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Comparative bioremediation of heavy metals and petroleum hydrocarbons co-contaminated soil by natural attenuation, phytoremediation, bioaugmentation and bioaugmentation-assisted phytoremediation

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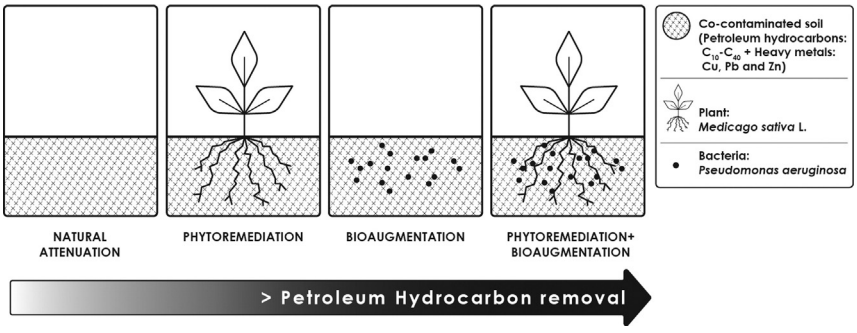
HIGHLIGHTS

- Knowledge on biological remediation of co-contaminated soils is not sufficient.
- Bioremediation strategies were compared through a pot experiment in growth-chamber.
- Alfalfa was able to tolerate and grow in a moderately co-contaminated soil.
- *Pseudomonas aeruginosa* promoted plant growth and alleviated plant stress.
- Alfalfa-*Pseudomonas aeruginosa* association enhanced petroleum hydrocarbon removal.

GRAPHICAL ABSTRACT



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ABSTRACT

Biological remediation technologies are an environmentally friendly approach for the treatment of polluted soils. This study evaluated through a pot experiment four bioremediation strategies: a) natural attenuation, b) phytoremediation with alfalfa (*Medicago sativa* L.), c) bioaugmentation with *Pseudomonas aeruginosa* and d) bioaugmentation-assisted phytoremediation, for the treatment of a co-contaminated soil presenting moderate levels of heavy metals (Cu, Pb and Zn at 87, 100 and 110 mg kg^{-1} DW, respectively) and petroleum hydrocarbons (3800 mg kg^{-1} DW). As demonstrated by plant biomass and selected physiological parameters alfalfa plants were able to tolerate and grow in the co-contaminated soil, especially when soil was inoculated with *P. aeruginosa*, which promoted plant growth (56% and 105% increase for shoots and roots, respectively) and appeared to alleviate plant stress. The content of heavy metals in alfalfa plants was limited and followed the order: Zn > Cu > Pb. Heavy metals were mainly concentrated in plant roots and were poorly translocated, favouring their stabilization in the root zone. Bioaugmentation of planted soil with *P. aeruginosa* generally led to a decrease of plant metal concentration and translocation. The highest degree of total petroleum hydrocarbon removal was obtained for bioaugmentation-assisted phytoremediation treatment (68%), followed by bioaugmentation (59%), phytoremediation (47%) and natural attenuation (37%). The results of this study demonstrated that the

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combined use of plant and bacteria was the most advantageous option for the treatment of the present co-contaminated soil, as compared to natural attenuation, bioaugmentation or phytoremediation applied alone.

1. Introduction

A recent European report estimates a total number of 2.5 million potentially contaminated sites in Europe and it is expected that 340,000 of these sites are contaminated and likely to require remediation, showing the significance of this problem (Van Liedekerke et al., 2014). The most frequent contaminants are heavy metals and mineral oils, affecting 35% and 24% of European soils, respectively (Van Liedekerke et al., 2014). Moreover, it is not uncommon that these pollutants are present together in polluted soils, rendering their remediation more difficult. These pollutants arise in the environment from various sources deriving from anthropogenic activities. Heavy metals originate mainly from human activities related to energy and mineral consumption (Kabata-Pendias, 2011), while petroleum hydrocarbons usually come from accidental spills of petroleum-based products commonly-used as fuels for transportation (Chartered Institute of Environmental Health, 2009). Both types of pollutants entail a danger for the environment and living organisms (Tchounwou et al., 2012; Todd et al., 1999).

In contrast to "traditional" soil remediation technologies (e.g. excavation, off-site disposal), biological methods are environmentally friendly and particularly attractive because of their low cost and relatively simple maintenance (Mirsal, 2008). Natural attenuation, bioaugmentation and phytoremediation are examples of biological remediation strategies and can be used for the remediation of soils affected by different types of pollutants. Natural attenuation consists of the use of natural processes (e.g. biodegradation, dispersion, sorption, volatilization, (bio)chemical stabilization) to contain and/or reduce the concentration of pollutants at contaminated sites (EPA, 1999; Mulligan and Yong, 2004). Biodegradation of target compounds by indigenous microbial communities is frequently considered to be the primary mechanism for attenuation of contaminants (Declercq et al., 2012). Bioaugmentation enables an increase of biodegradative capacities of contaminated sites by the introduction of single strains or consortia of microorganisms with the desired catalytic capabilities (Lebeau, 2011; Mrozik and Piotrowska-Seget, 2010). Finally, phytoremediation comprises a group of technologies that use plants and their associated microorganisms to remove pollutants from the environment or to make them harmless (Salt et al., 1998). Plant uptake, translocation and accumulation of heavy metals (phytoextraction), their stabilization in the root zone (phytostabilization) and the metabolism of organic pollutants by rhizosphere microorganisms (rhizodegradation) are examples of phytoremediation processes. Natural attenuation, bioaugmentation and phytoremediation approaches can be used not only as remediation technologies in themselves but also in combination. For instance, bioaugmentation can be coupled with phytoremediation to intensify clean-up processes (Glick, 2003; White, 2001). In particular, bioaugmentation-assisted phytoextraction optimizes the synergistic effect of plants and microorganisms and has been used for the cleaning-up of soils contaminated by metals (Huguenot et al., 2015; Lebeau et al., 2008). This enhanced trace element uptake by plants can be ascribed to an increase in root absorption ability and/or to an enhancement of trace metal bioavailability in the rhizosphere, mediated by microorganisms (Sessitsch et al., 2013).

Moreover, plant-microorganism associations can also be used to facilitate the removal of organic contaminants (Glick, 2010). In particular, some studies have addressed the combined use of plants and biodegradative bacteria with the aim to remove petroleum products (Lin et al., 2008b), which seems to be a promising remediation strategy.

A key aspect in biological remediation methods is the selection of appropriate plant-bacteria partnerships for the remediation of polluted soils (Khan et al., 2013). Among plants used in phytoremediation, alfalfa (*Medicago sativa* L.) is of particular relevance. It is a fast growing species (Hallam et al., 2001) that develops an extensive tap root system favourable for the establishment of rhizosphere microorganisms (Kirk et al., 2005) and can associate with symbiotic nitrogen fixing bacteria (Truchet et al., 1991). Alfalfa has been assessed for the remediation of several types of pollutants: heavy metals like Cd, Cr, Cu, Ni and Zn (Bonfranceschi et al., 2009; Peralta-Videa et al., 2002, 2004), petroleum hydrocarbons (Kirk et al., 2002; Wiltse et al., 1998), polycyclic aromatic hydrocarbons (PAHs) (Fan et al., 2008) or organochlorines (Li and Yang, 2013). Moreover, recent findings have shown promising results for alfalfa phytoremediation of co-contaminated soils (Ding and Luo, 2005; Ouvrard et al., 2011; Zhang et al., 2013).

