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FEMS Microbiology Ecology 48 (2004) 387–399

FEMS MICROBIOLOGY Ecology

www.fems-microbiology.org

Microbial community response to petroleum hydrocarbon contamination in the unsaturated zone at the experimental field site Værløse, Denmark

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Received 14 October 2003; received in revised form 16 February 2004; accepted 25 February 2004

First published online 20 March 2004

Abstract

This study investigates the influence of petroleum hydrocarbons on a microbial community in the vadose zone under field conditions. An artificial hydrocarbon mixture consisting of volatile and semi-volatile compounds similar to jet-fuel was emplaced in a previously uncontaminated vadose zone in nutrient-poor glacial melt water sand. The experiment included monitoring of microbial parameters and CO_2 concentrations in soil gas over 3 months in and outside the hydrocarbon vapor plume that formed around the buried petroleum. Microbial and chemical analyses of soil and vadose zone samples were performed on samples from cores drilled to 3.3 m depth on three dates and three lateral distances from the buried petroleum mass. Significantly elevated CO_2 concentrations were observed after contamination. Total cell numbers as determined by fluorescence microscopy were strongly correlated with soil organic carbon and nitrogen content but varied little with contamination. Redundancy analysis (RDA) allowed direct analysis of effects of selected environmental variables or the artificial contamination on microbiological parameters. Variation in biomass and CO_2 production was explained by soil parameters, to 46%, and by the duration of contamination, to 39.8%. The microbial community structure was assessed by community level physiological profiles (CLPP) analysis using Biolog™ Eco-Plates. In the CLPP data only 35.9% of the variation could be linked to soil parameters and contamination, however, the samples with greatest exposure to hydrocarbons grouped together on RDA plots. It is concluded that, at this nutrient-poor site, the microbial community was dominated by natural heterogeneity and that the influence of petroleum hydrocarbon vapors was weak.

Keywords: Unsaturated zone; Biolog method; Community level substrate utilization; Limiting factors; Redundancy analysis

1. Introduction

Contamination of soils and the underlying subsurface by petroleum spills is a widespread environmental problem. In situ bioremediation [1] for a cost-effective environmentally friendly cleanup and monitored natural attenuation [2] for risk management are two frequently applied strategies to cope with petroleum spills. Both rely on the potential of the autochthonous microbial communities to biodegrade petroleum hydrocarbons, which is supposed to be ubiquitous in oxic environments [3]. The addition of hydrocarbon-degrading microbes is usually not more effective for hydrocarbon removal than stimulating the growth of the indigenous microorganisms, and indeed, inoculation is usually not practiced. A

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doi:10.1016/j.femsec.2004.02.011

central question at polluted sites undergoing enhanced in situ bioremediation or natural attenuation is therefore the impact of petroleum contamination on the activity and on the structure of the indigenous microbial communities in the subsurface. Previous work, e.g. [4–7], focused on the bioremediation of petroleum-contaminated topsoils, covering microbiological, chemical and engineering aspects. The effects of petroleum pollution on terrestrial microbial communities have been studied at different experimental scales: in enrichment cultures [8], microcosm experiments [9,10], soil columns [11], lysimeters [12,13] and field plots or transects [5,14,15]. Various methods such as community level physiological profiles (CLPP) [4,16,17], phospholipid fatty acid (PLFA) profiling [5,10] and nucleic acid-based methods [15,18–20] have been used to study the composition of the microbial communities. Widmer and coworkers [21] applied three different methods (CLPP using Biolog GN plates, PLFA and restriction fragment length polymorphism (RFLP) analysis) to evaluate the community structure in three different non-polluted agricultural soils. All three methods allowed distinguishing the soils based on fingerprints. After contamination of pristine soil with petroleum, an increase in microbial activity, but a decrease in diversity, is generally observed (see [8–15]). However, most studies with artificially polluted soils were performed in the laboratory, and significant effects on the activity and composition of the microbial communities in controls without contamination were also observed, as a result of the incubation process [10,22]. Thus, the transferability of long-term laboratory studies to the field situation is often questionable. It can be concluded that the best way to exclude incubation bias is to work in situ.

The unsaturated or vadose zone is a fairly unknown compartment of the soil ecosystem [23]. Before the1970s a general agreement on a sterile or sparsely populated subsurface prevailed [24]. Since then, studies characterizing the microbial communities of deeper vadose zones between topsoil and groundwater were primarily focused on the influence of the water potential on the abundance and activity of microbial organisms (see [23] for review). Consistently with lower carbon and nutrient fluxes, as compared to aquifers and topsoil layers, microbial activity and diversity is lower in the intermediate unsaturated zone. At a pristine site in Oklahoma with an unsaturated zone of 3 m thickness [25], biomass and substrate-induced activity of bacteria and protozoa were constant in summer and winter, but varied sharply with depth, with a minimum in the deep unsaturated zone above the capillary fringe. In a pristine unsaturated zone of 26 m thickness in the US Midwest, the lowest biomass and smaller biodegradation rates of glucose and phenol were found in unsaturated tills as compared with both topsoil and the deeper saturated zone [26]. Only a few field studies [14,27] report on microbial populations in vadose zones at petroleum spill sites. In most studies the petroleum is floating on the groundwater and investigations were limited to the deep vadose and the saturated zone. The significance of the vadose zone for petroleum biodegradation remains unclear and better characterization is needed at contaminated sites. Pasteris et al. [28] performed a lysimeter experiment simulating an unsaturated zone above a groundwater table using pristine alluvial sand as a porous medium. The CO_2 concentration in the soil increased the day after addition of petroleum hydrocarbons and was linked to aerobic biodegradation of hydrocarbons and growth of microbial biomass. Hence, the microbial community in the sand adapted within a short time to petroleum and biodegradation started rapidly. However, the unsaturated alluvial sand had been disturbed physically during filling of the lysimeter, potentially activating the microbial community before contamination.

The main aim of this study was to monitor the activity and structure of an indigenous microbial community in a 3.3 m deep natural sandy unsaturated soil before and after artificial contamination with petroleum hydrocarbons. Biolog^{TM} EcoPlates results were used together with redundancy analysis (RDA) in order to test the relationship between functional diversity (using the term as defined in [29]) and site-specific variables. Detailed results on hydrocarbon migration were measured by P. Kjeldsen et al. (Technical University of Denmark, unpublished data).

2. Materials and methods

2.1. Experimental site and contamination

The field experiment was conducted from July 2001 to July 2002 at the site of the European project GRACOS at Airbase Værløse in Denmark, which is a location without prior contamination by petroleum products. At this site, a sandy dark brown topsoil of 0.3–0.5 m thickness is overlaying 2.0–3.3 m of homogeneous glacial melt-water sand, which in turn overlays 0.5–1.0 m of moraine sand/gravel, having visible white lime. An unconfined aquifer was found in July 2001 at a depth of 3.3 m. The water table was shown to rise between 1.2 and 1.6 m during wet winters. The site was covered with grass and had not been fertilized over the course of at least 50 years.

