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Effect of petrochemical sludge concentrations on microbial communities during soil bioremediation

María T. Del Panno [a,*], Irma S. Morelli [a], Bert Engelen [b], Luise Berthe-Corti [b]

[a] LBMH, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Calle 47 y 115, La Plata 1900, Buenos Aires, Argentina
[b] ICBM, University of Oldenburg, D-26111 Oldenburg, Germany

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Abstract

Qualitative and quantitative changes of microbial communities in soil microcosms during bioremediation were determined throughout one year. The soil was contaminated with 0%, 2.5%, 5%, 10% (wt/wt) of petrochemical sludge containing polynuclear aromatic hydrocarbons. We analyzed the hydrocarbon concentration in the microcosms, the number of cultivable bacteria using CFU and most probable number assays, the community structure using denaturing gradient gel electrophoresis, and the metabolic activity of soil using dehydrogenase activity and substrate-induced respiration assays. After one year of treatment, the chemical analysis suggested that the hydrocarbon elimination process was over. The biological analysis, however, showed that the contaminated microcosms suffered under long-term disturbance. The number of heterotrophic bacteria that increased after sludge addition (up to 10^8–10^9 cells ml^{-1}) has not returned to the level of the control soil (2–6×10^7 cells ml^{-1}). The community structure in the contaminated soils differed considerably from that in the control. The substrate-induced respiration of the contaminated soils was significantly lower (≈10-fold) and the dehydrogenase activity was significantly higher (20–40-fold) compared to the control. Changes in the community structure of soils depended on the amount of added sludge. The species, which were predominant in the sludge community, could not be detected in the contaminated soils.
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Keywords: Bioremediation; Soil bacterial community; Hydrocarbon degradation

1. Introduction

The long-term production and use of different petroleum derivatives have caused widespread contamination of soil around the facilities handling them [1]. Improper disposal of oily sludge leads to environmental pollution, particularly soil contamination [2]. Among the large variety of techniques for decontamination of affected sites, in situ bioremediation using indigenous microorganisms is by far the most widely used [2]. In hydrocarbon-contaminated soil, bioremediation is undertaken to reduce the hydrocarbon concentration, its mobility and toxicity [3]. Although, several studies of changes in microbial communities during bioremediation have been published [4–6], the microbial contribution to the effectiveness of bioremediation and the impact on ecosystems need to be better clarified [7,8]. When bioremediation is not successful, it is often difficult from a microbiological standpoint to identify the cause of the failure and to develop measures to counter it [7]. On the other hand, similarly as other segments of the biota, microorganisms are perceptible to pollution. An inhibition of microbial activity leads to reductions in ecological processes [9,10]. Since these processes are subtle and covert, this

* Corresponding author. Tel.: +54 221 4235333x38; fax: +54 221 4226947.
E-mail address: lbmh@biol.unlp.edu.ar (M.T. Del Panno).

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component of the biota is often overlooked by regulatory agencies when establishing environmental policies.

In previous investigations of soil bioremediation we found that, even though chemical analysis suggested that bioremediation had been completed, gram-negative bacterial communities in soil were characterized by diminished metabolic versatility compared to the pristine soil. This might be related to the selective pressure exerted by the accumulation of metabolites during the degradation process. Techniques based on studies of microbial isolates suffer from the fact that a large proportion of microorganisms in soil cannot yet be cultured. Of course, a limited number of isolates cannot represent the entire microbial soil community. It is possible that metabolic capabilities are lost in the cultivated part of the microbial community but are retained in the part that was not cultivated [10]. In contrast to this, culture-independent methods such as denaturing gradient gel electrophoresis (DGGE) can detect changes even in the non-cultivated part of the investigated communities. However, it is often not possible to deduce the physiology of the detected microorganisms. A combined approach of analyzing changes in microbial communities using both kinds of techniques is therefore favourable.

The aim of this study was to determine the qualitative and quantitative changes manifested by microbial communities during bioremediation of polynuclear aromatic hydrocarbon (PAH)-contaminated soils in order to better comprehend the effectiveness of biodegradation and its impact on soil bacterial communities. We therefore analyzed changes in composition and metabolic capability of the microbial community and complemented it with functional analyses (enzymatic activities, substrate-induced respiration) and a cultivation-independent technique (DGGE).

2. Materials and methods

The study was performed using API oil–water separator sludge from a petrochemical industry. The sludge was characterized previously by gas chromatography/mass spectrometry (GC/MS) after supercritical fluid extraction [11] as well as by gas chromatography (GC) after conventional Soxhlet-extraction using *n*-hexane as a solvent. The analysis revealed that more than 44 of the 114 compounds in the sludge were PAHs [11]; 60% of the 91 g kg^{-1} hydrocarbons, which were present in the sludge, were PAHs; 9.6% were *n*-alkanes; and 28% were other compounds [11,12]. Naphthalene, methylnaphthalenes, biphenyl, acenaphthylene, acenaphthene, fluorene, phenanthrene, antharacene, fluoranthene, pyrene, benzo-[a]anthracene and chrysene, were the predominant PAHs in the sludge [11,12].

The soil selected for the study was non-contaminated soil from an area near La Plata City, Argentina. It was analyzed in the Laboratory of Soil Science at the University of La Plata and showed the following physicochemical properties: a texture containing clay loam, a pH of 5.8–5.9; 3.60% organic carbon, 6.21% soil organic matter, 0.296% total nitrogen, 0.00042% available phosphorus, and 0.05 g hydrocarbons kg^{-1}.

Soil microcosms consisted of 2 kg of soil in a glass container with a 5 kg capacity. They were prepared at three different sludge concentrations (2.5, 5, and 10 g sludge per 100 g of dry soil) following the recommendations of Pramer and Bartha [13]. Soil without sludge added was used as a control microcosm. In addition, one contaminated soil microcosm was amended with $HgCl_2$ (2% wt/wt) [14] and was used for abiotic process measurements (abiotic control).

After the sludge has been added, the soil in the microcosms was mixed thoroughly using a sterile spatula and then sieved (2 mm mesh) to reduce heterogeneity. All microcosms were prepared in duplicate and incubated at 20 ± 2 °C (regional climate conditions) in the dark for one year. The microcosms were mixed weekly for aeration and the moisture content of the soils was determined by comparing the weight of wet soil sample (10 g) to the weight of dried soil sample (dried at 105 °C to weight constancy). Humidity was corrected when necessary to a 20 ± 2% water content by adding distilled water.

2.1. Chemical analysis

Hydrocarbon concentration in soil samples was quantified as total GC-available hydrocarbons (GCAHC). Soil sample (25 g) was mixed with anhydrous sodium sulfate (25 g) and hydrocarbons were extracted for 6 h with *n*-hexane in a Soxhlet apparatus, *n*-hexadecane (Merck, Schuchardt, Germany) was added as the internal standard. During the first month of the experiment, weekly determinations were performed and later on after 60, 70, 150, and 360 days. Three samples (25 g) from each microcosm were analyzed at each sampling time.

