1 and T2 within the 20 most abundant classes in both soils can be observed in Table S3 and Table S4 for soil A and B, respectively. In soil A, the abundance of 12 classes was found to be significantly different among T1 and T2. Among them, ten classes increased their abundance in T2 compared with T1, while two, *Betaproteobacteria* and *Bacilli* decreased. In soil B, the abundance of 13 classes show significant differences among T1 and T2. Ten classes showed an increased abundance at T2 compared with T1, while the three remaining classes, *Actinobacteria*, *Gammaproteobacteria* and *Spartobacteria* show a reduction in their abundances. Among the classes that have increased their abundance between T1 and T2, only four of them, *Deltaproteobacteria*, *Gemmatimonadetes*, *Planctomycetia* and

Thermomicrobia, coincided in the two different pollution scenarios (soils A and B).

The changes in the structure of bacterial communities during the twelve weeks of treatment was assessed by means of Principal Coordinate Analysis (PcoA; Fig. 3). The axis 1 is related to sampling times, with T1 showing a low richness of bacteria on the right side of axis 1, contrasting with T2 on the left, where there is a greater diversity of classes in both soils. The second axis illustrates the differences between treatments with and without biochar, with clear differences for soil B. For soil A axis 2 represents 18.1% of the total variability, and although the differences along this axis is visualized for biochar and non-biochar treatments, it is not as evident as for soil B, due to a major change in the SN2 treatment. This increase in abundance in bacterial diversity with respect to the other treatments was also observed in the diversity indexes (Table 2). For soil B, the second axis represents the 30.8% of the total variance. Treatments with biochar (BC, BC2, SN, and SN2) are located in the negative section of the axis whilst pots without biochar (NT, NT2, BA and BA2) are located in the positive part of axis 2. In general, soil B shows more homogeneous variability on Class abundances than in soil A.

3.3. Effectiveness in degradation of specific aliphatic and aromatic fractions by different microbial taxonomic classes

The relationships between the hydrocarbon fractions and the bacterial composition on both soils under the different treatments are showed in the Redundancy Analysis (RDA) in Fig. 4. The two canonical components, RDA1 and RDA2 were significant for both soils. After selecting the explanatory variables (C_{12}–C_{16} and EC_{12}-EC_{16} were removed in soil A to avoid a strong correlation), we obtained a canonical adjusted square correlation (R^2_{Adj}) of 0.4748 for soil A and 0.5974 for soil B (Fig. 4). Therefore, the 47.5% and the 59.7% of the variation observed in the bacterial communities is explained by the hydrocarbon fractions. In both soils, the highest variation is found in the first RDA1 component that differentiates the two sampling times, T1 and T2. In soil A, positive correlations are observed with T1 and short-chain hydrocarbon fractions, being associated to Class *Actinobacteria*. On the other hand, long-chain hydrocarbon fractions are associated with *Betaproteobacteria* and *Bacilli*. Negative correlations are observed with a higher bacterial diversity, which corresponds with treatments at T2 (Fig. 4A). In the case of soil B, positive correlations are found at both sampling times and hydrocarbon fractions are also distributed among the sampling times and treatments, although in T1 Class *Actinobacteria* is associated with long-chain aromatic hydrocarbon fractions (Fig. 4B). At T2 there is also a high bacterial diversity associated with C_{10}–C_{12}, C_{16}–C_{21} and EC_{16}-EC_{21} fractions, which have increased compared with T1, also shown in Table 2. It should also be noted that most of the represented classes in Fig. 4, have significantly changed their abundance between times T1 and T2 (Tables S3 and S4), in both soils (A and B).

