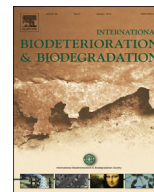




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Changes in respiration activities and bacterial communities in a bioaugmented oil-polluted soil in response to the addition of acyl homoserine lactones



Samiha Al-Kharusi^a, Raeid M.M. Abed^{a,*}, Sergey Dobretsov^{b,**}

^a Department of Biology, College of Science, Sultan Qaboos University, Oman

^b Department of Marine Science and Fisheries, College of Agricultural and Marine Science, Sultan Qaboos University, Oman

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ABSTRACT

The effect of bacterial quorum sensing (QS) signals on the respiration activity of an oil-polluted soil with and without the addition of an alkane-degrading bacterial consortium was investigated. The addition of C₄–C₁₂-HSL N-acyl homoserine lactones (AHLs) to the contaminated soil with the bacterial consortium resulted in a significant increase in CO₂ evolution rates. Experiments with 1, 10 and 100 μM of C₁₂-HSL exhibited an increase in respiration activities with decreasing concentrations. This increase was concomitant with the degradation of hydrocarbons. 93% of the alkanes were degraded in the bioaugmented soil after C₁₂-HSL addition. Illumina MiSeq sequencing of soil communities at the end of the treatments showed that bacteria belonging to the classes *Deinococci* and *Alphaproteobacteria* dominated (i.e. 62–87% of total sequences) all bioaugmented and non-bioaugmented soils. Addition of C₁₂-HSL did not cause major changes in the detected genera of bacterial communities. Out of the five bacterial strains of the consortium, only *Alcanivorax* sp. and *Parvibaculum* sp. survived after 42 days. An additional experiment demonstrated that C₁₂-HSL increased biofilm formation in the consortium bacteria. In conclusion, the addition of AHLs has a stimulative effect on bacterial respiration activities and degradation of hydrocarbons, hence can be useful in bioaugmentation treatments of oil-polluted soils.

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1. Introduction

Bioaugmentation, which relies on the addition of oil-degrading microorganisms to speed up the degradation of hydrocarbons, is an environmental friendly approach that has been widely used in the bioremediation of contaminated ecosystems (Boopathy, 2000; Tyagi et al., 2011; Zouboulis and Moussas, 2011). The main pitfall of this method is the inability of the introduced microorganisms to cope with the harsh environmental conditions in the field and to compete with the indigenous bacteria. Thus, the selection of appropriate bacterial strains, with the ability to survive and degrade hydrocarbons under *in situ* conditions, is a key prerequisite

for the success of bioaugmentation. Indeed, it was postulated that biofilm formation could potentially enhance biodegradation of pollutants, including hydrocarbons (Singh et al., 2006). Although several studies have tested bioaugmentation and reported on its success or failure (Ruberto et al., 2003; Abed et al., 2014; Colla et al., 2014; Fan et al., 2014; Hassanshahian et al., 2014; Qiao et al., 2014; Suja et al., 2014; Andreolli et al., 2015), very few studies have monitored the fate of introduced bacteria at the end of the treatment (Gertler et al., 2009a). Such studies are required in order to identify the most competent bacteria that are successful in the degradation of contaminants under field conditions.

In the environment, about 80–90% of soil microorganisms are sorbed to solid surfaces while enclosed in a matrix of hydrated extracellular polymeric substances (EPS) on soil particles (Maier et al., 2009; Flemming and Wingender, 2010). The formation of biofilms protects microorganisms from different environmental factors, such as desiccation, solar and UV radiation, heat, and allows cells to survive in hostile environments (Hall-Stoodley et al., 2004). The formation of biofilms is facilitated through the production, release and perception of QS signals, which allow cell-to-cell

* Corresponding author. Sultan Qaboos University, College of Science, Biology Department, P.O. Box 36, Al Khoud, PC 123, Muscat, Oman.

** Corresponding author. Sultan Qaboos University, College of Agricultural and Marine Sciences, Marine Science and Fisheries Department, P.O. Box 34, Al Khoud, PC 123, Muscat, Oman.

E-mail addresses: rabed@squ.edu.om (R.M.M. Abed), sergey@squ.edu.om (S. Dobretsov).

communication between bacteria (Waters and Bassler, 2005; Steinberg et al., 2011). Once QS signals accumulate in the environment to critical threshold concentrations they bind to the target receptors, which result in the activation of target genes and, thus, control “bacterial social behavior” (Crespi, 2001; Waters and Bassler, 2005; Hartmann and Schikora, 2012; Harder et al., 2014). The most common QS signals of Gram negative bacteria are AHLs (Decho et al., 2009). Different species of bacteria produce AHLs with the different side-chain length ranging from four to eighteen carbons (Harder et al., 2014). Due to the well-known role of AHLs in the formation of biofilms, we hypothesize that the addition of these compounds during bioaugmentation treatments will enhance the survival of introduced (exogenous) as well as native (endogenous) oil-degrading bacteria and consequently increase rates of oil degradation.

The main objectives of this study were to investigate the effect of five AHLs (i.e. N-butyl-L-homoserine lactone (C₄-HSL), N-hexanoyl-L-homoserine lactone (C₆-HSL), N-octanoyl-L-homoserine lactone (C₈-HSL) and N-decanoyl-L-homoserine lactone (C₁₀-HSL), dodecanoyl-L-homoserine lactone (C₁₂-HSL) on respiration activities of a bacterial consortium and on oil-polluted soils with and without the consortium. Based on the results from this experiment and since C₁₂-HSL is more stable in the environment than shorter side chain AHLs, the effect of different concentrations of C₁₂-HSL on respiration activities of a bacterial consortium and oil-polluted soils and on biofilm formation of bacterial strains was further investigated. The fate of the bacterial consortium and changes in the bacterial community composition of the soils at the end of the experiment were followed using illumina MiSeq sequencing. The degradation of hydrocarbons was compared in the presence and absence of the bacteria consortium with and without C₁₂-HSL.

