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ORIGINAL ARTICLE

Mesocosm-based simulations to optimize a bioremediation strategy for the effective restoration of wildfire-impacted soils contaminated with high-molecular-weight hydrocarbons

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bioaugmentation, bioremediation, biostimulation, biosurfactants, forest fires, high-molecular-weight hydrocarbons.

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Abstract

Aims: We obtained four microbial isolates from soil exposed to forest fire and evaluated their potential bioremediation activity when combined with a biosurfactant-producing bacterial strain for the decontamination of wildfire-impacted soil polluted with high-molecular-weight (HMW) hydrocarbons.
Methods and Results: We established mesocosm trials to compare three bioremediation strategies: natural attenuation, bioaugmentation and biostimulation. Chemical analysis, culture-dependent and culture-independent methods were used to evaluate the bioremediation efficiency and speciation of the microbial cenoses based on these approaches. After treatment for 90 days, bioaugmentation removed 75·2–75·9% of the HMW hydrocarbons, biostimulation removed 63·2–69·5% and natural attenuation removed ~22·5%. Hydrocarbon degradation was significantly enhanced in the mesocosm supplemented with the biosurfactant-producing bacterial strain after 20 and 50 days of treatment compared to the other bioremediation strategies.
Conclusions: We found that the bioaugmentation approach was more effective than biostimulation and natural attenuation for the removal of HMW hydrocarbons from fire-impacted soil.
Significance and Impact of the Study: Our study showed that microorganisms from wildfire-impacted soil show significant potential for bioremediation, and that biosurfactant-producing bacterial strains can be combined with them as part of an effective bioremediation strategy.

Introduction

Hydrocarbons are found in all ecosystem compartments, namely soil, groundwater and surface waters (including sediment), as well as the tropospheric air. They can reach these different ecological niches following accidental oil spills or due to anthropogenic activities such as power generation, industrial processes and vehicle use (Park and Park 2011). Furthermore, polycyclic aromatic hydrocarbons (PAHs) such as naphthalene, fluorene, anthracene, phenanthrene, pyrene, chrysene, tetraphene and to a minor extent—polychlorinated biphenyls and dioxin have been detected as a consequence of forest fires (Howsam *et al.* 2000; Martínez *et al.* 2000; Gabos *et al.* 2001; Kim *et al.* 2003; Kim *et al.* 2011; Vergnoux *et al.* 2011; Gennadiev and Tsibart 2013). High-molecular-weight (HMW) hydrocarbons represent up to 99·8% of the total hydrocarbons released onto the soil after a woodland fire (Andreolli *et al.* 2015). There is also a close relationship between the environmental persistence of aromatic hydrocarbons and the number of benzene rings in their molecular structure (Kanaly and Harayama 2000). The natural attenuation of HMW hydrocarbons after a forest wildfire takes much longer than the attenuation of

lower-molecular-weight (LMW) compounds (Vergnoux *et al.* 2011; Andreolli *et al.* 2015). Due to their toxicity, HMW hydrocarbons are not only a risk to human health, but can also extend the time required to restore resilience and biodiversity as close as possible to pristine ecological conditions (Delistraty 1997). Forest wildfires result in the formation of a continuous water-repellent layer beneath the soil surface, which reduces permeability and exposes the soil to erosion due to increased storm water runoff (Certini 2005). Significant amounts of hydrocarbons present in the ash bed can be removed by surface runoff and transported downstream to rivers or humid systems (Simon *et al.* 2016; Rey-Salgueiro *et al.* 2018).

The rapid and efficient removal of hydrocarbons (especially the HMW fraction) is highly desirable after a forest fire. More than 50,000 wildfires occur every year in the Mediterranean basin, destroying 0·6–0·8 Mha of forest and woodland (EEA (European Environment Agency) 2011; FAO 2013), but there has been little effort to develop reliable bioremediation strategies that remove hydrocarbons from forest soils. Pizarro-Tobías *et al.* (2015) demonstrated that rhizoremediation can completely restore burned soils in 8 months. Moreover field experiments carried out in woodland soils crossed by fire revealed that biostimulation treatments can achieve the more efficient degradation of HMW hydrocarbons compared to natural attenuation or bioaugmentation with allochthonous micro-organisms (Andreolli *et al.* 2015). These results suggest that surviving microbial communities in the soil possess innate metabolic capabilities for the removal of HMW hydrocarbons, and the first examples of such organisms were recently isolated and identified (Andreolli *et al.* 2015).

Here we compared the bioremediation potential of four micro-organisms (three bacteria and one fungus) from soil samples collected in a forest area crossed by fire, evaluating their suitability for the abatement of HMW hydrocarbons generated by wildfires. A series of mesocosm trials mimicking different conditions was established to compare three options: natural attenuation, bioaugmentation and biostimulation. Chemical analysis was combined with culture-dependent and culture-independent (PCR-DGGE) methods to assess both the overall bioremediation efficiency and the dynamic changes in soil microbial communities resulting from each bioremediation approach.

Materials and Methods

Soil collection and preparation

Soil was collected in a deciduous forest area located within the jurisdiction of the 'Comunità Montana della Val Trompia' in the Pezzaze municipality, north Italy. A wildfire swept the area on 2 April 2012 and the soil was collected 4 months later, according to the quartering procedure (Andreolli *et al.* 2016a). To increase the quantity of HMW hydrocarbons, the soil was spiked with 5 g kg^{-1} of V-Power Diesel (Royal Dutch Shell; The Hague, Netherlands), then carefully homogenized, collected in triplicate following the quartering procedure and used for both chemical and microbiological analysis at experimental time zero (T_0).

