RESEARCH ARTICLE

Flooding forested groundwater recharge areas modifies microbial communities from top soil to groundwater table

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Abstract

Subsurface microorganisms are crucial for contaminant degradation and maintenance of groundwater quality. This study investigates the microbial biomass and community composition [by phospholipid fatty acids (PLFAs)], as well as physical and chemical soil characteristics at woodland flooding sites of an artificial groundwater recharge system used for drinking water production. Vertical soil profiles to *c.* 4 m at two watered and one nonwatered site were analyzed. The microbial biomass was equal in watered and nonwatered sites, and considerable fractions (25–42%) were located in 40–340 cm depth. The microbial community structure differed significantly between watered and nonwatered sites, predominantly below 100 cm depth. Proportions of the bacterial PLFAs 16:1ω5, 16:1ω7, cy17:0 and 18:1ω9t, and the long-chained PLFAs 22:1ω9 and 24:1ω9 were more prominent at the watered sites, whereas branched, saturated PLFAs (iso/anteiso) dominated at the nonwatered site. PLFA community indices indicated stress response (*trans*/*cis* ratio), higher nutrient availability (unsaturation index) and changes in membrane fluidity (iso/anteiso ratio) due to flooding. In conclusion, water recharge processes led to nutrient input and altered environmental conditions, which resulted in a highly active and adapted microbial community residing in the vadose zone that effectively degraded organic compounds.

Introduction

Water purification through artificial groundwater recharge is widespread, and common routines comprise unvegetated slow sand filters, such as dunes or channels, in combination with long flooding periods (Peters *et al.*, 1998). In these systems a biofilm matrix, comprising microorganisms, microfauna and a range of aquatic insect larvae, develops at the surface of the sand filter, which is predominantly responsible for water purification (Duncan, 1988; Webershirk & Dick, 1997). The present study investigates the drinking water production of the city of Basel (Switzerland) at the former floodplain area called 'Lange Erlen'. This system is unique throughout the world. Embanked forest sites with either artificial landfill (loess loam) or fluvi-eutric cambisol are periodically flooded with water from the Rhine river to augment groundwater resources. Thereby river water is transformed into purified drinking water, but in contrast to slow sand filter systems no biofilm is generated. Nevertheless infiltration and purification capacities [i.e. removal of dissolved organic carbon (DOC)] remain constant and satisfying since the system has been established in 1912 (Rüetschi, 2004).

Generally, surface and subsurface microorganisms play an important role in soil biogeochemistry, contaminant degradation and maintenance of groundwater quality (Konopka & Turco, 1991). Thereby processes of pollutant biodegradation in the vadose zone, the unsaturated zone extending from the soil surface to the groundwater table, are of considerable importance (Holden & Fierer, 2005). The vadose zone is a three-phase system comprising solid, aqueous and gaseous compartments and the soil conditions can change dramatically from saturated conditions to desiccation over short time intervals (Kieft & Brockmann, 2001).

Because of the function of the vadose zone as a natural mechanism for pollutant attenuation, there is need to better

understand the microbial ecology of this unsaturated subsurface. However, as the density of microorganisms is highest in the top soil layer (0–20 cm), most studies have predominantly investigated abundance, community structure and physiological status there (Konopka & Turco, 1991; Hinojosa *et al.*, 2005; Williams & Rice, 2007). Nevertheless, the microbial biomass of a soil profile down to 2 m depth can comprise *c.* 35% of the biomass in surface layers (Fierer *et al.*, 2003), and thus should not be ignored in biodegradation processes. Impacts of flooding or water stress on microorganisms in the top soil (0–20 cm) resulted in a decline of fungi, increase of Gram-positive bacteria, and decrease in monoenoic fatty acids, an indicator for aerobic conditions (Bossio & Scow, 1998; Williams, 2007). In the upper layer of floodplain soils, Rinklebe & Langer (2006) observed the highest microbial biomass and proportion of monoenoic fatty acids in short-time, and the lowest in long-time flooded sites. Further, Williams & Rice (2007) documented a reduction in the physiological stress of the Gram-negative microbial community in continuous moisture treatments.

To date, the water purification processes in the artificial groundwater recharge system 'Lange Erlen' are not understood in detail. Presumably they consist of a combination of groundwater dilution, particle sorption and biodegradation. In this study, we analyzed soil depth transects (*c.* 4 m deep) comprising surface soil, vadose zone and capillary fringe for microbial composition as well as physical and chemical characteristics in two watered and one nonwatered site. Microorganisms were assessed using phospholipid fatty acid (PLFA) pattern to determine biomass, community structure, physiological conditions and presence of specific groups. Our goal was to verify the following hypotheses: firstly, microorganisms residing there are usually exposed to low water availability, and periodic flooding is expected to alter community structure, activity and physiological status. Secondly, the vadose zone is generally regarded as static entity with strong depth gradients determined by biogeochemical properties. Thirdly, we propose water recharge sites to have a different spatial organization, with biologically active layers below the rooted zone. Thirdly, the microbial communities located at these 'purification sites' are assumed to be distinct from the communities above and below, and to consist of defined groups adapted to available resource and environmental conditions.

Materials and methods

Study site

The study area 'Lange Erlen' (size *c.* 3 km^2) is situated northeast of the city of Basel, Switzerland (for map, see Schütz *et al.*, 2008). This former natural floodplain area extends along the river Wiese, a straightened tributary of the river Rhine, descending from the Black Forest, Germany. Since 1912, part of the 'Lange Erlen' serves as groundwater recharge area, and today approximately half of the drinking water for the city of Basel originates there (15×10^6 m^3 ha^{-1} $year^{-1}$).