4. Discussion

In this work, the bioremediation effectiveness of an autochthonous bacterial consortium on a freshly (A) and aged (B) diesel-polluted soil, was compared when applied alone and combined with biochar and other techniques. Regarding biostimulation and/or bioaugmentation, several authors (Johnsen et al., 2005; Makadia et al., 2011; Moreno and Rodríguez, 2011; Płociniczak et al., 2017; Wu et al., 2017, 2019) established that although these techniques, alone or in combination, generally improved soil remediation compared to natural attenuation, various factors determine that they are not always effective. On the other hand, biochar-based treatments have been widely used, alone or in combination with biostimulants (Aziz et al., 2020; Jiang et al., 2016; Qin et al., 2013; Wang et al., 2017). Specifically, biochar has been used as a bioaugmentation method for hydrocarbon polluted soils by means of immobilization of bacteria, for which biochar acts as a habitat. Thus, immobilized degrading bacteria, are more tolerant to physicochemical changes in the soil. Likewise, the effectiveness of the bacteria-hydrocarbon interaction is conditioned by the effective growth or abundance of functional bacteria in such habitat (Chen et al., 2012).

This work was conditioned with two environmental premises: i) the use of biochar, derived from tree pruning residue, and obtained at high

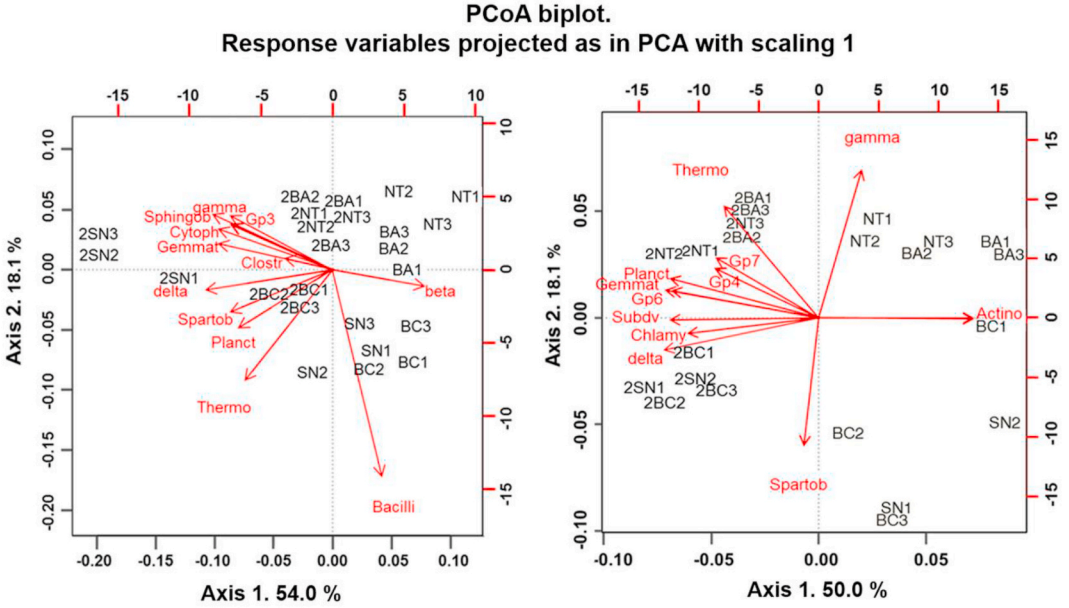

Fig. 3. Principal Coordinate Analysis (PCoA) of bacterial classes across treatments, sampling times and soils. For both soils: freshly polluted (A) and aged (B), red vectors represent bacterial classes. T1 treatments are NT; natural attenuation, BA; bioaugmentation, BC; biochar and SN; combination of bioaugmentation and biochar. For T2, the treatments are the same but 2NT 2BA, 2BC and 2SN, respectively. Numbers 1–3 indicate the replicates. The bacterial classes shown are abbreviated as follow: *Betaproteobacteria* (beta), *Gammaproteobacteria* (gamma), *Deltaproteobacteria* (delta), *Gemmatimonadetes* (Gemmat), *Clostridia* (Clostr), *Cytophagia* (Cytoph), *Sphingobacteriia* (Sphingob), *Planctomycetia* (Planct), *Thermomicrobia* (Thermo), *Spartobacteria* (Spartob), *Actinobacteria* (Actino), *Acidobacteria* Gp16 (GP16), *Acidobacteria* Gp6 (GP6), *Acidobacteria* Gp4 (GP4), Subdivision3 (Subdv), *Acidobacteria* Gp7 (GP7) and *Chlamydia* (Chlamy).