Among bacterial strains used for bioremediation, *Pseudomonas aeruginosa* is especially interesting because it can improve pollutant remediation through various mechanisms. Firstly, *P. aeruginosa* has been described to produce metal chelating siderophores, which could improve metal bioavailability (Visca et al., 2007). Secondly it produces biosurfactants (rhamnolipids) that can enhance the solubility of poor water-soluble organic compounds and the mobility of heavy metals (Mulligan, 2005; Zhang et al., 2012), improving their bioavailability. As a result, *P. aeruginosa* has been tested for bioremediation of metals (Singh et al., 2013) and hydrocarbons (Das and Mukherjee, 2007). Finally, a role as plant growth promoting rhizobacteria (PGPR) has been described for *P. aeruginosa*, which leads to improved plant growth and enhanced phytoremediation rates (Wang et al., 2011).

The aim of this study was to perform a comparative assessment of four bioremediation strategies: a) natural attenuation, b) phytoremediation with alfalfa, c) bioaugmentation with *P. aeruginosa* and d) bioaugmentation-assisted phytoremediation, for the treatment of a soil co-contaminated by moderate levels of heavy metals and petroleum hydrocarbons.

2. Materials and methods

2.1. Soil samples

Soil samples were collected from an urban area close to a fuel station with a history of contamination by heavy metals and petroleum hydrocarbons, mostly diesel. Samples were taken with a drill auger, which allowed collecting soil from different depths between 0 and 100 cm. This soil was sieved to pass through a 6 mm mesh and homogenized. To limit the level of pollutants, the contaminated soil was mixed (1:1 *w*/*w*) with soil from the same site but characterized by negligible hydrocarbon contamination. Before mixing, this soil was sieved through a 2 mm mesh. Selected chemical and physical properties of this unique composite sample (1:1 *w*/*w* mix of both soils) are presented in Table 1. Initial physicochemical characterization of soil samples was performed by a certified laboratory: ALcontrol Laboratories (The Netherlands). ALcontrol is accredited by the Cofrac (Comité français d'accréditation) and by the RvA (Raad voor Accreditatie) under number L028, in accordance with the criteria of laboratory analysis: ISO/IEC 17025:2005.

2.2. Plants

Alfalfa seeds (*M. sativa* L. v. La Bella Campagnola, purity: 99%, germinability: 85%) were surface disinfected by immersion in 2% (*v*/*v*) hydrogen peroxide for 8 min (Qu et al., 2011), in order to avoid the addition of non-indigenous microorganisms to the system. Then, seeds were thoroughly rinsed three times with sterile water and used for the pot experiment.

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Table 1
Chemical and physical properties of the soil.

Agronomic parameters	
pH (H_2O)(NF ISO 10693)	8.1
Cation exchange capacity at soil pH ($cmol^+ kg^{-1}$ DW)(NF X 31-130)	10.7
Organic matter ($g\ kg^{-1}$ DW)(NF ISO 14235)	49
Organic carbon ($g\ kg^{-1}$ DW)(NF ISO 14235)	28.3
Total nitrogen ($mg\ kg^{-1}$ DW)(Internal method, NEN 6604)	640
C/N ratio	44
P_2O_5 ($g\ kg^{-1}$ DW)(Joret-Hebert method, NF X 31-161)	0.10
K_2O ($g\ kg^{-1}$ DW)(NF X 31-108)	0.09
MgO ($g\ kg^{-1}$ DW)(NF X 31-108)	0.12
CaO ($g\ kg^{-1}$ DW)(NF X 31-108)	9.63
Fe* ($mg\ kg^{-1}$ DW)(NF X 31-121)	116
Mn* ($mg\ kg^{-1}$ DW)(NF X 31-121)	19.5
B* ($mg\ kg^{-1}$ DW)(NF X 31-122)	0.71
Sand (%)(NF X 31-107)	82.6
Silt (%)(NF X 31-107)	12.5
Clay (%)(NF X 31-107)	4.9
Total heavy metal content ($mg\ kg^{-1}$ DW) (Internal method: destruction in accordance with NEN 6961, analyses in accordance with ISO 22036 and NEN-ISO 16772)	
As	7.4
Cd	0.36
Cr	<10
Cu	87
Hg	1.0
Pb	100
Ni	8.7
Zn	110
Hydrocarbons ($mg\ kg^{-1}$ DW)	
C_{10}–C_{12}	130
C_{12}–C_{16}	1100
C_{16}–C_{21}	1600
C_{21}–C_{40}	830
Total C_{10}–C_{40}(Equivalent to NEN-EN-ISO 16703)	3600

DW: dry weight.
ISO: International Organization for Standardization.
EN: European Norm.
NF: French Norm.
NEN: Netherlands Standardization Institute.
* DTPA (diethylenetriaminepentaacetic acid) extraction.

2.3. Bacteria

The bacterial strain *P. aeruginosa* ATCC® 9027 was used as inoculum for the bioaugmentation treatments. This strain was bought as Vitroids™ discs of bacteria (Sigma-Aldrich Chimie S.a.r.l., Lyon, France) containing 1000 CFU (colony forming units).

2.4. Pot experiment

Disinfected alfalfa seeds were sown in a commercial soil (organic carbon: 20%, organic nitrogen: 0.4%, organic matter: 40%, dry matter content: 58%), where seedlings grew for 21 days in a growth chamber (Sanyo Versatile Environmental Test Chamber MLR-352). Growth conditions were as follows: photoperiod of 16 h light at 22 °C and 8 h dark at 18 °C, photosynthetic photon flux density (PPFD) of 130 μmol m^{-2} s^{-1}. Subsequently, ten seedlings of uniform size were selected and transplanted in plastic pots (8 × 10 cm) filled with 200 g of the contaminated soil. A pre-growth phase in the commercial soil allowed seedlings to develop certain biomass and favoured a better establishment in the co-contaminated soil after transplanting. Pots containing the transplants were put in the growth chamber (same conditions as described above) and received water daily. The location of pots was randomly changed daily. The experimental design included four experimental conditions: (a) natural attenuation (NA, intrinsic clean up ability of the soil), (b) phytoremediation (PR, soil vegetated with alfalfa), (c) bioaugmentation (BA, soil inoculated with *P. aeruginosa* strain), and (d) bioaugmentation-assisted phytoremediation (BA + PR, soil vegetated with alfalfa and inoculated with *P. aeruginosa* strain). Bioaugmentation was performed every 15 days, i.e., up to six times during the experiment, with the aim to maintain an elevated number of microorganisms throughout the experiment, as described by Huguenot et al. (2015). *P. aeruginosa* was added to pots as 5 ml of cell suspension (4.0×10^{11}–1.0×10^{12} CFU ml^{-1}). Non-bioaugmented pots received the same amount of sterile distilled water. Each condition was performed in triplicates. Plants were harvested after 30, 60 and 90 days of growth in the co-contaminated soil (the different treatments were grown in parallel) and every time bioaugmentation was performed three days before. Plants were removed from pots, and roots and shoots were separated. A fraction of fresh shoots was kept for subsequent analyses. Roots were first washed with distilled water to remove attached soil particles and with ethylenediaminetetraacetic acid (EDTA, 10 mM) afterwards to remove adsorbed metals. Roots were further rinsed with distilled water and blotted with tissue paper. The plant material was put at 70 °C for 3 days (Tabatabai, 1998) and dry weights of shoots and roots were recorded. Dried plant material was used for further analyses. Soil samples were collected at the end of the experiment (90 days) and kept at 4 °C until further microbiological and chemical analyses. In the case of vegetated pots, rhizosphere soil samples were taken. In order to collect rhizosphere soil, plant roots were vigorously shaken by hand, taking care of the roots' integrity. The external soil not attached to roots was removed, while the soil in the immediate vicinity of roots was kept for analyses.

