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Assessment of The Roles of Indigenous and Augmented Microorganisms in Bioremediation of Recent Diesel Pollution in Agricultural Soil: A Case Study

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The roles of indigenous and augmented microorganisms in the process of biodegradation of spilled diesel fuel (~2 years old contamination, ~2500 C_{10}-C_{40} mg/kg initial concentration) were assessed in ~2-month pot experiment as supporting information for selection of an applicable bioremediation method. Non-sterile soil and semi-sterile autoclaved soil were inoculated with *Comamonas acidovorans* strain with high biodegradation ability and mixed-culture extracted from the soil and enriched on mineral medium with diesel as a sole source of carbon and energy. Significant decrease of C_{10}-C_{40} concentration was observed in all variants except in the autoclaved control. Analyses of variance revealed that only the "augmentation" factor contributed to significantly more effective biodegradation, while factors "soil sterilization" and "inoculum type" were insignificant. With reference to microbial biomass, the results suggested that the soil was insufficient in number of microorganisms capable of petrol biodegradation and indicated that bioaugmentation was the important treatment in order to enhance bioremediation process.

Keywords: Diesel bioremediation; Indigenous microorganisms; Bioaugmentation; Phospholipid fatty acids; C_{10}-C_{40} hydrocarbon index.

Bioremediation is considered to be a cost-effective and environmental friendly approach for decontamination of organically polluted soils[1-3]. The process is dependent on the soil microorganisms, i.e. they need to be present in sufficient quantity, they need to harbor and express appropriate metabolic pathways, and they need to be in favorable conditions in order to exhibit sufficient metabolic activity[4]. If such microorganisms are not present in the soil, or are present in low quantities only, they need to be added externally. In past years a series of bioremediation methods (such as landfarming, bioventing etc.) has been developed, however all of them are based on two basic strategies, often

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combined together: biostimulation (i.e. support of indigenous microorganisms to higher metabolic biodegradation activity by means of aeration, supply of nutrients, induction of metabolic pathways etc.) or bioaugmentation (i.e. inoculation of soil by externally cultivated microorganisms capable of the biodegradation of pollution)[5]. Despite advances in the study of bioremediation processes, polluted soil is such a heterogenic and complex environment that prediction of the process course, its rate and results is difficult. Therefore carrying out a preliminary laboratory experiment before bulk bioremediation treatment presents more reliable strategy[3]. Such experiment should be fast and give clear recommendations. Nevertheless, the remediation technologies present a large business area nowadays. The preservation of know-how is likely the reason why the number of published case studies on real polluted soils is limited, despite their usefulness in terms of analogies and comparisons.

In our previous study we have followed the effect of bioaugmentation and abiotic treatments (addition of humates and zeolites) on the biodegradation of fresh diesel spill in loamy arable soil[6]. In this sequel study, two years after the spill, we aimed to assess the roles of indigenous and augmented microorganisms (pure culture of degrading strain *Comamonas acidovorans* and mixed culture isolated from the soil and enriched on the diesel as a sole source of carbon and energy) in the process of biodegradation of residual aliphatic hydrocarbons as a supporting information for decision of applicable bioremediation method. Biodegradation was followed by determination of C_{10}-C_{40} aliphatic hydrocarbons[7] while soil microbial community was assessed by phospholipid fatty acid (PLFA) content[8,9].

MATERIALS AND METHODS

Soil

A loamy field soil (ash content 7 %, moisture 10 %, pH 7.5) polluted ~2 years prior to experiment by diesel oil after a tank truck accident was used[6]. Some details of composition are shown in Table 1. Significant portion of stones was eliminated by sieving through a 1 cm sieve. Approx. 10 kg of the soil was semi-sterilized by single autoclaving (121°C, 0.1 MPa, 1 hour).

Bioaugmentation

Comamonas acidovorans strain capable of degrading both aliphatic as well as aromatic hydrocarbons was obtained from Dekonta a.s., Czech Republic. Indigenous mixed-culture was extracted from 1 g of the soil in Erlenmayer flask containing 100 ml of Bacterial Salt Medium (BSM[10], 150 rpm, 25°C, 1 hour) and 10 ml aliquot was used for further enrichment. Both cultures were cultivated with diesel oil (~1 %) serving as a sole source of carbon and energy to an early exponential phase (~10^7 CFU/mL).