The semi-natural forest sites of the 'Lange Erlen' are classified as Galio–Carpinetum oak–hornbeam forests (Burnand & Hasspacher, 1999). The flooded sites have been modified by human activities, such as afforestation with poplars (*Populus canadensis*), ash (*Fraxinus excelsior*), alder (*Alnus nigra*) and willows (*Salix* spp.). The upper soil is characterized as fluvi-eutric cambisol, and the aquifer consists of 80% Rhine gravel (lower part, mostly limestone) and 20% Wiese gravel (upper part, mostly silicates and limestone).

The sampling site 'Hintere Stellimatten' (HST) is the most recent flooding area and started operating after afforestation in 1977. Too high infiltration rates, and subsequently too short water retention periods, (11 m^3 m^{-2} day^{-1}) led to artificial landfill (loess loam) and soil compaction by bulldozing to gain sufficient water retention capacity in 1981. The soil profile down to *c.* 1 m consists of landfill loess loam, followed by sand and gravel with interspersed silt and clay lenses and partial FeMn deposition. Average pH across the soil depth profile is 7.3. The water content (% DW soil) increases from 81% to 94%, whereas the C/N ratio declines from 14.6 to 8.2 with depth. The site is mainly overgrown with an alder swamp forest (*P. canadensis, A. nigra, Salix* spp.) and the herb layer is dominated by *Rubus caesius* and *Urtica dioica*. Unvegetated sites comprise some spots of *Poa trivialis, Potentilla reptans, Agropyron repens* and *Geum urbanum*.

The sampling site, 'Verbindungsweg' (VW), was established as flooding area after afforestation and artificial landfill (loess loam) in 1970. Landfill loess loam is *c.* 0.5 m deep, and is followed by silt, sand and gravel with interspersed clay lenses and partial FeMn deposition. pH values and water content (% DW soil) increase with depth from 7.2% to 7.5% and 79% to 92%, respectively. C/N ratio decreases with soil depth from 9.3 to 7.6. The forest consists of some old poplars, ash-leaved maple (*Acer negundo*) and oak trees. The understory is dominated by *Lysimachia nummularia, U. dioica, Phalaris arundinacea, R. caesus/fruticosus, Duchesnea indica, Iris preudacorus, Seneco aquaticus* and *Cardamine pratensis*. In the shady northern part of the site, dominated by poplars, the soil is bare of vegetation.

The nonwatered site 'Bachtelenweg' (BW) is situated between these two flooding areas and is agriculturally used with extensive biological grassland management. The soil profile is similar to VW, pH values range between 7.5 in the top soil and 5.9 in the deepest soil layer. The C/N ratio

declines from 9.7 to 7.3, and water content (% DW soil) increases from 86% to 94% with depth.

Infiltration system

Rhine water, prefiltered by rapid sandfilters (80 cm quartz sand layer), is periodically seeped into 11 wooded flooding areas (total area 22 ha). Each flooding area (10 000–20 000 m^2) is divided into three fields (3000–8500 m^2) by small dams (height *c.* 50 cm). Watering cycles usually consist of 10 days flooding and 20 days drying and regeneration, however, longer interruptions due to revisions occur. The water fills up to variable heights depending on soil surface structure (maximum 50 cm) and seeps with a speed of 1–2 m day^{-1} through a mull humus and fluvial silt layer of 30–90 cm and a sand/gravel layer of 2–3 m before it reaches the groundwater table at 3–4 m depth. Subsequently, the water flows horizontally (from northeast to southwest) in the aquifer for 200–800 m. After 10–30 days, the average retention time in the aquifer, purified water is pumped out of groundwater wells, collected in the pumping station and, after a brief chemical treatment with chloride dioxide (ClO_2), delivered to consumers (Fig. 1).

Soil sampling

Soil sampling was conducted in triplicate at the two watered sites (HST, VW) and the nonwatered site (BW). The groundwater level at the watered sites was lowered by a stop of the flooding regime 4 weeks before sampling. Three vertical core drillings (diameter 25 cm) from the soil surface down to a maximum of 4.5 m depth, or alternatively the groundwater level, were conducted in HST and VW by the construction firm Glanzmann (Basel, Switzerland) in November 2005. Soil cores were excavated in 0.5 m intervals and placed carefully into soil core boxes, representing the full soil profile along the depth transect. Subsequently, soil samples (100 g) were taken every 30 cm along the transect with a shovel from the inner part of the cores, transported to the laboratory and frozen (− 20 °C) until analysis. Overall, 45 soil samples were taken in each, HST and VW.

Three horizontal soil samplings through the face of a trench down to 3.5 m depth (above groundwater level) were performed in the nonwatered site BW in connection with drain installations for flood protection in December 2005. This was our chance for deep soil sampling outside the flooding areas in the same time period. Three depth transects were randomly chosen, and, respective to the soil layers in HST and VW, soil samples were directly taken from the inner trench wall. A total of 34 soil samples were taken and frozen (− 20 °C) until analysis.

Before all analyses the required soil substrate was thawed in the fridge (24 h, 8 °C). Visible organic material (fine roots and leaves) and stones were removed, and soil was sieved (5 mm).