2. Materials and methods

2.1. Soil collection

Oil contaminated soils were collected from an area close to an oil production facility in Mina Al-Fahal, a coastal area in Muscat, Oman (23° 37' 49.92"N; 58° 31' 23.84"E). Soils from this area have been previously investigated for their physical and chemical properties as well as for their response to different bioremediation treatments including the addition of inorganic nutrients and exogenous microorganisms (Abed et al., 2014). In brief, the concentration of total petroleum hydrocarbons (TPH) in this soil was 22 ± 0.7 mg g⁻¹ soil and the pH was 7.46. The soils contained 62% silt, 30% sand and 8% clay and thus had a silt loam texture. The concentration of phosphate and nitrate reached 0.5 and 21 mg l⁻¹, respectively. Soils in sterile plastic boxes were brought back to the laboratory, where all experiments were performed.

2.2. Bioaugmentation experiments

2.2.1. Preparation of consortium

Prior to the experiment, a consortium composed of five alkane-degrading bacterial strains (i.e. *Alcanivorax* sp. MH3, *Parvibaculum* MH21, *Azospirillum* sp. AH2, *Marinobacter* sp. AH3 and *Marinobacter* sp. AH6) was prepared by cultivation in an artificial seawater medium supplemented with acetate at 30 °C. Detailed biochemical, physiological and phylogenetic characterization of the strains has been published elsewhere (Abed et al., 2014). The strains were selected based on their ability to degrade different alkanes and to represent different bacterial genera. The optical density of each strain was monitored at 600 nm until it reached 0.2. The cells were then collected at the exponential phase by centrifugation, washed three times with sterile seawater to remove traces of acetate and

re-suspended in carbon- and nutrient-free (i.e. without KH₂PO₄ and NH₄Cl) minimal salt medium. Equal volumes of each bacterial suspension were mixed together in artificial seawater medium and 1 ml of this mixture was added to 19 ml medium for each treatment (see below 2.2.2.). The artificial seawater medium contained MgCl₂·6H₂O (5.6 g l⁻¹), MgSO₄·7H₂O (6.8 g l⁻¹), CaCl₂·2H₂O (1.47 g l⁻¹), KCl (0.66 g l⁻¹), KBr (0.09 g l⁻¹) and was supplemented with trace elements mixture (Widdel and Rabus, 2001) and vitamins (Heijthuijsen and Hansen, 1986).

2.2.2. Experimental setup

The experiment was performed in glass bottles (volume 165 ml) containing 10 g of oil polluted soil mixed with 20 ml of carbon- and nutrient-free minimal salt medium. These soils were incubated, each in triplicate: 1) with five different acyl homoserine lactones (i.e. C₄-HSL, C₆-HSL, C₈-HSL, C₁₀-HSL and C₁₂-HSL), each added separately to the oil-polluted soils in the presence of the bacterial consortium at the concentration of 1 μM and 2) with different concentrations of C₁₂-HSL (i.e. 1 μM, 10 μM and 100 μM) added to the soil with and without consortium as well as to the consortium alone. A fresh dose of crude Arabian light oil 0.5% (v/v) was added to all soils, in order to follow the degradation of alkanes. The same incubations in the absence of AHLs and in the absence of extra oil served as controls. All used AHLs were ordered as pure compounds from Sigma–Aldrich (USA). The bottles were sealed with thick, black rubber stoppers to ensure no gas leakage and incubated for 42 days at 30 °C. CO₂ evolution was followed at different time intervals using gas chromatography (GC) (see below 2.2.3.).

2.2.3. Chemical analyses

CO₂ evolution was measured in the headspace of the gas tight serum bottles by withdrawing 250 μl of gas and injecting it manually into the GC (GC, Agilent model 6890N). The GC used helium gas as a carrier gas at a flow rate of 4 ml min⁻¹. The GC was equipped with a thermal conductivity detector and a 30 m × 250 μm capillary column (HP-PLOT Q). The thermal conductivity detector was maintained at 200 °C while the injector temperature was 210 °C. The oven temperature was programmed from 50 °C to 80 °C with a final hold time 3 min at a rate of 20 °C min⁻¹. Oil mineralization rates were calculated by comparing the experimentally quantified CO₂ with the theoretical amount of CO₂ that would be produced by complete oxidation of the present oil (Abed et al., 2014, 2015a, b). CO₂ evolution data over time were statistically analyzed using two-way ANOVA using the SPSS software (10th edition, Chicago, USA). The assumption of normality of data was verified with the Shapiro–Wilk's test (Shapiro and Wilk, 1965). Turkey post-hoc test was used in order to compare individual means. In all cases, the threshold for significance was 5%.

Oil degradation was assessed by extracting samples 3 times with 10 ml dichloromethane (DCM) and the pooled extract was filtered with non-absorbent cotton to remove solid particles. Anhydrous sodium sulfate was used to remove traces of water from the filtrate. The filtrate was then evaporated using a rotary evaporator. The dry extract was re-dissolved into DCM and passed through silica gel prior to injection. Gas chromatography-mass spectrometry (GC–MS) was equipped with a 30 m × 250 μm capillary column (Rtx®-5MS) and individual hydrocarbons were quantified by comparing them to an external standard (C₇–C₃₀). Helium gas was used as a carrier at a flow rate of 1 ml min⁻¹ and the injector and detector were maintained at 290 °C. The oven temperature was programmed from 80 °C (initial hold time 2 min) to 290 °C (final hold time 30 min) at a rate of 10 °C min⁻¹.