The cells were harvested by centrifugation (4000 rpm, 10 min), resuspended in sterile tap-water and augmented in concentration ~2×10^5 CFU/g wet soil respectively.

Experiment parameters

The experiment consisted of 6 variants, three with semi-sterile soil and three with non-sterile soil (Table 2) in triplicates. Conical pots (bottom diameter 135 mm, upper diameter 180 mm, height 155 mm) were filled with 1 kg of wet soil and other admixtures according to Table 2. The pots were regularly (~2 times per week) irrigated by distilled water and mixed. This ensured good aeration and maintenance of the soil moisture at optimal ~15 % throughout the experiment. The experiment was conducted in an air-conditioned laboratory at constant temperature 25±1°C.

Sampling

Soil samples (~90 g) were withdrawn from each pot at days 0-7-38-71 and frozen immediately at 30°C for later analyses.

Analyses

Aliphatic hydrocarbon content, expressed as the total concentration of C_{10}-C_{40}, was determined according to optimized method for soils and sludge[6,7].

PLFA were analyzed by the adopted method of Zelles (1995) a using frozen soil samples as described previously[6,11]. Briefly, total lipids were extracted by a single-phase mixture of methanol, chloroform and phosphate buffer, separated on silica solid-phase-extraction columns, the polar-lipid fraction was subjected to mild alkaline methanolysis and incurred fatty acid methylesters (FAME) were determined by gas chromatography with mass spectrometry detection (GC-MS). Soil microbial biomass was quantified using total PLFA concentration ($PLFA_{tot}$). Fungal biomass ($PLFA_{fun}$)

was quantified by concentration of 18:2ω6,9. Bacterial biomass ($PLFA_{bac}$) was quantified by a sum of concentrations i14:0, i15:0, a15:0, 16:1ω7t, 16:1ω9, 16:1ω7, 18:1ω7, 10Me-16:0, i17:0, a17:0, cy17:0, 17:0, 10Me-17:0, 10Me-18:0, cy19:0. Biomass of gram-positive bacteria ($PLFA_{G+}$) was represented by a sum of i14:0, i15:0, a15:0, i17:0, a17:0 while biomass of gram-negative bacteria ($PLFA_{G-}$) was represented by a sum of cy17:0, cy19:0, 18:1ω7. Ratios of fungal to bacterial biomass (F/B) and gram-positive to gram-negative bacterial biomass (G+/G-) were calculated as $PLFA_{fun}/PLFA_{bac}$ and $PLFA_{G+}/PLFA_{G-}$ respectively[12]. Stress indicator (*trans/cis*) was calculated as the ratio (18:1ω7+16:1ω7)/(18:1ω7t+16:1ω7t)[8]. Nutrition stress (*cy/pre*) was calculated as the ration (cy17:0+cy19:0)/(16:1ω7 + 18:1ω7)[13].

The concentration of cultivable heterotrophic microorganisms was determined by spreading on the Luria Broth Agar (Pepton 10 g/L, Yeast extract 5 g/L, NaCl 1 g/L, agar 20 g/L) after series of 10-fold dilutions. Soil samples (1 g) were extracted prior to spreading into 100 mL of the sterile saline (1% NaCl + 0.1% polysorbate 80). Contents of N_{tot}, P_{tot} (in Mehlich extract[14]), and C_{org} were determined according to European standards (EN 13342[15], EN ISO 11885[16], and ISO 14235[17] respectively).

Calculations and statistics

C_{10}-C_{40} hydrocarbon concentration was expressed as the percentage decrease relative to time 0 for each pot. All statistical tests were calculated using Statistica 10 (StatSoft, USA). Unless stated, all statistical tests and comparisons were evaluated at $\alpha = 0.1$ and $\alpha = 0.05$ for hydrocarbon and PLFA data respectively.

RESULTS

Soil microbial community during bioremediation

Soil properties and their changes upon autoclaving are summarized in Table 1. $PLFA_{tot}$, indicator of living microbial biomass, was comparable to other agriculture and meadow soils[18,19] and comparable to final values from preceding study[6]. Microbial community composition exhibited signs typical for disturbed soils; i.e. *trans/cis* ratio higher than 0.1 (indicating microbial stress), G+/G- ratio significantly lower than 1.0 (indicating a slight prevalence of more resilient gram-negative bacteria), and low F/B ratio (even lower than in similar arable soils)[8].

