Assessment of the efficiency of *in situ* bioremediation techniques in a creosote polluted soil: change in bacterial community.

Simarro R[1],González N.[1*], Bautista L.F.[2], Molina M.C.[1]

[1] Department of Biology and Geology, ESCET, Universidad Rey Juan Carlos. E-28933 Móstoles, Madrid, Spain.

[2] Department of Chemical and Environmental Technology, ESCET, Universidad Rey Juan Carlos. E-28933 Móstoles, Madrid, Spain.

[*] Corresponding author:

Natalia González: Department of Biology and Geology, ESCET, Universidad Rey Juan Carlos. E-28933 Móstoles, Madrid, Spain.

Phone: [34] 914888102

Fax: [34] 916647490

E-mail: natalia.gonzalez@urjc.es

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Abstract

This work aimed to assess the effectiveness of different *in situ* bioremediation treatments (bioaugmentation, biostimulation, bioaugmentation and biostimulation, and natural attenuation) on creosote polluted soil. Toxicity, microbial respiration, creosote degradation and the evolution of bacterial communities were analyzed. Results showed that creosote decreased significantly in all treatments, and no significant differences were found between treatments. However, some specific polycyclic aromatic hydrocarbons (PAH) were degraded to a greater extent by biostimulation. The dominance of low temperatures (8.9ºC average) slowed down microbial creosote and PAH uptake and, despite significantly creosote degradation (>60%) at the end of the experiment, toxicity remained constant and high throughout the biodegradation process. DGGE results revealed that biostimulation showed the highest microbial biodiversity, although at the end of the biodegradation process, community composition in all treatments was different from that of the control assay (unpolluted soil). The active uncultured bacteria belonged to the genera *Pseudomonas*, *Sphingomonas*, *Flexibacter*, *Pantoea* and *Balneimonas*, the latter two of which have not been previously described as PAH degraders. The majority of the species identified during the creosote biodegradation belonged to *Pseudomonas* genus, which has been widely studied in bioremediation processes. Results confirmed that some bacteria have an intrinsic capacity to degrade the creosote without previous exposure.

Keywords: *in situ* biodegradation, biostimulation, bioaugmentation, creosote, bacterial diversity

1. Introduction

Creosote is a complex mixture of persistent organic compounds derived from coal pyrolysis and the further distillation of the oily product obtained and is widely used as a wood preservative. It is composed of approximately 85% polycyclic aromatic hydrocarbons (PAH), 10% phenolic compounds, and 5% nitrogen and sulfur. As PAH are toxic, mutagenic, bioaccumulative and persistent in environmental compounds, they are considered priority pollutants by the US-EPA. Compared to physicochemical methods, bioremediation is a more effective, versatile and economical technique for removing PAH from the environment. Microbial degradation is the main process in natural decontamination and the biological removal of pollutants in chronically contaminated soils [1] where degrading bacteria are abundant [2]. However, recent studies have reported the potential ability of microorganisms to degrade PAH in soils which have not been previously exposed to these toxic compounds [3-5]. The technique based on the degradation capacity of indigenous bacteria is called natural attenuation. This method avoids damaging the habitat [6], allowing the ecosystem to revert back to original conditions and converting toxic compounds into harmless ones [7,8]. However, it takes a long time to remove toxic components, because degrading microorganisms in soils can represent about only 10% of the total population [9]. Thus, many bioremediation studies focus on bioaugmentation, which consists of adding allochthonous degrading microorganisms [10] which can be a pre-adapted pure bacteria strain or consortium, genetically engineering bacteria or the addition of relevant genes in a vector to be transferred by conjugation [11]. However, bioaugmentation is a complex technique, because a negative or positive effect depends on the interaction between the inocula and the indigenous population, due to resource competition, mainly for nutrients [12]. Biostimulation is another bioremediation technique which consists of increasing the degrading capacity of the indigenous community by adding nutrients to avoid metabolic limitations [13]. However, inconsistent results have been obtained with these techniques. Previous studies have shown that biostimulation can increase biodegradation rates [14] without negatively effecting degradation rates [9,15]. Similarly, when bioaugmentation was applied, biodegradation rates were enhanced [10], but not significantly [12,13].

It should be noted that each contaminated site can respond differently [13]. Therefore, laboratory-scale assays should be designed before carrying out an *in situ* bioremediation process to determine the most efficient technique and evaluate its effect on microbial diversity. Furthermore, previous works [16] have shown that although PAH were depleted, toxicity was still significant. Although most reported works did not perform toxicity assays, they should be carried out to assess the effectiveness of biodegradation on the final result. The main goal of the present study is to determine the most effective bioremediation technique in the decontamination of a creosote contaminated soil through microcosm assays, evaluating changes in the bacterial community and toxicity.

2. Materials and methods

2.1 Chemical, media and inoculated consortium

Creosote contained 87%wt of PAH and derived compounds thereof. For the purpose of the present work, 6 key PAH, representing a wide range of molecular weight and structures, were used to monitor the bioremediation process. The compositions of these key compounds in creosote were: 0.5%wt naphthalene, 5.1%wt phenanthrene, 12.2%wt anthracene, 3.1%wt pyrene, 1.3%wt dibenzofurane and 4.7%wt acenaphthene. Creosote was diluted in acetonitrile (Sigma-Aldrich. Steinheim, Germany) in a stock solution (0.439 g/mL final concentration) containing 0.117 gPAH/mL. Luria–Bertani (LB) and Bushnell-Haas Broth (BHB) media were purchased from Panreac (Barcelona, Spain). Biostimulation treatments were amended with BHB as a source of inorganic nutrients, whose composition was previously optimized [17] for a PAH-degrading consortium (C2PL05). Tween-80 (300 μl/mL) was used as optimum surfactant for PAH biodegradation using C2PL05 consortium [18]. Bioaugmentation treatments were inoculated with PAH-degrading consortium C2PL05, extracted from PAH-contaminated soil at a petrochemical plant and described elsewhere [19].

2.2 Experimental design

The efficiency of five different types of treatments for creosote removal was compared: control or untreated (C), natural attenuation (NA), biostimulation (BS), bioaugmentation (BA) and biostimulation and bioaugmentation (BS+BA). Experiments were carried out in duplicate microcosms

100 for five sampling times: 0, 6, 40, 145 and 176 days from December 2009 to May 2010. Thus, a total of
101 40 microcosms were prepared. Microcosms consisted of plastic trays containing 550 g of soil samples,
102 collected from unpolluted soil in the area of Rey Juan Carlos University. Soil samples were obtained
103 from the top 20 cm of soil and sieved to obtain the fraction with a particle size <2 mm. Mesocosms
104 were randomly arranged outdoors in a terrace and protected with plastic film to avoid direct rain and
105 snow. Except for the control treatment, each tray was spiked with 5.6 mL of a creosote solution (0.439
106 g/mL) in *n*-hexane to a final amount of 2.5 g of creosote per tray. All microcosms were maintained at
107 40% water holding capacity (WHC) [13]. For BS, microcosms were hydrated with the required
108 amount of optimum BHB, while deionized and sterilized water was used in treatments without BS. BA
109 microcosms were inoculated with 5 ml of C2PL05 consortium containing $2.0 \cdot 10^7 \pm 4.3 \cdot 10^6$ cells/g soil
110 of heterothrophic microorganisms and $1.8 \cdot 10^5 \pm 1.0 \cdot 10^5$ cells/g soil of creosote-degrading
111 microorganisms. Air temperature was continuously recorded during the whole experiment using
112 temperature data loggers (MicroLog EC650, Fourier Systems Ltd., Barrington, RI, USA).
113
114 *2.3 Characterization of soil and environmental conditions*
115 Soil NO_3^- concentration was estimated using a SKALAR San++ Analyzer (Skalar, Breda, The
116 Netherlands) after shaking the soil sample with distilled water (1:5 ratio) for one hour. Water holding
117 capacity (WHC) was measured following the method described by Wilke [20], and water content was
118 calculated as the difference between wet soil weight and soil weight after drying at 60ºC for 24 hours.
119 One gram of dried soil was suspended in deionized water (1:10) and incubated in an orbital shaker at
120 150 rpm and 25ºC for 1 h. Then, the pH of the suspension was measured using a GLP 21 micro *p*H
121 meter (Crison. Barcelona, Spain). Ambient temperature was continuously recorded with temperature
122 loggers (Tidbit Loggers. Onset Computer, Pocasset, MA, US) placed on site.
123 Total heterotrophic microorganisms (HM) and creosote-degrading microorganisms (CDM) of the
124 microbial population in the natural soil were counted using a miniaturized most probable number
125 (MPN) technique and 96-well microtiter plates with eight replicates per dilution [21]. The number of
126 cells was calculated with Most Probable Number Calculator software version 4.04 [22]. To extract
127 microorganisms from the soil, 1 g of soil was resuspended in 10 ml of phosphate buffer saline (PBS)

and shook at 150 rpm and 25ºC for 24 h. HM were determined in 180 µL of LB medium with glucose (15 g/L), and CDM were counted in 180 µl of BHB medium with 10 µL of creosote stock solution as a carbon source.