An artificial petroleum hydrocarbon mixture of 13 typical kerosene hydrocarbons (Table 1) was composed from pure products, to which the chlorofluorocarbon 1,1,2-trichloro-1,2,2-trifluoroethane (CFC-113) was added as a conservative volatile tracer. To form a source of hydrocarbons, 10.2 liters of this mixture was mixed with about 200 liters of sand from the site and buried at a depth of 0.8 to 1.3 m below the surface in a round hole

Table 1
Composition of the oil phase after site installation

Compound	Amount (wt%)
Benzene	1.02
Toluene	2.93
m-Xylene	4.57
1,2,4-Trimethylbenzene	10.99
n-Hexane	7.26
n-Octane	7.16
n-Decane	15.99
n-Dodecane	9.50
3-Methylpentane	7.45
Isooctane	15.36
Cyclopentane	1.59
Methylcyclopentane	5.79
Methylcyclohexane	10.23
CFC-113	0.16

of 0.70 m diameter. After filling the rest of the hole with the original soil material, a lid of 1.24 m diameter was installed 0.3 m above ground in order to prevent direct rainwater infiltration into the buried source. The migration of hydrocarbon vapors and the composition of the soil gas were monitored with high spatial and temporal resolution for the subsequent 12 months after contamination. Stainless steel capillaries (1/16 in.) were installed in the experimental area before kerosene source installation (Fig. 1). The compounds in the pore-gas were sampled on sorbent tubes packed with Tenax® and analyzed by automated thermal desorption to a gas chromatograph with flame ionization and electron capture detection (ATD-GC-FID and -ECD). Soil gas and groundwater were sampled along a main sampling axis within 20 m from the source center. The groundwater flow direction, determined before the experiment, defined the main sampling axis (Fig. 1). Hydrocarbon data measured along this main axis and along three secondary axes (90°, 180° and 270° to the main axis) showed that vapor migration was generally isotropic. The soil temperature was measured with fixed installed thermometers to a depth of 2.5 m.

At sampling distances of 0.7 (samples A, D and G), 2 (B, E and H) and 10 m (C, F and I) from the source center (for the spatial arrangement of coring sites see Fig. 1), three soil cores were sampled on three different days. Soil cores A–C were taken on the 24th day before contamination, soil cores D–F and cores G–I on the 23rd and 80th day after contamination of the site, respectively.

The soil cores D, E, G and H were taken within the vapor plume. Microbial populations in these cores had been more or less steadily exposed to hydrocarbon vapors for about 20 days (cores D + E) and 77 days (cores G + H). At 10 m from the source, most hydrocarbons were at all times below the detection limits, except *n*-hexane, isooctane, cyclopentane, methylcyclopentane, methylcyclohexane and 3-methylpentane which were detected in traces between day 13 and 17. Therefore, the microbial populations in samples from the soil cores F and I can be viewed as uninfluenced by petroleum hydrocarbons, in addition to those of soil cores A–C taken prior to contamination. A maximum petroleum vapor mass in the soil pore air of 450 g was reached after 17 days and then decreased to 2 g on day 350. The vapor concentrations of individual components generally decreased three orders of magnitude within 2–8 m lateral distance from the buried source. Only after 113 days did the most volatile compounds (cyclopentane, 3-methylpentane and methylcyclopentane) disappear completely from the vadose zone at 2 m distance from the source center and some compounds persisted at the site for more than one year.

2.2. Soil sampling

In order to avoid any disturbance of the soil by the installed gas or water sampling devices, soil samples were taken only at locations outside the buried petroleum and along an axis at an angle of 120–140° to the main sampling axis. Soil cores were taken using a stainless steel hollow-stem soil corer of 2.8 cm diameter

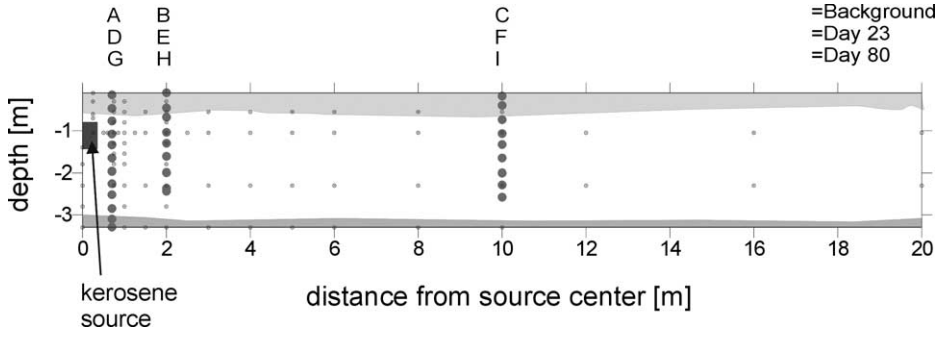

Fig. 1. Cross-section of field site, with location of kerosene source (black square), soil gas sampling ports along the main sampling axis (black circles) and location of soil cores drilled along an axis of 120–140° to the main sampling axis (grey dots). Soil cores A–C were taken on the 24th day before contamination, soil cores D–F and cores G–I on the 23rd and 80th day after contamination of the site, respectively, at distances of 0.7 (samples A, D and G), 2 (B, E and H) and 10 m (C, F and I) from the source center.

(LB system, Geoprobe, Salina, Kansas). The corer was driven into the ground by a motor hammer until the moraine gravel made further lowering impossible. Undisturbed samples were obtained in PVC liners of 60 cm length. The top 10–15 cm in each liner were discarded to exclude topsoil, which had fallen into the hole. Soil samples were transferred on site into sterile PE tubes and stored at soil temperature (15 ± 3 °C) for a maximum of 3 days before they were homogenized, sieved (<2 mm) and further analyzed. After coring, each remaining hole was filled with clean sand and bentonite.

2.3. Carbon dioxide and oxygen measurements

Soil gas for the measurement of oxygen and carbon dioxide was sampled at 107 gas sampling tubes represented as black circles in Fig. 1. These probes were purged of dead air before samples of 3 ml were withdrawn with a syringe. The gas samples were stored in evacuated blood collection tubes (Venoject tubes, Terumo, Leuven, Belgium) and analyzed using a portable Chrompack Micro GC (Middelburg, The Netherlands). The method has been previously described in [30]. Since the sampling days for CO_2 and O_2 did not correspond to the sampling days of the soil cores, adjustment was allowed for gas data by linearly interpolating between consecutive dates. Linear interpolation was considered accurate enough since the delay between gas sampling days and the core drilling never exceeded more than 3% of the total duration of the experiment. For the depths of the samples A10, A11, A12, D10 and G10, no CO_2 values were available. Average values of the corresponding samples were used for statistical analysis instead. Contour plots were drawn with Surfer 7.00 (Golden Software Inc., Colorado, USA) using Kriging for interpolation.