2.2.4. Illumina MiSeq 16S rRNA amplicon sequencing

At the end of the experiments, the fate of the bacterial

consortium and changes in the bacterial community composition in the bioaugmented and non-bioaugmented soils with (1 μM) and without C_{12} -HSL were followed using illumina MiSeq sequencing. DNA was extracted from triplicate soil samples at the end of experiments using skim milk protocol (Volossiuk et al., 1995). Purified DNA extracts were then submitted to Molecular Research MR DNA laboratory (www.mrdnalab.com, Shallowater, TX, USA) for illumina MiSeq sequencing of the bacterial 16S rRNA genes using the primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3') with barcode on the forward primer (Klindworth et al., 2013). Sequence analysis was carried out using the Mothur MiSeq SOP pipeline (Schloss et al., 2009). Briefly, barcodes were removed and sequences <200 bp and sequences with ambiguous base calls were eliminated. Sequences were denoised, OTUs generated and chimeras removed. Operational taxonomic units (OTUs) were defined by clustering at 3% divergence (97% similarity). Final OTUs were taxonomically classified using BLASTn against a curated GreenGenes database (DeSantis et al., 2006). Rarefaction curves and diversity indices (OTU richness, Chao and ACE) were calculated using the Mothur software. A multivariate analysis of all samples was performed to examine for significant changes in soil communities using multidimensional scaling (MDS) based on Bray–Curtis dissimilarities as described in Ramette (2007). Analysis of similarities (ANOSIM) was carried out to test for significant differences in bacterial communities. ANOSIM produces a sample statistic (R), which represents the degree of separation between test groups (Clarke, 1993). SIMPER (similarity percentage) analysis of the sequence data was performed using PAST program (version 2.17, Hammer et al., 2001), to find out the bacterial genera that accounted for the community differences in the different treatments.

2.3. Effect of AHLs on biofilm formation

An additional experiment was performed in order to find out if the addition of AHLs enhances the formation of biofilms. Prior to these experiments, individual strains of the consortium were grown in artificial seawater medium supplemented with acetate at 30 °C (see above 1.2.1). The impact of the different concentrations (1, 10 and 100 μM) of C_{12} -HSL on biofilm formation of each bacterial strain was investigated after 2 h at 30 °C (Djordjevic et al., 2002). Briefly, bound bacterial cells were stained with crystal violet solution and crystal violet absorbance was measured using a microplate photometer reader. Alteration between the control and the treatment color indicated differences in biofilm formation of tested bacteria due to the presence of AHLs. Biofilm formation is represented as the percent increase (%) in the attached cells in the presence of AHLs compared to the AHLs-free control.

3. Results

3.1. Effect of different AHLs on respiration activities

A significant increase in CO_2 evolution from 0.6 to 1.2 $\text{mg-CO}_2 \text{g}^{-1}$ of soil was observed in the soil bioaugmented with exogenous bacteria after 42 days of incubation in the presence of oil ($p < 0.005$). The addition of the five AHLs compounds (i.e. C_4 -HSL, C_6 -HSL, C_8 -HSL, C_{10} -HSL and C_{12} -HSL) to the soils resulted in a further increase in CO_2 evolution rates (Fig. 1). Analysis of variance (ANOVA) showed that the presence of different AHLs, the incubation time and their combination had a significant ($p < 0.05$) effect on the production of CO_2 (Table 1). Already after 12 days of incubation, the amounts of evolved CO_2 in the presence of the five AHLs were significantly higher than in their absence (Turkey HSD, $p < 0.05$). The five AHLs exhibited comparable temporal dynamics

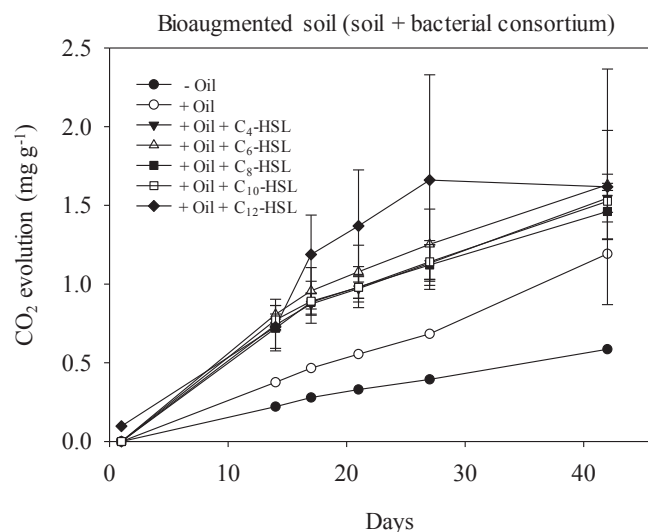


Fig. 1. The effect of different AHLs compounds (C_4 -HSL, C_6 -HSL, C_8 -HSL, C_{10} -HSL and C_{12} -HSL) on CO_2 evolution rates of an oil contaminated soil (amended with extra crude oil) in the presence of the bacterial consortium. As a control, the contaminated soil without additional oil was used.

Table 1

The effect of different AHLs agents and different concentrations of C_{12} -HSL on CO_2 evolution determined by ANOVA. An asterisk (*) denotes significant p -values at $\alpha = 0.05$.