2.4 MPN, respiration and toxicity assays

CDM in microcosms were estimated by MPN at 6, 40, 145 and 176 days. For respiration assays, 10 g of soil (moistened with deionized water to 40% of WHC) were incubated in duplicate in closed desiccators at 25ºC for14 days. Each replicate contained a vial with 14 ml 0.2 M NaOH to absorb and neutralize the CO_2 produced by microorganisms. The vials were periodically replaced and titrated with 0.1M HCl. To ensure carbonate precipitation, 0.1 M $BaCl_2$ was previously added in excess. The amount of CO_2 produced was then calculated as the difference between the initial and final amount of NaOH in the vial. Control assays were performed with sterile soil.

$$\text{Respiration}\left(\frac{\text{mmol } CO_2}{\text{g soil}}\right) = \frac{\left[1.4 - \left(0.05 \cdot V_{HCl}\right)\right]}{W_{soil}} \qquad \text{(Eq. 1),}$$

where, V_{HCl} is the volume (ml) of HCl used to titrate the residual NaOH not neutralized with CO_2 and W_{soil} is the weight (g) of soil, used for the assay. Toxicity during PAH degradation was monitored following the Microtox™ method using the luminescent bacterium *Vibrio fischeri* following the protocol suggested by Microbics Corporation [23]. Toxicity was expressed as the percentage of *V. fischeri* bioluminiscence inhibition after 15 min at 15ºC.

2.5 Creosote Degradation

Approximately 30 g of soil were extracted from microcosms with dichloromethane in a Soxhlet device for 3 hours. The solvent was then removed in a rotary evaporator, and the residue was dissolved in 1 ml of dichloromethane for GC-MS analysis in a CP3800 GC (Varian. Palo Alto, CA, USA) equipped with a Varian Factor Four VF-1ms capillary column (15 m length x 0.25 mm ID x 0.25 µm film thickness) and coupled to a Varian 1200L quadrupole mass spectrometer. The following temperature program was used: 80ºC for 2 min, 80-300ºC at 10ºC/min and 300ºC for 15 min. The concentration of key PAH and creosote was calculated from standard calibration curves.

2.6 Characterization of the microbial population in the microcosms: DNA extraction and molecular and phylogenetic analysis

Non culture-dependent molecular techniques such as denaturing gradient gel electrophoresis (DGGE) were used to identify non-cultured microorganisms and compare biodiversity between treatments and its evolution at 145 and 176 days of the process. Total community DNA was extracted using Microbial Power Soil DNA isolation kit (MoBio Laboratories. Solano Beach, CA, USA), and the V3 to V5 variable regions of the 16S rRNA gene were amplified using the primers set 16S 518R and 16S 338F-GC clamp (5´-CGC CCG CCGCGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-3´). DGGE was performed by loading the PCR product onto polyacrylamide gels following the method described elsewhere [16]. Predominant bands were excised to clone it in the pGEM-T Easy Vector (Promega, Madison, WI). Plasmid was purified using the High Pure plasmid Isolation Kit (Roche) and sequenced using internal primers 338F and 518R. However, some bands were considered "unidentified" when re-amplification or cloning was not possible due to DNA degradation by exposure to UV light. Uncultured bacteria (DUB) were edited and assembled using version 4.8.7 of the BioEdit program [24]. BLAST search [25] was used to find nearly identical sequences for the 16S rRNA sequences determined. All DUB identified sequences and 25 similar sequences downloaded from GenBank were used to build the phylogenetic tree. Sequences were aligned in a single step using the Q-INS-i algorithm [26] of the multiple sequence alignment software MAFFT version 6.611 [27]. Sequence divergence was computed as the number of nucleotide differences per site between sequences according to the Jukes and Cantor algorithm [28] .The distance matrix for all pair wise sequence combinations was analyzed with the neighbour-joining method (NJ) of phylogenetic tree construction with 1000 bootstrap replicates using version PAUP 4.0B10 [29]. Maximum parsimony (MP) was also analyzed [19]. Sequences of *Spirochaeta americana* belonging to Spirochaetes phylum were used as out-group [30]. Images of DGGE were digitalized, and DGGE bands were processed using the UN-Scan-It gel analysis software version 6.0 (Silk Scientific Inc. Orem, UT, USA).

2.7 Statystical analysis

Analysis of variance (ANOVA) was performed to evaluate the effects of treatment on specific growth rate (μ), toxicity, degradation of organic compounds and respiration. Variances were checked for homogeneity using the Cochran test. The Student-Newman-Keuls (SNK) test was used to discriminate among different treatments after significant F-test, representing these differences by letters in the graphs. Data were considered significant when p-values were <0.05. All tests were performed with Statistica 6.0 for Windows. To visualize multivariate patterns, non-metric multidimensional scaling (nMDS) ordinations were made on the basis of the Bray–Curtis dissimilarity matrix on each sampling date using PRIMER software [31]. The SIMPER procedure [31] was used to identify the percentage contribution (%) of each band (ribotype) to the measures of the Bray-Curtis dissimilarity between biostimulation plots and non-biostimulation plots, and between bioaugmentation plots and non-bioaugmentation plots at 145 days (cold period) and 176 days (warm period). Bands were considered "important" if they contributed to the first 60% of the cumulative percentage of average dissimilarity between treatments within time intervals (145 and 176 days). Distance-based permutational multivariate analyses of variance (PERMANOVA [32]) were used to analyze differences in the whole assemblage between biostimulation treatments and non-biostimulation treatments across time (2 factors analyzed: treatment and time).

In addition, Simpson's index (D_S) was calculated from DGGE bands by applying equation 1 to estimate the ecological diversity of each treatment at 145 and 176 days.

$$D_S = \frac{1}{\sum_i p_i^2} \qquad \text{(Eq. 2),}$$

where p_i is the fraction of the i^{th} band in the gel with respect to the total of all bands detected, calculated as the coefficient between band intensity and total band intensity.