A Perkin–Elmer autosystem gas chromatograph equipped with a flame ionization detector was used with nitrogen as the carrier gas. The injection port was maintained at 280 °C, and the detector temperature at 300 °C. The oven was set at 50 °C (initial time 4 min) and then raised to 150 °C (rate 4 K min^{-1}), and to 280 °C at a rate of 10 K min^{-1} (final time 15 min). A fused-silica capillary column PVMS/54 (50 m × 0.25 mm i.d.) was used. Data acquisition and handling was computer-assisted (PE NELSON Model 1022) [12].

The PAH composition was characterized using the following reference substances (all in the highest purity available): acenaphthylene, anthracene, benzo-[a]anthracene, biphenyl, chrysene, fluoranthene, fluorine, methylnaphthalene, naphthalene, phenanthrene,

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and pyrene (Carlo Erba, Divisione Chimica Industriale, Milano, Italy).

2.2. Plating of soil samples and analysis of isolated bacteria

Viable bacterial counts were determined weekly during the first month of the experiment, later on after every 40 days.

For this purpose, 10 g (wet weight) of soil sample was suspended in 100 ml 0.85% NaCl and homogenized for 30 min on a rotary shaker (150 rpm). Samples (0.1 ml) of 10-fold dilutions were spread on plates containing R2A-agar medium [15], which was supplemented with cycloheximide (7 mg per 100 ml) in order to count heterotrophic bacteria. The agar plates were incubated at 20 ± 2 °C for 10 days. The most probable number (MPN) of hydrocarbon-degrading bacteria was determined according to Song and Bartha [16] using mineral salts medium (LMM) [17] containing resazurin (1 mg l^{-1}). The medium was supplemented with 0.5% of *n*-hexane extract of the sludge as carbon source. The samples were incubated at 20 ± 2 °C for 21 days in 96-well microtiter plates, 4 dilutions each in eight parallels.

Two sampling points during the bioremediation process were chosen for the isolation of pure cultures: the first time when hydrocarbon-degrading bacteria were established in the soil; and the second time when the residual hydrocarbon concentration of the soil was within the legal limits for soils (Argentinean Nacional law 24051). The dilution plates prepared for counting heterotrophic bacteria were also used for the isolation of bacteria. About 30–40 colonies were picked from R2A-agar containing cycloheximide from the control and the contaminated microcosms. Replicates were made on R3A-agar until pure isolates were obtained. R3A-agar contains all the nutrients of the R2A-agar at double concentration, except the pyruvate, which is only 0.3 g l^{-1} as it is in R2A-agar [15].

Growth experiments, biochemical tests (catalase, oxidase, fermentation of glucose, Gram stain) and morphological analysis were performed with the predominant isolates from all microcosms using standard methods [18]. The gram-negative isolates that did not ferment glucose were analyzed by the bioMerieux API-20 NE identification system (Biomerieux, France). The data were used as a basis for classical biochemical determination of the isolates according to Bergey's Manual of Systematic Bacteriology [26].

The phenotypic relationships (morphologic characteristics of colonies and cells, biochemical tests and carbon utilization patterns (API-test)) between bacterial populations in different samples were described using the population similarity coefficient (S_p) [19]. Comparisons of several bacterial populations yielded a matrix of S_p coefficients [19] that was clustered according to the unweighted pair group method using arithmetic averages (UPGMA method) [20].

2.3. DNA extraction

Total DNA was extracted from 2-g soil aliquots of each microcosm after 2, 30, 60, 180, and 375 days of treatment as described by Kuske et al. [21]. The DNA pellets obtained were suspended in 100 µl of TE buffer, and the presence of DNA was analyzed by agarose gel electrophoresis and ethidium bromide staining [21]. For further molecular biological analyses, the crude DNA was purified from humic acid contaminants using Genomic-tips (20/G) from Quiagen (Quiagen Inc., Chatsworth, CA). After purification, one microliter of each sample and several DNA standards were spotted on an ethidium bromide containing agarose gel. The DNA yield was estimated by comparing the spots of the samples to the DNA standards on an UV transilluminator.

2.4. PCR amplification

The primers GC341f and 907r [22] were used to amplify a 626-bp fragment of the 16S rRNA gene. The PCRs contained 1 µl of target DNA, 1 U of AmpliTaq, the manufacturers' recommended buffer as supplied with the polymerase enzyme, 200 mM of BSA, 0.4 mM dNTPs and 20 pM of each primer at a total volume of 50 µl.

Amplification was performed on a Perkin Elmer Gene Amp System 9600, using a step-down PCR. The program included an initial denaturation step for 4 min at 95 °C, the first cycle step for 30 s at 94 °C; 45 s at 62 °C; and 1 min at 72 °C (10 cycles), followed by a step down of 30 s at 94 °C, 40 s at 57 °C; and 1 min at 72 °C (25 cycles), plus a final extension for 10 min at 72 °C.

The PCR products were analyzed with agarose gel electrophoresis and purified using a PCR Purification Kit (Quiagen Inc., Chatsworth, CA).

2.5. Denaturing gradient gel electrophoresis

DGGE (denaturing gradient gel electrophoresis) was performed on the BioRad D GENE System (BioRad, Munich Germany). The purified PCR amplicons were directly applied onto 6% (wt/vol) polyacrylamide gels, which were formed with acrylamide stock solution (acrylamide-*N*,*N*′-methylenbisacrylamide, 37.5:1). The gel contained a linear gradient of 40–70% denaturant (100% denaturant corresponds to 7 M urea and 40% (vol/vol) formamide). Electrophoresis was performed in 1 × TAE buffer (20 mM Tris–acetate (pH 7.4), 10 mM sodium acetate, 0.5 mM Na_2EDTA), at a

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temperature of 60 °C. A pre-run at 50 V for 30 min was followed by the main run at a constant voltage of 100 V for 16 h. After electrophoresis, the gel was stained for 1 h with SYBR-gold (Molecular probes, Eugene, USA). The gel was digitized using a CCD camera, stored as a tif file and analyzed using the software package GelComparII (Applied Maths, Kortrijk, Belgium). The optical densities of the lanes were determined and transformed into a densitogram. These density profiles served as a base for calculating a similarity matrix using Pearson's product moment correlation [23]. The dendrogram was calculated by the UPGMA [20].