Experimental design

Treatments were carried out in pots filled with 4 kg of diesel-spiked soil kept in a greenhouse maintained at 16°C at night and 28°C during the day (average temperatures) for 90 days. Each treatment was carried out in triplicate:

1 Natural attenuation: control soil (C).
2 Biostimulation treatments: (2i) watered/aerated (W/A) soil; (2ii) watered/aerated soil supplemented with commercial nutrients (W/A-N).
3 Bioaugmentation treatments: (3i) W/A-N soil further supplemented with *Bacillus subtilis* DSM 3256, a biosurfactant-producing strain (W/A-N-S); (3ii) W/A soil supplemented with a mix of selected autochthonous micro-organisms (W/A-Au); (3iii) W/A soil supplemented with a mix of selected autochthonous micro-organisms and with the biosurfactant-producing strain *B. subtilis* DSM 3256 (W/A-Au-S).

Description and preparation of different treatments

(C): The soil was left untreated and the moisture content was 3–5%.

(W/A): The soil was manually aerated and watered with double-distilled water twice weekly, and the moisture was maintained at 15–25%.

(N): The commercial nutrient supplement was derived from vegetable sources with the following composition: organic carbon (40%), carbohydrates (10%), organic nitrogen (1%) and phosphorus (1%), as well as trace elements, amino acids and growth factors.

(S): The *B. subtilis* DSM 3256 inoculum was added at a concentration of 10^9 CFU per g soil. The strain was purchased from the German Collection of Micro-organisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen) and maintained and grown on nutrient medium (Oxoid).

(Au): The autochthonous bacterial strains *Cellulomonas* sp. P5, *Dyella* sp. P15 and *Ochrobactrum* sp. P1d have been described in detail (Andreolli *et al.* 2015). These

strains were chosen on the basis of their representation of different genera and their ability to efficiently decolorize the dyes Congo red (CR) or malachite green (MG). We also added the fungal strain *Trichoderma* sp. fP6 based on its ability to transform both Congo red and methylene blue (MB) (Andreolli *et al.* 2015). These dyes represent three different classes of recalcitrant hydrocarbon compounds: (i) azo (CR), (ii) triphenyl methane (MG) and (iii) heterocyclic (MB) (Bandounas *et al.* 2011) and their decolorization may predict the microbial capacity to transform HMW hydrocarbon pollutants (Onofre and Steilmann 2012; Lee *et al.* 2014). Each microbial strain was inoculated at a final concentration of 10^9 CFU per g soil.

Physical and chemical analysis

The total organic carbon (TOC) and pH of the forest soil were measured using the standardized methods UNI-10501 (1996) and UNI-EN 13137 (2002) respectively. Soil samples from different bioremediation treatments were homogenized and collected from all the pots and analysed separately. HMW hydrocarbon compounds were identified using method UNI-EN ISO 16703 (2011), which is suitable for all hydrocarbons with a boiling point within the range 175–525°C, including isoalkanes, cycloalkanes, alkylnaphthalenes, alkylbenzenes, PAHs and *n*-alkanes from $C_{10}H_{22}$ to $C_{40}H_{82}$. Analysis was carried out by Dolomiti Energia Laboratories. HMW hydrocarbon abatement was calculated as the ratio of hydrocarbon concentrations measured after 20, 30, 50 and 90 days compared to the level at T_0.

Microbial analysis for the enumeration of heterotrophic bacteria and fungi

The total cultivable microbes in the soil were quantified by mixing 5 g soil with 45 ml 0·9% NaCl for 1 h and plating serial dilutions on R_2A-agar (Oxoid) for the bacterial count and on malt yeast extract agar comprising 20 g l^{-1} malt extract, 5 g l^{-1} yeast extract and 1·5% agar (pH 7·0) supplemented with 15 mg l^{-1} rifampicin for the fungal count. Triplicate plates were counted and mean values were presented.

PCR-DGGE analysis

Total microbial DNA was extracted from soil using the FastDNA Spin kit for soil (MP Biomedical; Irvine, CA, USA) according to the manufacturer's instructions. Each 25-μl PCR mix comprised 8·5 mg l^{-1} of each primer, 100 mg l^{-1} dNTPs, 1 unit GoTaq DNA polymerase (Promega; Milan, Italy) and 5 μl 5 × PCR buffer. The complete bacterial 16S rRNA gene (~1500 bp) was amplified using primers FD1 and rp2 (Weisburg *et al.* 1991). The V2-V3 region (~200 bp) was amplified using primers HDA1-GC (5′-GCA CTC CTA CGG GAG GCA GCA GT-3′, with the GC clamp underlined) and HDA2 (5′-GTA TTA CCG CGG CTG CTG GCA C-3′) (Walter *et al.* 2000). The reaction was heated to 95°C for 5 min followed by 30 cycles of 95°C for 1 min, 56°C for 1 min, and 72°C for 1 min and a final extension step at 72°C for 5 min. The fungal 18S rRNA gene (~1800 bp) was amplified using primers NS1 and EF3 (Oros-Sichler *et al.* 2006) and the resulting amplicons were diluted for a nested amplification (~1650 bp) using primers NS1 and FR1GC (Oros-Sichler *et al.* 2006).