3. Results

3.1 Physical, chemical and biological characterization of soil

The average monthly values of ambient during the experiment and during the last 30 years are detailed in Figure 1. During the experiment, average temperatures decreased from 16ºC in October to a minimum of 6ºC in January. Temperatures then increased progressively to reach an average value of

210 21ºC in June. During most of the *in situ* experiment (75% of total length), temperatures were below
211 9.1ºC. Collected soil had a pH of 8.4 and its water content was 10%, although it had a high WHC
212 (52.1%).
213 Nutrient media data of each treatment are indicated in Figure 2. The average NO_3^- concentration within
214 the C microcosm (natural soil) and microcosms without nutrient addition were around 1.1 ppm
215 whereas microcosms amended with nutrients (BS, BS+BA) were two fold (2 ppm).
216 Creosote-degrading microorganisms (CDM) from natural soil represented only 0.88% ($1.77 \cdot 10^5 \pm$
217 $1.01 \cdot 10^5$ cells/g soil) of the overall heterotrophic population with a total number of microorganisms
218 two orders of magnitude higher ($2.01 \cdot 10^7 \pm 4.27 \cdot 10^6$ cells/g soil).
219
220 *3.2 MPN and respiration of the microbial population*
221 CDM in the natural soil was virtually zero throughout the experiment (Fig. 2). CDM in NA, BS and
222 BS+BA microcosms increased progressively from the initial sampling time reaching a degrading
223 population above 4 10^5 cells/g^1 soil at the final sampling time. However, BA microcosms showed a
224 constant degrading population under 10^5 cells/g soil throughout the *in situ* experiment.
225 Table 1A shows the values of accumulated CO_2 produced in each treatment for the different time 6,
226 40, 145 and 176 days. Time was the only significant factor (Table 1A), and showed that CO_2
227 accumulation was significantly greater at 176 days than at any other time (Fig. 3).
228
229 *3.3 Toxicity assays*
230 Changes in toxicity during *in situ* creosote degradation (Fig. 4A) showed that all treatments followed a
231 similar trend. The control treatment (C) was not toxic (<20%), but soil toxicity in each treatment with
232 creosote increased steadily from an initial value of 26% to over 50%. Toxicity only started to decrease
233 slightly during the last time interval (145 to 176 days). Interaction between time intervals (time
234 interval 1 from 0 to 6 days, 2 from 40 to 145 days and 3 from 145 to 176 days) and treatment showed
235 that toxicity in all treatments was significantly lower in time interval 1 (Table 1B) than in the other
236 time intervals (Fig. 4B).
237

3.4 Biodegradation of creosote and polycyclic aromatic hydrocarbons

Creosote depletion in all contaminated treatments is shown in Figure 5A. The creosote removed was very high in all treatments between 60 and 80% (Fig. 5A). Furthermore, PAH (phenanthrene, anthracene and pyrene) and treatment had a significant effect on residual PAH concentration, while their interaction (treatment x PAH) did not (Table 1C). Anthracene degradation was significantly higher than that of the other monitored PAH (Fig. 5B), and total PAH depletion was significantly higher in BS than in NA (Fig. 5C).

3.5 Diversity and evolution of uncultivated bacteria and dynamics during PAH degradation

Thus, changes in bacterial community structure during the bioremediation process were investigated using DGGE fingerprints (Fig. 6) and MDS analysis (Fig. 7) at 145 days (cool period) and 176 days (warm period). Summary of the identification of bands, as well as the correspondence between bands and DUB identified is show in Table 2. PERMANOVA found no significant differences in microbial assemblages between biostimulation (BS, BS+BA) and non- biostimulation (C, NA, BA) treatments or between the different time intervals. However, the bacterial community experienced important changes (Fig. 6) once it was perturbed with creosote, except when the soil was not treated (NA) during the cool period. During the warm period (176 days), creosote concentration decreased greatly, but the bacterial community showed a very different pattern from that of the control community. Simpson's diversity index (D_S) show in Figure 8, increased in C, NA, BS and BS+BA treatments from the initial time (6 days) to the final time (176 days). In addition, the highest Simpson's diversity indexes were observed in treatments amended with nutrients (BS, BS+BA). However, it remained virtually constant throughout the *in situ* degradation process in BA.

3.6 Phylogenetic analyses

The phylogenetic relationships of the degrading uncultured bacteria are shown in Figure 9. The aligned matrix contained 1373 unambiguous nucleotide position characters of which 496 were parsimony-informative. Parsimony analysis of the data matrix yielded 87 parsimonious trees with CI = 0.671, RI = 0.767 and a length of 1452. Phylogenetic analysis also showed the topology of the

maximum parsimony (MP) tree with the bootstrap values of the maximum parsimony and neighbor-joining analyses. No inconsistencies were found between parsimony and neighbor-joining (NJ) topology.

The phylogenetic tree was composed of bacteria belonging to Proteobacteria (α- and γ- Proteobacteria) and Bacteroidetes phylum. DUB-12RS to DUB-17RS were located in the Pseudomonadaceae clade, in which five groups of species can be clearly observed. DUB-13RS and DUB-15RS, identified as *P. trivialis* (HM134251) and *P. poae* (HM640290) respectively, were in an undifferentiated group supported by *P. trivialis* and *P. poae* type-strains. DUB-14RS, similar to *Pseudomonas viridiflava* (HM190224), formed a group supported by *P. viridiflava*^{T} (HM190229). DUB-12RS, which was 98% similar to *P. fluorescens* (GQ 496662), was grouped with *P.fluorescens*^{T} (D84013). DUB-16RS was identified as uncultured *Pseudomonas* sp (HQ677222) and classified in an undefined group. Finally, the last group in the Pseudomonadaceae clade was formed by DUB17-RS, which was 98% similar to *P. parafulva* (HQ406758) and grouped with *P. parafulva*^{T} (D84015). DUB-21RS was nested in the Enterobacteriaceae clade, since it was 99% similar to *Pantoea Brenneri* (HM163514). This clade is supported by types-strains of other *Pantoea* species such as *Pa. agglomerans*^{T} (FJ613819) and other enteric bacteria such as *Enterobacter cloacae*^{T} (AJ251469). The DUB nested in the Enterobacteriaceae and Pseudomonadaceae clades were γ-Proteobacteria. The Bradyrihzobiaceae and Sphingomonadaceae clades were included in the α-Proteobacteria class DUB-19-RS, which was 99% similar to an uncultured Balneimonas strain (HM799006), was nested in the first clade formed by uncultured Balneimonas and Rihzobiales bacterium supported by *Balneimonas floculans*^{T}. DUB-20RS, identified as uncultured Sphingomonadales bacterium, was nested in the Sphingomonadaceae clade. DUB-18RS was 99% similar to uncultured Flexibacteriaceae bacterium and was nested in the Cytophagaceae clade, belonging to the Bacteroidetes phylum.

4. Discussion

4.1 Biodegradation efficiency of bioremediation processes

The duration of the experiment (almost 6 months) was considered adequate to evaluate the *in situ* biodegradation process, as previous studies developed at low temperatures (15ºC – 5ºC) found that

toxicity and PAH concentrations were reduced to low levels in approximately 3 months (33). However, the very low temperatures during a significant part of the *in situ* biodegradation experiment delayed complete creosote degradation and, therefore, toxicity reduction. It is important to highlight that toxicity measures are needed to evaluate the real status of polluted soil. Despite significantly lower creosote concentrations at the end of the experiment (<40%), toxicity remained constant and high throughout the biodegradation process (>80%). Low temperatures delayed microbial activities such as respiration, which only showed a significant increase at the end of the biodegradation period when ambient temperature increased. This delay may be because creosote was not completely mineralized to CO_2 but to intermediate metabolites showing a certain degree of toxicity. Furthermore, previous studies [33, 34] have shown that biodegradation can progress at low temperatures, although low biodegradation rates were achieved due to the limited diffusion rate, PAH bioavailability and metabolism rates [8, 35].

The current controversy about the efficiency of biostimulation or bioaugmentation in bioremediation [13, 36, 37] makes the present study more relevant. In accordance with previous works on creosote biodegradation [36], our results did not show significant differences between biostimulation, biaugmentation and natural attenuation treatments. Biostimulation may be effective in soils or media with low nutrient concentrations, in which the addition of inorganic nutrient sources is not a limiting factor for microbial metabolism. Some authors [38] state that nutrient supply is essential under all conditions in soil, especially when the process operates at low temperatures [39]. However, creosote degradation was higher than 60% in all treatments including the natural attenuation treatment. Therefore, these results could confirm the nutrient concentration in the present natural soil (1 ppm) was not a limiting factor for the creosote degrading bacteria.