Soil analysis

Chemical and physical soil characteristics

The soil samples were analyzed for a variety of physical and chemical characteristics to determine the gradients in pH, carbon (C), nitrogen (N), DOC, nitrate (NO_3^-), sulfate (SO_4^{2-}) and moisture content. Total carbon and nitrogen content were determined from oven-dried (65 °C, 3 days) and pulverized (swing mill, Retsch MM 200) aliquots of the bulk soil with an element analyzer (CHN 1000, Leco). Results are expressed in percentage per soil dry weight. Soil pH was measured in soil salt (0.01 M $CaCl_2$) solution (1 : 4 w/v) after stirring and incubating at room temperature for 1 h. Soil moisture content was determined by the mass difference before and after drying at 105 °C for 24 h.

DOC, NO_3^- and SO_4^{2-} were extracted from 5 g soil (fresh weight) for the 0–200 cm depths and from 10 g for the

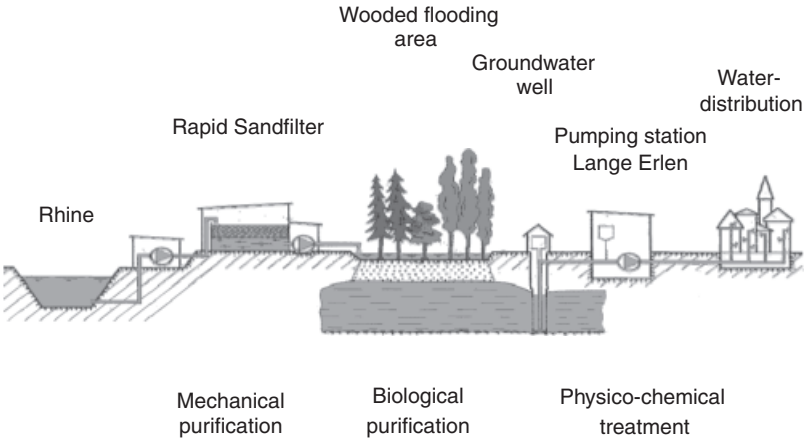

Fig. 1. The Basel Water Recharge System. Modified from IWB (2001).

200–450 cm depths. A solution was prepared with soil and 0.01 M $CaCl_2$ (1 : 4 w/v), shaken for 1 h (200 r.p.m.) and centrifuged for 15 min (450 ***g***). The supernatant of each soil solution was filtered through sterile 0.45 μm membrane filters (Millex HA) and stored in the fridge until analysis (maximum 1 h). To avoid contaminations, particularly in low concentrated soil solutions of deeper soil samples, only glass ware was used exclusively in the whole procedure. The soil solutions were analyzed for DOC after acidification and air-purging (N55, O45, Carbagas, Switzerland) with a TOC analyzer (TOC-5000 A, Shimadzu) in quintuplicate. NO_3^- and SO_4^{2-} were measured in an ion chromatograph (IC-690, Metrohm). Results are expressed in mg g^{-1} DW soil.

PLFA analysis

Lipid extraction and methylation was conducted on soil samples thawed for 24 h in a fridge (8 °C) using previously described procedures (Frostegård *et al.*, 1993a, b). From each layer PLFAs were extracted and quantified from 3 g soil (fresh weight) in 0–40 cm, from 6 g in 40–100 cm, and from 10 g in 100–400 cm depth in order to compensate for the decrease in microbial biomass with depth. PLFAs were identified and quantified using GC interfaced to an electron ionization mass spectrometer (GC/EI-MS). Analyses were performed with a 3400/Saturn 4D iontrap GC/MS system (Varian, Darmstadt, Germany). The split/splitless injector was operated in splitless mode for 2 min and kept at 225 °C. The carrier gas (helium, purity 5.0) was used at a constant pressure of 22.5 psi. A DB 5 cross-linked 5% phenyl 95% methyl polysiloxane column (50 m, 0.32 mm i.d., 0.17 μm d.f.; Varian Chrompack, Middelburg, the Netherlands) was installed in the GC oven. The GC oven temperature was programmed as follows: 50 °C (2 min hold time), then at 5 °C min^{-1} to 180 °C (2 min) and at 5 °C min^{-1} to 300 °C (50 min). The transfer line was heated to 300 °C. The manifold was set to 200 °C, and the multiplier voltage was set at 2400 V. At a filament emission current of 15 μA, the mass range was scanned from *m/z* 50 to 600 twice a second after a solvent delay of 5 min. One microliter of sample extract was injected each run.

PLFAs were determined using retention times and the occurrence of fragment ions: *m/z* 74 and 87 were used for saturated PLFAs, *m/z* 81 and 87 for monoenoic and cyclic PLFAs and *m/z* 79 and 81 for polyenoic PLFAs as described in Thurnhofer & Vetter (2005). A standard mixture composed of 37 different FAMEs comprising fatty acids from C11 to C24 (Sigma-Aldrich, St. Louis) was used to verify retention time. For the quantification of PLFAs, known concentrations of an internal standard (19 : 0) and the according fragment ion of the specific PLFA were included in the calculation. The abundance of individual PLFAs was expressed in nmol g^{-1} DW soil and in mol%. The total microbial biomass was expressed as sum of PLFAs. The ω notation (IUPAC-IUB, 1978) was used to classify fatty acids, where unsaturated acid are ascribed according to the number of carbon atoms from the terminal methyl group (ω end) to the nearest double bond, i.e. ω9, ω6, ω3 (IUPAC-IUB, 1978). Four PLFA ratios were calculated as indicators. The fungal to bacterial ratio (F/B) comprised 18:2ω6, 18:3ω6 as representatives of fungal and i15:0, a15:0, i16:0, i17:0, cy17:0, cy19:0 and 16:1ω7 of bacterial PLFAs (according to Frostegård *et al.*, 1993a, b, 1996; Zelles, 1999). The PLFA 16:1ω5 as biomarker for mycorrhizal fungi was excluded, because this exclusively fits for the rooted zone. Furthermore, the bacterial Gram-positive/Gram-negative ratio (i.e. iso+anteiso/cyclo PLFAs) and the iso/anteiso ratio of the PLFAs 15:0 and 17:0 as temperature (Kaneda, 1991; Petersen & Klug, 1994) and aerob/anaerob indicators (Parkes & Taylor, 1983; Mauclaire *et al.*, 2007) were estimated. The *trans*/*cis* ratio for 18:1ω9 was used as stress indicator (Heipieper *et al.*, 1992). As a measure of membrane fluidity the unsaturation index (UI, Ruess *et al.*, 2007) was calculated as $UI = ((C{:}1 \times 1) + (C{:}2 \times 2) + (C{:}3 \times 3) + (C{:}4 \times 4) + (C{:}5 \times 5))/100$, where C:1, C:2, C:3, C:4 and C:5 represent the proportion (%) of FAs with 1, 2, 3, 4 and 5 double bonds, respectively.