Denaturing gradient gel electrophoresis (DGGE) was carried out using the DCode universal mutation detection system (Bio-Rad). Amplicons derived from the bacterial 16S rRNA gene were loaded on a gel containing a 30–60% denaturing gradient of urea and formamide plus 8% polyacrylamide prepared from a 37·5 : 1 ratio of acrylamide and bisacrylamide stock solutions (Bio-Rad; Segrate (MI), Italy). The fully denaturing end of the gradient was 40% formamide and 420 g l^{-1} urea. The samples were fractionated at a constant 130 V at 60°C for ~5 h. Amplicons derived from the fungal 18S rRNA gene were loaded on a gel containing a 18–38% denaturing gradient of urea and formamide and a 4–7·5% gradient of polyacrylamide. The samples were fractionated at a constant 120 V at 58°C for ~17 h. The buffer in all experiments was 1 × TAE (40 mmol l^{-1} Tris-acetate, 1 mmol l^{-1} EDTA, pH 8). The gels were stained with SYBR Green I (EuroClone; Pero (MI), Italy) for 30 min and visualized using the Gel Documentation System (UVITEC; Cambridge, UK). Similarity indexes were calculated by unweighted pair group method with arithmetic mean (UPGMA) cluster analysis and the dendrogram was generated using UVIbandmap software (UVITEC; Cambridge, UK).

Sequencing of DGGE fragments

Well-defined DNA bands in the DGGE profiles were excised from the gel with a sterile razor blade, transferred to vials and mixed with 20 μl of sterile water. The samples were incubated at 37°C for 4–5 h, allowing the passive diffusion of the DNA. We used 2 μl of the eluate as the template DNA for re-amplification by PCR using primers without GC clamps, under the conditions described above. PCR products were transferred to the pGEM-T vector (Promega, Milan, Italy) and introduced into *Escherichia coli* XL1-Blue competent cells (Agilent Technologies; Santa Clara, CA, USA) according to the manufacturer's instructions. The cloned plasmids were

recovered and sequenced on both strands (Primm) and the insert sequences were screened against National Center for Biotechnology (NCBI) GenBank using BlastN and EzBioCloud databases (Altschul *et al.* 1997; Yoon *et al.* 2017). Sequences obtained by PCR-DGGE analysis were assigned accession numbers MW349698 and MW404683–MW404690.

Statistical analysis

Data were analysed by one-way ANOVA and significant differences were confirmed by applying Tukey's test ($P < 0{\cdot}05$).

Results

Bacterial and fungal counts

The total number of cultivable bacteria was determined at 0, 10, 20, 30, 50 and 90 days from the start of the experimental trials (Table 1). The bacterial count in the control soil was ~7·5 $\log_{10}$ (CFU per g) without any significant variation throughout the test run. In contrast, soil samples in the biostimulation experiments (W/A, W/A-N) showed an increase in bacterial counts from ~7·2 to ~8·1 $\log_{10}$ (CFU per g) during the first 10 days, maintaining the higher concentration until the end of the trial, whereas those in the bioaugmentation experiments (W/A-N-S, W/A-Au, W/A-Au-S) showed counts of 8·25–8·96 $\log_{10}$ (CFU per g) throughout the tests. Initially, the total fungal count in the control soil was 6·2–6·6 $\log_{10}$ (CFU per g) with no significant difference compared to the treatment groups. The fungal count remained within the range 5·9–6·7 $\log_{10}$ (CFU per g) throughout the experimental trial in all groups (Table 2).

PCR-DGGE analysis

The persistence of microbial inoculums in the soil throughout the bioremediation treatments was monitored by PCR-DGGE. Initially, the DGGE profile from the bioaugmented soil featured clear bands representing each of the bacterial and fungal inoculants: *B. subtilis* DSM 3256, *Cellulomonas* sp. P5, *Dyella* sp. P15, *Ochrobactrum* sp. P1d (Fig. 1, T_0) and *Trichoderma* sp. fP6 (Fig. 2, T_0). The allochthonous biosurfactant-producing strain *B. subtilis* DSM 3256 disappeared after 30–50 days, but all the autochthonous bacteria were clearly detected throughout the first 50 days (Fig. S1). However, only *Ochrobactrum* sp. P1d (Fig. 1, T_{90}) and *Trichoderma* sp. fP6 (Fig. 2, T_{90}) were still present after 90 days. Statistical analysis of bacterial DGGE profiles at the beginning and end of the experimental trials was visualized in a dendrogram (Fig. 3) but the fungal community was not similarly evaluated due the limited number of bands visible on the gel. DGGE profiles from control samples formed a single cluster with ~20% similarity to profiles representing the other treatments. DGGE profiles from the bioaugmented and biostimulated samples were assigned to two different clusters with ~80% similarity to each other.