Bioaugmentation with the PAH-degrading consortium C2PL05 did not significantly increase the biodegradation of the pollutant. However, many studies [9,12,13] have concluded that the relationship between inoculated and autochthonous consortium in terms of, for instance, resource competition and/or predation [40,41] greatly influences the effect of bioaugmentation. Some authors [38,42,43] consider this a positive treatment when the native population does not possess the necessary metabolic pathways to metabolize compounds or does not have enough microbial cells. The indigenous bacterial

soil consortium in this study showed a high initial number of creosote-degrading microorganisms with an efficient capacity to degrade creosote (Fig. 4A), but they showed a negative interaction with the allochthonous consortium as indicated by Simpson's diversity index (Fig. 6). All treatments except BA showed an increment of biodiversity with time, the majority being those biostimulated. This result suggests, indigenous microbial community and not the allocthonous is responsible for increasing the richness and/or the evenness during the period of the creosote degradation. In addition the Allocthonous consortium may not be suited to the cold temperatures during most of the experiment period. Also it may compete with the natural microbial community which is adapted to the environmental conditions and has the intrinsic and efficient capacity to degrade creosote. These results could confirm the low efficiency of the bioaugmentation treatment under these conditions. There is also great controversy as to whether pre-exposure to a pollutant is required for bacteria degradation [44] or whether it is an intrinsic characteristic present in some species of the microbial community that is expressed when the community is exposed to a pollutant [3,33,45,46]. Our results confirm the intrinsic capacity of bacteria to degrade PAH without previous exposure to these pollutants.

4.2 Bacterial community response to bioremediation treatments and to perturbation by creosote addition

As selective pressures such as physical disturbance, pollution or nutrient addition shape the diversity of bacterial communities [47], these parameters should be used to assess the impact of treatments. It has been previously demonstrated [13,48] that microbial communities in contaminated soils are typically less diverse than those in non-stressed systems [1,13]. However, the diversity index (Fig. 6) found that the polluted and biostimulated soil was more diverse than the unpolluted soil. Diversity is influenced by type of pollutant and time of exposure. In this study, creosote contamination for almost 6 months changed the community [49], which adapted to the new conditions. The control soil (uncontaminated) had a bacterial community with an important resistance and the capacity to grow and survive at the high level of toxicity produced by the creosote. The degradation process is carried out in different metabolical steps in which some microbial populations start degrading actively, and primary pollutants generate secondary products which other populations can access more easily,

increasing microbial abundance [50]. As previously reported, major changes in the microbial community may occur to a greater extent from the time biodegradation starts [50]. Creosote depletion increased greatly, and subsequently D_S, when ambient temperature exceeded 15ºC (145 days). Biostimulation (treatments BS and BS+BA) increased biodiversity and the number of degrading microorganisms during the period of highest activity (from 145 to 176 days). An increase in the abundance of degrading bacteria was also observed in the natural attenuation and biostimulation treatments (Fig. 1), suggesting that an intrinsic degrading capacity in some of the soil microorganisms was expressed when exposed to the pollutant. Furthermore, important changes in the bacterial community took place from 145 to 176 days. These changes were more intense in nutrient addition treatments BS and BS+BA with the appearance of 24 and 19 new bands, respectively, at 176 days. These results are in agreement with other authors [51] who state that nutrient addition may maintain bacterial community diversity. When autochthonous communities are able to degrade pollutants, nutrient addition improves community development, consequently increasing diversity and abundance. Diversity and creosote degrading microorganisms decreased when bioaugmentation was applied, most likely due to competition or incompatibility between the inoculated consortium and the autochthonous population [9,12,13]. Although inoculation of a consortium into the native population should increase diversity and creosote depletion capacity, the ability of both populations to degrade creosote and PAH would result in them competing for this resource. Therefore, the most effective population in metabolizing the pollutants, which is the autochthonous community, prevails over the other, whose diversity and abundance decrease. The results of this study also show that the period of time used for the bioremediation process was not long enough for the microbial assemblage to recover from creosote perturbation, i.e. resilience did not occur even though creosote concentration was highly depleted.

In spite of the differences between treatments at 176 days, DGGE (Fig. 5) showed that bands 22, 23 and 26 (*Pseudomonas fluorescens*, unidentified and *P. trivialis*, respectively, Table 2) were present in all treatments including natural soil. Furthermore, only polluted treatments with creosote (NA, BS, BA and BS+BA) share intense bands 11 and 13 (unidentified bands). Our results showed that 60% of the DUB identified (DUB-12RS to DUB-17RS) at 145 and 176 days belonged to *Pseudomonas* genus, which has been widely studied in bioremediation processes [52,19]. Our results showed that this genus

was the only representative group at 145 days and the most representative at 176 days. However, we also identified some *Pseudomonas* species grouped in *P. trivialis* (DUB-13RS), *P. poae* (DUB-15RS) and *P. Viridiflava* (DUB-14RS) clades, less commonly described in biodegradation processes [53]. α-Proteobacteria class was composed by DUB-19RS (uncultured *Balneimonas*) and DUB-20RS (uncultured *Sphingomonadales* bacterium). DUB-18RS, belonging to phylum Bacteroidetes, was previously identified in the degradation of high molecular weight organic matter in marine ecosystems, in petroleum degradation processes at low temperatures and in PAH degradation during bioremediation of creosote-contaminated soils [13,54,55]. We highlight the identification of the *Pantoea brenneri* (DUB-21RS, *Enterobacteriaceae* clade) and uncultured *Balneimonas* bacteria (DUB-19RS, *Metylbacteriaceae,* Rhizobiales clade), as they have not been previously related to creosote degradation. However, there are very few reports of the ability of some genera of the enteric bacteria group such as *Enterobacter* to degrade PAH [19, 56, 57].

Conclusions

Temperature is a very influential factor in *in situ* biodegradation processes, controlling biodegradation rates, toxicity, availability of contaminant and bacterial metabolism. Therefore, it should be considered in the design of bioremediation processes. The use of bioremediation techniques, such as biaugmentation, to increase the effectiveness of the process *in situ* involves certain changes in the composition and structure of bacterial communities. However, we should keep in mind that these changes do not always benefit the degrading community and may even decrease the effectiveness of the whole process. Preliminary studies under laboratory or small-scale ambient conditions should be carried out to assess community response to different treatments to select the most appropriate in each case. Most of the species identified during the creosote biodegradation belonged to *Pseudomonas* genus, which has been widely studied in bioremediation processes. Therefore, pre-exposure to a pollutant is not required for some bacteria because they have an intrinsic capacity to degrade the pollutants without previous exposure.

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551

552 **Table 1**

553 Analysis of variance (ANOVA) of the effects on accumulated CO_2 respiration (A), toxicity (B) and

554 residual PAH concentration (C).

Factor	d.f	S.S	*F*	*p*
A) Accumulated value of CO_2 (n=40)				
Time period	3	6.5^{-5}	31.12	***
Treatment	4	6.0^{-6}	2.02	n.s
Interval x Treatment	12	1.1^{-5}	1.34	n.s
Error	20	1.4^{-5}		
B)Toxicity (n=24)				
Time period	2	9071.33	110.754	***
Treatment	3	120.90	0.98	n.s
Interval x Treatment	6	1221.38	4.97	**
Error	12	491.43		
C) Residual PAH concentration (n=24)				
Treatment	3	951.48	5.48	*
PAH	2	1681.13	14.52	***
Treatment x PAH	6	178.47	0.51	n.s
Error	12	694.86		

555 * *p*-value < 0.05; ** *p*-value < 0.01; *** *p*-value < 0.001

556

557 **Table 2**

558 Correspondence between bands and degrading uncultured bacteria (DUB) and bacteria identification

559 and percentage of similarity from the GenBank data base.