As expected, autoclaving did not cause complete soil sterilization and it only resulted in a slight reduction of several PLFA indicators (Table 1). Nevertheless the decrease of the total PLFA by

Table 1. Soil parameters (average ± standard deviation) and their change upon autoclaving

Parameter	Non-sterile	Autoclaved	Significance
C_{org} [%]	2.29 ± 0.14	2.21 ± 0.23	ns
N_{total} [%]	0.17 ± 0.02	ND	-
P [mg/kg]	159 ± 16	ND	-
C_{10}-C_{40} [mg/kg]	2733 ± 564	2592 ± 517	ns
$PLFA_{tot}$ [mg/kg]	19.1 ± 3.0	13.6 ± 3.6	*
F/B []	0.07 ± 0.02	0.09 ± 0.02	ns
G+/G- []	0.75 ± 0.09	0.83 ± 0.10	ns
trans/cis []	0.53 ± 0.04	0.27 ± 0.15	***
cy/pre []	0.52± 0.02	0.53 ± 0.07	ns

ND = not determined, ns = not significant (P>0.05), * P<0.05, ** P<0.01, *** P<0.001

C_{10}-C_{40} – concentration of soil aliphatic hydrocarbons of chain length C_{10}-C_{40}, $PLFA_{tot}$ – total phospholipid fatty acids, F/B – ratio of fungal/bacterial PLFA, G+/G- – ratio of indicator PLFA of gram-positive and gram-negative bacteria, *trans/cis* – stress indicator based on the ratio of *trans* and *cis* monounsaturated fatty acids[8], *cy/pre* – stress indicator based on the ratio of fatty acids with cyclopropyl ring and their metabolic precursors[13]

29% was significant (t-test; $P<0.05$) and comparable to other studies utilizing similarly autoclaved soil[20-22]. Ratios of F/B and G+/G- were not affected by autoclaving thus indicating its non-specific impact on the followed microbial groups. Of interest is the significant decrease of *trans/cis* ratio. This observation indicates preferential extinction of stressed bacteria and supports the hypothesis of stronger effect of autoclaving on active microorganisms than on the resting ones. Abiotic soil parameters (hydrocarbon index, major nutrients) were not affected by the autoclaving.

The soil microbial community remained predominantly stable throughout the experiment (Table 3), which was the reason for grouping the experiment variants according to the factor "autoclaving" in order to pronounce differences. The most remarkable is the increase of $PLFA_{tot}$ in autoclaved variants back to non-autoclaved levels at day 71. The F/B ratio was significantly increased in autoclaved variants. This indicates that the autoclaving distorted the microbial competition by

Table 2. Experiment setup (variants)

Variant	Soil	Bioaugmentation
A	Autoclaved	None
AC	Autoclaved	*C. acidovorans*
AE	Autoclaved	enriched mixed-culture
N	Non-sterile	None
NC	Non-sterile	*C. acidovorans*
NE	Non-sterile	enriched mixed-culture

Table 3. PLFA indicators in the beginning and at the end of the experiment (average ± standard deviation). Letters denote homogenous groups according to Bonferoni test (α=0.05). In order to pronounce differences the variants were joined according to "autoclaving" factor.

Day Autoclaving (variants)	0 Autoclaved (A+AC+AE)	0 Non-autoclaved (N+NC+NE)	71 Autoclaved (A+AC+AE)	71 Non-autoclaved (N+NC+NE)
$PLFA_{tot}$	14.4 ±1.7 a	19.6 ± 1.5 b	18.0 ± 1.7 b	21.0 ±1.5 b
trans/cis	0.38 ± 0.11 a	0.52 ± 0.10 a	0.36 ± 0.11 a	0.41 ± 0.10 a
cy/pre	0.62 ± 0.07 a	0.52 ± 0.06 a	0.59 ± 0.07 a	0.59 ± 0.06 a
F/B	0.09 ± 0.02 bc	0.07 ± 0.01 ab	0.10 ± 0.02 c	0.06 ± 0.01 a
G+/G-	0.76 ± 0.08 a	0.76 ± 0.07 a	0.66 ± 0.08 ab	0.60 ± 0.07 b