The most representative bands were excised and the amplicons were cloned and sequenced, revealing the presence of multiple genera including *Granulicella* sp. (band $C_0$1), *Arthrobacter* sp. (band $C_0$2), *Rhodococcus/Saccharothrix* sp. (band $C_0$3), *Nitrobacter/Afipia* sp. (band $C_0$4), *Mesorhizobium* sp. (band $C_0$5) and *Dyella* sp. (band C_{90}1). Bands C_{90}2 and C_{90}3 showed high levels of identity with uncultured bacteria (Table 3). Finally, the main PCR-DGGE band representing the fungal population ($F_0$1) was assigned to *Lecythophora/Coniochaeta* sp. (Table 3).

The abatement of HMW hydrocarbons

The TOC content and pH of the soil before spiking were 432·3 mg kg^{-1} and 6·3 respectively. The impact of the different bioremediation treatments on the abatement of HMW hydrocarbons is shown in Fig. 4. At the beginning of the experiment, the HMW hydrocarbon content was 2186·25 ± 25·7 mg kg^{-1} in all groups. During the first

Table 1 Total bacterial cell counts expressed as $\log_{10}$ (CFU per g) in soil at different times during the experimental treatments. Results are means (n = 3) ± SD. The (–) symbol indicates no measurement was taken

	0 days $\log_{10}$ (CFU per g)	10 days $\log_{10}$ (CFU per g)	20 days $\log_{10}$ (CFU per g)	30 days $\log_{10}$ (CFU per g)	50 days $\log_{10}$ (CFU per g)	90 days $\log_{10}$ (CFU per g)
Control	7·54 ± 0·38	–	–	–	7·77 ± 0·10	7·58 ± 0·09
W/A	7·21 ± 0·02	8·25 ± 0·25	8·34 ± 0·27	8·79 ± 0·03	8·65 ± 0·17	8·53 ± 0·21
W/A-N	7·28 ± 0·10	8·11 ± 0·19	8·31 ± 0·02	8·83 ± 0·01	8·83 ± 0·13	8·77 ± 0·17
W/A-N-S	8·25 ± 0·11	8·70 ± 0·22	8·61 ± 0·10	8·91 ± 0·14	8·96 ± 0·04	8·61 ± 0·09
W/A-Au	8·45 ± 0·10	8·79 ± 0·09	8·84 ± 0·14	8·68 ± 0·15	8·56 ± 0·13	8·78 ± 0·17
W/A-Au-S	8·36 ± 0·11	8·66 ± 0·09	8·46 ± 0·26	8·47 ± 0·22	8·38 ± 0·18	8·71 ± 0·23

Table 2 Total fungal cell counts expressed as $\log_{10}$ (CFU per g) in soil at different times during the experimental treatments. Results are means (n = 3) ± standard deviation. The (–) symbol indicates no measurement was taken

	0 day $\log_{10}$ (CFU per g)	10 days $\log_{10}$ (CFU per g)	20 days $\log_{10}$ (CFU per g)	30 days $\log_{10}$ (CFU per g)	50 days $\log_{10}$ (CFU per g)	90 days $\log_{10}$ (CFU per g)
Control	6·33 ± 0·18	–	–	–	6·51 ± 0·06	6·05 ± 0·09
W/A	6·38 ± 0·19	6·68 ± 0·09	6·70 ± 0·06	6·62 ± 0·13	6·14 ± 0·04	6·21 ± 0·13
W/A-N	6·21 ± 0·10	6·72 ± 0·17	6·76 ± 0·08	6·59 ± 0·12	5·96 ± 0·14	6·28 ± 0·07
W/A-N-S	6·21 ± 0·31	6·72 ± 0·02	6·74 ± 0·08	6·87 ± 0·05	6·14 ± 0·23	6·10 ± 0·09
W/A-Au	6·58 ± 0·13	6·58 ± 0·06	6·60 ± 0·07	6·55 ± 0·19	6·23 ± 0·04	6·12 ± 0·18
W/A-Au-S	6·44 ± 0·31	6·50 ± 0·02	6·56 ± 0·08	6·53 ± 0·07	5·99 ± 0·04	6·12 ± 0·16