2.4. General soil characteristics

Soil pH (free acidity) was measured in H_2O (soil to water ratio 1:2.5 w/w) after shaking for 1 h. All soil samples were ground, mineralized by the method of Kjeldahl and analyzed colorimetrically for total nitrogen (N_{tot}) and phosphorus (P_{tot}) on a Technicon Autoanalyzer II (Dublin, Ireland). Total soil carbon was combusted at 1000 °C to CO_2 which was analyzed on a CASUMAT analyzer (Wöstmann, Bochum, Germany). The CO_2 release from soil boiled in 33% phosphoric acid was measured in order to get the total inorganic carbon content and the total organic carbon (TOC) content was calculated by subtracting the inorganic carbon content, from the total carbon content. The temperature profiles in the soil were measured on selected days at 4 p.m. with buried thermometers. Missing values were interpolated from the closest measurements. Water content was measured by weight loss due to evaporation after 24 h at 105 °C.

2.5. Direct counts of soil bacteria

For enumeration of total bacteria, the <2 mm sieved fraction of 0.3 g of each soil sample was suspended in 4% paraformaldehyde, washed with phosphate-buffered saline (0.13 M NaCl, 7 mM Na_2HPO_4 and 3 mM NaH_2PO_4, pH 7.2) and stored in 96% EtOH at −20 °C [14]. The suspensions were then diluted between two- and tenfold (depending on cell density) in pyrophosphate buffer (0.1% $Na_4P_2O_7 \times 10\ H_2O$), and ultrasonicated to disrupt biofilms and permeabilize cell membranes. Three times 20 μl of the dispersed suspension were spotted on gelatin-coated slides dried on heating plates at 70 °C and finally dehydrated sequentially in 50%, 80% and 96% ethanol for 3 min each. The dry samples were stained for 10 min with an aqueous mixture of 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI, 5 $\mu g\,ml^{-1}$) and acridine orange (AO, 0.001%), dried again, covered with a drop of citifluor AF1 (Citifluor Ltd., London, UK) and a cover slip and stored at −20 °C. Bacteria were counted with a microscope (Olympus BX-60, Olympus Optical Co. Ltd., Tokyo, Japan) equipped for epifluorescence. Staining with a DAPI/AO mixture allowed easy distinction between orange-stained soil particles and blue-stained bacteria. Cell counts are average cell numbers of 16 equally distributed fields on a microscope slide. Averages and standard deviations presented in Fig. 3 were calculated from three of these measurements based on 16 fields.

2.6. Protein quantification in soil samples after Bradford

Protein was quantified in order to test the correlation between total cell numbers and microbial biomass. To prepare 1 l of Bradford Reagent, 100 mg of Coomassie brilliant blue G-250 was dissolved in 50 ml ethanol (96%). Deionized, filtered H_2O and 100 ml H_3PO_4 (85%) were added to give a volume of 1000 ml. Protein standards in the range of 0.5–0.005 $mg\,ml^{-1}$ were prepared from a stock solution of 1 mg bovine serum albumin (BSA) per ml. Sieving soil samples (<2 mm) allowed to separate roots and worms from the soil before protein analysis. One ml of 1 M NaOH was added to 1.5 g sample soil and cooked for 10 min at 95 °C [14]. The soil particles were subsequently settled by centrifugation (5 min at 4000 rpm). After chilling, 0.5 ml of the supernatant was neutralized by addition of 1 M HCl (sterile, filtered) in a new tube. The OD_{590} was measured after incubation for 5 min with Bradford reagent. Particles and dissolved material were accounted for by a blank for each sample. Since the pH was lowered to 1 by addition of the Bradford reagent to the sample, some soil material was dissolved. To have the same conditions in the blank and in the sample, a solution prepared identically to Bradford reagent without Coomassie brilliant blue was added.

2.7. Characterization of community-level physiological profiles

Microorganisms for physiological profiles were extracted by shaking 30 g soil for 30 min in 75 ml sterile deionized water at 180 rpm on a rotary shaker. Deionized water was chosen since, according to the DLVO (Derjaguin, Landau, Verwey and Overbeek) theory of colloid stability, low ion concentration favors detachment of particles from surfaces [31]. The samples were allowed to settle for 10 min. Ten ml suspensions were put on ice until used. To estimate inoculated cell numbers and extraction efficiency, 100 μl of the suspension was stained by 20 μl of a DAPI/AO mixture described above, filtered and dried in ethanol on a polycarbonate filter. Extracted and inoculated cell numbers were determined by fluorescence microscopy. The extraction efficiency of 18–80%, expressed as percentage of total count cell numbers, strongly depended inversely on the organic matter content. CLPP were obtained from all samples of cores A, D and G, and from a few selected samples of the remaining cores taken at the depth of the buried source.

Biolog^{TM} EcoPlates (Biolog Inc., Hayward, CA, USA) [32] are commercially manufactured microtiter plates loaded with mineral medium, a tetrazolium dye and 31 different carbon sources in triplicates. CLPP are based on reduction of the dye linked to carbon source utilization in the wells of those plates [33]. None of the carbon sources is a fuel compound or expected to occur significantly in hydrocarbon-polluted soils due to contamination. By means of a 8-channel pipette, one plate per sample depth was inoculated with 125 μl per well of the 1:100 (topsoil) or 1:19 diluted extract. Plate incubation occurred at steady temperature of 21 °C in the dark, absorption was automatically measured at 595 nm every 12 h with a plate reader (Dynex Technologies Inc. MRX II, Chantilly, USA). Values of the respective blanks were subtracted and negative values that occasionally resulted were set to zero. Average well color development (AWCD) was calculated for each well but not used for further data transformation for reasons described in the results section. Values after 72 h incubation and absorbance areas under curves to 120 h were used for further analysis. The areas were obtained by calculating a 'Riemann's sum' as also suggested in [34]. The substrate richness, S, is the total number of utilized C-sources out of 31 and utilization being ascribed to absorbance values of samples minus absorbance of blanks exceeding 0.25 after 72 h. A coefficient of variation (CV) was introduced in order to gain information on the reliability of the mean values of the three substrate replicates on one plate. The percentages of all 31 plate substrates with a $CV = \sigma/\mu$ (σ being the standard deviation and μ the mean value of the three replicates) smaller than 100% are listed in Table 3.