2.5. Plant analyses

2.5.1. Parameters to assess plant physiology

Chlorophyll and flavonol contents were measured every 15 days during the course of the experiment using the sensor DUALEX SCIENTIFIC + ™. This portable battery-powered fluorimeter with a light-emitting diode possesses a leaf-clip to measure in an instantaneous and non-destructive way chlorophyll content in plant leaves and flavonol content in plant epidermis. Each measurement was performed on two leaves per pot in order to have a representative sample. A consensus equation was used for the transformation of optical chlorophyll readings into surface-based specific units (μg of chlorophyll cm^{-2}) (Cerovic et al., 2012). The content of flavonols was expressed in arbitrary units (a.u.).

The maximum quantum yield of photosystem (PS) II was measured every 15 days during the course of the experiment with a portable pulse modulated fluorimeter (Hansatech Fluorescence Monitoring System, FMS1) able to detect chlorophyll fluorescence emissions. Leaves were dark adapted for at least 15 min using leaf clips designed for use with the FMS1. Following dark adaptation the modulated light was turned on, the minimal fluorescence (F_0, with all PSII reaction centres fully open) signal recorded and then a saturating pulse applied to measure the maximal fluorescence (F_m, with all PSII reaction centres fully closed). The maximum quantum yield of PSII, which quantifies the maximal efficiency of photon capture by open PSII reaction centres, was calculated as $F_v/F_m = (F_m - F_0)/F_m$ (Papageorgiou and Govindjee, 2004).

Malondialdehyde (MDA) content was measured according to the colorimetric method in which MDA contained in fresh plant tissue extracts reacts with thiobarbituric acid (TBA) at 95 °C for 25 min to form a coloured product whose absorbance is recorded at 532 nm. The modified method described by Hodges et al. (1999) allows correcting for plant interfering compounds that also absorb at 532 nm. The effect of these interferences is avoided by subtracting the absorbance at 532 nm of a solution containing plant extract incubated without TBA from an identical solution containing TBA. Results of MDA concentration are expressed as nmol MDA equivalents per g of fresh plant tissue.

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2.5.2. Heavy metal content in plant parts

Prior to elemental analyses, dried plant material was wet digested as described by Tabatabai (1998). Briefly, plant material was mixed with 5 ml concentrated nitric acid and 2 ml 30% hydrogen peroxide in a digestion block (LabTech DigiBlock Digester ED16S) at 125 °C for 1 h. Heating cycles and hydrogen peroxide additions were repeated three times to obtain a clear digest. To remove residual particles, mineralized samples were filtered through cellulose filters (pore size 2.5 μm) and brought to a final volume of 20 ml. Samples were additionally filtered through nitrocellulose syringe filters (pore size 0.45 μm) and stored at 4 °C until heavy metals were analysed by Inductively Coupled Plasma-Optical Emission Spectrometry (PerkinElmer Optima 8300 ICP-OES Spectrometer). Cu, Pb and Zn were analysed at the respective wavelengths of 324.752 nm, 220.353 nm and 213.857 nm.

2.6. Calculation of phytoextraction parameters

To evaluate the performance of metal phytoextraction the following parameters were considered: a) plant biomass, b) metal concentration in plant tissues, c) total metal uptake per pot calculated as: $C_{plant} \times M_{plant}$ where C_{plant} is the metal concentration in the plant tissues ($\mu g\ mg^{-1}$) and M_{plant} is the plant biomass (mg) per pot produced in one harvest, d) translocation factors: $TF = C_{shoot}/C_{root}$ and e) bioconcentration factors (BCF): $BCF_{shoots} = C_{shoot}/C_{soil}$ and $BCF_{roots} = C_{root}/C_{soil}$, where C_{shoot} and C_{root} are metal concentrations in the shoot ($mg\ kg^{-1}$) and root of plants ($mg\ kg^{-1}$), respectively, and C_{soil} is the metal concentration in the soil ($mg\ kg^{-1}$).

2.7. Soil analyses

2.7.1. Total petroleum hydrocarbons

Total petroleum hydrocarbons (TPH) and their fractions (C_{10}–C_{12}, C_{12}–C_{16}, C_{16}–C_{21}, C_{21}–C_{40}) were quantified in soil samples by a certified laboratory: AlControl Laboratories, which performed an internal analytical method based on the standard procedure NEN-EN-ISO 16703: soil acetone-hexane extraction was followed by purification and analysis by gas chromatography with flame ionization detector (GC-FID).

2.7.2. Microbiological indicators

Total heterotrophic microflora was counted in microwell plates, filled with 200 μl of lysogeny broth (LB) and inoculated with 20 μl of appropriate dilutions of a soil suspension prepared mixing 1 g of fresh soil and 10 ml of potassium chloride solution (9 g l^{-1}, pH 7.0). Plates were incubated for 5 days at 25 °C. The number of positive wells (visible turbidity) was scored and the microbial concentrations in soil were calculated by using the most probable number method (MPN) designed by Briones and Reichardt (1999).

Alkane-degrading microorganisms were enumerated by the MPN method described by Wrenn and Venosa (1996), using 96-well microtiter plates. Bushnell-Haas medium supplemented with 2% sodium chloride was used as the growth medium (180 μl per well) and n-hexadecane (5 μl per well) was added as the selective growth substrate. 10-fold serial dilutions were performed from a suspension of 1 g of fresh soil and 10 ml of 0.1% sodium pyrophosphate (pH 7.5) and 2% sodium chloride. Plates were inoculated by adding 20 μl of the dilutions from 10^{-2} to 10^{-7}, in five replicates. Microplates were incubated for two weeks at room temperature. Afterwards, 50 μl of iodonitrotetrazolium violet (INT, 3 g l^{-1}) was added to identify positive wells, in which INT is reduced to an insoluble formazan that deposits intracellularly as a red precipitate. The scoring was done after incubating overnight with INT at room temperature. MPN of alkane-degrading microorganisms per g of soil was calculated according to Briones and Reichardt (1999).

Soil lipase activity was measured through the colorimetric method described by Margesin et al. (2002). 0.1 g of fresh soil was mixed with 5 ml 100 mM sodium dihydrogen phosphate/sodium hydroxide buffer, pH 7.25, and pre-warmed at 30 °C for 10 min. 50 μl of substrate solution (100 mM p-nitrophenyl butyrate (pNPB) in 2-propanol) was added and tubes were incubated at 30 °C for 10 min. The tubes were cooled for 10 min on ice to stop the reaction and centrifuged at 2000 *g* for 5 min. The absorbance of the released p-nitrophenol (pNP) in the supernatants was measured spectrophotometrically (PerkinElmer LAMBDA 10 UV/Vis Spectrophotometer) at 400 nm against the reagent blank. A standard solution of pNP (100 μg pNP ml^{-1} phosphate buffer) was used to prepare a calibration curve in the presence of soil. In order to measure the pNP released from the substrate, a control was prepared without soil. After subtracting the control reading (hydrolysis in the absence of soil) from the sample reading (hydrolysis in the presence of soil), soil lipase activity was calculated and expressed as μg pNP $(g\ soil \times 10\ min)^{-1}$.

2.8. Statistical analysis

The experiment was arranged in a completely randomized design. All data reported were averaged values of three independent replicates. Statistical analysis of the data was carried out using both one-way (in Figs. 4 and 5) and two-way (in Table 2 and Figs. 1–3) analysis of variance (ANOVA) and multiple comparisons of means were analysed by Tukey test. Differences were considered statistically significant at $p < 0.05$. The statistical analysis was accomplished using R software, version 3.0.2 (R Developement Core Team, 2014).