2.6. Reamplification and sequencing of DGGE bands

DGGE bands were excised from the gel by a sterile scalpel, transferred into 50 μl PCR water (Ampuware) and incubated for 48 h at 4 °C. After centrifugation, 1 μl of the supernatant was taken as a template for reamplification of the bands using the same reaction mix as described above. The PCR protocol was adjusted for reamplification: 96 °C for 30 s, 55 °C for 45 s, and 72 °C for 1 min (25 cycles) and a final elongation at 72 °C for 10 min. The amplicons (10 μl) were loaded onto a second denaturing gel for purification and were excised again. The bands were treated as described above and served as a target for sequencing. Before sequencing the amplicons were purified using a PCR purification kit (Qiagen, Hilden, Germany) and eluated in 30 μl PCR water. Sequencing was performed on the LiCor DNA Sequencing System 4000 (MWG Biotech) using IRD modified primers (forward: 341f, reverse: 907r) and the DYEnamic Direct Cycle Sequencing Kit (Amersham Biosciences) according to manufacturer's instructions. Highest similarities of the 16S rRNA gene sequences obtained from the DGGE bands to those of other bacteria were determined using the BLASTN tool of GenBank [24]. The sequenced DGGE bands have the Accession Nos. AJ870470–AJ870485.

2.7. Dehydrogenase activity (reduction of 2,3,5-triphenyl-2H-tetrazoliumtrichloride, TTC to triphenyl formazan)

The dehydrogenase activity was analyzed as described elsewhere [25]. During the first 70 days of the experiment, weekly determinations were performed and later on after 85, 125, 155, and 330 days. Three samples (5 g) from each microcosm were analyzed at each sampling time.

2.8. Substrate-induced respiration

The SIR response to glucose and glutamic acid was analyzed in the control microcosms at the beginning and at the end of the experiment; in the microcosms containing 10% sludge this was done only at the end of the treatment. The CO_2 production was determined by the biometer flask method [27]. Soil samples (50 g of dry wt) received 1 ml of an NP solution (equivalent to a ratio of C:N:P = 15:1:0.3 wt/wt with respect to the carbon content of the added substrate). Subsequently, they were mixed and preincubated for 10 days at 20 °C [10]. Afterwards, the soil samples were placed in the biometer flask, and the carbon source (equivalent to 500 μg C per g soil) and 0.4 g talcum powder were mixed with the soil [10]. The SIR response was determined after 5 h of incubation at 20 °C [28]. Three samples (50 g wet soil) from each microcosm were analyzed at each sampling time.

3. Results

3.1. Chemical analysis

The relative concentration (percent of the initial concentration) of the total hydrocarbon content in the sludge-contaminated microcosms is shown in Fig. 1. After one year of treatment, all microcosms showed considerable hydrocarbon elimination (79–95%). One exception was the abiotic control (10% contaminated microcosm amended with $HgCl_2$), which showed an elimination of only 4%. The difference in the hydrocarbon elimination of the biotic and the abiotic microcosms indicated that the hydrocarbons were removed by microbial degradation.

When analyzing the GCAHC-elimination curves in Fig. 1 using the two-phase model of Loehr et al. [29], we obtained two phases with clearly distinct elimination rates. A first phase had a rather high elimination rate of $k = 0.074–0.085\ d^{-1}$ which lasted about 50 days; a second phase had a constant and infinitely slow elimination

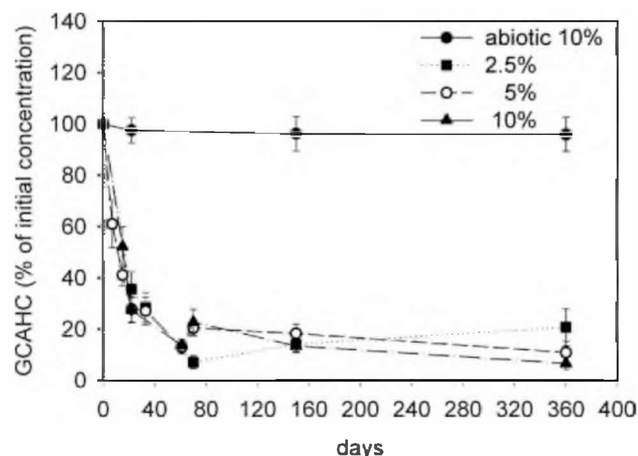

Fig. 1. Relative concentration (percentage of initial concentration) of the total hydrocarbon content (GCAHC) in the sludge-amended microcosms during bioremediation. Mean values (inclusive standard deviations) of six determinations are represented.

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rate. Both rates were independent of the hydrocarbon charge of the microcosms.

In all contaminated microcosms, naphthalene, methylnaphthalene, biphenyl, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene and benzo-[a]anthracene were completely eliminated (5 mg kg^{-1} detection limit) after a year of treatment. Acenaphthylene, pyrene and chrysene were still present in the 10% systems, although their concentrations were below the cleanup standards for soils (10 mg kg^{-1}) (Argentinean Nacional law 24051).

3.2. Cell density of soil samples and analysis of isolated cultures

The addition of different concentrations of sludge to the soils provoked an increase in the density of heterotrophic and the hydrocarbon-degrading bacteria in all microcosms (Fig. 2(a) and (b)). The preparation of soil itself (sieving, adjusting for a defined humidity, and temperature) provoked an increase in the bacterial density even in the control microcosms (unamended soil), an effect, which has also been described by Degens and Harris [30]. Immediately after the addition of sludge to the soils, the number of cultivated hydrocarbon-degrading bacteria significantly increased in all contaminated microcosms and remained higher throughout the whole experiment (Fig. 2(a)). The number of cultivated heterotrophic bacteria of the contaminated systems was also higher than in the non-contaminated soil (Fig. 2(b)). One exception was the number of cultivated heterotrophic bacteria, which were present in the microcosms containing 2.5% and 5% (wt/wt) of sludge, immediately after the sludge was added. We also observed a relative decrease ($\log N_{\text{contaminated}}$ $(\log N_{\text{control}})^{-1}$) in cultivated hydrocarbon-degrading bacteria after one week of incubation. Table 1 shows the ratio of the logarithm of the cell concentration of the contaminated microcosms to the logarithm of the cell concentration of the control microcosms during the first 30 days of incubation. The above-mentioned decrease was a pseudo-inhibiting effect, resulting from the increase in the number of hydrocarbon-degrading bacteria in the control microcosm. This increase may be due to the fact that the mixing and sieving of soil liberated substrates that formerly had not been bioavailable [30].