2.8. Statistical treatment of data

The whole data set of 33 objects/samples was subdivided in five files according to the variables: 'Biolog 72h' (absorption after 72 h: 31 quantitative variables), 'Biolog area 120h (areas under absorbance curves after 120 h: 31 quantitative variables), 'Biology data' (CO_2 concentration log of cell numbers, protein quantification: 3 quantitative variables), 'Soil parameters' (depth, pH, temperature, N_{tot}, P_{tot}, total carbon, TOC and volumetric water content: 8 quantitative variables) and 'Time' (days after contamination, given as three binary variables). In order to quantify and test effects of various sets of explanatory variables on the Biolog and Biology data variation, RDA was applied using the CANOCO 3.12 software [35,36]. RDA is a canonical method that allows simultaneous analysis of two or more data tables. Like Principal Component Analysis (PCA), RDA is used to calculate ordination of samples, with the difference that in RDA the explanatory variables intervene in the ordination of the response variable matrix. The ordination vectors are forced to be maximally related to linear combinations of the explanatory variables [37] and the canonical model can be tested. Variables of the data matrix have been centered by CANOCO, those of the explanatory variables were standardized. A correlation matrix was used to map the various types of variables in the data matrix. During the analysis the most discriminating variables were selected by the 'forward selection' procedure of the program. Statistical tests were run using the Monte Carlo permutation procedure of CANOCO [38]. The same statistical package was used for preliminary PCA.

3. Results

3.1. Soil characteristics

Results from chemical soil analysis are presented in Table 2. The total organic carbon (TOC) content declined from 4–9 g kg^{-1} in topsoil to less than 1 g kg^{-1} in the vadose zone at 80–130 cm depth where the contamination was buried. However, in selected samples such as D7 at 1.97 m depth, visible organic matter lenses led to a high TOC content. Total nitrogen and phosphorus were distributed similarly to TOC. Volumetric soil water content was influenced by recharge in the first meter, but remained stable at a depth of 2.50 m. The maximum water content was found around 2.9 m and 3.3 m depth. The soil pH showed a distinct vertical gradient with values ranging from 5 in the topsoil to nearly 7 in the sandy unsaturated zone. However, pH values higher than 8.5 caused by carbonate minerals in the moraine gravel were found at 3.36 m depth in core A and at 3.2 m depth in core G.

Table 2
Selected properties of the soil samples

Label	Depth (m)	pH	TOC ($g\,kg^{-1}$)	N tot ($g\,kg^{-1}$)	P tot ($g\,kg^{-1}$)	Wat Cont[a]	HCarb[b] ($g\,m^{-3}$)
Core A, background							
A1	−0.23	5.3	9.5	0.822	0.56	0.113	0.00
A2	−0.55	6.2	2.6	0.166	0.62	0.132	0.00
A3	−0.85	6.4	1.0	0.050	0.24	0.074	0.00
A4	−1.16	6.3	1.9	0.093	0.30	0.057	0.00
A5	−1.42	6.6	1.1	0.045	0.26	0.078	0.00
A6	−1.73	6.8	0.6	0.029	0.20	0.104	0.00
A7	−2.04	6.7	0.7	b. d. l.	0.16	0.069	0.00
A8	−2.33	6.7	b. d. l.	b. d. l.	0.15	0.107	0.00
A9	−2.59	6.8	0.6	b. d. l.	0.14	0.076	0.00
A10	−2.92	6.9	0.6	b. d. l.	0.22	0.190	0.00
A11	−3.17	7.0	1.1	0.041	0.20	0.096	0.00
A12	−3.36	8.8	1.3	0.046	0.34	0.228	0.00
Core D, day 23							
D1	−0.24	5.0	4.0	0.281	0.73	0.071	0.65
D2	−0.57	6.0	3.5	0.214	0.71	0.108	13.44
D3	−0.88	6.5	1.8	0.089	0.27	0.068	20.81
D4	−1.18	6.6	0.6	0.029	0.21	0.043	62.24
D5	−1.39	6.6	0.6	b. d. l.	0.24	0.079	99.73
D6	−1.75	6.7	0.6	0.013	0.21	0.082	36.65
D7	−1.97	6.5	5.1	0.375	0.49	0.078	47.27
D8	−2.27	6.7	0.4	b. d. l.	0.19	0.066	42.65
D9	−2.63	7.0	0.7	0.020	0.18	0.078	–
D10	−2.90	6.8	0.7	0.012	0.16	0.164	–
Core G, day 80							
G1	−0.30	5.7	8.3	0.664	0.61	0.162	2.16
G2	−0.55	6.1	2.2	0.189	0.51	0.188	5.74
G3	−0.85	6.5	0.8	0.046	0.27	0.110	11.33
G4	−1.20	6.5	0.4	0.042	0.23	0.112	19.56
G5	−1.45	6.7	0.3	b. d. l.	0.15	0.017	16.79
G6	−1.75	6.8	0.4	0.030	0.21	0.115	15.39
G7	−2.00	6.7	0.3	0.016	0.18	0.089	11.62
G8	−2.35	6.7	0.3	0.013	0.18	0.101	6.33
G9	−2.60	6.8	0.3	0.025	0.15	0.079	–
G10	−2.90	7.1	0.5	0.018	0.22	0.133	–
G11	−3.20	8.6	0.4	0.033	0.29	0.054	–

–, not measured.
b. d. l., below detection limit.
[a] Water content (v/v).
[b] Hydrocarbon (kerosene) content in the soil gas.

3.2. Microbial activity

Carbon dioxide (Fig. 2) was chosen as the factor indicating microbial activity in the vadose zone. Background CO_2 concentrations in the soil gas were heterogeneously distributed in the depth profile, but were lower than 0.9% everywhere (Fig. 2). A maximum was found in the topsoil at 4 m horizontal distance due to mineralization of natural soil organic matter. On day 22 after contamination, a maximum of 1.24% was found below the buried source, at 3.3 m depth just above the groundwater table, suggesting an increased mineralization activity in the capillary fringe below the source. On day 71 highest CO_2 concentrations were found at 1 m distance between 0.3 and 0.8 m depth as well as at 3.3 m depth. The maximum values of 2.12% were found at day 87 at the same location as on day 22. There was a distinct lateral gradient of CO_2 more than 10 m radially away from the source. The measurements of CO_2 at 20 m horizontal distance showed that there was a seasonal variation in the CO_2 concentrations. The O_2 consumption (data not shown) was small and inversely correlated to CO_2 production indicating that mineralization was essentially linked to aerobic respiration. The measurements ubiquitously showed more than 17% O_2 in the soil air.