Factor	Mean square	F	p -value
Experimenty with different AHLs compounds			
AHLs agent	9.845	81.362	>0.0001*
Time	38.277	316.315	>0.0001*
AHLs agent x time	0.353	2.921	>0.0001*
Residuals	0.121		
Experiment with different concentrations of C_{12} -HSL			
Concentrations	48.089	431.03	>0.0001*
Time	38.897	348.643	>0.0001*
Concentrations x time	2.019	18.097	>0.0001*
Residuals	0.112		

of CO_2 evolution (Fig. 1). After 42 days, all bioaugmented soils evolved around $1.6 \pm 0.03 \text{ mg-CO}_2 \text{g}^{-1}$ of soil.

3.2. Effect of different concentrations of C_{12} -HSL on respiration activities

The respiration activity of the bacterial consortium, as indicated by the evolved CO_2 significantly increased (Turkey HSD, $p < 0.05$) with the addition of 1 μM of C_{12} -HSL reaching $1.95 \pm 0.16 \text{ mg-CO}_2 \text{g}^{-1}$ after 42 days (Fig. 2). Higher concentrations of C_{12} -HSL resulted in much lower amounts of evolved CO_2 . In the case of the non-bioaugmented polluted soil, the addition of C_{12} -HSL at 1 μM concentration resulted in a significant (Turkey HSD, $p < 0.05$) increase in respiration activity in comparison with the HSL-free soil (Fig. 2) with a total amount of $1.58 \pm 0.7 \text{ mg-CO}_2 \text{g}^{-1}$ after 42 days. In contrast, the addition of 100-times higher concentrations of C_{12} -HSL to the soil did not have any effect on the CO_2 production. The addition of C_{12} -HSL to the bioaugmented soil exhibited a similar pattern of CO_2 evolution to that of the bacterial consortium alone (Fig. 2). The highest CO_2 evolution rate (i.e. $3.54 \pm 0.08 \text{ mg-CO}_2 \text{g}^{-1}$) of the contaminated soil with the consortium was observed in the

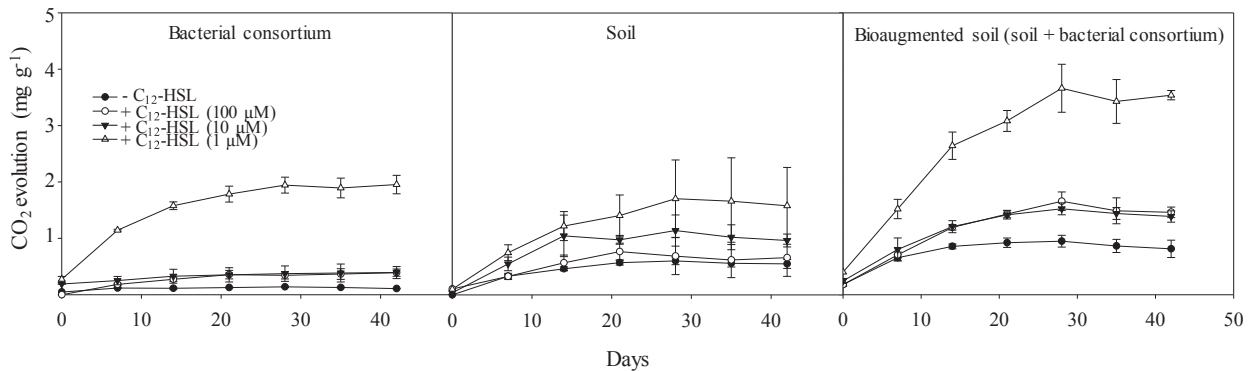


Fig. 2. The effect of different concentrations (1, 10 and 100 μM) of $\text{C}_{12}\text{-HSL}$ ($+\text{C}_{12}\text{-HSL}$) on CO_2 evolution rates of the contaminated soil, the contaminated soil with the bacterial consortium and the bacterial consortium alone. Samples without AHLs ($-\text{C}_{12}\text{-HSL}$) were used as controls.

presence of 1 μM of $\text{C}_{12}\text{-HSL}$. ANOVA suggested that different concentrations of $\text{C}_{12}\text{-HSL}$, the time of the experiment and their combination had a significant ($p < 0.05$) effect on the production of CO_2 (Table 1).

3.3. Effect of addition of 1 μM $\text{C}_{12}\text{-HSL}$ on soil bacterial community

A total of 1,246,999 16S rRNA gene sequences were obtained by MiSeq sequencing from all studied contaminated soils with numbers ranging between 26,178 and 152,882 sequences per sample (Table 2). The calculated OTU richness and Chao-1 index showed variations among the triplicates of each sample (Table 2). When variations in bacterial community composition were visualized in a two-dimensional space using multivariate analyses of OTUs (Fig. 3A), bacterial communities of differently-treated samples formed separate clusters (ANOSIM = 0.2–0.55), however this variation was not statistically significant (Bonferroni-corrected $p > 0.6$). The rarefaction curves revealed a good coverage of the community richness by the number of obtained sequences (Fig. 3B).

The bacterial classes *Deinococci* and *Alphaproteobacteria* dominated all bioaugmented and non-bioaugmented soils, with a relative abundance of 62–87% of total sequences (Fig. 3C). While sequences affiliated to the genus *Truepera* dominated the class *Deinococci* ($\geq 99\%$), most sequences belonging to the *Alphaproteobacteria* were related to the genus *Sphingomonas* ($< 20\%$). The remaining sequences belonged to *Bacilli*, *Gammaproteobacteria*, Candidate division tm7, *Phycisphaerae*, *Gemmatimonadetes* and *Actinobacteria* (Fig. 3C).