Table 4. Changes of aliphatic hydrocarbons C_{10}-C_{40} in individual experiment variants and grouped according to joint factors (average ± standard deviations). Letters denote homogenous groups according to Bonferoni test (α=0.1). Bold-face indicates values significantly lower than 0 (i.e. significant decrease of hydrocarbon concentration, t-test, α=0.1)

	7	38	71
a) Experiment variants			
A	6.2% ± 5.8% a	-10.3% ± 13.3% abcd	-6.9% ± 1.4% abc
AC	-13.9% ± 9.2% abcd	-17.8% ± 2.6% abcd	-27.0% ± 6.7% bcd
AE	-14.4% ± 10.1% abbc	-14.0% ± 11.7% abcd	-28.1% ± 3.9% cd
N	2.1% ± 6.1% ab	-12.5% ± 5.4% abcd	-22.9% ± 2.5% abcd
NC	-9.2% ± 11.4% abcd	-8.4% ± 2.1% abcd	-37.4% ± 5.6% d
NE	2.6% ± 4.1% a	-28.0% ± 14.8% cd	-28.2% ± 6.5% cd
b) Effect of autoclaving			
Autoclaved(A+AC+AE)	-7.4% ± 12.9% ab	-14.0% ± 10.8% a	-20.7% ± 10.7% b
Non-autoclaved(N+NC+NE)	-1.5% ± 9.5% a	-16.3% ± 12.5% abc	-29.5%± 7.9% bc
c) Effect of augmentation			
Augmented(AC+AE+NC+NE)	-8.7% ± 11.4% ab	-17.0% ± 12.0% b	-30.2%± 7.2% c
Non-augmented(A+N)	4.2% ± 6.3% a	-11.4% ± 10.2% ab	-14.9%± 8.2% b
d) Effect of inoculum type			
C. acidovorans(AC+AE)	-11.5% ± 10.6% ab	-13.1% ± 5.3%a	-32.2%± 8.1% c
Enriched(AE+NE)	-11.5% ± 12.0% a	-21.0% ± 15.1% abc	-28.2%± 5.4% bc

slight favoring of the soil fungi. The G+/G- ratio decreased throughout the experiment, which indicates higher biodegradation activity and proliferation of G- bacteria.

Aliphatic hydrocarbon biodegradation

Decrease of C_{10}-C_{40} aliphatic hydrocarbons as a function of time and variant is summarized in Table 4. Significant decrease (i.e. compared to 0, t-test, α=0.1) was achieved in all experiment variants (bold-face values) except for the non-augmented autoclaved control (variant A). At day 71 the highest hydrocarbon decrease was achieved in the NC variant. Bonferoni intervals (comparing all individual variants from all sampling times) showed significant differences only between autoclaved control A and other variants. Nevertheless the Bonferoni test is rather conservative and prone to producing false-negatives[23]. Based on stronger pair comparisons (Mann-Whitney test, α=0.1), more suitable for such short study, all four augmented variants (NC, NE, AC, AE) were comparable to each other, but all were higher than the non-augmented control N, which was in turn higher than the autoclaved control A (not shown).

Significant difference was detected for the effect of augmentation (regardless of inoculum type) – bioaugmented variants exhibited higher hydrocarbon decrease (Table 4c). Contrary, no differences were detected between autoclaved and non-sterile variants (Table 4b) as well as between variants augmented by pure *C. acidovorans* culture and enriched mixed culture (Table 4d).

Table 4

DISCUSSION

Both indigenous and augmented microorganisms contributed to biodegradation. Obtained results require deeper discussion in order to interpret properly the roles of indigenous and augmented microorganisms.

In the autoclaved control A no significant decrease of C_{10}-C_{40} aliphatic hydrocarbons was detected even after 71 days of the experiment; in spite of the regular mixing and optimal humidity control. On the contrary, the hydrocarbon decrease in the non-sterile control N was significant. This observation confirms the biological nature of the hydrocarbon elimination as well as the participation of the indigenous microorganisms in this process. In addition, the existence of the petrol-degrading microorganisms in the soil is confirmed by the fact that it was possible to isolate them from the soil. Upon enrichment and reinoculation back to the soil, these former indigenous microbes performed comparably to externally prepared culture of allochtonic petrol-degrading strain of *C. acidovorans* (Table 4d).