3.3. Distribution of microbial cells

The total number of cells counted before contamination decreased from 7×10^8–3×10^9 cells g^{-1} in the topsoil to 10^7–4×10^7 cells g^{-1} in the sandy vadose zone

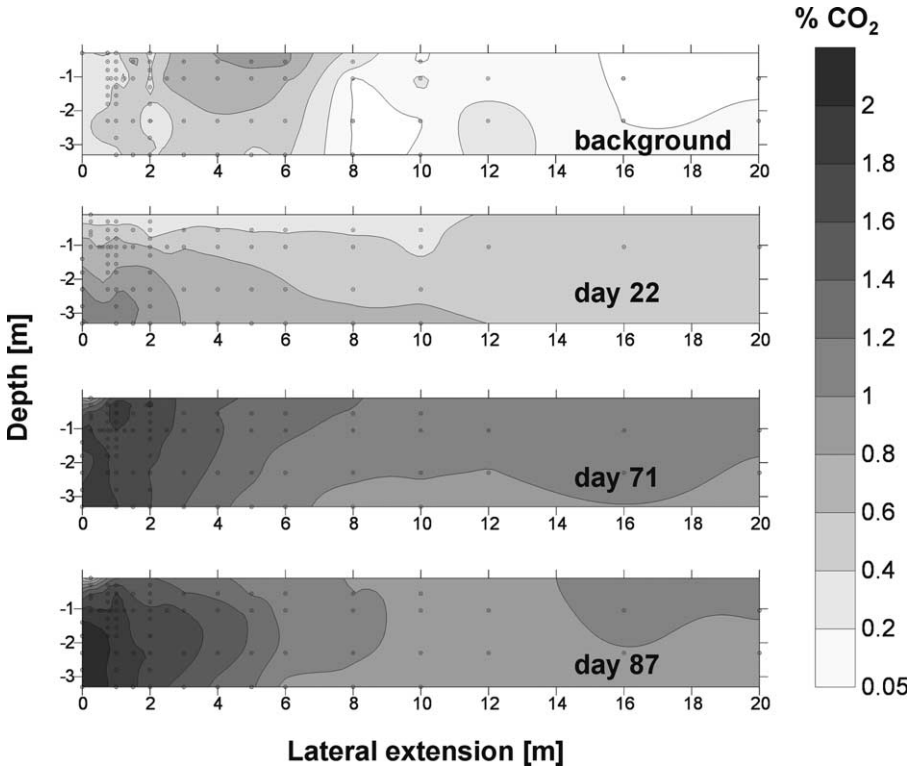

Fig. 2. Contour plots of CO_2 concentration (v/v) in soil gas measured 7 days before (background), 22, 71 and 87 days after the burial of the artificial fuel mixture.

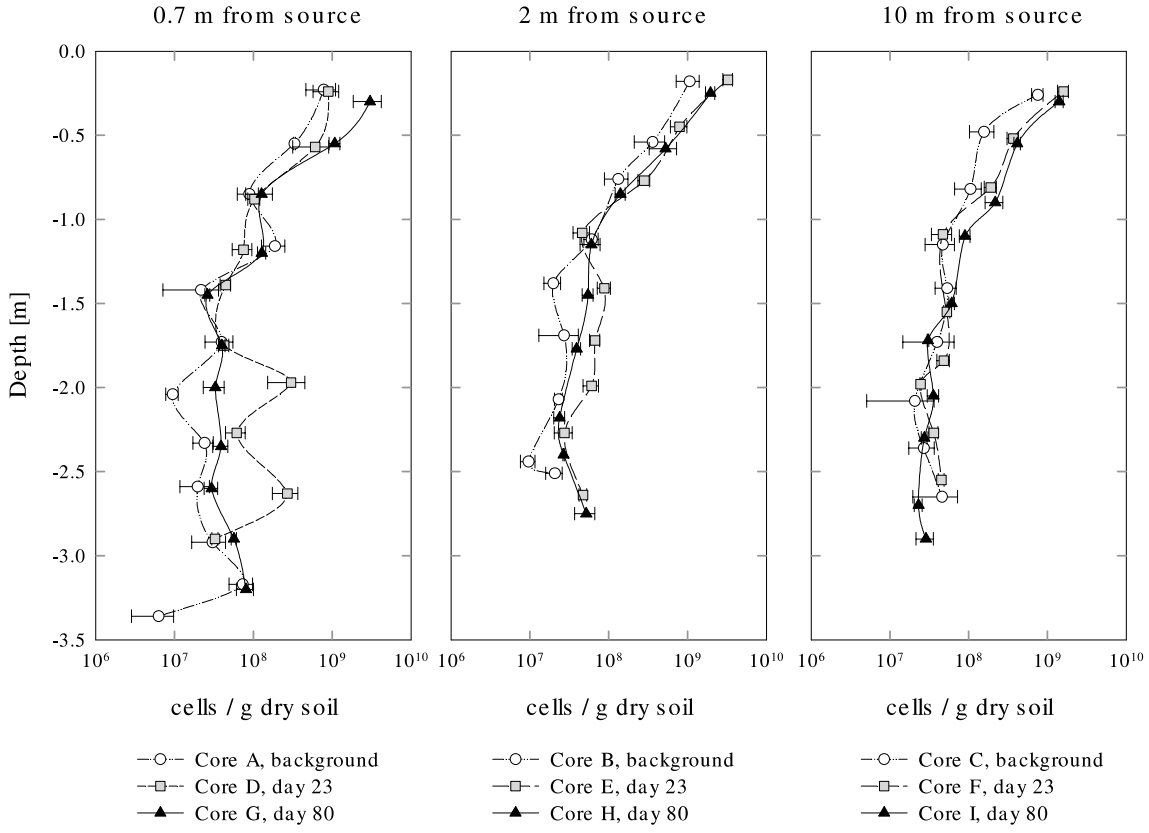

Fig. 3. Depth profiles of total microbial cell numbers measured 7 days before (background, A), 22 (D) and 87 days (G) after the burial of the artificial fuel mixture determined as direct microscopic counts by fluorescent microscopy. Error bars represent standard deviation.

between 140 and 280 cm depth (Fig. 3). Increased cell numbers were found in samples taken from the capillary fringe (samples A11 and G11). In the saturated zone the numbers decreased again (sample A12, see Fig. 3). Two samples of the vadose zone of core D contained unexpectedly high numbers of bacteria: At 2 m depth the corer was drilled across a local organic matter lens easily visible by eye. Organic matter, N and P analyses confirmed the observation (Table 2). The reason for the occurrence of high bacterial numbers at the depth of 2.6 m was less evident, since no visible structural change accompanied the observed higher contents of organic matter, P and N. Cell numbers were best correlated with the N_{tot} (R^2 for all cores >0.9, $p < 0.001$) and organic matter content. Protein and cell counts were generally well correlated with the exception of some surface samples where the protein content was underestimated.