Statistical analyses

Overall, sampling was conducted in triplicate for each field site and summarized in seven depths: The surface soil (rooted zone) with 0–40 and 40–100 cm, the upper vadose zone with 100–160 and 160–220 cm and the lower vadose zone with 220–280, 280–340 and 340–400 cm. Calculations were conducted on LOG transformed environmental data, which also included the sum of PLFA, mol% PLFA and the different PLFA ratios. Environmental variables, PLFA amounts and ratios were analyzed for field- and depth-differences using repeated ANOVA procedures. As the 340–400 cm depth was only sampled at the watered sites HST and VW, this layer was excluded from analysis. Pair wise comparison of means was performed using Tukey's honestly significance difference test (HSD). Pearson's correlation coefficients for depth gradients were calculated for single PLFAs including all depth layers. Correlations and repeated measure ANOVAS were calculated using SAS (SAS Institute, Cary, NC).

Multivariate data analysis of PLFA data were carried out by analysis of similarities (ANOSIM), the similarity percentages procedure (SIMPER) and nonparametric multidimensional scaling (nMDS) to determine whether the microbial assemblages differed between sites and/or depths. Before analyses, the Bray–Curtis similarity index was applied to the data set. Two-way ANOSIM was calculated on the mol% data

of the overall PLFA pattern (two-way nested ANOSIM with the factors 'depth' nested in 'field'). One-way ANOSIM with 'field' as factor was applied to the bulked functional layers: surface soil, upper vadose zone and lower vadose zone (without the 340–400 cm layer). On this tripartite soil profile, nMDS was applied for visualizing the differences between fields and depths on the basis of mol% PLFA data. The nature of community groupings identified by ANOSIM was further explored by applying SIMPER procedure to determine the contribution of individual PLFAs to the average dissimilarity between sites in single soil layers (presented in Supporting Information, Table S1). Multivariate data analyses were calculated with PRIMER 6.1.5 (Clarke & Warwick, 2006).

To visualize differences in PLFA composition in relation to environmental variables biplot ordinations of redundancy analysis (RDA) and the Monte Carlo permutation test (CANOCO 4.5, Ter Braak & Smilauer, 2002) was used. First, data were analyzed by detrended canonical correspondence analyses (DCCA) to determine the length of gradient. Because this gradient was rather short, data were analyzed and visualized by linear RDA.

Results

Soil parameters

Concentrations of DOC, total carbon, total nitrogen and the C/N ratio in the soil significantly decreased with soil depth at all three sites by 85% and 87% (for statistics see Table 1). DOC ranged from 2.2 (HST) to 42.1 mg g^{-1} DW (BW) in the top soil layer, and from 0.2 (HST) to 6.0 mg g^{-1} DW (BW) in 280–340 cm depth (data not shown). The concentrations for DOC were significantly higher in the nonwatered site BW at all depths (average increase 15-fold), compared with the watered sites, HST and VW, which contained comparable amounts.

Total soil carbon content declined within the soil profile to about 88% in the watered sites, and 82% in the nonwatered site, with an average of 2.0 (top soil) to 0.3% DW soil (lower vadose zone, data not shown). Soil nitrogen content decreased with depth by about 80% from 0.2% to 0.03% DW soil. Carbon and nitrogen content were significantly reduced with depth, whereas the corresponding C/N ratio was significantly influenced by both, soil depth and field site, without interaction (Table 1).

NO_3^- concentrations in the top soil layer ranged between 2.3 (HST) and 3.1 (VW) mg g^{-1} DW soil of the watered sites, and 0.5 mg g^{-1} DW in the nonwatered site BW. SO_4^{2-} concentrations in the top soil layer ranged between 2.7, 2.5 and 0.8 mg g^{-1} DW soil in HST, VW and BW, respectively (data not shown). At all sites NO_3^- and SO_4^{2-} concentrations significantly decreased with soil depth (for statistics see Table 1). Water recharge treatment resulted in significantly higher nutrient concentrations in -40 to -220 cm depth for NO_3^-, and -40 to -340 cm depth for SO_4^{2-}.

pH values ranged between 7.5 and 6.6 and displayed contrasting depth gradients with lowest pH values in the lower vadose zone at BW and highest at VW, which are reflected in a significant depth × field interaction (Table 1).