After 21 days of treatment, an increase in the hydrocarbon-degrading population proportional to the initially applied sludge concentration was observed in the microcosms supplied with 2.5% and 5% of sludge (Fig. 2(a)). This was not so in the microcosm with 10% sludge. In this system we observed less cultivated hydrocarbon-degrading bacteria after three weeks of incubation. This could be explained by toxicity of high sludge concentrations. But the hydrocarbon elimination rate (k) was not significantly different from that of the other microcosms

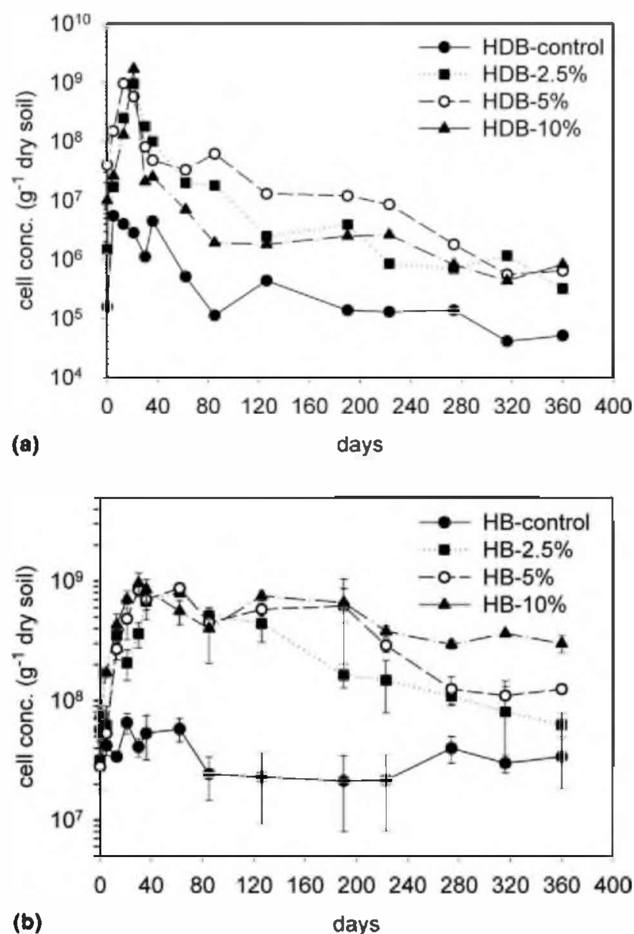

Fig. 2. Cell concentration in the sludge-amended microcosms during 360 days of treatment. (a) Cell concentration of hydrocarbon-degrading bacteria (HDB). Data are calculated on the basis of MPN, which were obtained with LMM medium supplemented with a *n*-hexane extract of API sludge as carbon source. (b) Concentration of heterotrophic bacteria (HB). Data are calculated on the basis of CFU, which were obtained on R2A-agar.

($p < 0.05$). This suggests that at high sludge concentrations a small part of the viable population was selected and could survive and increased its hydrocarbon-degrading activity.

From the isolates that were obtained after 21 days of incubation, 266 colonies were characterized phenotypically. The same was done with the isolates that were obtained after 360 days of incubation. This time, 243 bacterial isolates were characterized.

Table 2 shows the relative abundance of genera and bacterial groups, which were isolated from R2A agar plates. From these data it can be derived that adding sludge to the soils provoked a change in the predominant bacterial communities, which became very clear after 21 days of incubation and that the cultivated bacterial communities of the pristine and of the contaminated microcosms are subjected to fluctuations over time. Fig. 3 shows the dendrogram derived by UPGMA

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Table 1
Relative concentration of heterotrophic bacteria (HB) and hydrocarbon-degrading bacteria (HDB) in sludge-amended microcosms during the first 30 days of treatment

Microcosms		Incubation time (d)				
		0	5	13	21	30
2.5%	HDB	0.975	0.490	1.800	2.520	2.210
	HB	−0.249	0.170	1.015	0.500	0.947
5%	HDB	2.400	1.440	2.380	2.310	1.860
	HB	−0.306	0.104	0.900	0.869	1.313
10%	HDB	1.800	0.675	1.510	2.780	1.280
	HB	0.130	0.609	1.102	1.032	1.369

Data are expressed as $\log N$ of contaminated soil per $\log N$ of the control soil.

Table 2
Relative abundance of bacterial genera and groups that form part of the heterotrophic bacteria (HB) in the microcosms

Microcosms	Incubation time							
	Day 21				Day 360			
	0%	2.5%	5.0%	10%	0%	2.5%	5.0%	10%
Acinetobacter	–	–	–	–	–	–	–	4
Agrobacterium	15	3	2	–	5	–	–	2
Alcaligenes	–	7.5	5	7	8	4	4.3	4
Brevundimonas	2	4.5	2	–	–	6	6.4	2
Burkholderia	–	–	–	7.5	–	–	–	–
Chryseomonas	8	9	19	5	6	–	–	–
Comamonas	1.5	3	3	–	–	13	8	2
Moraxella	3	1.5	–	2.5	–	–	4	9
Pasteurella	–	–	–	–	–	–	–	4
Pseudomonas	7	10.5	14	37	11	11	10	6
Sphingobacterium	–	–	–	–	–	–	–	4
Sphingomonas	2	–	2	2.5	–	–	2	–
Stenotrophomonas	–	–	–	2.5	7	9	–	2.5
Weeksella	4.5	–	–	–	–	–	4.3	–
Bacilli G(+) only rods	21	22	21	12	13	20	8	16
Bacilli G(+) pleomorph	10.4	10	9	7.2	4	8	2	–
Bacilli G(+) spore forming	6	–	–	–	12	13	35	34
Bacilli G(+) filamentous	3.2	16	16	4.8	13	6	6	–
Cocci G(+)	8.2	13	7	12	4	6	6	5
Streptomyces	8.2	–	–	–	17	4	4	5

The data are given as percentage of the total cell number isolated from each system.

of S_p coefficients from the microcosms after 21 and 360 days of treatment. After 21 days the isolated species obtained from contaminated soils formed a cluster, which was clearly separated from the cluster by the control soil. The contaminated microcosms shared very similar populations, which differed from the control soil. The formation of a separate cluster indicates that the hydrocarbon-degrading population was established in the contaminated microcosms after 21 days of incubation. This is also reflected in the data of Table 2 that show a relative increase in the abundance of the gram-negative community (principally in favour of the genus *Pseudomonas*), and a significant reduction in the number of gram-positive genera (especially *Bacillus* and *Streptomyces*). After one year of incubation, two clearly separated clusters were formed: (i) the bacterial population of the microcosms that contained 5% or 10% of sludge; (ii) the bacterial populations of the control microcosm and the microcosm that contained 2.5% of sludge. The latter cluster also formed part of an additional bigger cluster, which included all of the microcosms (after 21 days of treatment), irrespective of whether they were contaminated or not. This can be attributed primarily to the number of *Bacillaceae* in the soil community.

3.3. Community structure

Fig. 4(a) shows the DGGE patterns of different contaminated microcosms at different stages of the bioremediation (lanes 2–20) and of the API sludge itself (lane 1) (see also the table below). The patterns indicate the changes in microbial community structures over time.