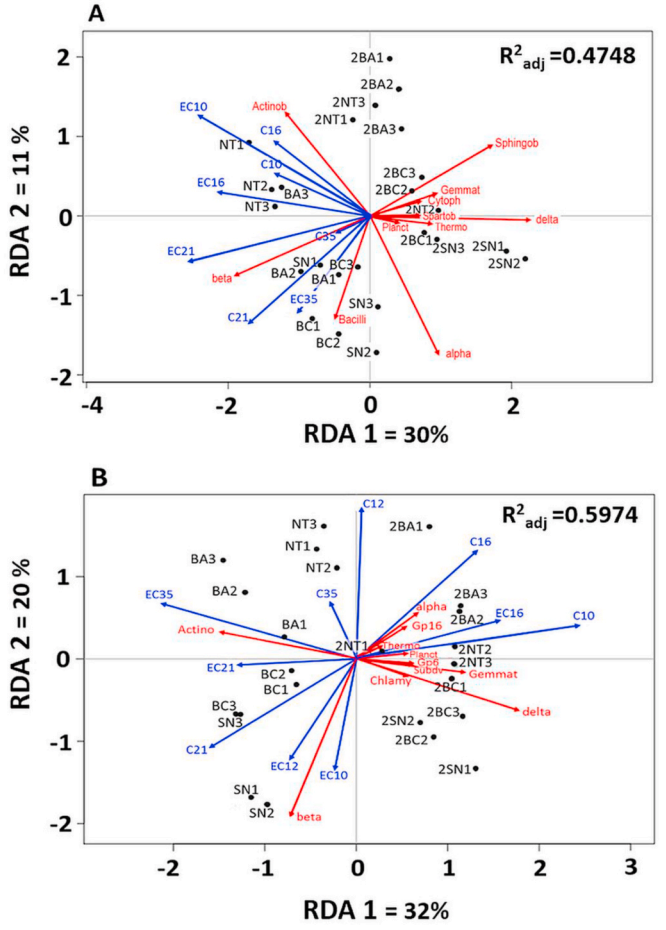

Fig. 4. Redundancy analysis (RDA) of hydrocarbon fractions and most abundant bacterial classes among the two soils and treatments. For both soils A (A) and B (B), blue vectors represent the hydrocarbon fractions and red vectors represent bacterial classes with a goodness of fit > 0.5. T1 treatments are NT; natural attenuation, BA; bioaugmentation, BC; biochar and SN; combination of bioaugmentation and biochar. For T2, the treatments are the same but 2NT, 2BA, 2BC and 2SN, respectively. Numbers 1–3 indicate the replicates. The bacterial classes shown are abbreviated as follow: *Actinobacteria* (Actino), *Betaproteobacteria* (beta), *Alphaproteobacteria* (alpha), *Gemmatimonadetes* (Gemmat), *Sphingobacteriia* (Sphingob), *Cytophatia* (Cytoph), *Spartobacteria* (Spartob), *Deltaproteobacteria* (delta), *Planctomycetia* (Planct), *Thermomicrobia* (Thermo), *Acidobacteria* Gp6 (GP6), Subdivision3 (Subdv) and *Clamydia* (Chlamy). Specific fractions of aromatic and aliphatic hydrocarbons are abbreviated as shown in Table S2.

temperature being potentially useful in bioremediation, ii) the use of a degrading consortium isolated from the polluted soil for the purpose of achieving a lower impact on the soil biota. In order to preserve most of the consortium's bacteria in the bioaugmentation process, it was decided to repeatedly inoculate it as free cells in the contaminated soil, favoring its growth and survival. The assessment of the efficiency of this technique would allow us to take the bioremediation process to a higher spatial scale and for the time necessary to achieve the specific objectives of hydrocarbon degradation.