3. Results and discussion

3.1. Plant growth in co-contaminated soil and the effect of bioaugmentation

After transplanting alfalfa seedlings in the contaminated soil, all plants survived and no plant mortality was evidenced throughout the 90-day experiment. Fig. 1 presents the experimental data on plant biomass for alfalfa plants growing in bioaugmented and non-bioaugmented soil as a function of experimental time. Alfalfa growth was not hindered and both above and below ground biomasses continuously increased for both treatments. After 90 days there was a *statistically* significant enhancement in plant biomass, with respect to that at the moment of transplanting. Shoot biomass increased by a factor of 25 and 39 for alfalfa growing in non-bioaugmented and bioaugmented soil, respectively. In addition, root biomass increased by a factor of 166 and 340 for alfalfa growing in non-bioaugmented and bioaugmented soil, respectively. The fact that alfalfa biomass continuously increased and no plant mortality was observed *indicates* that alfalfa was able to tolerate a co-contaminated soil presenting moderate levels of heavy metals and petroleum hydrocarbons, which is an essential condition for phytoremediation. Fast growth and high above ground biomass yield are among the requisites needed for phytoextraction purposes. In addition, the establishment of a rich root system could create a favourable niche for rhizosphere microorganisms, which is required for rhizodegradation. The results of this study seem to be consistent with those obtained by Kirk et al. (2005), who reported no alfalfa phytotoxicity up to 15,000 mg kg^{-1} *dry weight* (DW) of TPH, while they observed that growth of seedlings was stressed and stunted at higher TPH levels (31,000 mg kg^{-1} DW). Although pollutant concentration is certainly a key factor determining plant tolerance/sensitivity towards pollutants, other factors such as metal speciation, composition of heterogeneous petroleum hydrocarbon fractions, soil-pollutant and pollutant–pollutant interactions must also be considered (Salanitro et al., 1997). For instance, previous studies reported that heavy metals had a distinct effect on alfalfa when present individually at more than 50 mg kg^{-1} DW (Peralta et al., 2001) or in a mix of Cd, Cu, Ni, and Zn at 50 mg kg^{-1} DW each (Peralta-Videa et al., 2002). A combined stress that affected the shoot length of alfalfa was observed in the latter case, showing the influence of multi-metal contamination. Mechanisms underlying heavy metal and petroleum hydrocarbon phytotoxicity may be related both to direct effects on plant physiology (e.g. cell membrane disruption, damage of photosynthetic apparatus) or indirectly, altering the physical

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Table 2
Heavy metal phytoextraction parameters.

Heavy metal	Parameter	30 days		60 days		90 days	
		Alf	Alf + Pa	Alf	Alf + Pa	Alf	Alf + Pa
Cu	TF	0.49 ± 0.19 b	0.41 ± 0.18 ab	0.36 ± 0.07ab	0.22 ± 0.04 ab	0.29 ± 0.03 ab	0.14 ± 0.02 a
	BCF_{shoots}	0.19 ± 0.05 a	0.17 ± 0.09 a	0.15 ± 0.03 a	0.15 ± 0.01 a	0.24 ± 0.01 a	0.13 ± 0.01 a
	BCF_{roots}	0.40 ± 0.07 a	0.41 ± 0.04 a	0.44 ± 0.03 a	0.69 ± 0.12 b	0.81 ± 0.10 b	0.88 ± 0.12 b
Pb	TF	0.83 ± 0.63 a	0.63 ± 0.40 a	0.69 ± 0.21a	0.41 ± 0.05 a	0.87 ± 0.02 a	0.28 ± 0.10 a
	BCF_{shoots}	0.17 ± 0.10 a	0.11 ± 0.08 a	0.09 ± 0.02 a	0.05 ± 0.00 a	0.17 ± 0.01 a	0.04 ± 0.00 a
	BCF_{roots}	0.23 ± 0.06 b	0.18 ± 0.02 ab	0.13 ± 0.02 a	0.13 ± 0.02 a	0.20 ± 0.02 ab	0.13 ± 0.04 a
Zn	TF	0.46 ± 0.07 a	0.41 ± 0.08 a	0.37 ± 0.06 a	0.42 ± 0.11 a	0.40 ± 0.06 a	0.43 ± 0.04 a
	BCF_{shoots}	0.71 ± 0.14 b	0.61 ± 0.11 b	0.52 ± 0.05 ab	0.61 ± 0.03 b	0.52 ± 0.01 ab	0.36 ± 0.03 a
	BCF_{roots}	1.53 ± 0.06 b	1.50 ± 0.11b	1.42 ± 0.13 b	1.49 ± 0.32 b	1.32 ± 0.19 b	0.84 ± 0.08 a

Translocation factor (TF) and bioconcentration factor (BCF) for alfalfa plants growing in not bioaugmented soil (Alf) and in soil bioaugmented with *P. aeruginosa* (Alf + Pa).
Values are expressed as mean ± standard deviation (n = 3). Statistical analysis of the data was performed using two-way analysis of variance (ANOVA) for the effects of treatment and time on the specific phytoextraction parameter. Means with the same letters are not statistically different according to the Tukey test with $p < 0.05$.

and chemical properties of the soil where plants are growing (Baker, 1970; Kabata-Pendias, 2011).

Bioaugmentation with *P. aeruginosa* had a positive effect on plant biomass production. There was an initial trend to improve plant biomass, which became *statistically* significant for shoots and roots after 90 days. Soil inoculation with *P. aeruginosa* enhanced shoot biomass by 15, 33 and 56% at 30, 60 and 90 days, respectively. Similarly, root biomass was also increased by 13, 19 and 105% at 30, 60 and 90 days, respectively. These results are in accordance with previous studies, in which plant growth promoting ability of *P. aeruginosa* was assessed for other plant species. For instance, it has been demonstrated that *P. aeruginosa* promoted not only dry matter accumulation but also symbiotic attributes (e.g. nodule numbers and leghemoglobin content), grain yield and protein of chickpea (*Cicer arietinum* L.) growing in a soil contaminated with Cr (Oves et al., 2013). PGPR may facilitate plant growth directly by assisting in resource acquisition and modulating plant hormone levels, or indirectly by decreasing the inhibitory effects of pathogens (Ahemad and Kibret, 2014; Puga-Freitas and Blouin, 2015). Increases of P solubilisation and indole acetic acid production have been reported for *P. aeruginosa* (Oves et al., 2013), as well as the synthesis of secondary metabolites with antibiotic activity (Spago et al., 2014). Finally, PGPR may also improve plant growth in polluted soils mitigating phytotoxicity (Khan et al., 2013). In this sense, *P. aeruginosa* has been reported to promote green pea (*Pisum sativum* L.) growth alleviating Pb toxicity through the production of metallothioneins (Naik et al., 2012). Similarly, inoculation with *P. aeruginosa* in a phenol-spiked soil vegetated with corn (*Zea mays*) resulted in plant growth promotion, which correlated with the decrease in soil phenol content (Wang et al., 2011). Although the scope of the present study was limited in terms of establishing which are the mechanisms responsible for alfalfa growth promotion by *P. aeruginosa* in the present co-contaminated soil, one or more of the above-mentioned mechanisms might have been involved.