145 days		176 days	
Band	Degrading Uncultured Bacterium (DUB)	Band	Degrading Uncultured Bacterium (DUB)
3	DUB-12RS, DUB-17RS	4	DUB-19RS DUB-20RS
4	DUB-12RS, DUB-15RS DUB-16RS	11.2	DUB-13RS DUB-21RS
10	DUB-14RS DUB-15RS DUB-17RS	11.3	DUB-18RS
8.10	DUB-15RS DUB-13RS	22	DUB-12RS
16	DUB-17RS	26	DUB-13RS
17	DUB-12RS DUB-16RS		
26	DUB-15RS		
27	DUB-12RS DUB-15RS		
Clone ID DUB	Similarity (%)	Mayor relationship with bacteria of Gen Bank (acc. No)	
DUB12-RS	98	*P. fluorescens* (GQ 496662)	
DUB13-RS	99	*P. trivialis (HM134251)*	
DUB14-RS	99	*P. viridiflava* (HM190224)	
DUB15-RS	99	*P. Poae* (HM640290)	
DUB16-RS	98	*Pseudomonas* sp. (HQ677222)	
DUB17-RS	98	*P. parafulva* (HQ406758)	
DUB18-RS	99	Uncultured Flexibacteriaceae	
DUB19-RS	99	Uncultured Balneimonas	
DUB20-RS	99	Uncultured Sphingomonadales	
DUB21-RS	99	*Pa. brenneri* (HM163514)	

560

Figure captions

Fig. 1. Evolution of the average monthly values of ambient temperature during the last 30 years (■) and observed during the experiment (●).

Fig. 2. Number of creosote-degrading microorganisms cells at 6 (▬), 40 (▭), 145 (▬) and 176 (▬) days for each treatment (C control, NA natural attenuation, BS biostimulation, BA bioaugmentation, BS+BA biostimulation and bioaugmentation). Numbers above each treatment show the nutrients media data ± standard error (ppm).

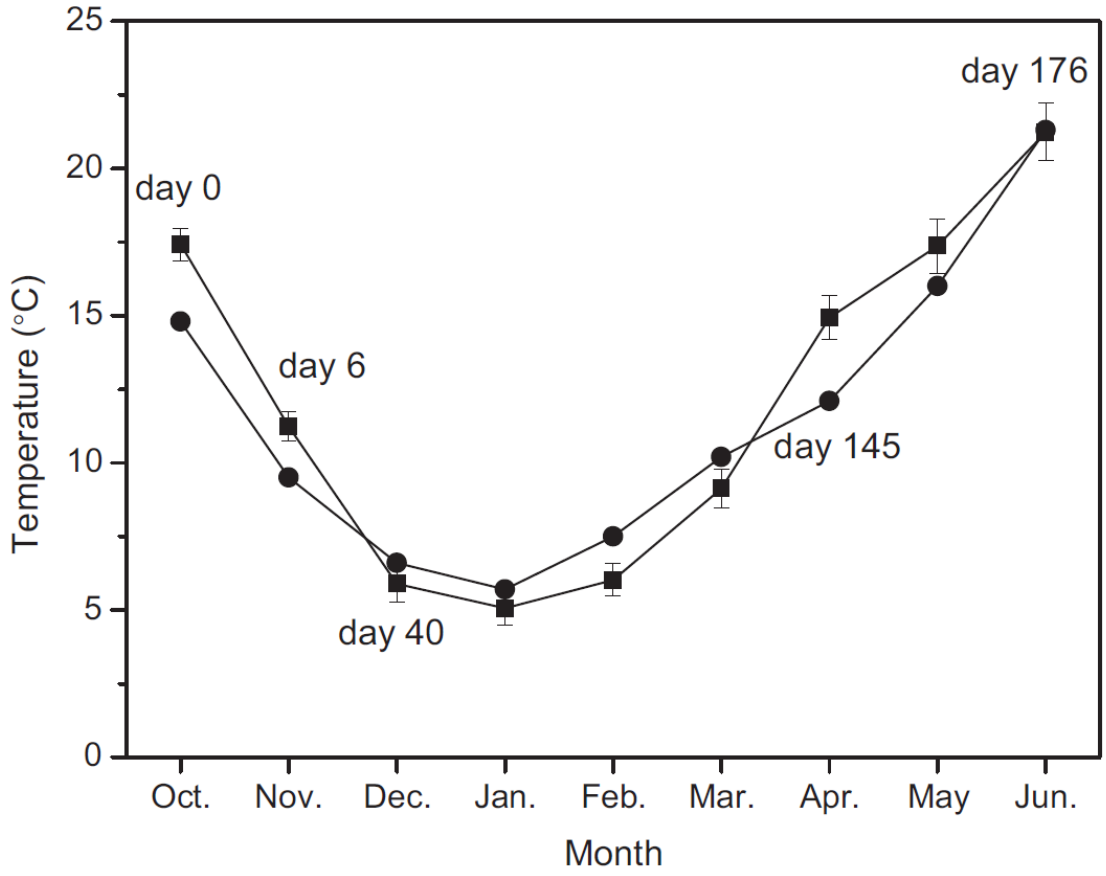
Fig. 3. Accumulated CO_2 production during respiration assays at 6, 40, 145 and 176 days. Error bars show standard deviation and letters show significant differences between groups ($p<0.05$, SNK).

Fig. 4. (A) Toxicity (%) of each treatment C (control ■), NA (natural attenuation ●), BS (biostimulation ▲), BA (bioaugmentation ▼) and BS+BA (biostimulation and bioaugmentation ◆) during the creosote degradation process under *in situ* conditions. (B) Toxicity (%) of each treatment at time interval 1 (day 0 to 6, white columns), time interval 2 (day 7 to 40, grey columns) and time interval 3 (day 41 to 176, black columns). Error bars show standard deviation and letters correspond to differences between groups ($p<0.05$, SNK).

Fig. 5. (A) Evolution of creosote depletion at *in situ* conditions at 6 days (white bars) and 176 days (black bars). (B) Average residual concentration of some key PAH at 176 days. (C) Average residual total PAH concentration for each treatment: control (C), natural attenuation (NA), biostimulation (BS), bioaugmentation (BA) and biostimulation + bioaugmentation (BS+BA). Error bars show the standard error and letters show significant differences between groups ($p<0.05$, SNK).

Fig. 6. Denaturing gradient gel electrophoresis (DGGE) at 145 days (left) and 176 days (right) of PCR-amplified 16S rDNA gen fragments from the consortium C2PL05 (B), control (C), natural attenuation (NA), biostimulation (BS), bioaugmentation (BA) and biostimulation + bioaugmentation

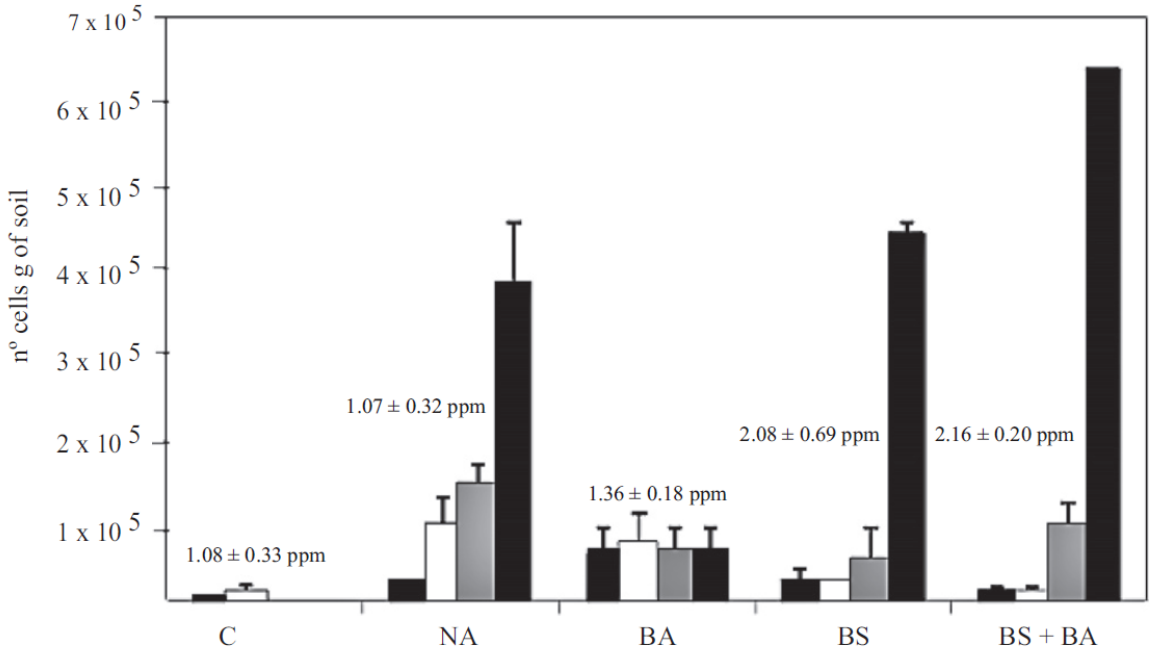
589 (BS+BA). Lane A shows molecular weight markers. Numbers correspond to the bands cloned. The
590 relationship between each band and the degrading uncultured bacterium (DUB) is shown in Table 2.
591
592 **Fig. 7.** Non-metric multidimensional scaling ordinatios (nMDSs) on the basis of Bray-Curtis
593 dissimilarity measure for the treatments: natural attenuation (NA), bioaugmentation (BA),
594 biostimulation (BS), bioaugmentation + biostimulation (BA+BS) and control (C) at 145 days (A) and
595 176 days (B).
596
597 **Fig. 8.** Change in Simpson's diversity index (D_S) with time (6, 40, 145 and 176 days) for the following
598 treatments: control (C), natural attenuation (NA), biostimulation (BS), bioaugmentation (BA) and
599 biostimulation + bioaugmentation (BS+BA).
600
601 **Fig. 9.** Phylogenetic relationships of the degrading uncultured bacteria (DUB) obtained from the
602 DGGE of treatments at 145 and 176 days of the process. Phylogenetic relationships showing the
603 topology of the maximum parsimony (MP) tree with the bootstrap values of the neighbor joining and
604 maximum parsimony higher than 50% showed on the branch of the tree (NJ/MP). No incongruence
605 was detected between parsimony and neighbour joining topology. ^{T}= type strain.
606