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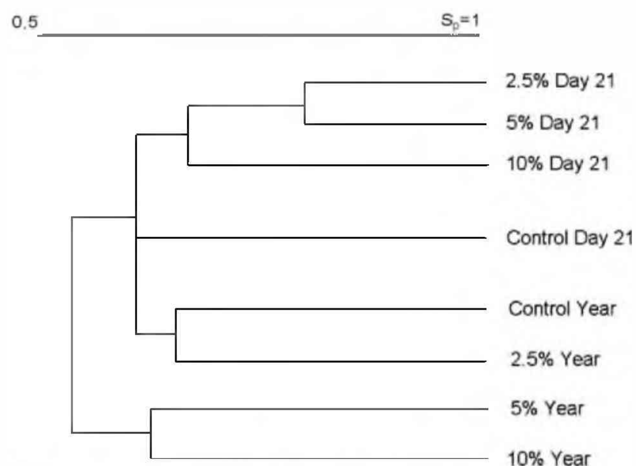

Fig. 3. UPGMA cluster analysis of the population similarity (S_r) based on the phenotypic characteristics of the R2A-cultivable bacteria of the microcosms after 21 and 360 days of treatment.

Successions in community composition are shown by bands that appear or disappear during the experiment. Sequence analysis of DGGE bands provides more detailed information on these pattern variations. The similarities between representatively sequenced bands and their nearest relatives indicate that the phylogenetic affiliation of the bacteria that were affected by the contamination.

Main DNA bands of the API sludge are not represented in the DGGE gels of the contaminated soils. Comparing the sequence of these bands (bands 1.1–1.4) to bacterial sequences in the NCBI data bank revealed high similarities to bacteria that were isolated from highly contaminated environments or mineral oil compounds. The origin of the species represented by further DNA bands in the DGGE gel cannot clearly be assigned to the soil or to the API sludge. Species, which had a very low concentration in the API sludge and which therefore were below the detection limit of our method, may have proliferated when they got into the soil and may thus have reached a concentration that is within the detection limit of the method.

To attain a more objective view of the variations of the DGGE patterns, the gel was digitized, and band positions as well as band intensities were determined. The resulting densitometric curves served as a data set to calculate a similarity matrix and a dendrogram for comparing the DGGE patterns. The dendrogram of the cluster analysis is represented in Fig. 4(b). It shows two main clusters. The first cluster includes the DGGE profiles of freshly contaminated soils (day 2), the 2.5% and 5.0% contaminated microcosms (days 30 and 60), and microcosms containing 10% sludge at all times. The profiles included in this cluster describe the microbial community structure of the microcosms during the period with a high elimination rate.

The second cluster includes the DGGE profile of the control microcosm and the 2.5% and 5% contaminated microcosms between 60 and 360 days of treatment. During this period the hydrocarbon elimination rate was slow (Fig. 1), and the abundance of cultivated hydrocarbon-degrading bacteria diminished considerably (Fig. 2(a)).

Considering the effect that the addition of sludge and the incubation had on the bacterial communities during the bioremediation process, we might refer to the first cluster as High impact (Hi) and to the second one as Low impact (Li).

Analyzing the Hi cluster, we see that the sludge produces an immediate change in the bacterial community, as the relative cell numbers show (Table 1). During the early stage of the treatment, significant changes were detected in the structure of the communities. This led to the formation of two different sub-clusters, one corresponding to the microcosms with 2.5% and 5% sludge in the early stage of treatment, and one corresponding to the microcosm with 10% sludge during the entire investigation period (Fig. 4(b)).

In the Li cluster, no significant changes in the unamended soil bacterial community were observed during the year of treatment. The samples from the control soil formed one distinct sub-cluster. Another sub-cluster included the profiles of the soils containing 2.5% and 5% sludge in the late stage of treatment (Fig. 4(b)). After treatment, the community structure of all contaminated microcosms differed from the structure observed in the unamended control microcosm. But the fact that the low-contaminated microcosms (2.5% and 5% sludge) and the unamended microcosms formed the Li cluster at the end of the treatment, showed that the original soil communities had recovered considerably.

3.4. Enzymatic activities

3.4.1. Dehydrogenase activity

In all microcosms, the dehydrogenase activity was determined by analyzing the reduction of TTC to triphenyl formazan (TPF). The influence of the added sludge on the enzyme activity was determined by subtracting the TPF formation obtained in the control microcosms from TPF formation obtained in the contaminated microcosms. In all of the systems, regardless of whether sludge had been added or not, the dehydrogenase activity increased during incubation (data not shown); the preparation and incubation of the soil thus enhanced the dehydrogenase activity. However, adding sludge produced an additional initially stimulatory effect, which decreased within the first 50 h of incubation to the level of the control microcosm (Fig. 5). One exception was the microcosm with 10% sludge, which showed a second increase in dehydrogenase activity. The data indicate that the dehydrogenase activity in soil is nearly

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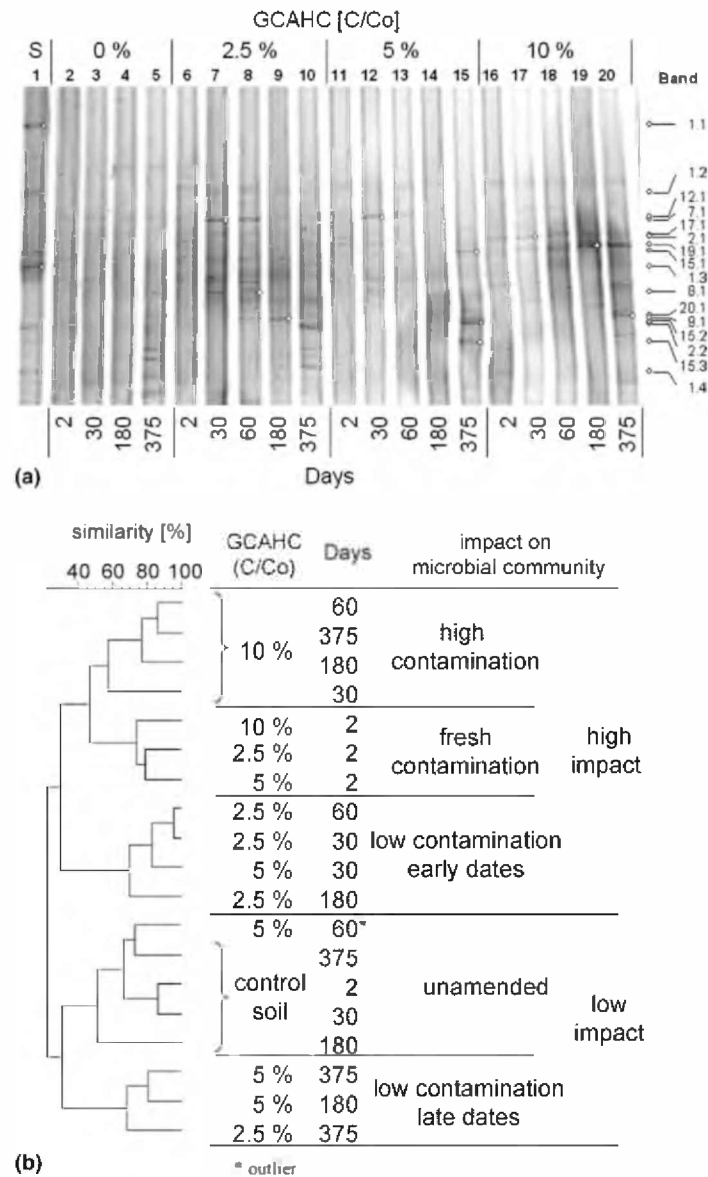