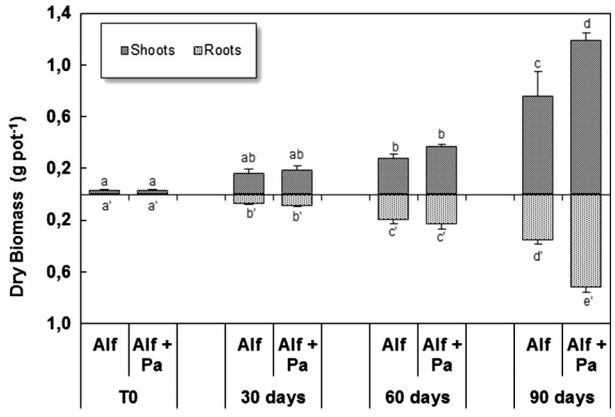

Fig. 1. Dry biomass (g pot^{-1}) for alfalfa plants growing in not bioaugmented soil (Alf) and in soil bioaugmented with *P. aeruginosa* (Alf + Pa). Values are expressed as mean ± standard deviation (n = 3). Statistical analysis of the data was performed using two-way analysis of variance (ANOVA) for the effects of treatment and time. Means with the same letters are not statistically different according to the Tukey test with $p < 0.05$. The symbol ' distinguishes root from shoot statistical analysis.

3.2. Physiological parameters of plants growing in co-contaminated soil and the effect of bioaugmentation

Fig. 2 presents selected physiological parameters of alfalfa plants growing in the co-contaminated soil alone or bioaugmented with *P. aeruginosa*.

There was a *statistically* significant decrease in chlorophyll content (Fig. 2a) and maximum quantum yield of PSII (F_v/F_m, Fig. 2b) 15 days after transplanting seedlings to the co-contaminated soil. Afterwards, chlorophyll content and F_v/F_m values gradually increased up to levels equal to or greater than the initial values. Interestingly, when bioaugmentation was performed, the recovery of these parameters appeared to be favoured. The content of chlorophyll in leaves as well as chlorophyll fluorescence measurement as marker to estimate the photochemical activity of PSII ($F_v/F_m < 0.8$ revealing plant stress) (Pessarakli, 1996) are two useful parameters to assess plant health. In the present study, photosynthetic performance of alfalfa was affected as observed by the decrease in both parameters after transplanting seedlings to the co-contaminated soil.

The highest levels in MDA content (Fig. 2c) were found at 30 days in plants growing in the non-bioaugmented soil (23% higher than the plants growing in the bioaugmented soil). Subsequently MDA content tended to decrease. MDA is a secondary end product of the oxidation of polyunsaturated fatty acids (Del Rio et al., 2005), used as an index of general lipid peroxidation (Hodges et al., 1999). Oxidative processes are responsible for cell membranes damage, which can result in a reduction of photosynthetic efficiency (Del Rio et al., 2005; Demidchik, 2014). The increase of lipid peroxidation, as observed by an increase in MDA content at 30 days, is indicative of toxicity that resulted in oxidative stress, possibly responsible for physiological perturbations on alfalfa plants. Stress processes take place when transplanting seedlings, not only as a result of moving plants but also due to the hostile characteristics of co-contaminated soil in terms of the presence of heavy metals and petroleum hydrocarbons, poor nutrient state, low organic matter content, texture, etc., which may influence plant performance.

Flavonol content (Fig. 2d) tended to increase over time in alfalfa plants. From 45 days on, and especially at 75 days, the highest leaf flavonols were found in plants growing in the soil that was not inoculated with *P. aeruginosa*. In response to oxidative stress plants are able to develop antioxidant defence systems, which comprise the synthesis of protective compounds with antioxidant activity (Gill and Tuteja, 2010). Among them, flavonols are a class of flavonoids, which are secondary metabolites able to inhibit the generation of reactive oxygen

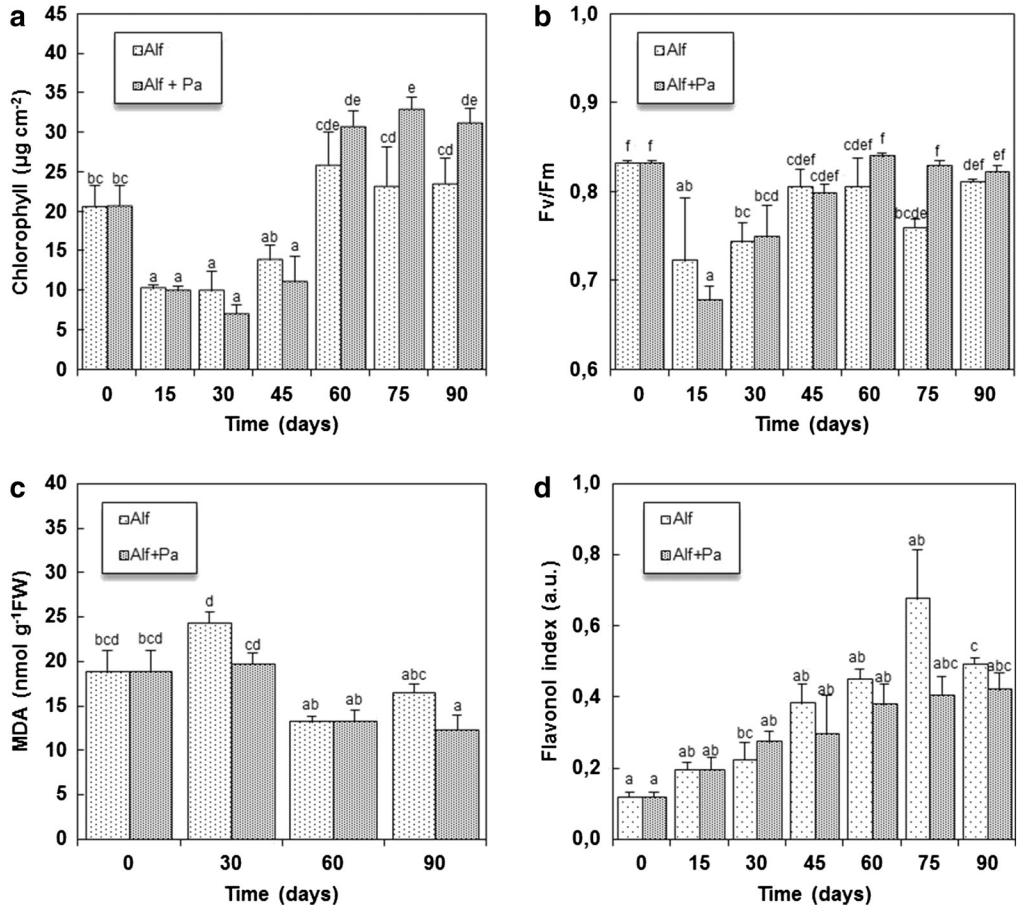

Fig. 2. Physiological parameters: (a) chlorophyll content, (b) maximum quantum yield of photosystem II (F_v/F_m), (c) malondialdehyde (MDA) content and (d) flavonol content, of alfalfa plants growing in not bioaugmented soil (Alf) and in soil bioaugmented with *P. aeruginosa* (Alf + Pa). Values are expressed as mean ± standard deviation (n = 3). Statistical analysis of the data was performed using two-way analysis of variance (ANOVA) for the effects of treatment and time on the specific physiological parameter. Means with the same letters are not statistically different according to the Tukey test with $p < 0.05$.

species (ROS) and reduce the levels of ROS once they are formed (Agati et al., 2012). The findings of the current study suggest that the synthesis of flavonols occurred in alfalfa plants after transplanting to the co-contaminated soil, possibly in response to oxidative stress. It could be hypothesized that the synthesis of such molecules helped to counteract the oxidative events taking place, although probably many other antioxidative processes and molecules could have also contributed. This hypothesis could be supported by the decrease in MDA content observed at 60 and 90 days. As a consequence of plant acclimatization to the co-contaminated soil, other physiological parameters (F_v/F_m ratio and chlorophyll content) returned to the initial levels as well.