607

608

609 **Figure 1**



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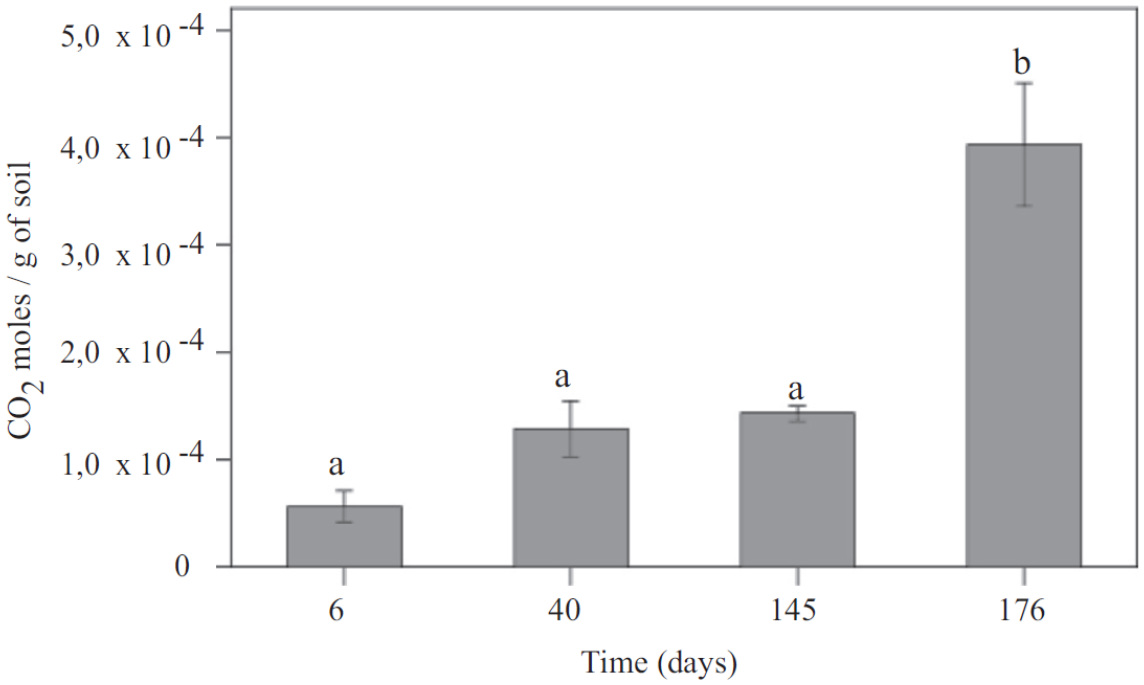
611 **Figure 2**

612



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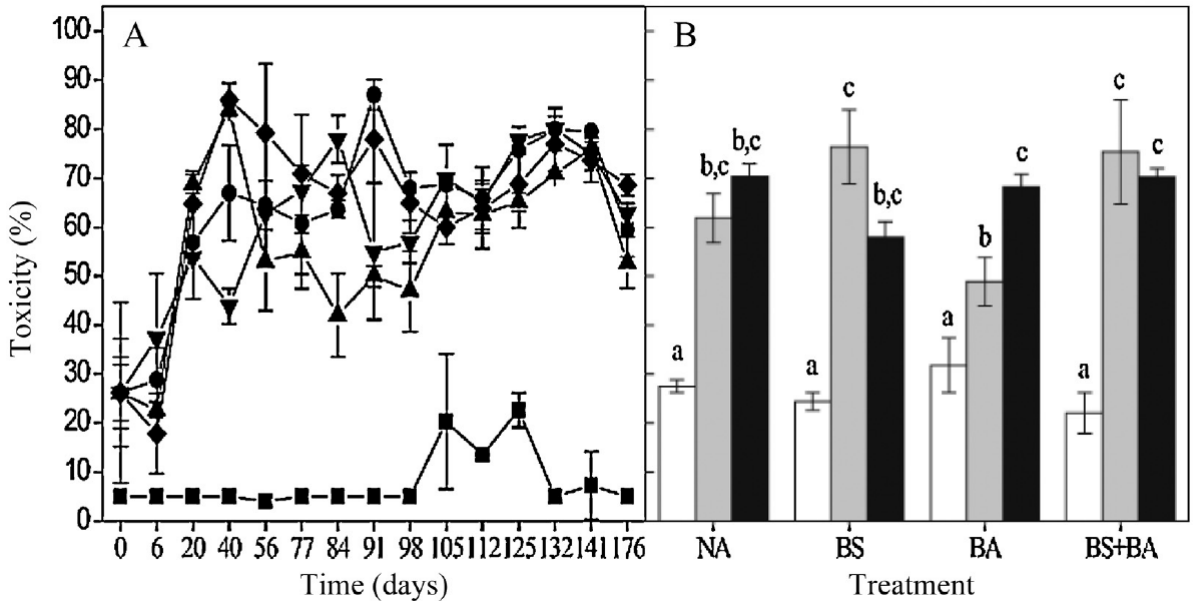
614 **Figure 3**