Fig. 4. (a) PCR-DGGE patterns of the different contaminated microcosms (2.5%, 5% and 10% sludge) and of the unamended control microcosm (0%) during the observation period (2–375 days) and the microbial community profile of the API sludge (S). Total DNA was amplified by universal 16S rRNA gene primers (positions 357–907) and loaded onto a DGGE gel containing 40–70% of denaturant. (b) Dendrogram of the cluster analysis of the DGGE patterns as calculated by using the Pearson correlation and UPGMA. The DGGE profile of the soil with 5% API sludge after 60 days of treatment is marked as an outlier (*).

Band	Similarity (%)	Affiliation	Firstly isolated from
1.1	89	*Bacteroides*	Uranium mining waste piles
1.2	94	*Deferribacterales*	Petroleum
12.1	99	*Sphingomonas* sp.	Olive-mill-wastewater
7.1	98	*Sphingomonas* sp.	Olive-mill-wastewater
17.1	93	*Xanthomonas* sp.	Hydrocarbon-contaminated soils
2.1	90	*Bradyrhizobium* sp.	Australian soil sample
19.1	90	*Halochromatium* sp.	Microbial mats
15.1	90	*Halochromatium* sp	Microbial mats
1.3	95	*Sinorhizobium* sp.	Soil
8.1	95	*Rhodanobacter* sp.	Carbofuran-degrading soil bacteria
20.1	91	*Actinobacteridae*	Subtropical Australian soil
9.1	95	*Acidobacterium* sp.	Heavy metal contaminated environment
2.2	90	α-Poteobacterium	Heavy metal contaminated environments
15.2	93	*Actinomycetes*	Subtropical Australian soil
15.3	92	α-Poteobacterium	Unknown
1.4	95	*Spirochaetes*	Trichloroethene-dechlorinating culture

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unaffected by "small" concentrations (<10%) of API sludge.

3.4.2. Substrate-induced respiration

Fig. 6 shows the SIR values obtained at the beginning of the experiment (control microcosm) and after one year of soil treatment (control microcosm and microcosm contaminated with 10% sludge). The respiration rates of both the control microcosm and the contaminated microcosm containing 10% sludge were higher after one year of incubation than that of the control microcosm at the beginning of the experiment. This indicates that the incubation conditions enhanced the respiratory activity of the microbial community. However,

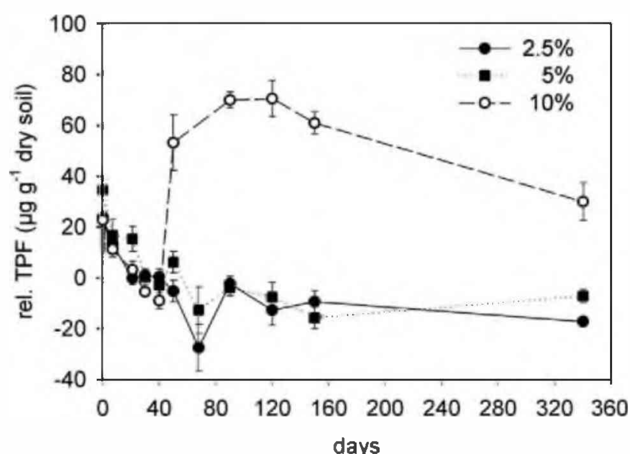

Fig. 5. Changes in net dehydrogenase activity of contaminated microcosms during the bioremediation process. The data are calculated as follows: µg of TPF per g of dry contaminated soil minus µg of TPF per g of dry control soil.

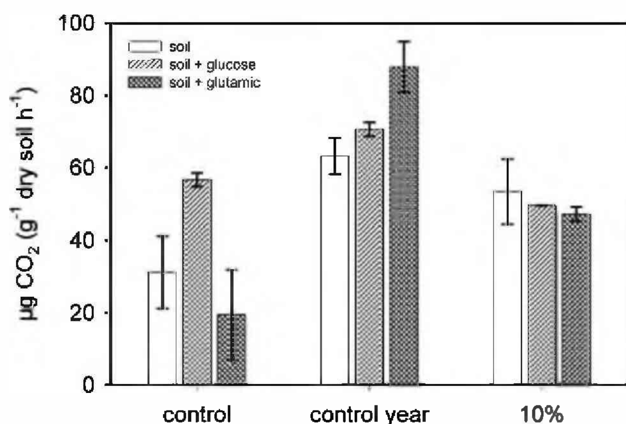

Fig. 6. Substrate-induced respiration (SIR values) measured as specific CO_2– production rate (µg CO_2 per g of dry soil and per h) 5 h after carbon source addition. Control: control microcosm (unamended soil) at the beginning of the experiment. Control year: control microcosm (unamended soil) after one year of incubation. 10%: microcosm (amended with 10%, wt/wt) of API sludge after one year of incubation. Mean values (inclusive standard deviations) of three determinations are represented.

the addition of different carbon sources provoked differences in the responses of the microcosms. The addition of glucose at the beginning of the experiment to the control microcosm increased its respiration rate considerably, whereas the increase in the respiration rate after 375 days of incubation was minor. The addition of gluconic acid only increased the respiration rate of the control microcosm after one year of incubation. The microcosm with 10% sludge showed no response in terms of respiration rate upon addition of carbohydrates. The activity of the microbial community was not stimulated by added carbohydrates. This may indicate that the community was not able to metabolize glucose or gluconic acid, or it may indicate that the community showed its maximal activity irrespective of whether hydrocarbons or carbohydrates were consumed as a carbon and energy source.

4. Discussion

Knowledge about the changes in composition and activity of microbial communities in contaminated soils is of particular interest in respect to the implementation of bioremediation in the field. In recent years, authors have investigated the response of bacterial soil communities, especially after the addition of pollutants such as crude oil, agricultural chemicals, and pure hydrocarbons [4–6,31]. However, the response of the bacterial community to soil pollution due to activities of refineries and petrochemical industry is not understood well enough to predict its long-term effect on the structure and activity of soil communities.

We examined the composition and the structure and the enzymatic activity of bacterial communities in API sludge-contaminated soil microcosms throughout one year. We profiled the dynamic of this community and compared it to the hydrocarbon elimination curves. In the API sludge-contaminated microcosms, we observed an 80% elimination of GCAHC within 45 days, followed by an extremely slow reduction of the hydrocarbon concentration, independent of the initially added hydrocarbon load. This is similar to the degradation rate for PAH under favourable conditions in soils or sediments, as described by other authors as well. MacNaughton et al. [5], for example, observed that in oil-contaminated blocks of beach 70% of the applied PAHs were degraded within a period of 42 days. In the study of MacNaughton et al. [5], as well as in our study, degradation could be attributed to a biological activity and was not due to abiotic losses.