Concerning the effect of soil inoculation with *P. aeruginosa* on alfalfa physiology, it seemed that bioaugmentation could favour plant performance. Plant growth promoting bacteria have been demonstrated to enhance plant tolerance to biotic and abiotic stresses, mitigating the levels of ROS (Jebara et al., 2005; Rodrigues et al., 2013). Hence, it could be assumed that soil inoculation with *P. aeruginosa* alleviated oxidative stress in alfalfa plants, which is in accordance with the observed decrease in MDA content and the increase in F_v/F_m values and chlorophyll content, relative to those found in alfalfa growing in the non-bioaugmented soil.

3.3. Phytoremediation treatments and uptake of heavy metals

Fig. 3a, c and e shows the data of Cu, Pb and Zn concentrations in alfalfa tissues depending on treatment and experimental time. Heavy metal concentrations in shoots and roots of alfalfa growing in non-bioaugmented soil were, in decreasing order: Zn > Cu > Pb. Maximum metal concentration in roots reached 169, 71 and 23 mg kg^{-1} DW while in shoots they did not exceed 78, 21 and 17 mg kg^{-1} DW for Zn, Cu and Pb, respectively. Peralta-Videa et al. (2002) have previously reported higher metal concentrations in the shoots of alfalfa (160 and 105 mg kg^{-1} DW for Zn and Cu, respectively) growing in a soil artificially contaminated with a mixture of Cu and Zn (each one at 50 mg kg^{-1} soil DW, i.e. half of the concentrations found in the present work). This discrepancy could be attributed to certain soil characteristics that may negatively influence plant uptake of metals (Kabata-Pendias, 2011) like soil alkaline pH and sorption to organic matter (Gobran et al., 2000) as well as metal speciation (Van Hullebusch et al., 2005). Moreover, antagonistic effects between metals in multi-metal contaminated soils (Flogeac et al., 2007) in addition to the simultaneous presence of organic pollutants and soil ageing could have also contributed (Lin et al., 2008a). The extent of metal accumulation in alfalfa shoots was not influenced by the bioaugmentation treatment. In contrast, soil bioaugmentation had a statistically significant effect on root metal concentrations: Zn content was decreased at 90 days, while Cu content was increased at 60 days. The decrease of metal accumulation when plants were growing in bioaugmented soil could be the consequence of a 'dilution effect' of the increase of plant biomass facilitated by bacteria. In addition, metal immobilization onto bacteria due to biosorption processes could have also contributed to reduce metal availability, as *P. aeruginosa* has been reported to biosorb metals like Cu, Pb and Zn (Gabr et al., 2008; Pérez Silva et al., 2009). The distinct impact of bioaugmentation on Cu accumulation by alfalfa may be explained by specific

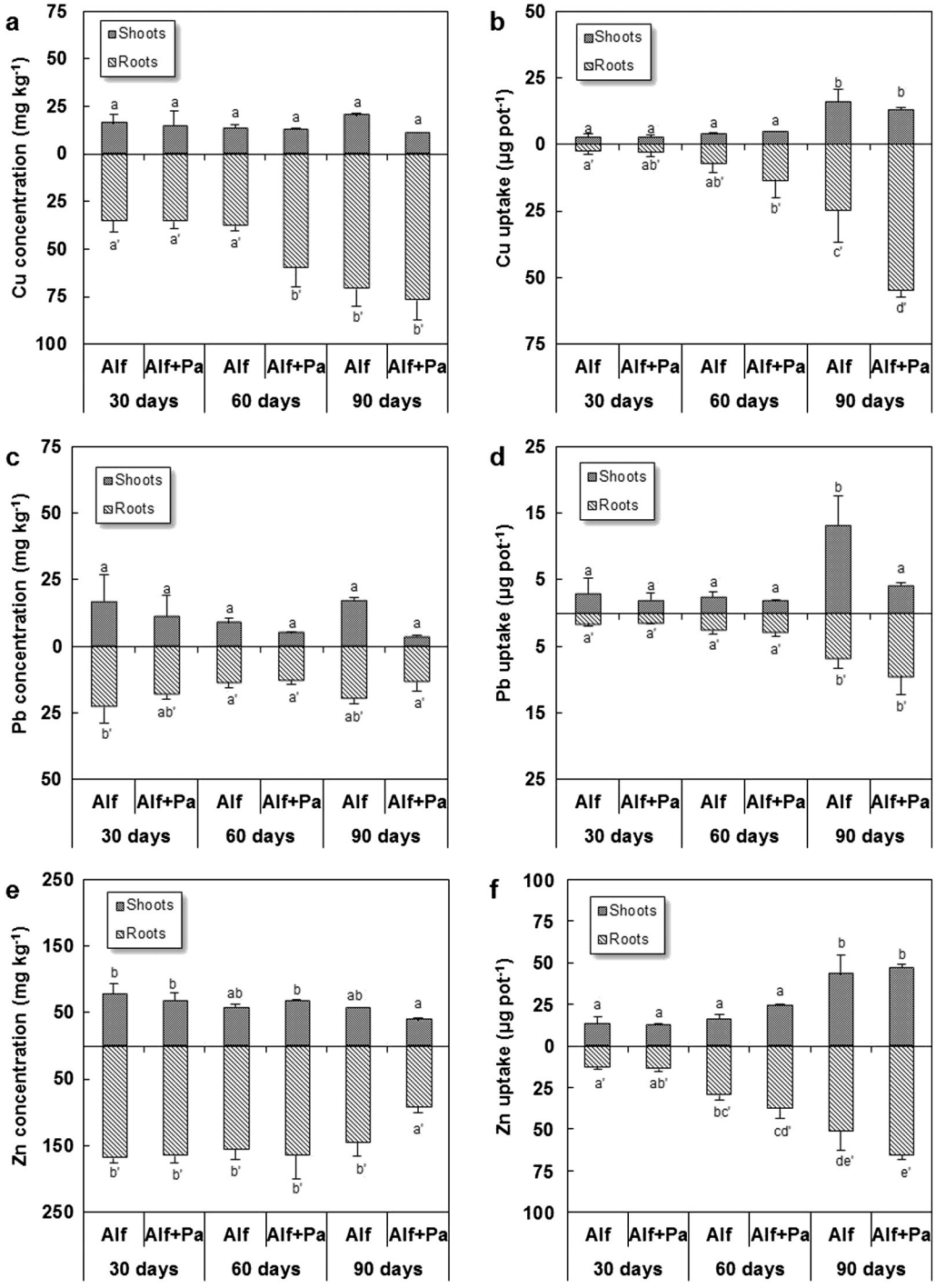

Fig. 3. Heavy metal concentration (a, c, e) and total uptake (b, d, f) by alfalfa plants growing in not bioaugmented soil (Alf) and in soil bioaugmented with *P. aeruginosa* (Alf + Pa). Values are expressed as mean ± standard deviation (n = 3). Statistical analysis of the data was performed using two-way analysis of variance (ANOVA) for the effects of treatment and time on the specific phytoextraction parameter. Means with the same letters are not statistically different according to the Tukey test with $p < 0.05$. The symbol ' distinguishes root from shoot statistical analysis.

coordination properties (i.e. stability constant of chelating molecule–metal complex) of siderophores (e.g. pyoverdine) produced by *P. aeruginosa* towards particular metals like Cu (Cornu et al., 2014).

As shown by TF data (Table 2), Pb was the most translocated element (average TF value of 0.80), followed by Zn and Cu (average TF values of 0.41 and 0.38, respectively). Average BCF of shoots and roots were in the following decreasing order: Zn > Cu > Pb. BCF values of roots were higher than for shoots. BCF and TF were calculated to evaluate the ability of the plant to accumulate metal in the tissues from soils and transfer them from roots to shoots, respectively. The fact that TF and BCF values were low (<1) suggests that alfalfa was unable to extract significant amounts of metals from the soil and translocate them to the shoots. As a result this species could be rather used for phytostabilization purposes than for phytoextraction, which would not be feasible in a reasonable frame-time (Robinson et al., 2015). Moreover, both TF and BCF values were generally lower in bioaugmented treatment. These results seem to be consistent with Lebeau et al. (2008), who reviewed several experiments of phytoextraction-assisted bioaugmentation with bacteria and found that PGPR always decrease TF, while BCF vary irrespective of bioaugmentation.