615



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617 **Figure 4**

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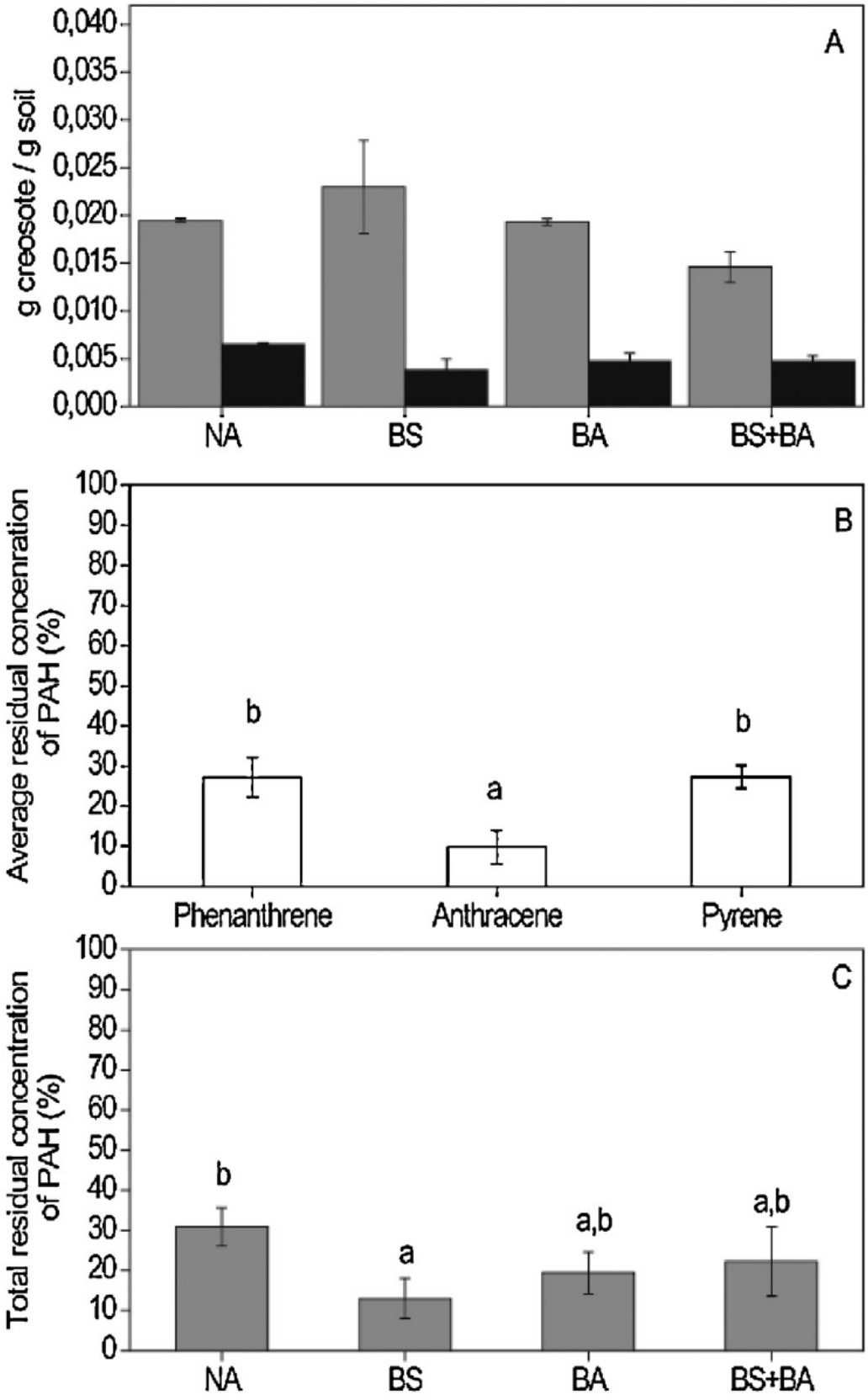

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620 **Figure 5**

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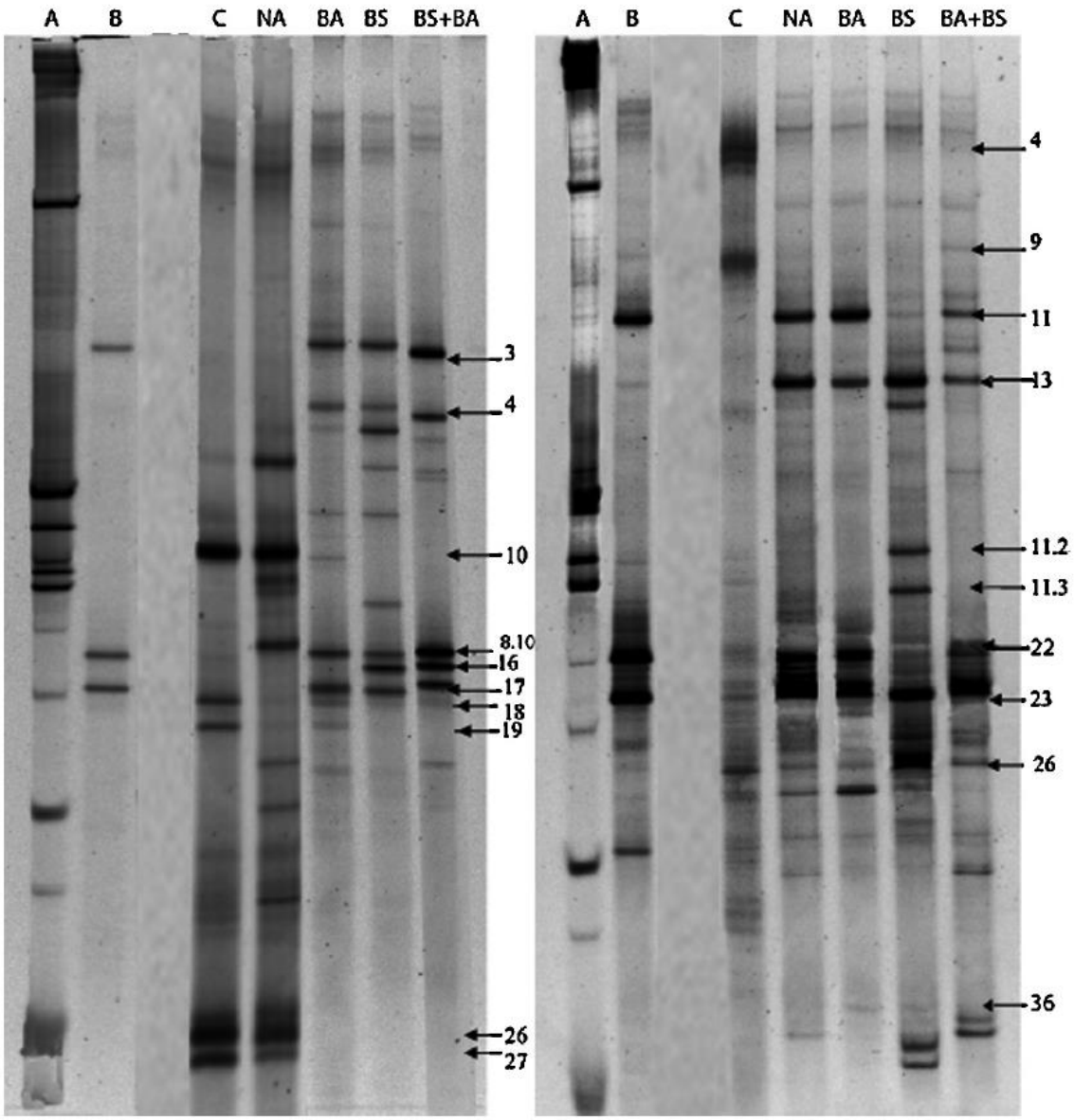