3.4. Community-level physiological profiles

Within a core, the AWCDs were very heterogeneous and sometimes not exceeding a mean absorption of 0.4. To compare plates at such low AWCDs was not reasonable in this experiment since surface samples had already reached this value after a few hours, although bacteria had not started to mineralize all of the degradable substrates by that time. Since the cell numbers in the vadose zone were very low it seemed to be unreasonable to dilute extracts of other samples to the cell density of those poor ones, which would have led to losses of bacterial strains. Neither, did we divide absorbance values by the AWCD since the activity of bacteria in surface soil and deeper vadose zone is very different, and we would have lost information as described in [29,39]. Hence, PCA profiles of the areas under the curve up to 120 h were compared with those using data at 72 and 96 h and no obvious differences were detected, indicating that an incubation time of 72 h was long enough to level out differences in inoculum densities. The substrate richness (S) at 72 h is indicated in Table 3. All three cores show a similar pattern of S and CV. Generally, S and CV tended to decrease with depth. Unexpectedly high values were, however, found in samples A11 and G11 from the capillary fringe, indicating high functional diversity. In core D, high values for both parameters were found in the organic matter rich sample D7 at 1.97 m depth. The low cell number in some of the deeper samples led to only few degraded substrates and to considerable variations between replicates.

3.5. RDA ordination

The correlation structure between the variables 'Biology data', 'Soil parameters' and 'Time' is summarized in Fig. 4, whereas Fig. 5 shows the correlation structure between 'Biolog area 120h', 'Soil parameters', 'Time' and 'Biology data'. Hydrocarbon concentrations in soil gas were not used since the variance inflation factors in the canonical regression model were very high and the 13 variables (petroleum hydrocarbons) had to be reduced to two. Instead, the time can be used as an indirect measure of the exposure by hydrocarbon pollution.

The variance in the 'Biology data' (Fig. 4(a)), consisting of cell counts, protein quantification and CO_2 measurements, could be explained to 46% by the 'Soil parameters', and 'Time' explained another 39.8% of variance, 5.7% of those values being shared. This result is significant overall ($p < 0.001$). Forward selection was used in order to reduce the number of variables of 'Soil parameters' to approximately the same as in 'Time'. Variables explaining the largest statistically significant amount of variation were P_{tot} ($p < 0.001$), N_{tot} ($p < 0.001$), temperature ($p < 0.005$) and pH ($p < 0.046$). With these variables, 51.2% of variance was explained, but we eliminated 'temperature' since some missing values were calculated. The next most significant variable, 'water content', was instead included in the further RDA. Depth, TOC and total carbon (TC) were all highly correlated with the above-mentioned variables. In the triplots Fig. 4(b) and 5(b), quantitative variables are represented by arrows, qualitative explanatory variables (centroïds) are indicated by their label. The angle between two arrows and between an arrow and an axis or between an arrow and inter-connecting line between the origin and an object-point are proportional to the correlation. Distance between object-points and between centroïd-points, or between each other, are interpreted, respectively, as a mutual similarity or contribution. The projection of a centroïd-point onto an arrow is proportional to its contribution. High correlations between cell number, N_{tot}, P_{tot} and protein measurements and the first RDA axis are observed. The pH is negatively correlated with those variables. CO_2 is correlated with axis 2. It is remarkable that the arrow 'CO_2 content' is perpendicular to the arrow 'cell numbers', meaning that they are independent. Cores are clearly separated by CO_2, and, indeed, the centroïd for day 80 is opposed to day 23 and the one representing the background. The samples of each core are arranged according to depth, selected soil parameters and biomass related variables.

In the analysis of 'Biolog area' (Fig. 5), the number of soil variables was reduced by forward selection for the same reason as described above. Again four variables were retained for the RDA: P_{tot} ($p < 0.014$), depth ($p < 0.036$), volumetric water content ($p < 0.047$) and N_{tot} ($p < 0.26$). The total explained variance in this canonical model is 42.7%, 'Time' explains 9.45%, 'Soil parameters' 27.05% and 'Biology' 15.6%. Shared variance occurred only between 'Soil parameters' and 'Biology' and was 9.4% of the previously mentioned values. This result is overall significant ($p < 0.007$). Descriptors

Table 3
Substrate richness S and repeatability of the substrate use (CV) of samples analyzed on Biolog EcoPlates™

Sample number	Depth (m)	Substrate richness (S)	Sum of CV < 1[a]
Core A, background			
A1	−0.23	18	65
A2	−0.55	18	81
A3	−0.85	19	56
A4	−1.16	22	68
A5	−1.42	19	55
A6	−1.73	6	10
A7	−2.04	21	74
A8	−2.33	22	58
A9	−2.59	20	52
A10	−2.92	20	23
A11	−3.17	28	100
A12	−3.36	16	58
Core D, day 23			
D1	−0.24	18	58
D2	−0.57	22	81
D3	−0.88	17	52
D4	−1.18	16	55
D5	−1.39	13	49
D6	−1.75	15	45
D7	−1.97	22	71
D8	−2.27	10	32
D9	−2.63	17	52
D10	−2.90	17	36
Core G, day 80			
G1	−0.30	18	68
G2	−0.55	20	61
G3	−0.85	17	71
G4	−1.20	14	36
G5	−1.45	16	61
G6	−1.75	3	3
G7	−2.00	17	42
G8	−2.35	13	36
G9	−2.60	16	56
G10	−2.90	12	13
G11	−3.20	24	68

[a] Percentage of all substrates having a coefficient of variation smaller than 1.

which are well correlated with the two first RDA axes were either polymers or carboxylic acids, according to the grouping of [31]. 'Biolog area' data did not separate the cores well but arranged samples in the following ways: in the bottom right quadrant are samples with high organic matter, which proved to be positively correlated with the selected soil variables N_{tot} and P_{tot}, in the bottom left quadrant are samples, which were exposed to high petroleum concentrations and which is in accordance with the CO_2 variable (see Table 2), in the top left quadrant are deep samples with a high water content, and in the first quadrant are less contaminated samples with small cell numbers. A11 and G11 have rather high cell numbers, have a high pH and are situated between two soil layers with high volumetric water contents.

Variance in 'Biology data' in samples of all soil cores (A–I) at the source depth was to 98% explainable by 'Soil parameters' when all variables were included. By reducing the 'Soil parameters' by forward selection but including 'Time' for explanation, 96.8% of the variance was explained with a significant overall model and one significant axis (data not shown).