The results of this study showed that soil A pollution presented a high degree of instability, probably caused by volatilization of diesel fuel components, as well as other abiotic degradation processes, including photo-oxidation that partially degrade or reduce the diesel constituents; such processes determine the evolution towards smaller fractions of hydrocarbons (Chikere et al., 2011). On the other hand, it has been verified that soil B, with aged diesel, shows greater resistance to degradation of hydrocarbon since they are strongly retained in the soil matrix after decades of aging (Semple et al., 2003).

Results in soil B suggests a lower bioavailability of the pollutant, mainly associated with soil organic matter (Koshlaf and Ball, 2017). Negative values in TPHs degradation were observed as well, indicating an increment in the concentration of specific fractions. These values can be explained by the breaking down of certain chains in orders of lower number of carbons, heterogeneity of the samples or retention in the soil matrix (Dandie et al., 2010; Rhodes et al., 2008; Wu et al., 2017). Interestingly, these negative values are generally more common in the biochar treatments, since this high-temperature biochar could increase sorption efficiency of long hydrocarbon chains in both polluted soils (Ahmad et al., 2014).

Bacterial diversity in oil-polluted soils has been observed by other authors (Gałązka et al., 2018; Shen et al., 2018), verifying the development of microbial communities adapted to contamination. In this work, soil A showed a high reduction in diversity in all treatments, up to 47% compared to the initial soil, which may be partially explained by means of the higher toxicity of petroleum hydrocarbons in a freshly contaminated soil (Jiang et al., 2016; Tang et al., 2012) compared with aged hydrocarbons. High volatility and physicochemical degradation is probably occurring within this soil, all of which could explain the observed changes in the structure of the bacterial community. On the contrary, the diversity found in soil B increased within the treatments to 20% in the first six weeks, which could be explained by the improvement of the environmental conditions in the configuration of the treatments, and the indigenous bacterial populations already adapted to the contamination present in soil B.

The bioremediation process depends on the bioavailability of the petroleum hydrocarbon and the biodegradation performed by the microbial communities. During a recent pollution event, the bioavailability of hydrocarbon fractions is higher, but the bacterial communities need to adapt and survive to the new conditions in order to biodegrade the pollutant (Chikere et al., 2011; Johnsen et al., 2005). Soil A, after the first six weeks of treatments (natural attenuation, biochar and bioaugmentation) showed similar concentrations of aromatic and aliphatic hydrocarbon fractions, while the combined biochar and bioaugmentation resulted in a higher reduction of these fractions, as reflected in Table 1. After twelve weeks, a significantly higher degradation was observed in all treatments, compared to the first sampling time (T1), except in the bioaugmentation treatment (Fig. 2A). This behaviour in this period corresponds to a greater diversity in the abundance of certain bacterial classes (Fig. 3A). In aged contaminated soil B, the growth factors, adaptability and permanence in time of the degrading bacterial, are key for a slower desorption in the strongly retained hydrocarbon (Jenkins et al., 2017; Semple et al., 2007; Truskewycz et al., 2019). None of the treatments applied, within the first six weeks, showed a significant decrease in hydrocarbons. At twelve weeks, a significant reduction with respect to T1 in diesel chains occurred, with the exception of natural attenuation treatment (Fig. 2B). Similarly, in soil B, a greater abundance of certain bacterial classes was observed in this period T1-T2 (Fig. 3B).

After twelve weeks, the bioaugmentation treatment with the autochthonous bacterial consortium in the artificially polluted soil A, did not enhance hydrocarbon degradation compared with biochar or natural attenuation treatments, even displaying a lower degradation of aliphatic fractions. This could be explained by the existence of competition for the aliphatic hydrocarbons between the bacterial consortium members and endogenous soil bacteria. In such a soil, with an extremely low content of easily oxidizable carbon (0.1% of carbon before induced pollution, and 0.5% after it), hydrocarbons constitute a key carbon source for microbial development, which is supported by the increase observed in certain taxonomic classes in T2 (Brzeszcz et al., 2020; Dagher et al., 2019). Although a 16S rRNA taxonomic profiling is not sufficient to explain the competition between bacteria, it must be taken into account that *Gammaproteobacteria* class is the most abundant in the consortium used in this study, and that the aliphatic metabolism falls mainly on *Pseudomonas* (Garrido-Sanz et al., 2019), the most abundant