The addition of $\text{C}_{12}\text{-HSL}$ to the non-bioaugmented soil resulted

in a pronounced shift in the bacterial community structure of *Gammaproteobacteria* (Fig. 4). Sequences belonging to genera such as *Gilvimirinus*, *Thermomonas*, *Acidimicrobiales* and *Phycisphaera* appeared after the addition of $\text{C}_{12}\text{-HSL}$, while sequences belonging to the genera *Gemmatimonas*, and *Aneurinibacillus* were not detected anymore (Fig. 4). A similar community structure to the original soil was encountered in the bioaugmented soil, except for the detection of sequences belonging to the introduced bacterial consortium. Out of five members of the consortium, only sequences of the *Alcanivorax* sp. MH3 and *Parvibaculum* sp. MH21 persisted and were detected at the end of the experiment at an average relative abundance of $10.2 \pm 6.7\%$ and $3.7 \pm 2.2\%$ of the total sequences, respectively. After the addition of $\text{C}_{12}\text{-HSL}$, there was no significant change in the average relative abundance of the bacterial classes. In this treatment, sequences belonging to *Alcanivorax* sp. MH3 and *Parvibaculum* sp. MH21 were still detectable, however at a relative abundance of $3.7 \pm 2.3\%$ and $3.9 \pm 2.0\%$ of total sequences.

SIMPER analysis revealed that the major genera contributing to the dissimilarity between communities developed in the different treatments were *Alcanivorax* and *Truepera* (Table 3). The contribution of other genera did not exceed 3.7%. In the presence of the consortium, the *Alcanivorax* group had a more pronounced effect on the dissimilarity between communities (Table 3).

3.4. Effect of $\text{C}_{12}\text{-HSL}$ addition on oil biodegradation

Since oil was presumably the only available carbon source in our experiments, oil mineralization could be calculated from the

Table 2
Amplicon library sizes and bacterial diversity estimators for the soil samples using MiSeq.

Sample ID	Replicates	No. of sequences	No. of OTUs 0.03 ^a	Chao-1	ACE	SSO	DSO
Soil	A	98,402	420	769	816	6.7	7.0
	B	146,687	476	858	881	7.5	7.8
	C	120,261	537	941	1005	8.6	10.5
Soil + $\text{C}_{12}\text{-HSL}$	A	77,880	453	806	855	6.9	7.6
	B	140,163	576	1008	1010	8.8	9.3
	C	41,002	334	621	666	6.1	6.5
Bioaugmented soil	A	152,882	632	1102	1148	11.3	11.1
	B	132,855	510	923	950	7.9	7.8
	C	96,543	416	766	813	6.6	6.5
Bioaugmented soil + $\text{C}_{12}\text{-HSL}$	A	102,764	450	801	839	8.4	8.7
	B	111,382	811	1338	1382	13.9	14.4
	C	26,178	359	671	743	8.4	7.0

SSO Singletons sequences that were observed once.

DSO Doubletons are sequences that were observed twice.

^a Operational taxonomic unit at 3% sequence dissimilarity based on equal subsets of sequences for all samples.

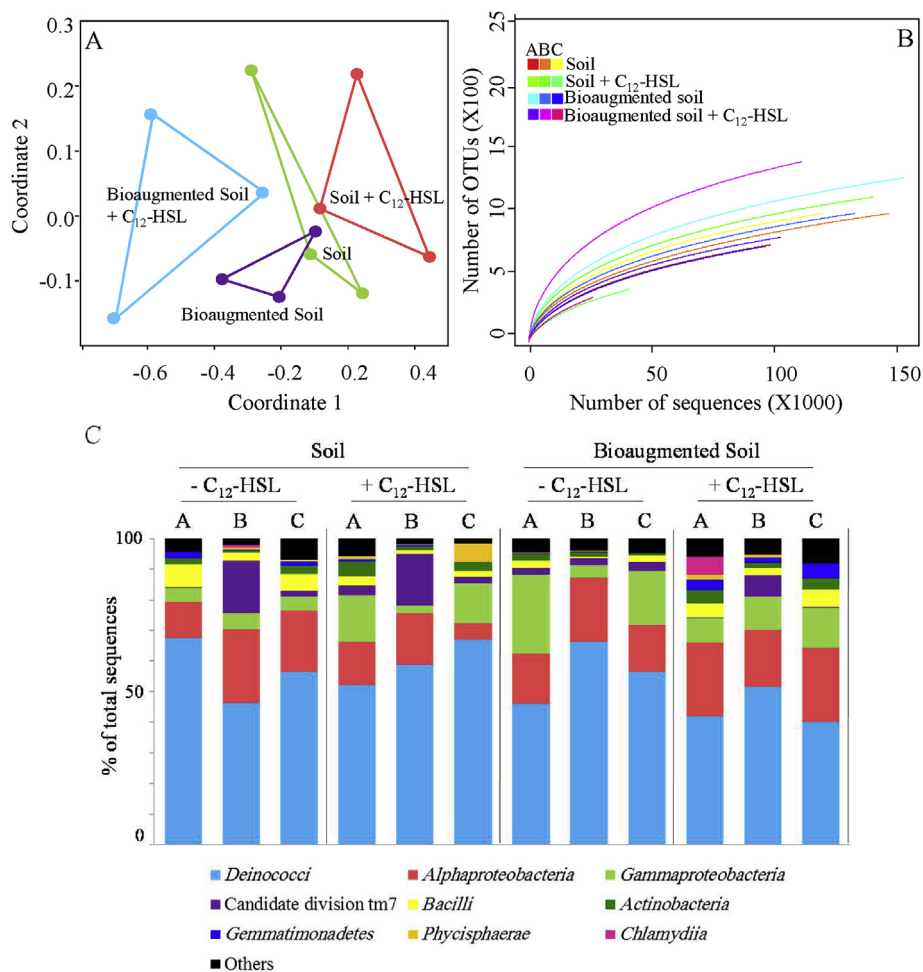


Fig. 3. The impact of C_{12} -HSL at 1 μM and addition of the bacterial consortium on the diversity of soil microbial communities. A. Two-dimensional nMDS ordinations of formed bacterial communities. B. Rarefaction curves. C. the relative abundance (%) of major classes of bacteria present in all treatments. The bioaugmented and non-bioaugmented soils were incubated with (+ C_{12} -HSL) and without the addition of AHLs (- C_{12} -HSL).