The density and composition of the bacterial community in the soil changed after the microcosms were prepared. Immediately after addition of the API sludge, the community began to diverge from that of the uncontaminated soil. This fact could be derived from the

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increase in the number of bacteria cultivated on R2A-agar (Fig. 2(b)), and the ratio of hydrocarbon-degrading bacteria to heterotrophic bacteria cultivated on R2A-agar (Table 1). The community's development over time could be attributed to two distinct phases: first, a phase which lasted about 40–60 days, correlating with the phase in which the hydrocarbon degradation rate was high; and one phase after 60 days, which correlated with the phase of extremely low hydrocarbon degradation (Figs. 1 and 2). After the initial increase, the bacterial community in the microcosms developed differently, depending on the amount of API sludge added (Fig. 2). After one year, microcosms that had been contaminated with hydrocarbon concentrations of 2.5% or lower reached viable cell counts that were in the same range as was shown for the untreated microcosms. Microcosms contaminated with higher concentrations reached cell concentrations that were significantly higher than that of the control soil. A higher sludge concentration (10%) revealed an inhibitory effect on the hydrocarbon-degrading community fraction (Table 1). This difference in response was also observed in the structure of the bacterial community (Figs. 3 and 4).

The structure of the bacterial community diverged quickly from that of the control microcosm after the API sludge was added to the microcosms. Two days after starting the experiment, all contaminated microcosms had formed one cluster of 16S rRNA gene band patterns. This cluster was clearly distinct from that of the control microcosm (Fig. 4(b)). Further divergence in the community structures varied, depending on the different concentrations of API sludge that had been applied to the microcosms. The microcosm with 10% sludge suffered a long-term disturbance of the community structure, which still continued even after one year of incubation. The main band within this DGGE pattern (band 19.1, Fig. 4(a)) can be affiliated to the genus of *Chromaciaceae* (*Thioalkalivibrio* sp., 90% sequence similarity). Members of this genus are involved in the sulfur cycle [32]. This is in good accordance with the fact that sludge contains sulfur at varying concentrations (unpublished data). The DNA band clusters of the microcosms containing a smaller hydrocarbon load revealed that the community was only able to achieve equilibrium after 180 days, finally regaining the structure that was relatively stable and diverse, with limited similarity to that of the pristine soil. The patterns of the contaminated microcosms did not reflect those patterns of the original API sludge. One of the main bands (7.1 and 12.1, Fig. 4(a)) that was detectable within the DGGE patterns of microcosms contaminated with 2.5% and 5% sludge at early stages of bioremediation was clearly affiliated to a *Sphingomonas* species (98–99% sequence similarity). The corresponding strain was first isolated from oil-mill wastewater [33].

However, we have to note that the number and intensity of the bands make it difficult to deduce the number and abundance of species within the community because of possible unknown PCR bias [34,35]. Furthermore, studies have revealed that only bacterial populations, which represent 1–10% of the species, can be detected [36]. For this reason, especially in complex microbial communities as typically found in soil samples, DGGE analysis can only serve as an ''observation window'' to microbial community structures. The general overview can sometimes be distorted because of misinterpretation of band patterns. In our results, the soil sample that was contaminated with 5% of petrochemical sludge (after 60 days of treatment) joined the cluster of unamended microcosm.

The changes in soil community, which were revealed by the community density and structure, were also reflected in the community activity, analyzed as enzyme activity (dehydrogenase) and as substrate-induced respiration rate. The addition of hydrocarbons at concentrations smaller than 10% allowed the microcosm community to recover its previous metabolic function (dehydrogenase) within one year. Addition of hydrocarbons at concentrations of 10% resulted in a soil community in which the dehydrogenase activity was enhanced, compared to the control microcosm. The increase in dehydrogenase activity may be explained as an enzyme induction process, since metabolic pathways of hydrocarbon degradation (such as alkane and PAH) involve dehydrongenases [37]. Thus, hydrocarbon-degrading bacteria may have a selective advantage over other bacteria in soil. Independently of the API-sludge concentration in the microcosms, the dehydrogenase activity was affected during the entire one-year treatment. This effect may be a true toxic effect. Morelli et al. [38] reported a genotoxic transformation of hydrocarbons resulting from API sludge in soil microcosms that were kept under conditions similar to those in our experiments. The contaminated microcosms maintained a residual mutagenic activity even after soil treatment for one year. We regarded the changes of API sludge-contaminated soil microcosms in hydrocarbon concentration, community structure of the cultivated part of the population, community structure in soil microcosms and in the enzymatic activity of the soil community. After one year of treatment, the chemical analysis suggested that the hydrocarbon elimination process was over. The hydrocarbon concentrations were within the legal limits in Argentina. However, the biological analysis showed that the soil never returned to its original state, even though soils, which had received a comparably low sludge load (⩽5%), came very close to the originally pristine state. This divergence in the bacterial community is also reflected in the dehydrogenase activity and SIR response, which in the case of the highly contaminated microcosms (10% sludge), continued to differ considerably

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from the control microcosm. This indicates that the high API-sludge contaminations cause an intense selection process in soil, resulting in a community of diverse metabolic activity.