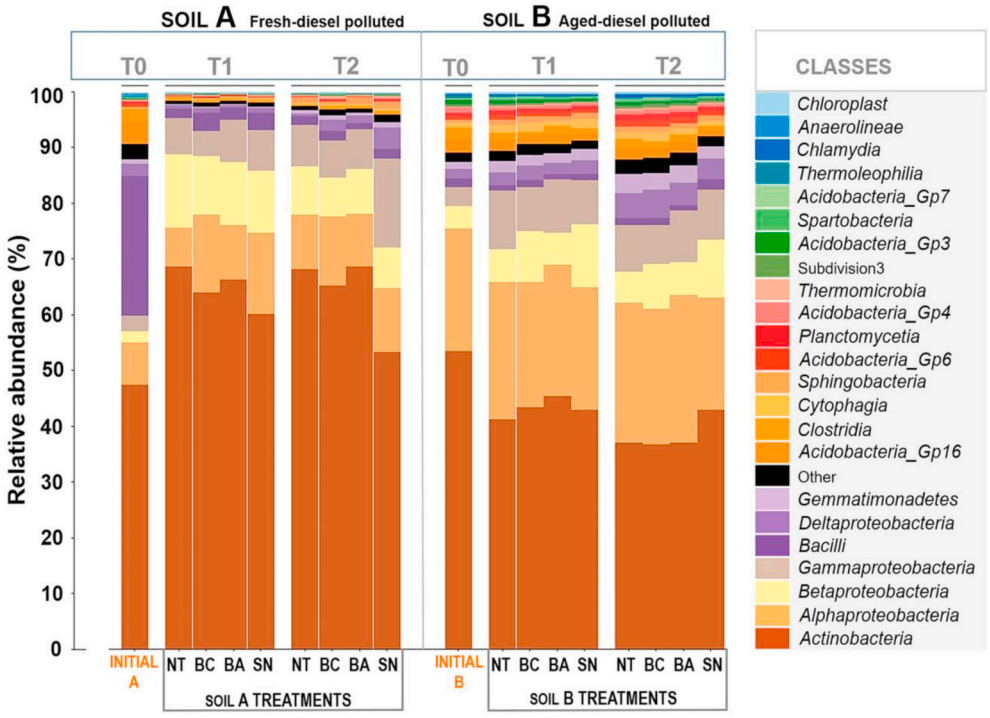

Fig. 5. Relative class abundances based on 16S rRNA amplicon sequencing at the different soils, treatments and sampling times. The relative Class abundance, in both soils A and B, over the different sampling times, is represented: "T0" (initial), "T1" (six weeks), and "T2" (twelve weeks). The different treatments are as follows: "NT", natural attenuation; "BC", biochar addition; "BA", bioaugmentation, and "SN", biochar addition and bioaugmentation. Samples in T0 are the initial communities, without treatments. Mean values of the tree replicates per sample are represented. Only taxa with a minimum relative abundance of 2%, across samples, are represented.

genus within this class. Thus, in soil A, it can be observed (Table S1) how *Gammaproteobacteria* should have increased in a similar way to the attenuation treatment, as a consequence of the incorporation of the consortium. This competition can also be observed at the diversity indexes (Table 2, Fig. S1A). The diversity in abundance of classes (N1) between six and twelve weeks remains constant for the bioaugmentation treatment with respect to the other treatments, in which a trend to increase diversity was observed. On the contrary, in soil B, after 12 weeks of treatment, bioaugmentation shows greater efficiency in degrading aliphatic chains than aromatic ones (Table 1). In this treatment, *Gammaproteobacteria* remained constant between both times compared to its decrease in the natural attenuation treatment (Table S1). This may be due to the greater persistence of the autochthonous consortium of this soil (Lawniczak et al., 2020; Thompson et al., 2005), from which, ultimately, the consortium was obtained.