evolved CO_2 . The extent of oil mineralization was highest in the presence of 1 μM C_{12} -HSL in all incubations. The amount of total petroleum hydrocarbons (TPH) mineralized after 42 days increased from 0.3 ± 0.04 to 0.9 ± 0.4 and from 0.06 ± 0.01 to 1.2 ± 0.09 mg g^{-1} soil when C_{12} -HSL was added to the soil and to the bacterial consortium, respectively. The maximum oil mineralization rate was calculated in the case of the soil amended with the bacterial consortium and C_{12} -HSL and reached 2.1 ± 0.05 mg TPH g^{-1} soil after 42 days of incubation. This value corresponds to 4.5% of the initial oil.

Biodegradation of oil at the end of the experiment was further verified by GC-MS analysis of the residual oil. The concentrations of different alkanes were significantly higher ($p < 0.05$) in the bioaugmented soil with and without C_{12} -HSL in comparison to the non-bioaugmented soils (Fig. 5). The decrease in the concentration of most alkanes was higher in the presence of C_{12} -HSL (Fig. 5). The addition of both the bacterial consortium and C_{12} -HSL to the soil resulted in the degradation of around 93% of the C_{14} – C_{30} alkanes.

3.5. Effect of AHLs on biofilm formation of the bacterial consortium

The same concentrations of C_{12} -HSL, which were used in the bioaugmentation experiment (i.e. 1, 10 and 100 μM) stimulated biofilm formation of the strains (Table 1S), however to different extents. The maximum biofilm formation was observed at 10 μM

concentration and reached up to 63.4% in *Marinobacter* sp. AH6 strain in 2 h. The addition of C_{12} -HSL at 1 μM concentration increased biofilm formation of only *Parvibaculum* sp. MH21 and *Marinobacter* sp. AH6, whereas its addition at 100 μM concentration increase biofilm formation of only *Azospirillum* sp. AH2 and *Marinobacter* sp. AH6.

4. Discussion

The success of any biodegradation approach relies on the presence of efficient hydrocarbon degraders in sufficient concentrations (Dellagnezze et al., 2014). Since QS compounds, such as AHLs, have been shown to facilitate the aggregation of microorganisms in biofilms and microbial mats (Decho et al., 2009), we proposed that the use of external AHLs in bioremediation can enhance the survival and activity of microorganisms, including hydrocarbon-degraders and consequently enhance biodegradation rates. Our data demonstrated a significant stimulatory effect of different AHLs on respiration activities of bacteria in the bioaugmented and non-bioaugmented oil-contaminated soils. This increase in respiration activities was concomitant with the degradation of hydrocarbons. The soils amended with AHLs having side-chain length ranging from four to twelve carbons had comparable rates of evolved CO_2 , indicating a similar mode of action.

A strong negative correlation between the concentration of C_{12} -

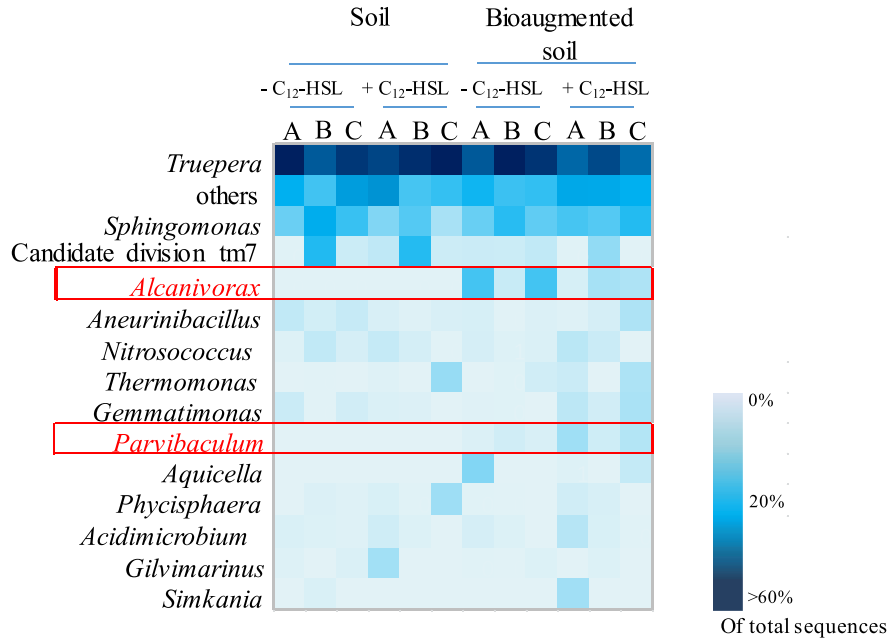


Fig. 4. A heatmap showing the relative abundance (%) of the most dominant bacterial genera detected in the bioaugmented and non-bioaugmented soils with (+C₁₂-HSL) or without (-C₁₂-HSL) the addition of C₁₂-HSL.

Table 3
The contribution of particular bacteria to total dissimilarity (as percentages) between the bacterial communities of the non-bioaugmented (S) and bioaugmented soils (BS) with and without C₁₂-HSL using SIMPER (similarity of percentage) analysis.