Table 1. Repeated measure ANOVA table of *F*-values and degrees of freedom (d.f.) on the effect of field (watered sites: HST, VW; nonwatered site: BW) and soil depth (0–40, 40–100, 100–160, 160–220, 220–280 and 280–340 cm) for soil parameters and PLFA ratios

	Within subjects effects		Between subjects effects
	Depth (d.f. 5)	Depth × field (d.f. 10)	Field (d.f. 2)
Soil parameters			
pH	1.68	3.40**	3.11
%C	24.66***	0.75	2.61
%N	20.86***	1.46	0.05
C/N	5.66***	1.15	11.1**
DOC (mg g^{-1}DW)	11.35***	1.03	163.31***
NO_3^- (mg g^{-1} DW)	41.2***	6.91***	8.68*
SO_4^{2-} (mg g^{-1} DW)	15.4***	0.73	36.53***
PLFA (nmol g^{-1} DW)	64.58***	1.94	10.46*
PLFA-ratios			
Fungi*/bacteria†	0.35	1.01	0.47
Gram+/Gram − ‡	2.14	5.73***	11.85**
Unsaturation index	2.42	3.19**	38.31***
i/a 15:0	1.22	3.36**	10.18*
i/a 17:0	12.96***	2.72*	14.31**
Trans/cis§	1.66	4.26**	31.18***

*18:3ω6, 18:2ω6.
†i15:0, a15:0, 15:0, 16:1ω7, i17:0, a17:0, cy17:0, 17:0, cy19:0.
‡i15:0+a15:0+i17:0+a17:0)/(cy17:0+cy19:0.
§18:1ω9t/18:1ω9c.

PLFAs and soil depth

The total amount of PLFAs, a measure for the microbial biomass, uniformly decreased from 53.2 ± 28.1 to 1.8 ± 1.1 nmol g^{-1} DW soil with depth across sites (Tables 1 and 2). Highest amounts of PLFAs were observed in the top soil layer (0–40 cm). However, an average of 42%, 26% and 25% of the total microbial biomass was located in -40 to -340 cm depth in HST, VW and BW, respectively. Differences between sites occurred in the 40–100 and the 100–160 cm layer where the sum of PLFAs in HST surpassed VW and BW (Table 2).

In total, 25 predominant PLFAs were determined in the soils of the investigated sites (Table 3). The concentrations of individual PLFAs decreased significantly with soil depth, and only minor fractions were located in the lower vadose zone. Proportions (%) of individual PLFAs within the profile of single soil layers distinctly varied with depth

Table 2. Total amount of PLFA (nmol g^{-1} DW) and different PLFA ratios in six soil depths (0–40, 40–100, 100–160, 160–220, 220–280, 280–340 cm) at the watered sites HST and VW, and the nonwatered site BW

	Depth (cm)	HST	VW	BW
Total amount (nmol g^{-1} DW)	0–40	53.77 (49.0)	54.86 (23.8)	50.95 (13.5)
	40–100	18.10 (2.27) a	7.03 (0.63) b	6.82 (1.61) b
	100–160	13.33 (1.20) a	3.89 (1.57) b	3.22 (0.86) b
	160–220	4.17 (2.13)	3.29 (0.43)	3.08 (1.32)
	220–280	2.14 (0.58)	2.60 (1.14)	1.48 (1.31)
	280–340	1.76 (0.78)	1.58 (0.11)	1.95 (1.96)
Fungi*/Bacteria†	0–40	0.06 (0.00)	0.07 (0.03)	0.08 (0.04)
	40–100	0.05 (0.01)	0.07 (0.03)	0.12 (0.10)
	100–160	0.06 (0.08)	0.05 (0.03)	0.09 (0.04)
	160–220	0.07 (0.03)	0.05 (0.06)	0.05 (0.05)
	220–280	0.07 (0.06)	0.07 (0.09)	0.07 (0.05)
	280–340	0.04 (0.04) b	0.14 (0.04) a	0.07 (0.04) ab
Gram+/Gram − ‡	0–40	2.71 (0.58)	2.67 (0.67)	2.79 (0.74)
	40–100	2.79 (0.76)	2.37 (0.39)	4.07 (2.38)
	100–160	1.90 (0.42) c	3.00 (0.34) b	5.28 (0.50) a
	160–220	1.88 (1.43) b	2.55 (1.45) b	8.25 (1.23) a
	220–280	2.05 (0.58) b	1.68 (0.17) b	9.50 (4.98) a
	280–340	1.73 (1.23) b	1.06 (0.37) b	10.52 (5.04) a
UI§	0–40	0.50 (0.06)	0.52 (0.04)	0.50 (0.06)
	40–100	0.39 (0.04)	0.47 (0.05)	0.47 (0.11)
	100–160	0.66 (0.04) a	0.56 (0.03) a	0.37 (0.07) b
	160–220	0.55 (0.04) a	0.64 (0.03) a	0.28 (0.09) b
	220–280	0.55 (0.09)	0.60 (0.06)	0.43 (0.17)
	280–340	0.61 (0.16)	0.69 (0.06)	0.43 (0.10)
I/a 15:0	0–40	1.25 (0.03)	1.28 (0.19)	1.22 (0.44)
	40–100	1.15 (0.07)	1.18 (0.06)	1.07 (0.29)
	100–160	1.26 (0.06) a	1.23 (0.05) a	0.67 (0.26) b
	160–220	1.41 (0.03) a	1.27 (0.07) a	0.46 (0.20) b
	220–280	1.30 (0.38)	1.19 (0.08)	0.76 (0.36)
	280–340	1.54 (0.31) a	1.38 (0.27) ab	0.53 (0.46) b
I/a 17:0	0–40	1.00 (0.08)	0.94 (0.16)	0.98 (0.24)
	40–100	0.98 (0.05)	0.80 (0.03)	0.85 (0.21)
	100–160	0.90 (0.04) a	0.72 (0.04) b	0.57 (0.06) c
	160–220	0.84 (0.13) a	0.70 (0.06) ab	0.49 (0.09) b
	220–280	0.70 (0.19) a	0.68 (0.04) ab	0.36 (0.14) b
	280–340	1.00 (0.09) a	0.61 (0.26) ab	0.39 (0.09) b
Trans/cis¶	0–40	1.10 (0.10)	1.09 (0.22)	0.98 (0.18)
	40–100	0.84 (0.21)	1.04 (0.11)	0.94 (0.19)
	100–160	1.26 (0.08) a	1.13 (0.17) a	0.45 (0.22) b
	160–220	1.32 (0.09) a	1.04 (0.04) a	0.54 (0.27) b
	220–280	1.33 (0.22) a	1.16 (0.36) a	0.24 (0.06) b
	280–340	1.00 (0.41) a	1.00 (0.06) a	0.39 (0.20) b