Biochar amendment has been reported to change the physicochemical properties of the soil and modify bacterial communities, creating a distinct bacterial structure (Jenkins et al., 2017; Yu et al., 2018); this supports the fact that the physicochemical characteristics of the biochar used in this work could promote the adsorption on its surface of different hydrocarbon fractions, mainly those of long chains such as C_{21}–C_{35} and EC_{21}-EC_{35} (Bao et al., 2020; Chikere et al., 2011; Rhodes et al., 2008). This retention effect implies the lower bioaccessibility of long chains fractions, which also negatively impact their bioavailability and their use by microbes as a source of carbon and energy. However, in soil A, after twelve weeks, there was a significant reduction in the hydrocarbon concentration in the treatment with biochar (BC), similar to that observed in other treatments, which is possibly related to the greater bioavailability, due to the freshly contamination, as well as a faster desorption of the fractions adsorbed on the biochar. At the same time, a greater abundance of individuals has been found within different taxonomic classes (Marchal et al., 2013a, 2013b). In soil B, at 6 weeks (T1), an increase in long chain fractions was observed in the biochar treatment with respect to the other treatments. The degradation at 12 weeks was significant, although compared to soil A, this decrease occurred to a lesser extent. This was possibly due to the intrinsic characteristics of the aged hydrocarbon, more recalcitrant, which would imply, as already mentioned, a slower desorption or a lower accessibility of the soil microbiota. The role of biochar as a support for bacterial activity depends on its physicochemical characteristics and those of the amended soil, as well as the abundance and composition of the endogenous bacteria after the addition of biochar (Gorovtsov et al., 2019). In this sense, the effect produced in bacterial communities by the addition of biochar has been observed in this work by a change in the composition of the bacterial communities, suggesting a possible selection of certain specific groups of bacteria (Fig. 3). A significant relationship of *Deltaproteobacteria* and *Gemmatimonadetes* classes, among others, with the degradation of hydrocarbons was also observed (Fig. 4).

The lack of greater degradation in BC with respect to the natural attenuation treatment (NT) can be explained by a possible retention of the contaminant by the biochar (Ahmad et al., 2014; Moreno Jiménez et al., 2018; Rhodes et al., 2008; Zhang et al., 2010). Additionally, soil factors such as the dominant fine texture (clay-loam) or the presence of organic matter together with the aged diesel (Yu et al., 2018), could restrict the bioavailability of the hydrocarbon, thus limiting the role of the biochar in the process (Galitskaya et al., 2016; Zhang et al., 2010). The hydrocarbon degradation obtained with the biochar treatment could be improved by means of a greater diversity or abundance of the involved taxa (Aziz et al., 2020), and by increasing the bacterial colonization of the biochar and therefore a potential mineralization and/or desorption of hydrocarbons (Rhodes et al., 2012). In any case, a longer time than the 12 weeks would be necessary to confirm this assumption. The most efficient treatment in the degradation of the petroleum hydrocarbon was the combination of biochar and bioaugmentation in both soils A and B. In freshly polluted soil A, after the first six weeks, this treatment was more effective than the other treatments (Table 1, Fig. 2), suggesting a synergistic effect. This effect could be explained by the ability of the biochar to adsorb the hydrocarbon on its surface, favoring the adhesion of the consortium bacteria to the biochar, and thus facilitating the degradation of the hydrocarbon (Marchal et al., 2013a, 2013b; Rhodes et al., 2008, 2012). In this way, the weekly addition of the bacterial consortium to both soils could progressively improve their survival and their bioaccessibility to hydrocarbons, thus favoring the degradation of the pollutant.

After twelve weeks, the combined biochar and bioaugmentation treatment in soil A was associated with a significant increase in microbial diversity, as well as degradation of aromatic fractions. In the aliphatic fractions, this increase is less than expected compared to the natural attenuation treatment, which again suggests possible competition from the bio-augmented microbial consortium with native bacteria. In contrast, during the first six weeks, the combined treatment showed very limited degradation of hydrocarbons in the B (aged) soil, probably due to the recalcitrant nature of these hydrocarbons. At this time (T1), significant degradation of the short chain aromatic and aliphatic fractions such as C_{16}–C_{21}, EC_{16}-EC_{21} fractions, were observed. After twelve weeks, in the combined treatment in B soil, the degradation increased significantly in the C_{21}–C_{35} and EC_{21}-EC_{35} fractions, reaching 17% and 31% degradation in aliphatic and aromatic hydrocarbon fractions, respectively. The combined treatment was able to degrade more diesel fractions than the simple treatments, which suggests its greater catabolic capacity considering the objective of the degradation of such a complex fuel composition (Mrozik and Piotrowska-Seget, 2010). In summary, for both scenarios, the combined treatment was more effective in the degradation of TPHs, and mainly in aromatic fractions.