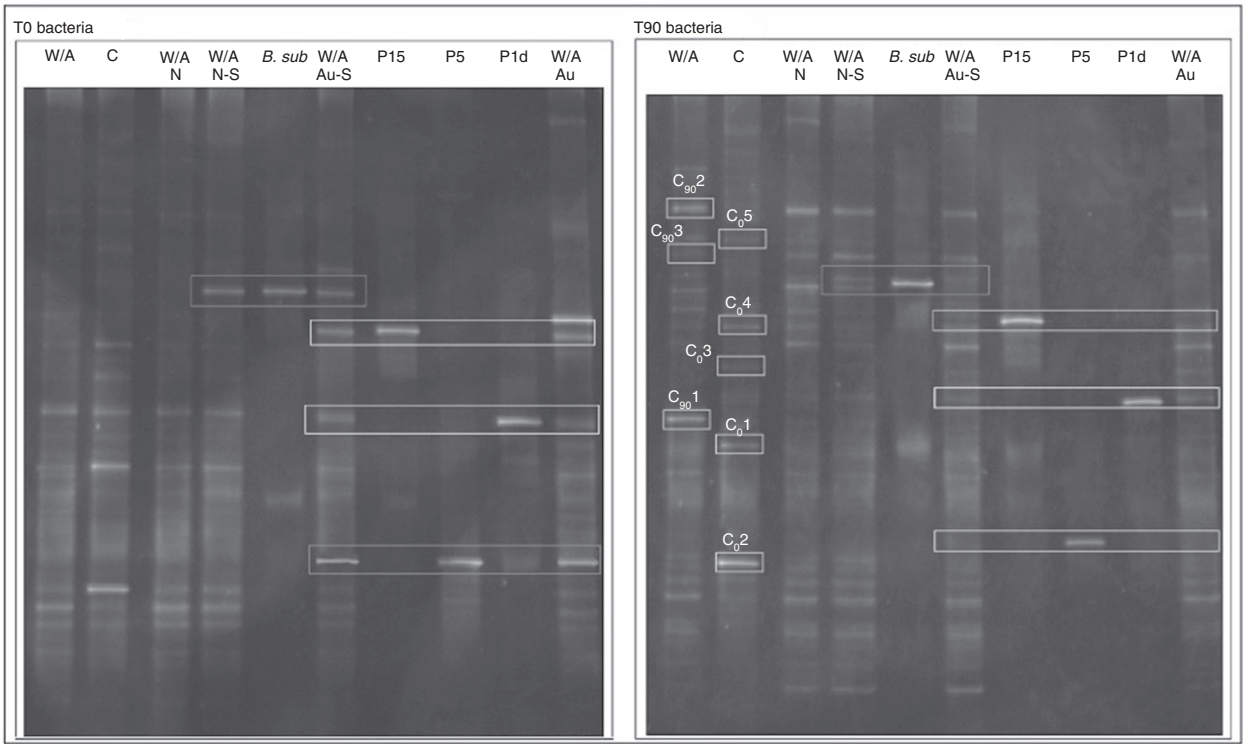

Figure 1 PCR–DGGE analysis of the bacterial community at the beginning of each bioremediation treatment (W/A, C, W/A-N, W/A-N-S, W/A-Au-S and W/A-Au) and after 90 days. DGGE analysis was based on amplicons from the 16S rRNA gene of *Dyella* sp. P15, *Cellulomonas* sp. P5 and *Ochrobactrum* sp. P1d (P15, P5 and P1d respectively). Bands that were excised for sequencing are indicated.

20 days, similar abatement efficiency was observed in the W/A, W/A-N and W/A-N-S treatments, with overall hydrocarbon levels falling by 14·3–24·4% with no significant fluctuations ($P > 0·05$). Thereafter, the presence of the autochthonous micro-organisms significantly promoted hydrocarbon abatement ($P < 0·05$), reducing overall levels by 30·6–37·2%. After 30 days, the hydrocarbon content of the W/A-N and W/A-N-S mesocosms was reduced by ~48·0%, significantly more than the 36·9% depletion observed in the W/A mesocosms ($P < 0·05$). During the same period, the hydrocarbon content in the bioaugmented mesocosms (W/A-Au and W/A-Au-S) fell by 57·0%, significantly more than observed in the biostimulated mesocosms ($P < 0·05$). After 50 days, the abatement of total hydrocarbons was efficient in the W/A-N (~59·9%), WA-N-S (60·2%), W/A-Au (63·3%) and W/A-Au-S (67·3%) mesocosms, but less so in the W/A mesocosms (51·5%) and inefficient in the control (8·4%). These results confirmed the significant abatement ($P < 0·05$) of HMW hydrocarbons in all treatment groups compared to the W/A and control mesocosms. At the end of the experiment, the most efficient hydrocarbon abatement (75·2–75·9%) was observed in the bioaugmented mesocosms (W/A-Au and W/A-Au-S), compared

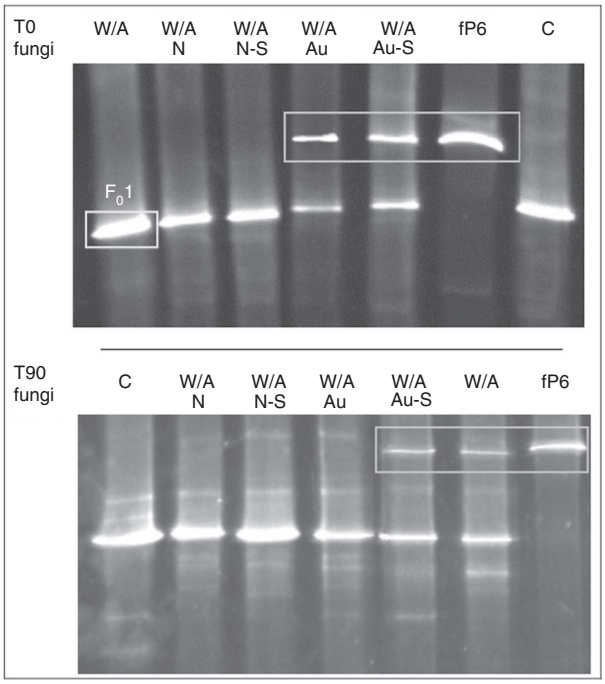

Figure 2 PCR–DGGE analysis of the fungal community at the beginning of each bioremediation treatment (W/A, C, W/A-N, W/A-N-S, W/A-Au-S and W/A-Au) and after 90 days. DGGE analysis was based on amplicons from the *Trichoderma* sp. fP6 (fP6) 18S rRNA gene. Bands that were excised for sequencing are indicated.

to 65·2–69·5% in the biostimulated soils (W/A-N and W/A-N-S). After 90 days, statistical analysis clearly revealed that all the treatments except W/A-N-S were significantly more effective than the W/A treatment.