*18:3ω6, 18:2ω6.

†i15:0, a15:0, 15:0, 16:1ω7, i17:0, a17:0, cy17:0, 17:0, cy19:0.

‡Gram-positive: i15:0, a15:0, i17:0, a17:0; gram-negative: cy17:0, cy19:0.

§UI, unsaturation index.

¶18:1ω9t/18:1ω9c.

Values in parentheses are the standard deviation. Means within a row with the same or no letter are not significantly different (Tukey's HSD, $P < 0.05$).

(Table 3). A uniform decrease in mol% occurred for the PLFAs 16:1ω5, i17:0, cy19:0 and 24:0, and an increase for 18:3ω6 at all sites. In contrast, the proportions of the mainly Gram-positive bacterial PLFAs a15:0 and a17:0 were negatively correlated with depth at the watered sites HST and VW, and positively correlated at the nonwatered site BW. Decreasing proportional amounts in 18:1ω9t, 20:4ω6 and 20:5ω3 were detected in BW soils only. Contrasting distributions were observed for the bacterial PLFAs i15:0 and i16:0, where proportions declined with depth in the

Table 3. Pearson correlation coefficients of single PLFA (%) with depth the watered sites HST and VW, and the nonwatered site BW

	Predominant origin	HST	VW	BW
14 : 0	Eucaryotes, widespread	0.26	− 0.36	0.17
I15:0	Gram-positive bacteria	− 0.62**	− 0.62**	− 0.02
A15:0	Gram-positive bacteria	− 0.68***	− 0.63**	0.55*
15 : 0	Bacteria	− 0.19	0.04	0.01
i16:0	Gram-positive bacteria	− 0.53*	− 0.69***	− 0.35
16:1ω7	Gram-negative and anaerobe bacteria	0.18	0.61**	0.24
16:1ω5	VA mycorrhiza fungi or bacteria	− 0.63**	− 0.67***	− 0.89***
16 : 0	Eucaryotes, widespread	− 0.12	0.01	0.08
i17:0	Gram-positive bacteria	− 0.74***	− 0.79***	− 0.76***
a17:0	Gram-positive bacteria	− 0.5*	− 0.65**	0.63**
cy17:0	Gram-negative or anaerobe bacteria	0.2	0.63**	− 0.23
17:0	Bacteria	− 0.26	− 0.74***	− 0.75***
18:3ω6	Fungi	0.55*	0.49*	0.47*
18:2ω6	Fungi	0.15	0.04	− 0.41
18:1ω9c	Bacteria, fungi, plants	0.35	0.25	0.38
18:1ω9t	Bacteria, fungi, plants	0.23	0.35	− 0.76***
18:0	Eucaryotes, widespread	0.28	− 0.17	− 0.09
cy19:0	Gram-negative or anaerobe bacteria	− 0.75***	− 0.49*	− 0.86***
20:4ω6	Eucaryotes, widespread	− 0.24	− 0.27	− 0.73***
20:5ω3	Algae	0.28	− 0.16	− 0.77***
20:0	Eucaryotes, widespread	− 0.13	0.08	− 0.12
22:1ω9	Fungi, plants	0.23	0	0.02
22:0	Plants	0.32	0.5*	− 0.13
24:1ω9	Eucaryotes	0.24	0.13	
24:0	Plants	− 0.65**	− 0.59**	− 0.57*

*,**,***with $P < 0.05$, 0.01, 0.001, respectively. For denoted predominant origin see Materials and methods.

watered sites, but remained constant in the nonwatered site. Exclusively in VW the PLFAs 16:1ω7, cy17:0 and 22:0 reached higher proportions with depth.

Multivariate analysis of PLFA patterns and soil parameters

RDA and Monte Carlo permutation test of PLFA proportions (%) and LOG transformed environmental variables separated the watered sites from the nonwatered site, with HST and VW being closer related to each other compared with BW (Fig. 2a and b). The first axis ($F = 14.01$, $P = 0.001$) explained 56.1% and the second axis 14.5% of the variation in the PLFA and soil parameter data set. Gram-positive bacterial PLFAs (iso/anteiso) were more prominent in the nonwatered site, whereas monoenoic PLFAs, including the stress attributed *trans* version of 18:1ω9 and cy17:0, with the latter as marker for Gram-negative and anaerobe bacteria, occurred predominantly in the watered sites. Higher DOC concentrations ($F = 12.04$, $P = 0.001$) in BW, and higher pH values ($F = 3.13$, $P = 0.009$) and SO_4^{2-} concentrations ($F = 2.7$, $P = 0.017$) in HST and VW, significantly contributed to the separation of watered and nonwatered sites (Fig. 2b). Higher microbial biomass, expressed as sum of PLFAs ($F = 2.35$, $P = 0.038$), as well as higher carbon concentrations ($F = 1.9$, $P = 0.087$) separated the top soil (0–100 cm depth) from deeper soil layers, which is supported by the contrasting vector of depth ($F = 3.66$, $P = 0.005$).