During the bioremediation process, the observed enhanced degradation at twelve weeks in both soils coincided with an increase in the diversity of specific microbial Classes. Other authors (Wu et al., 2017) did not find a relationship between higher degradation rates and diversity. However, the analysis developed in this work, which includes bioaugmentation with native bacterial communities, has found a significant correlation between the most abundant taxonomic classes and the greatest degradation of both aliphatic and aromatic fractions. This allowed us to affirm that, at least part of the degradation observed in both soils, was produced both by native microbial communities and autochthonous bacterial consortium added in the bioaugmentation process.

The taxa included in the most abundant classes found in this study have previously been described as hydrocarbon degrading microorganisms (Brzeszcz and Kaszycki, 2018; Koshlaf and Ball, 2017). Likewise, some of these classes, mainly *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, *Sphingobacteriia* and *Cytophagia*, are part of the bacterial consortium used in this work and previously characterized (Garrido-Sanz et al., 2019). The percentage distribution (Fig. 5) of the most abundant taxonomic classes is similar in the two pollution scenarios; this taxonomic similarity can be explained by the fact that they are samples from the same type of soil and polluted by the same type of diesel; the difference between the two scenarios is based on the time the soil has been in contact with the diesel: short term (soil A) and long term (soil B). On the other hand, in soil A, after treatments, a lower value of alpha diversity was observed, associated with the loss of some of the less abundant bacterial classes and the persistence of the main ones (Fig. 5). This same effect was observed by Galitskaya et al. in hydrocarbon polluted soils (Galitskaya et al., 2021). In treated soil A, an increase in the *Actinobacteria* Class was observed with respect to T0, as well as in *Alphaproteobacteria*, *Gammaproteobacteria* or *Betaproteobacteria*; on the contrary, it decreased in the *Bacilli* Class (Fig. 5). These same dominant classes were found in soil B, although in different relative proportions, probably due to the long contact time of the soil with the pollutant and the metabolic adaptation of bacteria to it. These two assumptions would also explain the greater diversity of soil B with respect to freshly polluted soil A.

The results of this study suggest, for both soils, that members of the aforementioned dominant taxa, as well as others, more abundant after 12 weeks treatments (*Gemmatimonadetes*, *Thermomicrobia*, *Sphingobacteriia*, *Spartobacteria* or *Chlamydia*, among others; Fig. 4), could be involved in the hydrocarbon degradation, either directly or indirectly through co-metabolism (Gałązka et al., 2018). Likewise, higher soil biodiversity in the two pollution scenarios could have been driven, either by lower toxicity, or by better bacterial adaptation to the pollutant over time. Future studies will allow us to clarify these relationships in more detail.

5. Conclusions

In this work, we have shown that a combination of biochar with a bioaugmentation based on an autochthonous bacterial consortium, is more effective for hydrocarbon bioremediation than the sole bioaugmentation. The higher effect on hydrocarbon degradation of combined biochar with bioaugmentation, is significant in short-term conditions and without the addition of chemical biostimulants or surfactants. Our results show that a bioremediation process can be effective exclusively using resources from the polluted soil area in an environmentally friendly context. Increases in bacterial diversity as well as relationships between hydrocarbon fractions and bacterial classes, have been found to be significant in our study. Future functional analysis at the metagenomic level of the bacterial populations undergoing these bioremediation treatments, could provide deeper insights by identifying the genes involved in the hydrocarbon degradation that could explain the specific roles of bacteria within the bioremediation process.

The authors declare no conflict of interest.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibiod.2021.105259.

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