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References

[1] Barbee, G.C., Brown, K.W. and Donnelly, K.C. (1992) Fate of mutagenic chemicals in soil amended with petroleum and wood preserving sludges. Waste Manag. Res. 10, 73–85.
[2] Mishra, S., Jyot, J., Kuhad, R.C. and Lal, B. (2001) Evaluation of inoculum addition to stimulate in situ bioremediation of oily-sludge-contaminated soil. Appl. Environ. Microbiol. 67, 1675–1681.
[3] Ritter, W.F. and Scarborough, R.W. (1995) A review of bioremediation of contaminated soils and groundwater. J. Environ. Sci. Health Part A Tox. Hazard. Subst. Environ. Eng. A 30, 333–357.
[4] Engelen, B., Meinken, K., von Wintzingerode, F. Heuer, H., Malkomes, H. and Backhaus, H. (1998) Monitoring impact of a pesticide treatment on bacterial soil communities by metabolic and genetic fingerprinting in addition to conventional testing procedures. Appl. Environ. Microbiol. 64, 2814–2821.
[5] MacNaughton, S.J., Stephen, J.R., Venosa, A.D., Davis, G.A., Chang, Y. and White, D.C. (1999) Microbial population changes during bioremediation of an experimental oil spill. Appl. Environ. Microbiol. 65, 3566–3574.
[6] Juck, D., Driscoll, B.T., Charles, T.C. and Greer, C.W. (2003) Effect of experimental contamination with the explosive hexahydro-1,3,5-triazine on soil bacterial communities. FEMS Microbiol. Ecol. 43, 255–262.
[7] Iwamoto, T. and Nasu, M. (2001) Current bioremediation practice and perspective. J. Biosci. Bioeng. 92, 1–8.
[8] Watanabe, K. and Baker, P.W. (2000) Environmentally relevant microorganisms. J. Biosci. Bioeng. 89, 1–11.
[9] Barkay, T., Shearer, D.F. and Olson, B.H. (1986) Toxicity testing in soil using microorganisms (Dutka, B.J. and Bitton, G., Eds.), Toxicity Testing Using Microorganisms, vol. 2, pp. 133–155. CRC Press, Boca Raton, FL.
[10] Witter, E., Gong, P., Baåth. E. and Marstorp, H. (2000) A study of the structure and metal tolerance of the soil microbial community six years after cessation of sewage sludge applications. Environ. Toxicol. Chem. 19, 1983–1991.
[11] Remmler, M., Kopinke, F.D. and Stottmeister, U. (1995) Thermoanalytical methods for characterizing hydrocarbon–sludge–soil mixtures. Thermochim. Acta 263, 101–112.
[12] Vecchioli, G.I., Costanza, O.R., Giorgieri, S.A. and Remmler, M. (1997) Extent of cleaning achievable by bioremediation of soil contaminated with petrochemical sludges. J. Chem. Technol. Biotechnol. 70, 331–336.
[13] Pramer, D. and Bartha, R. (1972) Preparation and processing of soil samples for biodegradation studies. Environ. Lett. 2, 217–224.
[14] Song, H.G., Wang, X. and Bartha, R. (1990) Bioremediation potential of terrestrial fuel spills. Appl. Environ. Microbiol. 56, 652–656.
[15] Reasoner, D.J. and Geldreich, E.E. (1985) A new medium for the enumeration and subculture of bacteria from potable water. Appl. Environ. Microbiol. 49, 1–7.
[16] Song, H.G. and Bartha, R. (1990) Effects of jet fuel spills on the microbial community of soil. Appl. Environ. Microbiol. 56, 646–651.
[17] Vecchioli, G.I., Del Panno, M.T. and Painceira, M.T. (1990) Use of selected autochthonous soil bacteria to enhance degradation of hydrocarbons in soil. Environ. Pollut. 67, 249–258.
[18] Gerhard, P.M.R., Costilow, R., Nester, E., Krieg, N. and Phillips, G. (1981) Manual of Methods for General Bacteriology. American Society of Microbiology, Washington, DC, p. 524.
[19] Kuehn, I., Allestam, G., Stenstroem, T.A. and Moellby, R. (1991) Biochemical fingerprinting of water coliform bacteria, a new method for measuring phenotypic diversity and for comparing different bacterial populations. Appl. Environ. Microbiol. 57, 3171–3177.
[20] Sokal, R.R. and Michener, C.D. (1958) A statistical method for evaluating systematic relationships. Univ. Kansas Sci. Bull. 38, 1409–1438.
[21] Kuske, C.R., Barns, S.M. and Busch, J.D. (1997) Diverse uncultivated bacterial groups from soils of the arid southwestern United States that are present in many geographic regions. Appl. Environ. Microbiol. 63, 3614–3621.
[22] Muyzer, G., Brinkhoff, T., Nübel, U., Santegoedes, C., Schäfer, H. and Wawer, C. (1998) Denaturing gradient gel electrophoresis (DGGE) in microbial ecology In: Molecular Microbial Ecology Manual (Akkermanns, A.D.L., van Elsas, J.D. and de Bruijn, F., Eds.), pp. 1–27. Kluwer Academic Press, Dordrecht.
[23] Pearson, K. (1926) On the coefficient of racial likeliness. Biometrika 18, 105–117.
[24] Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.
[25] Thalmann, A. (1968) Zur Methodik der Bestimmung der Dehydrogenaseaktivität im Boden mittels Triphenyletetrazoliumchlorid (TTC). Landwirtsch. Forsch. 21, 249–258.
[26] Garrity, G.M., Ed., (2005). Bergey's Manual of Systematic Bacteriology. Springer, New York.
[27] Bartha, R. and Pramer, D. (1965) Features of a flask and method for measuring the persistence and biological effects of pesticide in soil. Soil Sci. 100, 68–70.
[28] Nordgren, A., Bååth, E. and Söderström, B. (1988) Evaluation of soil respiration characteristics to assess heavy metal effects on soil microorganisms using glutamic acid as a substrate. Soil Biol. Biochem. 20, 949–954.
[29] Loehr, R.C., Martin, J.H.J. and Neuhauser, E.F. (1992) Land treatment of an aged oily sludge – organic loss and change in soil characteristics. Wat. Res. 26, 805–815.
[30] Degens, B.P. and Harris, J.A. (1997) Development of a physiological approach to measuring the catabolic diversity of soil microbial communities. Soil Biol. Biochem. 29, 1309–1320.
[31] Zucchi, M., Angiolini, L., Borin, S., Brusetti, L., Dietrich, N., Gigliotti, C., Barbieri, P., Sorlini, C. and Daffonchio, D. (2003) Response of bacterial community during bioremediation of an oil-polluted soil. J. Appl. Microbiol. 94, 248–257.
[32] Sorokin, D.Y., Tourova, T.P., Lysenko, A.M. and Kuenen, J.G. (2001) Microbial thiocyanate utilization under highly alkaline conditions. Appl. Environ. Microbiol. 67 (2), 528–538.
[33] Di Gioia, D., Barberio, C., Spagnesi, S., Marchetti, L. and Fava, F. (2002) Characterization of four olive-mill-wastewater indigenous bacterial strains capable of aerobically degrading hydroxylated and methoxylated monocyclic aromatic compounds. Arch. Microbiol. 178 (3), 208–217.

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[34] Suzuki, M.T. and Giovannoni, S.J. (1996) Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl. Environ. Microbiol. 62, 625–630.
[35] von Wintzingerode, F., Gobel, U.B. and Stackebrandt, E. (1997) Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol. Rev. 21, 213–229.
[36] Murray, A.E., Hollibaugh, J.T. and Orrego, C. (1996) Phylogenetic compositions of bacterioplankton from two California estuaries compared by denaturing gradient gel electrophoresis of 16S rDNA fragments. Appl. Environ. Microbiol. 62, 2676–2680.
[37] Atlas, R.M. (1984) Petroleum Microbiology. Macmillan Publishing Company, New York, p. 692.
[38] Morelli, I.S., Vecchioli, G.I., Del Panno, M.T. and Painceira, M.T. (2001) Effect of petrochemical sludge concentrations on changes in mutagenic activity during soil bioremediation process. Environ. Toxicol. Chem. 20, 2179–2183.

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