Taxon	Species contribution to dissimilarity (%)					
	S & BS	S & S + HSL	S & BS + HSL	BS & BS + HSL	BS + HSL & S + HSL	BS & S + HSL
<i>Alcanivorax</i>	5.0	0.0	1.8	3.8	1.8	5.0
<i>Truepera</i>	4.7	4.5	6.7	6.5	7.4	4.4
Candidate division tm7	2.9	3.7	3.3	1.5	3.4	2.6
<i>Sphingomonas</i>	2.2	3.6	2.0	1.4	2.7	2.4
<i>Aquicella</i>	1.4	0.0	0.5	1.6	0.5	1.4
<i>Thermomonas</i>	0.3	1.2	1.1	1.0	1.5	1.2
<i>Parvibaculum</i>	0.5	0.0	1.9	1.4	1.9	0.5
Others	82.9	87.0	82.6	82.7	80.7	82.5

The taxa that contribute to >3% to the total dissimilarities in microbial communities are shaded.

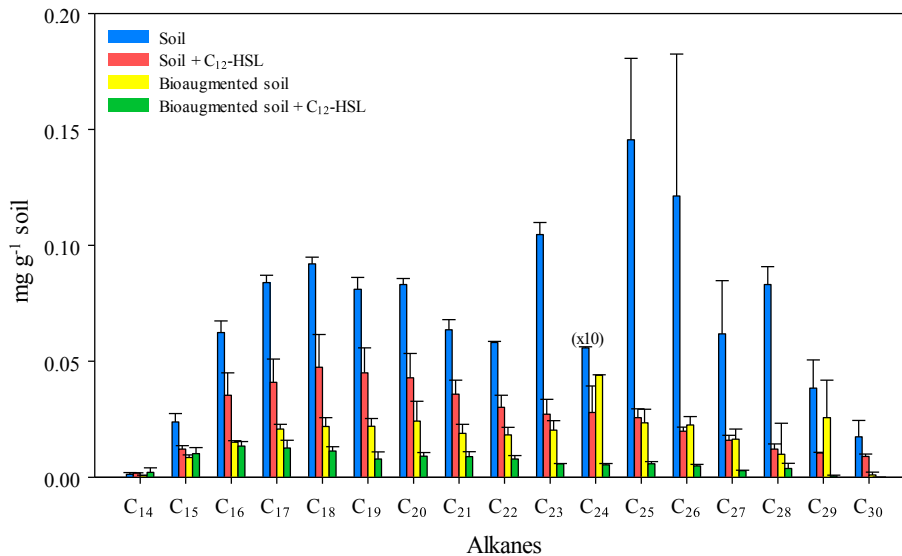


Fig. 5. Degradation of individual alkanes (C₁₄–C₃₀) at the end of the bioaugmentation experiments in bioaugmented and non-bioaugmented soils with and without C₁₂-HSL. Error bars represent ± standard deviation (n = 3). Note that the concentration of C₂₄ in the soil was 10 times higher than the displayed bar.

HSL and respiration activities was consistently observed in the bacterial consortium and in the contaminated soils with and without the consortium. It is known that even nm concentrations of AHLs can significantly affect bacterial QS, cell density and activity (Decho et al., 2011; Harder et al., 2014). It is also known that AHLs with the longer side chains ($>C_{10}$ -HSL) at higher concentrations can interfere with bacterial QS signaling using shorter chain ($<C_6$ -HSL) AHLs (McClean et al., 1997; Dobretsov et al., 2009). Thus, it is possible to speculate that C_{12} -HSL at the concentration of $>10 \mu\text{M}$ inhibited QS of some bacteria that used short chain AHLs for their communication. Additionally, high concentrations of C_{12} -HSL could be toxic to some bacteria, thus resulting in low CO_2 evolution. On the other hand, the enhancement of CO_2 evolution at $1 \mu\text{M}$ concentration could be attributed to increased activity of bacteria, although it should be kept in mind that even low number of bacteria can have high respiration rates. Indeed, MiSeq sequencing clearly demonstrated an increase in the relative abundance of the consortium member *Parvibaculum* sp. in the presence of C_{12} -HSL. The biofilm formation assay on individual strains of the consortium, although performed for only 2 h, showed that C_{12} -HSL addition was stimulatory to biofilm formation. AHL signals are known to enhance the formation of biofilm matrix (EPS) that protects the cells from environmental factors, facilitates chemical interaction among bacteria and increase the survival rate of bacteria (Gilan et al., 2004; Singh et al., 2006; Das et al., 2013).

Previous studies have shown that the degradation of environmental pollutants is more efficient by microorganisms in biofilms than by free-living bacteria (Singh et al., 2006; Verhagen et al., 2011; Shimada et al., 2012). For example, *Pseudomonas stutzeri* biofilm-associated cells degraded naphthalene and successfully survived in petroleum contaminated soil (Shimada et al., 2012). Although biofilm cultures degraded the pesticide chloroprotham slower than planktonic cultures, they did not produce toxic intermediate compounds (Verhagen et al., 2011). Relation between hydrocarbon degradation and biofilm formation by bacterial isolates was demonstrated by Dasgupta et al. (2013). Biofilms of *Marinobacter hydrocarbonoclasticus* enhanced hexadecane assimilation (Klein et al., 2010). AHLs compounds were found to play an important role in the degradation of sinking hydrocarbons in oceans (Buchan et al., 2005; Zierovogel and Arnosti, 2008; Hmelo and Van Mooy, 2009). An AHL-producing marine bacterium *Croceicoccus naphthovorans* was able to degrade polycyclic aromatic hydrocarbons (Huang et al., 2015). Similarly, AHL producing isolates belonging to the genera *Sphingomonadales* and *Rhizobiales* were able to degrade phenanthrene and pyrene (Huang et al., 2013). Rhl QS system in *Pseudomonas aeruginosa* was involved in regulation of aromatics biodegradation (Yong and Zhong, 2013). These examples suggest that AHLs compounds can be directly involved in the degradation of hydrocarbons and enhance the process by stimulation of bacterial activities and survival.