Discussion

Studies focusing on the biodegradation of hydrocarbons in natural environments have intensified in recent years and a number of technological approaches have been proposed (Badu *et al.* 2019). Mesocosm tests can determine the time-dependent effects of such approaches on ecological structure and function, reflecting the addition of nutrients, supplements and/or microbial inoculums (Hassanshahian *et al.* 2014). It is difficult to predict the extent to which bioremediation strategies will achieve hydrocarbon degradation because the outcome depends on climatic and edaphic conditions such as seasonal temperature, pH, redox potential, mineral and organic nutrient composition, and the size and structure of microbial cenoses that influence a variety of environmental matrices (Tyagi *et al.* 2011). Nevertheless, a number of comparative studies have demonstrated that bioaugmentation is more effective than either biostimulation or natural attenuation for the degradation of total petroleum hydrocarbons in soil (Agnello *et al.* 2016; Sarkar *et al.* 2017; Ramadass *et al.* 2018). Biostimulation has been shown to achieve the most efficient hydrocarbon degradation in other studies (Ruberto *et al.* 2008; Al-Mailem *et al.* 2017; Guarino *et al.* 2017), but Coulon *et al.* (2010) found that the addition of different nutrients to soil had little impact on hydrocarbon depletion and some authors have even reported that biostimulation treatments may suppress hydrocarbon degradation (Braddock *et al.* 1997; Sarkar *et al.* 2005; Wang *et al.* 2012; Akbari and Ghoshal 2014; Ramadass *et al.* 2018).

Here we found that bioaugmentation with selected autochthonous micro-organisms isolated from wildfire-impacted soil was superior to both biostimulation and natural attenuation in terms of HMW hydrocarbon degradation. It is challenging to compare our results to earlier studies due to the different experimental approaches used, as well as differences in the initial concentrations of hydrocarbons and the quantity and specific characteristics the experimental soils. One earlier investigation, carried out under bioaugmentation conditions similar to those reported here (with a similar initial concentration of hydrocarbons and the same experimental duration of 90 days), reported an abatement efficiency of 60% (Agnello *et al.* 2016), which is significantly lower than the >75% abatement we observed. Furthermore, Khudur *et al.* (2019) reported a mesocosm experiment in which biostimulation depleted the HMW hydrocarbon content by almost 50% within 84 days. Likewise, a land-

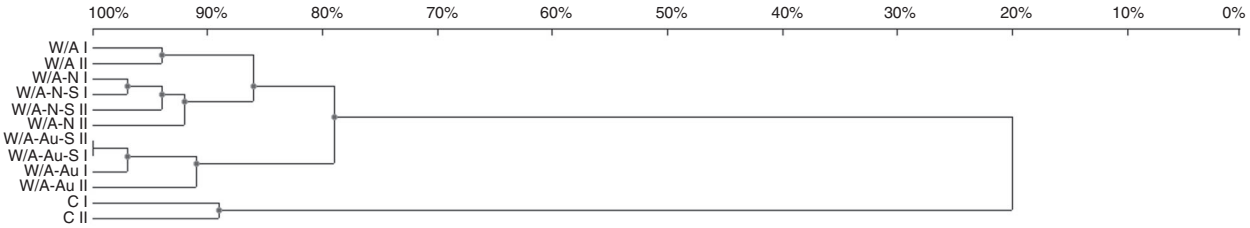

Figure 3 Dendrogram showing the relationships among different bacterial DGGE profiles at the beginning of each bioremediation treatment (W/A, C, W/A-N, W/A-N-S, W/A-Au-S and W/A-Au) and after 90 days.

Table 3 Sequencing analysis of the most representative bands excised from bacterial and fungal DGGE gels

Band	Organism	Accession number	Sequence similarity (%)	Phylogenetic affiliation
$C_0$1	*Granulicella rosea* TPO1014(T)	AM887759	100	Acidobacteria
	Granulicella aggregans TPB6028(T)	AM887756	100	Acidobacteria
	Granulicella sapmiensis S6CTX5A(T)	HQ687090	100	Acidobacteria
$C_0$2	*Arthrobacter pascens* DSM 20545(T)	X80740	99·43	Actinobacteria
	Arthrobacter oryzae KV-651(T)	AB279889	99·43	Actinobacteria
$C_0$3	*Rhodococcus erythropolis* DSM 43066(T)	X79289	100	Actinobacteria
	Rhodococcus qingshengii djl-6(T)	DQ090961	100	Actinobacteria
	Actinokineospora *diospyrosa* DSM 44255(T)	jgi.1048863	100	Actinobacteria
	Saccharothrix longispora NRRL B-116116(T)	AF114809	100	Actinobacteria
$C_0$4	*Nitrobacter winogradskyi* Nb-255(T)	CP000115	98·82	Alphaproteobacteria
	Afipia felis ATCC 53690(T)	KB375270	98·82	Alphaproteobacteria
$C_0$5	*Mesorhizobium tianshanense* CGMCC 1.2546(T)	jgi.1041444	99·41	Alphaproteobacteria
	Mesorhizobium loti ATCC 33669(T)	D14514	99·41	Alphaproteobacteria
C_{90}1	*Dyella thiooxydans* ATSB10(T)	EF397574	98·97	Gammaproteobacteria
C_{90}2	Uncultured bacterium clone NC42b4_14107	JQ386959	100	—
	Sphingomonas oligoaromativorans SY-6(T)	FJ434127	96·47	Alphaproteobacteria
C_{90}3	Uncultured bacterium clone FL11c5_16798	JQ381592	100	—
	Occallatibacter riparius 277(T)	HQ995659	94·97	Acidobacteria
$F_0$1	*Lecythophora lignicola* CBS267.33T	AJ496246	99·00	Ascomycota
	Coniochaeta velutina MA3370	AJ496244	99·00	Ascomycota