4. Discussion

4.1. Microbial activity and abundance vs. nutrient limitation

Measurable CO_2 production started 16 days after contamination of our experimental field site. This was later than expected since the lag phase in the lysimeter experiment described in [28] lasted only one day. The production of CO_2 was spatially closely related to the extension of the hydrocarbon vapor plume. However, although CO_2 concentration clearly increased at 0.7 m from the source (Fig. 2), the bacterial cell numbers increased only insignificantly (Fig. 3). This finding was unexpected since Ainsworth et al. [23] reported on 10^4–10^7 cells per gram of uncontaminated, dry soil and orders of magnitude higher cell numbers when contaminants were present at relatively high but not toxic concentrations. Zarda and co-workers [14] drilled cores through the unsaturated zone above a xylene plume. They found elevated numbers of bacteria and protists, especially in the xylene-affected zone just above the groundwater table and in the saturated zone. Pasteris et al. [28] observed increasing cell numbers in a lysimeter study after contamination by a similar petroleum mixture as the one used here, using the same counting method. Since in our study neither a significant increase in cell numbers nor a correlation between CO_2 production and cell numbers could be detected, it is likely that growth was limited by soil parameters such as nutrient concentrations and that degradation was essentially due to the metabolism of non-growing cells. Zhou and Crawford [40] showed that biodegradation of gasoline vapors in soil depended on the C:N ratio. The authors suggested that lower C:N ratios favored microbial growth. A good C:N ratio for their soils was 50:1 or lower. The natural ratio of TOC:N in the unsaturated zone of the Værløse soils is between 20:1 and 50:1. Taking into account the hydrocarbon contamination, which exceeds 1780 ppm described in [40], the ratio becomes higher than 50:1 and is thus outside the reported optimal range. Batch experiments carried out with Værløse soil showed increasing degradation when ammonium vapor was added (N. Dakhel and P. Höhener, personal communication), which confirms the assumption of N-limitation. This finding is in agreement with the better correlation of cell numbers with N_{tot}

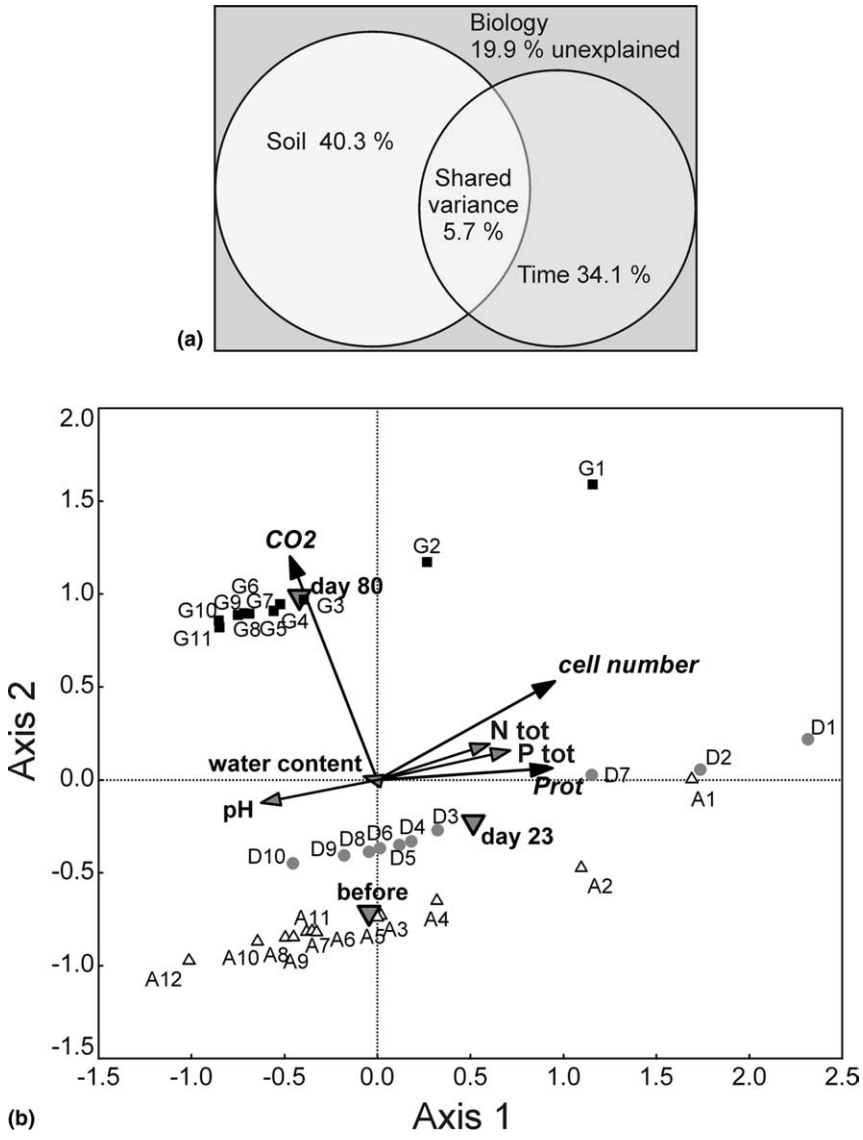

Fig. 4. (a) Variance decomposition of the redundancy analysis (RDA) of 'Biology data'. The percentage of variance explained by either the soil or the contamination (expressed as time) are given. (b) Triplot diagram of the redundancy analysis (RDA) of 'Biology data'. Descriptors (arrows) are the number of cells per gram dry soil (cell number), μg protein g^{-1} dry soil (Prot) and the carbon dioxide emission (CO_2). Total nitrogen (N_{tot}), volumetric water content (water content), phosphorus content (P_{tot}) and pH (pH) are used as a quantitative explanatory variables (arrows), Time 0 (before), day 23 and day 80 (after) are used as qualitative explanatory variables (centroïds). Axis 1 (45.1%, λ: 0.451, $p < 0.001$) and 2 (33.3%, λ: 0.33, $p < 0.001$) are represented. Total of explained variance in the correlative model is 80.1%. Samples are labeled according to the time of contamination (A: before, D: 23 days after and G: 80 days after) and numbered according to increasing depth (for details see Table 2).

than with any other soil parameter ($R^2 > 0.9$) and is concordant with results of [41], who found N limitation for toluene degradation in subsurface soils.