Two-way nested ANOSIM ('depth' nested in 'field') revealed different PLFA patterns between all fields (global $R = 0.608$, $P = 0.001$; HST-VW: $R = 0.445$, $P = 0.002$; HST-BW: $R = 0.667$, $P = 0.002$; VW-BW: $R = 0.751$, $P = 0.001$) and at all depths (Global $R = 0.41$, $P = 0.001$). Grouping PLFAs into the depth compartments surface soil (0–100 cm), upper vadose zone (100–220 cm) and lower vadose zone (220–340 cm) indicated distinct differences due to water recharge practice by one-way ANOSIM and nMDS (Fig. 3a–c). In the surface soil all fields displayed comparable PLFA profiles, i.e. microbial community patterns (global $R = 0.115$, NS; Fig. 3a). In the upper vadose zone, the model was significant (global $R = 0.63$, $P = 0.001$) and separated the watered sites from the nonwatered site (HST-VW: $R = -0.085$, NS; HST-BW: $R = 0.939$, $P = 0.002$; VW-BW: $R = 0.974$, $P = 0.002$; Fig. 3b). This corresponds to the differences in microbial community pattern of the lower vadose zone (global $R = 0.41$, $P = 0.001$; HST-VW: $R = -0.074$, NS; HST-BW: $R = 0.504$, $P = 0.002$; VW-BW: $R = 0.807$, $P = 0.001$; Fig. 3c).

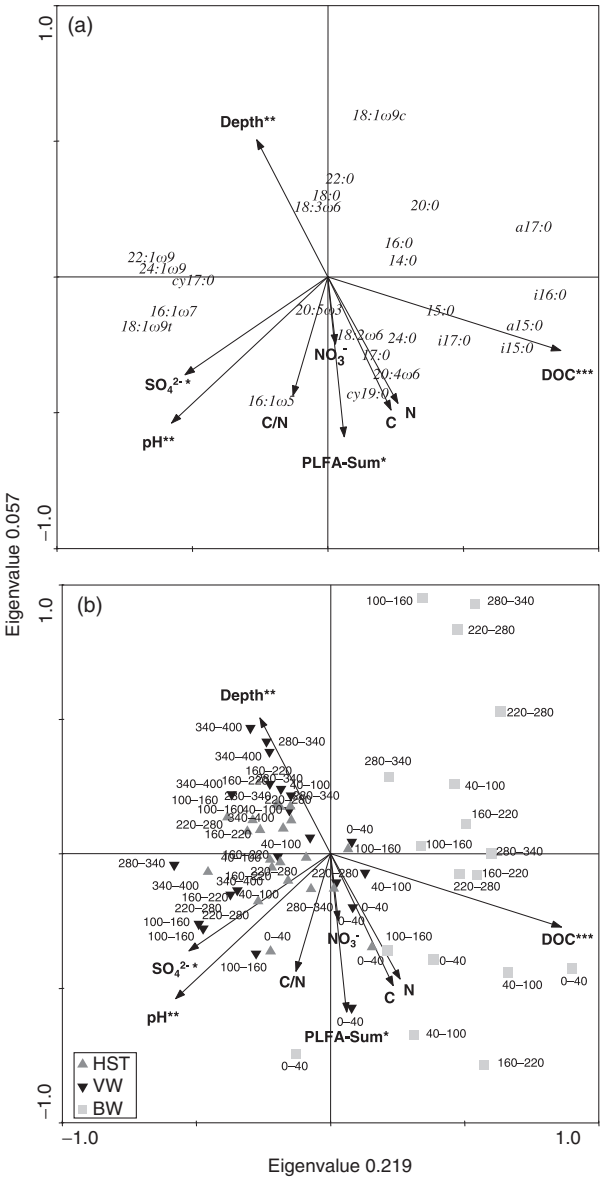

Fig 2. RDA ordination biplots of phospholipid fatty acid pattern (%) and LOG transformed soil parameter data. (a) Species score plot with individual PLFAs, (b) sample score plot. *, **, ***Significant contribution to separation with $P < 0.05$, 0.01 and 0.001, respectively.