Considering joint factors, the positive effect of augmentation (regardless of inoculum type, Table 4c) is in contrast to insignificant differences between autoclaved and non-sterile variants (Table 4b). In other words the effect of augmentation overwhelmed the effect of autoclaving which indicates higher contribution of augmented microorganisms to hydrocarbon biodegradation as compared to indigenous. This result was achieved in spite of the fact that the microbial counts of augmented microorganisms (2×10^5 CFU/g; both enriched and *C. acidovorans*) were two orders of magnitude lower compared to the overall microbial counts in the soil ($5\pm3\times10^7$ CFU/g). Also the initial concentration of the $PLFA_{tot}$ of non-sterile control N (19.1±3.0) was comparable (t-test, α=0.05) to non-sterile augmented variants (average 18.3±2.4), i.e. the initial biomass of augmented microorganisms was negligible compared to indigenous biomass. This indicates that the original soil contained microorganisms capable of diesel biodegradation but in very low quantities only (according to CFU counts less than 1%).

Summarized, presented comparisons show that both indigenous and augmented microorganisms contributed to the overall biodegradation of aliphatic hydrocarbons. Nevertheless, the augmented microorganisms were more effective in this process since they were concentrated aliphatics degraders while among the indigenous microorganisms the number of such degraders was limited.

Implications for bulk applications

The presented study was primarily carried out in order to decide on the necessity of bioaugmentation for possible bioremediation treatment. Results showed that the soil contained a limited number of aliphatics degrading microorganisms and confirmed that bioaugmentation was the most important treatment

in order to enhance aliphatic hydrocarbons biodegradation. The concentration of the inoculum was rather low, so we can presume that using of the higher microbial concentration should serve even better. On the other hand the data revealed no differences between augmentation of exogenous *C. acidovorans* strain with high degradation ability and mixed-culture isolated from the soil and enriched by cultivation on sterile diesel.

Since the soil had adequate content of major nutrients (Table 1) as well as their ratio, no nutrients addition was carried out and the microbial cultures were inoculated as nutrient-less suspension only. Despite that the successful bioremediation was achieved thus confirming that the nutrients content was not the limiting factor and that biostimulation was not necessary.

Bioaugmentation as a preferred bioremediation treatment was found in several similar studies[5,24-27] compared to others where biostimulation[28,29] or natural attenuation[30] seemed more suitable strategies or where both strategies were equal. In a generalizing attempt bioaugmentation-concluding studies were usually carried out on recently polluted soils, implicating low abundance of indigenous degrading microorganisms and insufficient time for their selection and enrichment. On the contrary in cases of older pollution, biostimulation is a more usual treatment[5,31]. In our previous study we have treated this particular soil directly after diesel-spill and found that bioaugmentation was the most important treatment affecting the rate of hydrocarbon removal[6]. The ~2-year period of air-dried storage of polluted soil therefore seems to be an insufficient time for development of strong community of indigenous degraders.

A laboratory case study serves as a simulation of the planned bulk process. However it cannot copy it in full details. One of the main problems is the limiting time scale due to pressure for gaining of the results as soon as possible. The 71 days limit indeed seemed to be a rather short time, which showed interesting trends but resulted in lower differences between variants, especially if using conservative Bonferoni test for multiple comparisons. This was the main reason for the necessity of stronger mutual comparisons, factors grouping, and using of the less usual α=0.1 significance level for the hydrocarbon decrease data. A few more weeks of the experiment might have given more pronounced results with higher significance.

On the other hand the laboratory-scale setup enables treatments that can not be carried out in bulk and which can aid in overcoming the short time period. This was especially the case of the soil autoclaving, unfeasible in bulk, which served as a useful tool for better discrimination between the roles of augmented and indigenous microorganisms in the process.

CONCLUSIONS

A model laboratory case study was carried out in order to discriminate the roles of indigenous and bioaugmented microorganisms during bioremediation of two-years-old diesel spill in arable soil. Detailed analysis of the data revealed that both microbial groups contributed to successful elimination of aliphatic hydrocarbons; however the role of augmented microorganisms was dominant due to limited number of indigenous degraders in the soil. This result implicates bioaugmentation as the recommended treatment for proposed bulk bioremediation application.

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