From our experiments, it remains unclear whether the evolved CO_2 was due to the growth of microorganisms on AHLs compounds or on hydrocarbons. Since the tested soils were originally polluted, we could not maintain a similar incubation in the absence of oil. However, incubations of the bacterial consortium alone without the soil in the presence of either oil or C_{12} -HSL as the sole carbon source (not shown) showed a similar temporal increase in the evolved CO_2 in the first 18 days of incubation. This suggests that the bacteria in the consortium, and maybe even in the soil, could probably use C_{12} -HSL as a carbon source, at least at the initial phase of growth, until its concentration is diminished and then switched to grow on hydrocarbons. This assumption is supported by several arguments. Firstly, the degradation of hydrocarbons in the presence of the consortium was indeed confirmed by our GC-MS analysis at the end of the experiment. Secondly, AHLs degradation has been

identified in a wide range of soil bacteria including strains from the genera *Bacillus*, *Arthrobacter*, *Variovorax*, *Ralstonia* and *Rhodococcus* (Leadbetter and Greenberg, 2000; Huang et al., 2003; Lin et al., 2003; Dong et al., 2000; Lee et al., 2002; Park et al., 2003, 2005; Uroz et al., 2003). Sequences affiliated to these genera were also detected in our studied soils. These bacteria use enzymes such as oxido-reductase, AHL-acylase and AHL-lactonases to degrade AHLs and use them as a carbon source (Uroz et al., 2003, 2005; Park et al., 2006). Thirdly, AHLs are known for their instability in the environment, which depends on their chemical structure, pH and presence of AHL-degrading microorganisms (Horswill et al., 2007). AHLs are not stable at high pH (>7) and in some cases can be mineralized in soil within minutes (Wang and Leadbetter, 2005). Preliminary laboratory experiments showed that C_{12} -HSL at concentration of $100 \mu\text{M}$ is fully degraded in 5–10 days in natural conditions (Dobretsov, personal communication). In our experiments, pH did not show a dramatic shift at the end of the incubation and was always between 7.4 and 7.6.

Illumina MiSeq sequencing provided interesting information on the fate of the added bacterial consortium and helped us to select the most competent strains for future bioremediation treatments. Only *Alcanivorax* sp. MH 3 and *Parvibaculum* sp. MH21 out of five members of the added exogenous consortium could persist until the end of the experiment. This indicates that these two species were not outcompeted by the indigenous soil bacteria and enhanced the degradation of alkanes, which was demonstrated by GC-MS. These results highlight the importance of species belonging to *Alcanivorax* and *Parvibaculum* genera in bioaugmentation of oil-polluted soils. Indeed, previous studies have reported that *Alcanivorax* spp. is a key player in many bioaugmentation treatments (Golyshin et al., 2003; Gertler et al., 2009a, 2009b; Martins dos Santos et al., 2010; Hassanshahian et al., 2012) and this species has been used quite often in the construction of hydrocarbon degrading consortia (Gertler et al., 2009a; Hassanshahian et al., 2012). The importance of *Parvibaculum* species in degrading alkanes (Wang and Shao, 2013; Nie et al., 2014) and polycyclic aromatic hydrocarbons (i.e. pyrene, phenanthrene and fluoranthene) has also been reported earlier (Hilyard et al., 2008; Sipilä et al., 2008).

Unlike their effect on respiration activities, the addition of C_{12} -HSL to the polluted soils did not induce dramatic changes in the bacterial community composition. All soils were dominated by bacteria belonging to the classes *Deinococci* and *Alphaproteobacteria*. While *Alphaproteobacteria* has been previously detected in oil-contaminated soils (Bordenave et al., 2007), the dominance of *Deinococci* in such ecosystems is not very common. Although the class *Deinococci* is known to include species that are resistant to the harsh environmental conditions in deserts (Albuquerque et al., 2005; Jun, 2010; Li et al., 2014), there is no evidence that these bacteria play any role in the degradation of hydrocarbons, which deserves further investigations. In opposite, MiSeq sequencing demonstrated presence of *Alphaproteobacteria* and *Gammaproteobacteria* that are known to include hydrocarbon-degraders. Strains belonging to *Sphingomonas*, *Halomonas*, *Bacilli*, *Actinobacteria* contain species known to degrade hydrocarbons (Ilori et al., 2000; Daane et al., 2002; Gauthier et al., 2003; Cubitto et al., 2004; Story et al., 2004; Perfumo et al., 2010; Prince et al., 2010; Ichikawa et al., 2010; Najafi et al., 2011; Lin et al., 2012; Chandankere et al., 2013).

5. Conclusion

In conclusion, our investigation showed that the addition of AHLs in appropriate concentrations has a stimulatory effect on the respiration activity of microorganisms. This stimulatory effect was observed when AHLs were added to the soil alone, to the

consortium alone and to the soil and consortium together. The increase in respiration activities was concomitant with the degradation of hydrocarbons. The highest level of degradation was observed when both AHLs and exogenous bacteria were simultaneously added to the soil. Although the addition of AHLs stimulated the bacterial respiration activities and degradation of hydrocarbons, the exact mechanism of action is still unknown. Therefore, further research is still needed to elucidate the exact mode of action of QS signals on the activity of microorganisms in soils and on the survival of exogenous bacteria used in bioaugmentation treatments.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.ibiod.2015.11.021>.

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