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The total amount of metals uptaken by alfalfa plants (Fig. 3b, d and f) depends both on plant biomass and metal concentration in plant tissues. As time passed plant uptake increased, probably as the result of the biomass increase, which varied more over time than heavy metal content. The most favourable scenario (increase both in plant biomass and metal concentration) that will indeed result in enhanced metal extraction by plants was the case of Cu, whose total uptake by alfalfa roots was significantly enhanced over time and also in the presence of *P. aeruginosa*.

3.4. Bioremediation treatments and removal of petroleum hydrocarbons

Removal percentages of different petroleum hydrocarbon fractions after 90 days of experiment were calculated for the four bioremediation treatments (Fig. 4). Irrespective of the bioremediation treatment, the pattern of removal of the light petroleum hydrocarbon fractions was higher than that of the heavy fractions and the degree of removal was in the following decreasing order: C_{10}–C_{12} > C_{12}–C_{16} > C_{16}–C_{21} > C_{21}–C_{40}. This pattern of removal is possibly related to the chemical structure of n-alkanes. Hydrocarbons with shorter carbon chain length are more readily biodegradable (Ji et al., 2013), while hydrocarbon fractions with longer C chains exhibit higher hydrophobicity and octanol–water partition coefficient (Kow) being less bioavailable due to higher sorption onto organic matter (Guo et al., 2010). In addition, a higher dissipation through evaporation could be expected for short n-alkanes owing to a low boiling point (Mehta and Mehta, 2005).

Indigenous microorganisms of the present soil appeared to be adapted and functional for petroleum hydrocarbons degradation, as demonstrated by the 37% TPH removal in natural attenuation treatment. Although biodegradation is generally considered as the main pathway for pollutant removal in natural attenuation (Declercq et al., 2012) other mechanisms (e.g. volatilization) could have also been involved. Serrano et al. (2008) have demonstrated that natural attenuation of diesel aliphatic hydrocarbons can occur to significant extents attaining aliphatic hydrocarbon concentrations below the legal contamination threshold for soil 400 days after a diesel fuel spill. They observed that this was mainly due to an initial phase of volatilization followed by biodegradation.

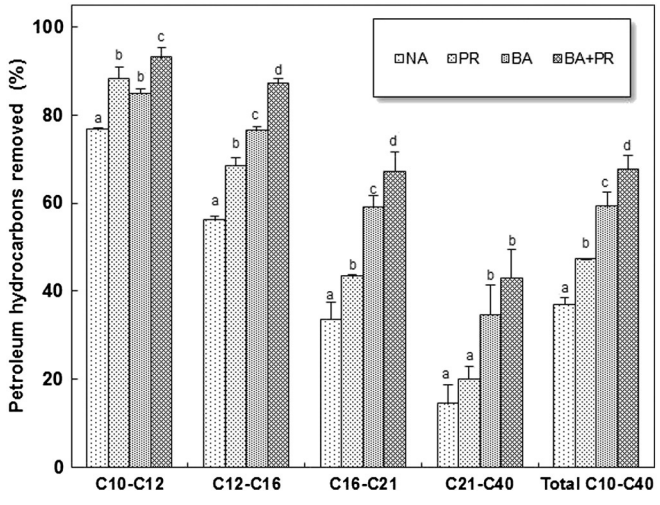

Fig. 4. Petroleum hydrocarbons removed (%) in the treatments: natural attenuation (NA, intrinsic clean up ability of the soil), phytoremediation (PR, soil vegetated with alfalfa), bioaugmentation (BA, soil inoculated with *P. aeruginosa* strain), and bioaugmentation-assisted phytoremediation (BA + PR, soil vegetated with alfalfa and inoculated with *P. aeruginosa* strain) after 90 days of experiment. Values are expressed as mean ± standard deviation (n = 3). Statistical analysis of the data was performed using one-way analysis of variance (ANOVA) for the effects of treatment on the specific petroleum hydrocarbon fraction. Means with the same letters are not statistically different according to the Tukey test with $p < 0.05$.

Vegetation with alfalfa species (phytoremediation treatment) led to higher petroleum hydrocarbon removal than natural attenuation (47%). There are several possible explanations for this result. Plants could enhance biodegradation processes by stimulating microbial biomass and/or activity in the rhizosphere (Pinton et al., 2001; Segura and Ramos, 2013). Moreover, recent studies support the idea of an active role of alfalfa plants in the rhizospheric degradation of hydrocarbons as the result of the action of plant enzymes released in root exudates (Muratova et al., 2014). Finally, an abiotic contribution could be attributed to root exudates, which have been demonstrated to enhance soil desorption of pollutants, improving bioavailability and subsequent biodegradation potential as a result (Lefevre et al., 2013; Sun et al., 2013).

Bioaugmentation with *P. aeruginosa* resulted in even greater remediation efficiency (59%). The present results seem to be consistent with a previous comparative study which demonstrated that bioaugmentation was more effective than natural attenuation on the degradation of light (C_{12}–C_{23}) and heavy (C_{23}–C_{40}) fractions of TPH in soil samples (Bento et al., 2005). It can be hypothesized that the observed increase in TPH removal when soil inoculation was performed are due to *P. aeruginosa* hydrocarbon-degrading ability (Ji et al., 2013; Liu et al., 2012; Ueno et al., 2006). Nevertheless, specific techniques (e.g. fluorescence in situ hybridization, FISH) are required to attribute petroleum hydrocarbon degradation particularly to *P. aeruginosa*. The observed increase in TPH removal when bioaugmentation was performed could be further facilitated by the production of biosurfactants that increase organic pollutant bioavailability (Zhang et al., 2012).

The combination of plants and microorganisms in bioaugmentation-assisted phytoremediation treatment gave rise to the best performance in TPH removal: 68%. The association between alfalfa and *P. aeruginosa* appeared to be particularly effective in terms of TPH removal as a result of the processes mediated by both bacteria and plants. Their contribution seemed to be additive rather than synergistic, as the bioaugmentation-assisted phytoremediation treatment resulted in a 31% increase of TPH removal, relative to natural attenuation, and a 10% and 22% increase in TPH removal was obtained for phytoremediation and bioaugmentation, respectively, in the individual treatments. Although the contribution to TPH removal of bacteria (bioaugmentation treatment) was greater than that of plants (phytoremediation treatment), the advantageous effect of plants is not only limited to the enhancement of pollutant dissipation in the rhizosphere. The presence of plants makes several noteworthy further contributions (e.g. control of soil erosion, surface water runoff and infiltration, improvement of physico-chemical properties of the soil as well as ecosystem functioning and landscape aesthetics) (Jiao et al., 2011; Ouvrard et al., 2011; Reid and Lewis, 2009), which renders advisable the vegetation of large areas presenting moderate levels of pollutants. The reduction of soil TPH in the presence of *P. aeruginosa*, could have positively influenced alfalfa growth, although other plant-growth promoting mechanisms could have also occurred.