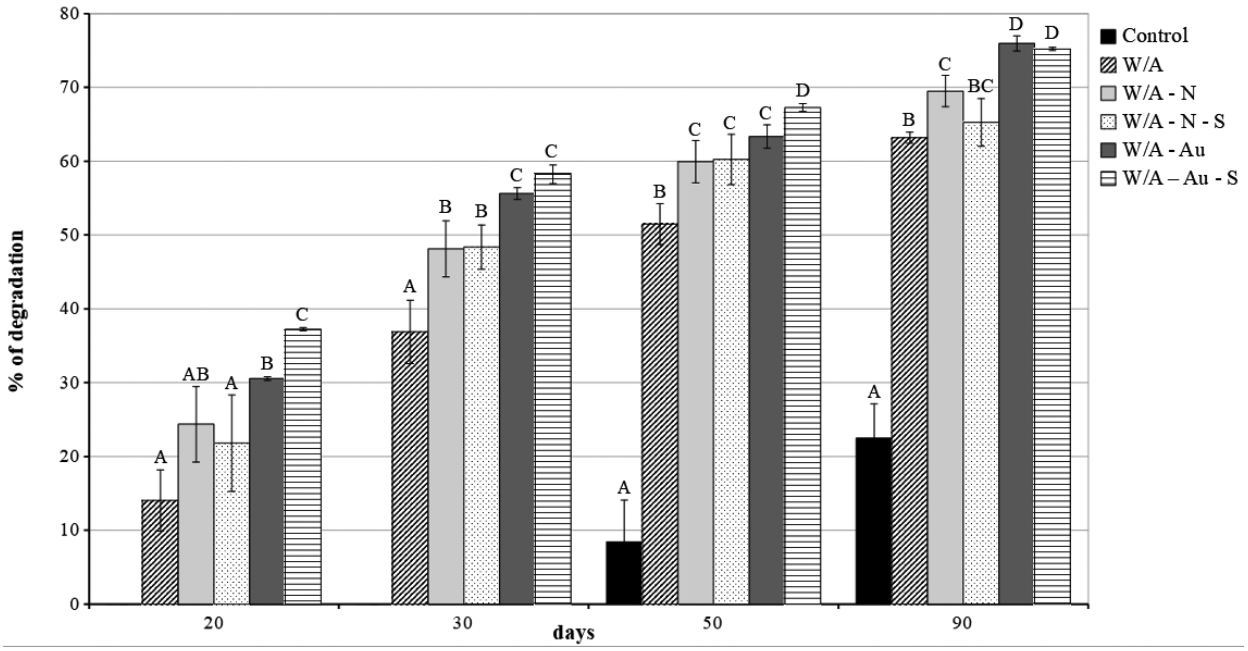

Figure 4 Efficiency of HMW hydrocarbon abatement after 20, 30, 50 and 90 days of different bioremediation treatments. Mean percentage abatement values marked by the same letter are not significantly different ($P < 0·05$). (■) Control; (▨) W/A; (▢) W/A–N; (▦) W/A–S; (■); W/A Au (▤) W/A Au–S.

farming procedure including the addition of an inoculum containing 22 different indigenous bacterial strains depleted total petroleum hydrocarbons by ~86% (Guarino *et al.* 2017). Finally, bioaugmentation in a microcosm environment was shown to remove ~60% of diesel added to the soil (Lahel *et al.* 2016; Bosco *et al.* 2020).

Several concomitant factors may explain the success of our bioaugmentation strategy, including the development

of an appropriate screening method for the selection of microbial strains capable of degrading HMW hydrocarbons, the use of autochthonous micro-organisms as an inoculum, and the use of a consortium of different microbes rather than individual strains. The selection of functional micro-organisms is necessary for effective bioaugmentation. Although the ability to decolorize aromatic organic dyes has been proposed as a method to predict whether fungi can metabolize other recalcitrant xenobiotic pollutants (Lee *et al.* 2014; Andreolli *et al.* 2016b), to the best of our knowledge this protocol has not been used before to screen bacterial isolates. We found that the use of three dyes representing different classes of aromatic compounds is a simple, reliable and inexpensive method for the selection of bacterial strains that can break down HMW hydrocarbons in bioremediation approaches.