4.2. Parameters influencing community size, activity and structure

The forward selection for the RDA model assessed the following order from the most to the least explicative variable of the 'Biology data': $P_{tot} > N_{tot} >$ temperature > pH > water content > TOC > C_{tot} > depth. PLFA data ordination in a study from [42] about sheep grazing on grassland shows that the microbial biomass variation among soils as determined by PLFA quantification is mostly correlated to differences in organic matter content and soil pH. Cell numbers in our study correlated negatively with pH. N_{tot} and TOC were highly correlated so that only the former was used for RDA. Neither CO_2 nor hydrocarbons were detected in topsoil since the gases diffused out to the atmosphere. Hence, topsoil samples in Fig. 4(b) are apart from the CO_2 axis. The uncontaminated core A is more dispersed along the cell number–pH axis than contaminated samples. Samples A11 and G11, which are rich in nitrogen and bacteria

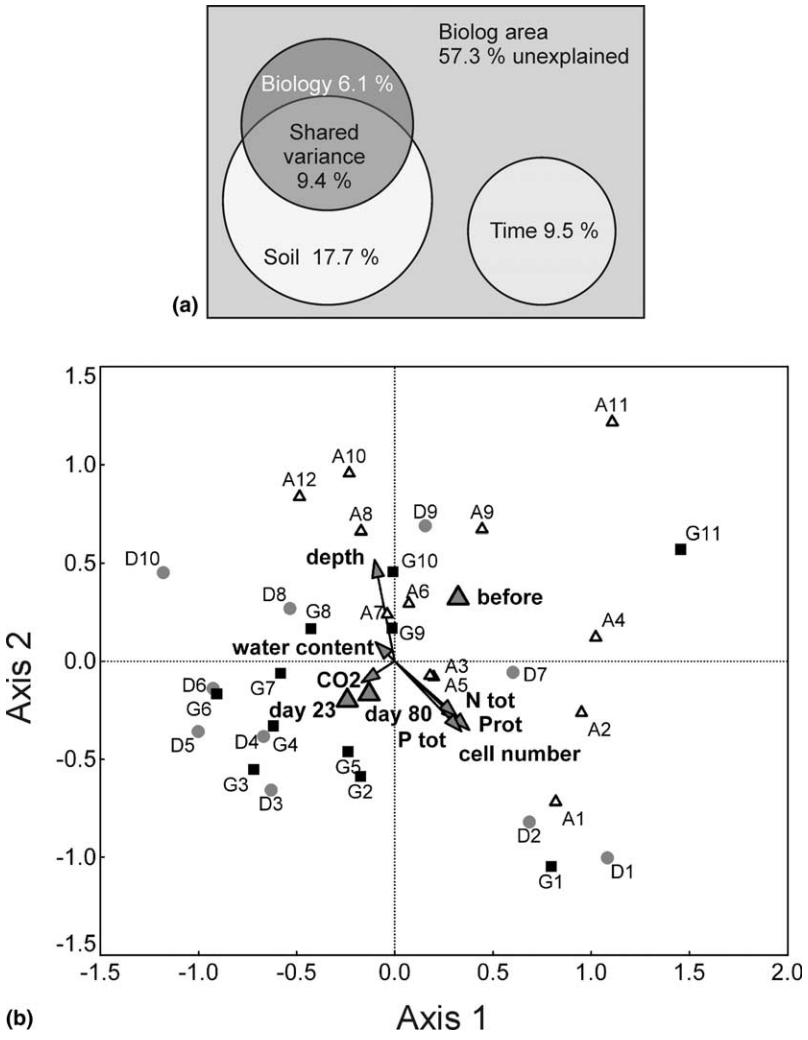

Fig. 5. (a) Variance decomposition of the RDA on 'Biolog area 120h' data. 'Soil parameter', 'Time' and 'Biology data' explain together 42.7% of the total variance. (b) Biplot diagram of the redundancy analysis (RDA) of 'Biolog area 120h' data. Descriptors are the average areas under the absorption curves of the Biolog plates after 120 h (not shown). Total phosphorous (P_{tot}), volumetric water content (water content), total nitrogen (N_{tot}), depth, protein quantification (Prot), log of cell numbers (cell number) and CO_2 emission (CO_2) are used as quantitative explanatory variables (arrows). Time 0 (before), day 23 and day 80 are used as qualitative explanatory variables (centroïds). Axis 1 (23.3%, λ: 0.233, $p < 0.07$) and 2 (10.3%, λ: 0.103, $p > 0.1$) are represented. Samples are labeled according to the time of contamination (A: before, D: 23 days after and G: 80 days after) and numbered according to increasing depth (for details see Table 2).

and have a lower water content than their neighboring samples, are not clearly grouped apart of the other data points in Fig. 4(b). This is different from what is inferred from the 'Biolog area' graph (Fig. 5(b)). Bacteria in these samples were able to degrade more substrates than in surface samples. The CV shows that this finding was reproducible. The reciprocal Simpson index [43] was 25.6 for A11 and 20.7 for G11 and, hence, significantly higher than the average of the subsurface samples (samples 5–10, $A = 13.6$, $D = 11.4$ and $G = 9.07$) and the surface samples (samples 1–3, $A = 15.34$, $D = 14.53$ and $G = 16.4$). It is remarkable that samples exposed to highest hydrocarbon doses are clustering together in the RDA plot since distinctive grouping of these exposed samples with PCA did not occur. The kerosene vapor concentration of these samples in the third quadrant was higher than 10 $g\,m^{-3}$ at the sample day. The distance from the origin to the data point for day 23 is longer than to the point of day 80. This may be indicative of a higher influence of the contamination on functional diversity after 3 weeks. Other authors reported on distinction due to hydrocarbon contamination but in most cases they applied higher oil concentrations [4]. Bundy and coworkers [10] incubated three different soils with diesel oil for 103 days in jar microcosm experiments. By performing PCA on Biolog GN data, they found that contaminated samples were clearly separated from uncontaminated samples, but that controls were also altered.

Biolog plates have in the last few years become a widely used tool in soil microbiology and they are generally considered as a sensitive tool to fingerprint

communities [4,44,45], however, the method for analyzing the data is still open to debate. In most studies the classical GN2 plates with their 95 substrates plates are used [29]. The reduction of substrates to 31 by using Biolog™ EcoPlates proved to be useful in our study because more repetitions could be made on one plate and the weak pattern reproducibility in the samples at depths around −1.75 and −2.90 m became apparent. This weak reproducibility as reported in the CV in Table 3 occurred at all sampling times and was probably not a methodic artifact but was due to the low microbial activity. A more sensitive tetrazolium dye in Biolog™ EcoPlates would probably increase the reproducibility. However, RDA based on Biolog™ EcoPlates data allowed the distinction between major influences on the community structure. But we should not forget that 57.3%, of the variance in the 'Biolog area' data remained unexplained with our measured variables and the grouping was hence influenced by unknown factors. These factors might be related to either the whole extraction method or the data analysis. For further studies, a CLPP method that does not require extraction of microorganisms from soil would be welcome.

Acknowledgements

Financial support is provided by the Swiss Federal Office for Education and Science (BBW No. C99.0025 and 99.0412). The study benefited from the co-operation within the European project *Groundwater risk assessment at contaminated sites GRACOS*. The authors thank Gabriele Pasteris, Nathalie Dakhel, Antonis Chatzinotas and David Werner for data collection, and Peter Kjeldsen and Mette Broholm for their support in Denmark.

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