3.5. Soil microbiological indicators

Fig. 5a shows the MPN of total heterotrophs for the bioremediation treatments after the sixth and last inoculation was done, at 80 days. Maximum counts in the MPN of total heterotrophs g^{-1} soil reached up to 1.3×10^{12} (bioaugmentation treatment) and 2.3×10^{12} (bioaugmentation-assisted phytoremediation treatment). The statistically significant difference in microbial counts of total heterotrophs between bioaugmented and non-bioaugmented treatments may indicate that the inoculated microorganisms were competitive towards the native indigenous bacteria. However, in order to be conclusive in that hypothesis particular analyses that specifically follow the fate of the inoculated strain are needed.

The number of alkane-degrading microorganisms (Fig. 5b) and lipase activity (Fig. 5c) at 90 days of experiment varied among bioremediation treatments being significantly higher in bioaugmentation than in natural attenuation treatments. These results are in agreement with

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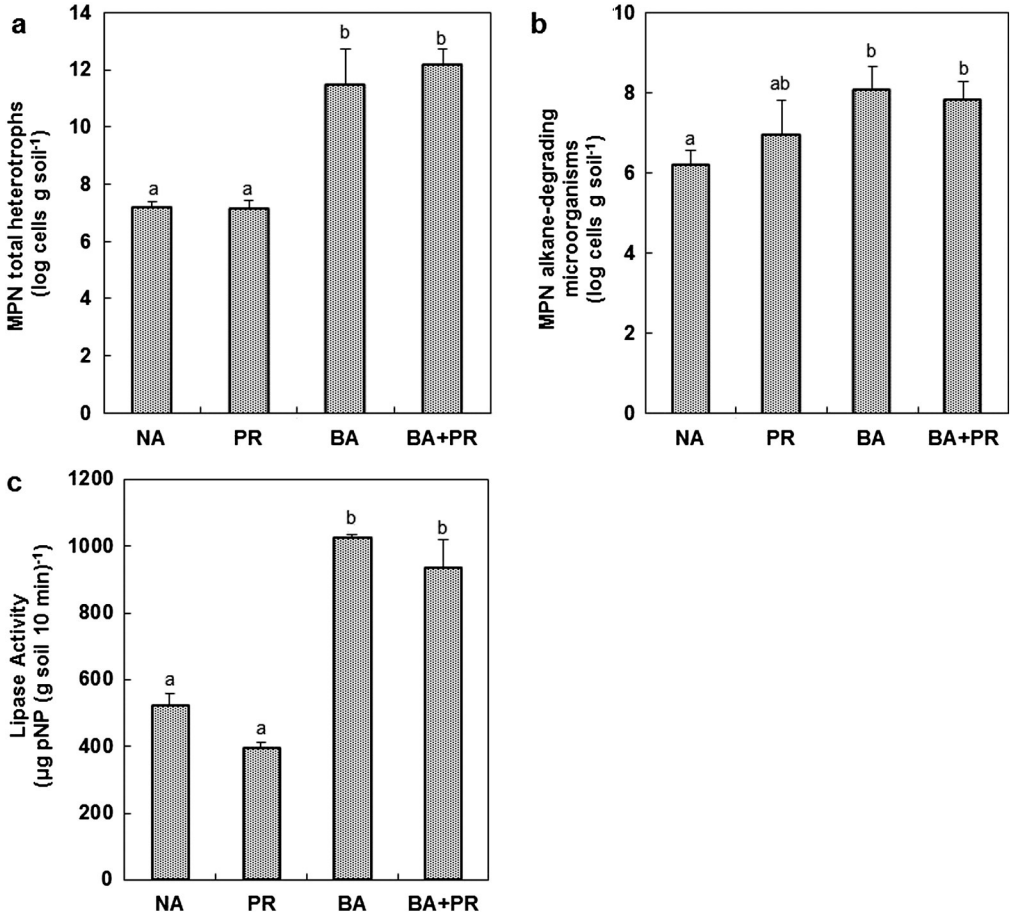

Fig. 5. Most probable number of total heterotrophs (a), alkane-degrading microorganisms (b) and lipase activity (c) in the soil for the treatments: natural attenuation (NA, intrinsic clean up ability of the soil), phytoremediation (PR, soil vegetated with alfalfa), bioaugmentation (BA, soil inoculated with *P. aeruginosa* strain), and bioaugmentation-assisted phytoremediation (BA + PR, soil vegetated with alfalfa and inoculated with *P. aeruginosa* strain) after 80 (a) and 90 (b, c) days of experiment. Values are expressed as mean ± standard deviation (n = 3). Statistical analysis of the data was performed using one-way analysis of variance (ANOVA) for the effects of treatment on the specific microbiological parameter. Means with the same letters are not statistically different according to the Tukey test with $p < 0.05$.

the *ability* of this species to degrade petroleum hydrocarbons by means of the suitable enzyme pathways (Ji et al., 2013) as well as the ability of *P. aeruginosa* to produce and secrete extracellular lipases (Gilbert and Leicester, 1993; Jaeger et al., 1994). The number of soil aliphatic hydrocarbon degrading bacteria and soil lipase activity are two useful indicators to quantify the population of microorganisms able to metabolize aliphatic hydrocarbons (Wrenn and Venosa, 1996) and to monitor oil biodegradation in soil (Margesin et al., 1999), respectively.

The rhizosphere effect refers to the positive influence of plant roots on microbial population and activity in the rhizosphere (Mukerji et al., 2006). In this study, alfalfa contribution to enhance microbial number and lipase activity appeared to be limited, as the presence of vegetation (phytoremediation and bioaugmentation-assisted phytoremediation treatments) did not result in a statistically significant improvement, with respect to unvegetated soil (natural attenuation and bioaugmentation treatments). As a result, the observed increase in the removal of petroleum hydrocarbons by alfalfa could be attributed to the action of plant enzymes different to lipases or to an abiotic contribution of root exudates enhancing desorption of hydrocarbons.

Contrary to expectations, this study did not find evident consistency between the number of alkane-degrading microorganisms, soil lipase activity and TPH removal. At 90 days the following divergent trends were obtained in 1) number of alkane-degrading microorganisms: NA < PR < BA + PR < BA, 2) soil lipase activity: PR < NA < BA + PR < BA and 3) TPH removal: NA < PR < BA < BA + PR. The three parameters were higher in bioaugmented treatments that in non-bioaugmented treatments. However, the presence of plants leads to a more variable behaviour. The lack of a clear correlation indicates that the population of microorganisms able to degrade alkanes and the soil lipase activity were not the only factors determining the removal of TPH.

4. Conclusions

The present study was designed to assess four bioremediation strategies for the treatment of a soil co-contaminated by moderate levels of heavy metals (Cu, Pb and Zn) and petroleum hydrocarbons.

As plant tolerance to polluted soil is essential in phytoremediation, it could be expected a viable application of alfalfa species with this purpose, especially in combination with *P. aeruginosa*, which significantly promoted plant growth and alleviated the stress of alfalfa growing in the co-contaminated soil. In contrast, metal accumulation and translocation from alfalfa roots to shoots appeared to be limited and not enhanced by *P. aeruginosa*. As a result, this system shows potential for metal stabilization rather than for phytoextraction. Bioaugmentation-assisted phytoremediation treatment presented the highest degree of TPH removal, followed by bioaugmentation, phytoremediation and natural attenuation alone. The present study provided novel evidence with respect to bioaugmentation-assisted phytoremediation of co-contaminated soils demonstrating that plant-bacteria partnerships and in particular alfalfa-*P. aeruginosa* association could be a suitable approach to reduce clean-up time. Nevertheless, successful results obtained in lab experiments under controlled conditions do not guarantee reproducibility at the field level, which implies a multitude of possibilities with highly variable physical, chemical and biological uncontrollable conditions. As a

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result, a direct extrapolation from such experiments to real-full scale applications is not advisable. Further studies, which take more variables into account, will need to be undertaken i.e. tests in an intermediate set up of field plots and for longer terms.

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