Another requirement for successful bioaugmentation is the persistence of the microbial inoculum once added to the soil. The permanence of exogenous microbial inoculums in the soil matrix is affected by biotic constraints such as predation, competition and antibiosis, as well as abiotic (edaphic) factors such as oxygen concentration, pH, moisture and organic matter content (Devinny and Chang 2000; El Fantroussi and Agathos 2005). The autochthonous, hydrocarbon-degrading microbial strains we tested could still be detected after 50 days (*Cellulomonas* sp. P5 and *Dyella* sp. P15) or even after 90 days (*Ochrobactrum* sp. P1d and *Trichoderma* sp. fP6), whereas the allochthonous *B. subtilis* DSM 3256 disappeared within the first 50 days. By monitoring the hydrocarbon-degrading micro-organisms we found that native microbial populations persisted for longer during the experimental trials than newly introduced strains, suggesting that microbial adaptation had occurred in the burned soil (Pizarro-Tobías *et al.* 2015). Moreover both the microbial counts and HMW hydrocarbon content analysis revealed that the fire-impacted forest soil used in this study contained enough nutrients to support bacterial growth and metabolism. Although the total soil nutrient pool (mainly the organic components) declines due to volatilization as a consequence of the combustion of both vegetative plant biomass and litter, the bioavailability of NH_4^+, PO_4^{3-} and Mn significantly increases after fire (Bret-Harte *et al.* 2013; Blank *et al.* 2017).

A microbial consortium is more suitable for bioremediation than individual microbial strains added to an environmental matrix because the degradation of a mixed population of hydrocarbons is promoted by metabolic diversity (Kumari *et al.* 2018). The increase in metabolic diversity makes the full-scale application of this approach in the open field less prone to failure (Nyer *et al.* 2002; Rahman *et al.* 2002; Tyagi *et al.* 2011). In addition, the inclusion of biosurfactant-producing microbes in bioaugmentation protocols may contribute to the degradation of hydrocarbons by emulsifying the nonpolar contaminants, making them more available to microbial enzymes. Accordingly, we found that the bioaugmentation treatment including *B. subtilis* DSM 3256 significantly promoted the degradation of HMW hydrocarbons 20 and 50 days after the start of the experimental trials, with far greater efficiency than the other bioremediation protocols. The synthesis of surfactin by *B. subtilis* is also stimulated by the presence of hydrocarbons (Abdel-Mawgoud *et al.* 2008), which can promote the detachment of oils from contaminated matrices (Vaz *et al.* 2012; Sen 2008), thus enhancing hydrocarbon biodegradation (Lai *et al.* 2009). Although *B. subtilis* DSM 3256 has already been used as an inoculum to promote the biotransformation of toxic metal compounds (Braud *et al.* 2006), this is the first time the strain has been used as a bioaugmentation strategy for the removal of hydrocarbons.

Even the W/A mesocosm and control achieved the abatement of HMW hydrocarbons with efficiencies of ~22·5 and ~63% after 90 days of treatment respectively. This may reflect the natural presence of autochthonous micro-organisms that metabolize hydrocarbons. Accordingly, DGGE analysis of the soil communities revealed the presence of various bacterial genera previously reported in analogous forest soils crossed by fire (Weber *et al.* 2014; Otsuka *et al.* 2008; Uroz *et al.* 2013; Andreolli *et al.* 2015), suggesting they may be able to break down HMW hydrocarbons efficiently. Strains belonging to *Arthrobacter* sp. (DGGE band $C_0$2) have already been tested for their ability to break down PAHs, HMW hydrocarbons and even diesel mixtures (Plotnikova *et al.* 2011; Sivaram *et al.* 2019; Abdulrasheed *et al.* 2020). Members of the genus *Rhodococcus* (DGGE band $C_0$3) can degrade organic xenobiotic pollutants, and are often used as an inoculum for the bioremediation of diesel-contaminated soils (Lee *et al.* 2006; Kuyukina and Ivshina 2019; Roslee *et al.* 2020). Finally, even strains related to the genera *Mesorhizobium* (DGGE band $C_0$5) and *Djella* (DGGE band C_{90}1) have been shown to transform some toxic hydrocarbon compounds (Qu *et al.* 2016; Teng *et al.* 2016).

One of the advantages of mesocosm tests is the ability to follow the dynamics of microbial cenoses over time in relation to the impact of different treatment conditions on the environmental matrix. During this investigation, the mesocosms exposed to different bioremediation treatments were therefore monitored by DGGE. Statistical analysis of the profiles revealed that all W/A treatments caused a drastic change in the bacterial community compared to the control, supporting our data on the abatement of HMW hydrocarbons. Moreover the bioaugmentation and

biostimulation treatments formed separate clusters with 80% similarity, suggesting a large proportion of common microbial species as previously reported (Ogino *et al.* 2001; Coppotelli *et al.* 2008; Madueño *et al.* 2015; Hassanshahian *et al.* 2016; Wu *et al.* 2016).

In conclusion, our results contribute to a better understanding of the transient effects of different bioremediation treatments on the microbial structure and activity of soils impacted by fire. Moreover we found that screening for the ability to decolorize various dye molecules was a reliable method for the selection of microbial strains that degrade HMW hydrocarbons. We also confirmed the beneficial effects of biosurfactant-producing microbial strains such as *B. subtilis* DSM 3256 on the efficiency of bioaugmentation. Our work provides a strategy to select optimal consortia of bacteria and fungi that can degrade HMW hydrocarbons with great efficiency, facilitating the development of more effective bioremediation methods.

Conflict of Interest

No conflict of interest declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. PCR–DGGE analysis of the bacterial community at the beginning of each bioremediation treatment (W/A, C, W/A-N, W/A-N-S, W/A-Au-S and W/A-Au) and after 20, 30 and 50 days.