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623 **Figure 6**

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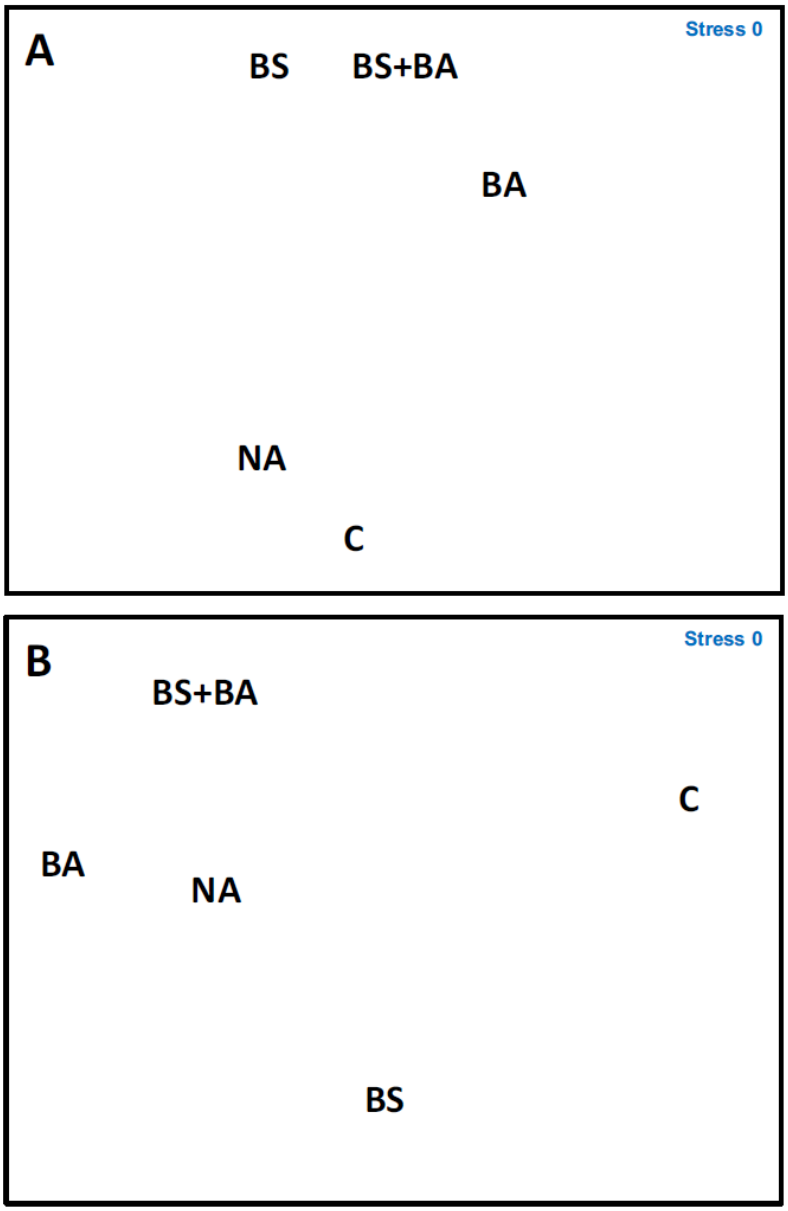

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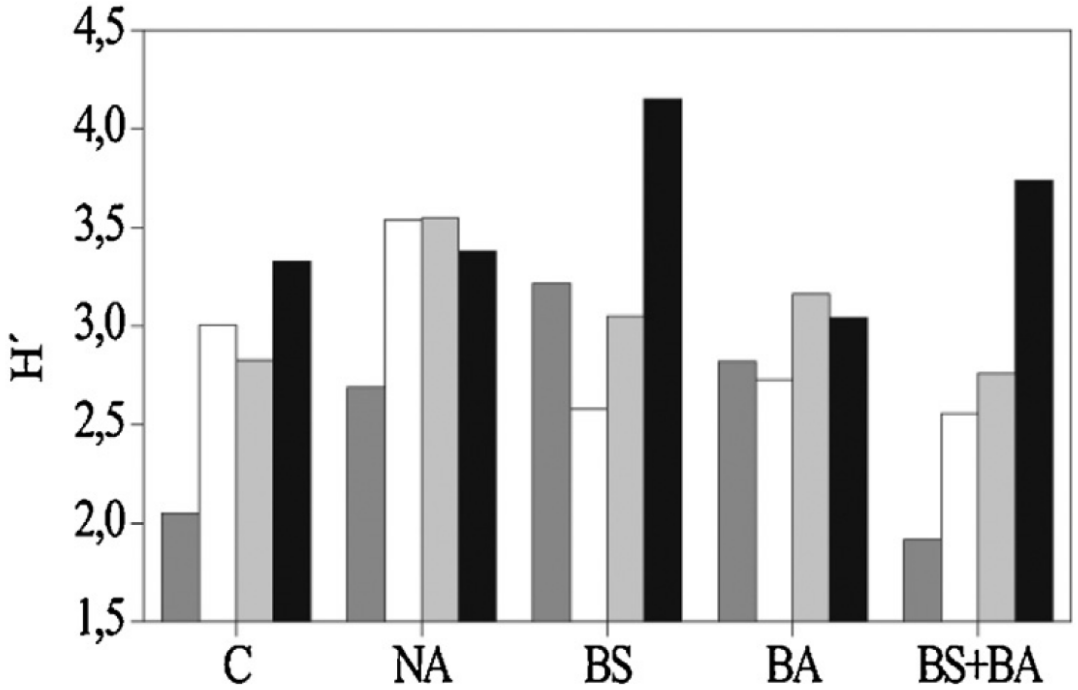
626 **Figure 7**

627



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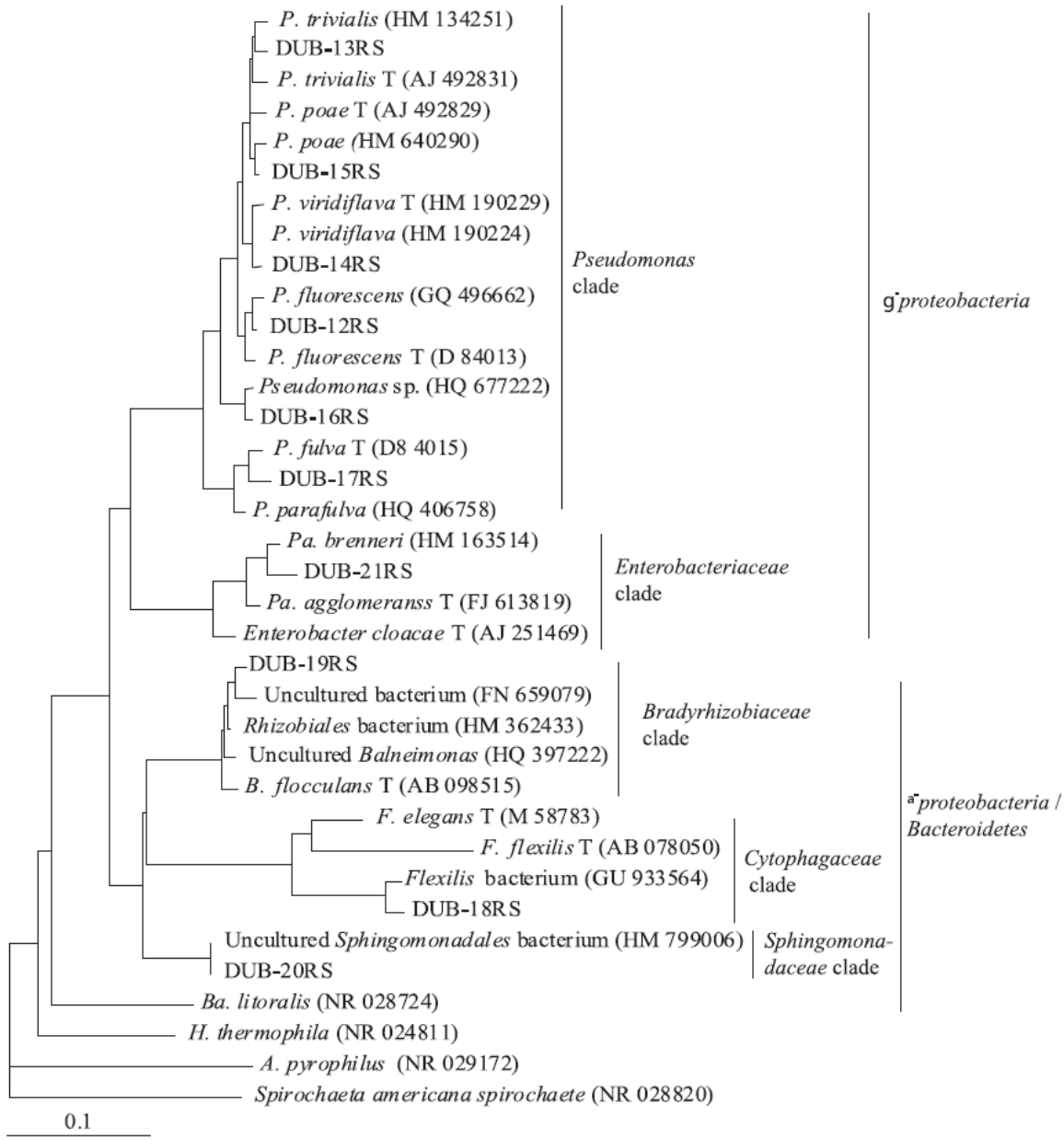
629 **Figure 8**

630



631

632 **Figure 9**