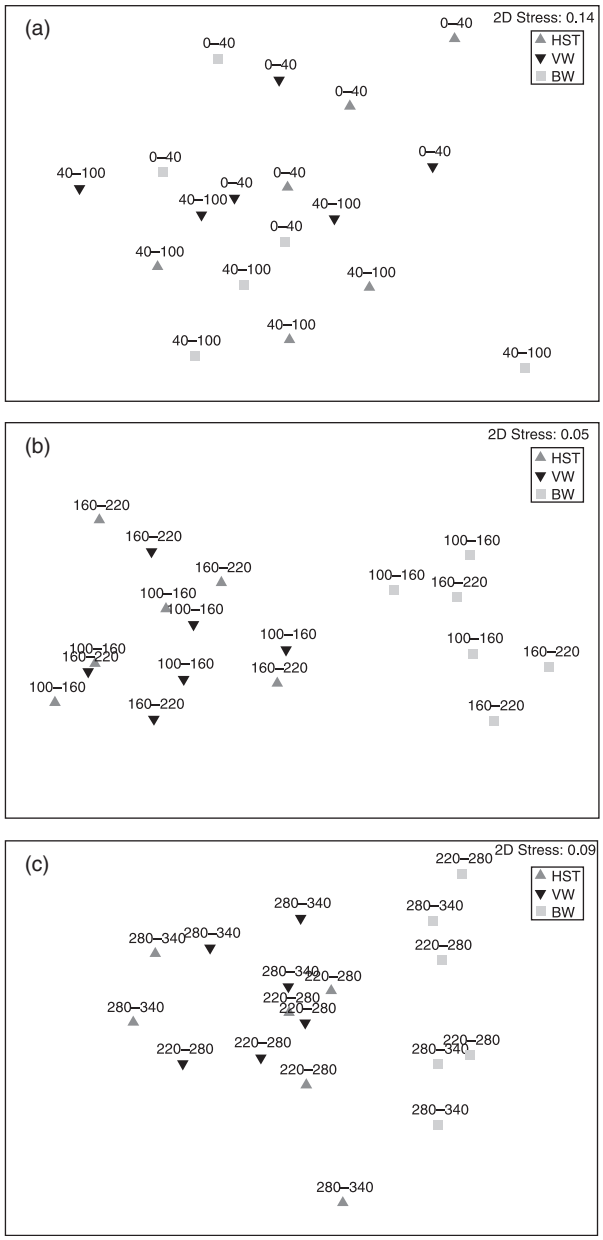

Fig 3. nMDS of the watered sites HST and VW, and the nonwatered site BW in three depths based on the proportion (%) of individual PLFAs. (a) Shallow soil layer: 0–100 cm. (b) Upper vadose zone: 100–220 cm. (c) Lower vadose zone: 220–340 cm. Similarity index: Bray curtis.

PLFA indicator ratios

Generally, all PLFA-based indices applied showed no differences between sites in the top soil layer from 0 to 100 cm (Table 2). Moreover, we found no differences in the fungal to bacterial PLFA ratio with depth or field (Tables 1 and 2). On the other hand, the ratio of PLFAs reflecting the contribution of Gram-positive to Gram-negative bacteria increased strongly with soil depth in BW, whereas it slightly decreased in HST and VW. The UI, a measure of membrane fluidity, showed a reversed pattern in the upper vadose zone (100–220 cm depth) with significantly lower values in the nonwatered site BW. The iso to anteiso ratios of the PLFAs 15:0 and 17:0, which denote changes in temperature and oxygen conditions, strongly decreased in BW, but not in the watered sites. Differences were most pronounced in the upper and lower vadose zone for i/a15:0 with higher values in watered compared with nonwatered sites. Generally, the i/a17:0 ratio had highest values in HST, followed by VW and the nonwatered site BW. The stress indicating *trans*/*cis* ratio

of 18:1ω9 decreased with depth in BW, but remained constant in HST and VW, resulting in significantly higher ratios in the watered sites in the upper and lower vadose zone (Table 2).

Discussion

The physical and chemical soil characteristics at all sites showed a strong depth dependency. This was particularly evident for DOC, C, N, NO_3^- and SO_4^{2-}, suggesting mineralization processes in the surface soil. DOC, as a measure for groundwater quality, was reduced to about 86% in all fields, likely due to biodegradation and sorption processes (Thurman, 1985). However, DOC concentrations of the nonwatered site BW significantly exceeded both watered sites. We hypothesize this to be caused by the artificial groundwater recharge procedure, where higher water turnover rates may function as flush, diluting river- and litter-borne DOC in the interstitial water of flooded fields.

In contrast to DOC, SO_4^{2-} and NO_3^- concentrations in watered exceeded those of nonwatered sites, which was also apparent by the occurrence of nitrogen indicator plants such as *U. dioica*, *R. caesius/fruticosus*, and *G. urbanum*. That also applies to the groundwater received from the area in relation to river water (-71% for DOC, but $+19\%$ and $+60\%$ for SO_4^{2-} and NO_3^-, respectively; Rüetschi, 2004). This indicates that the nutrient input due to water recharge is too high for an entire degradation from surface soil to groundwater table. Moreover, the observed increase in SO_4^{2-} and NO_3^- could be a result of ammonia and sulfide oxidation after release of these compounds during DOC mineralization. On average pH values in both watered sites tended to be higher as in the nonwatered site, most pronounced in deeper soil layers. Apparently, this is caused by the regular flooding with Rhine water at a relatively constant pH value of 8.2 (Waterworks Basel, Rhine water monitoring).

High recharge-vadose zones are characterized by high numbers of microorganisms due to the amendment with metabolizable organic substrates (Balkwill *et al.*, 1998; Ludvigsen *et al.*, 1999; Kieft & Brockmann, 2001). However, in the present study microbial biomass did not differ across sites and depths 4 weeks after the stop of flooding. In contrast, submerged surface soil (0–10 cm) at the 'Lange Erlen' had higher microbial biomass compared with the nonwatered site (Schütz *et al.*, 2008) indicating that differences due to recharge treatment may occur. Overall, this indicates that at comparable biomass to the nonwatered field, the microorganisms at the watered fields effectively degraded supplementary substrate inputs, suggesting a more active microbial community at these sites. Presumably, the improved nutritional status led to a rapid degradation of DOC by microorganisms using the additional nutrients as cosubstrates. This is in line with Aelion & Bradley (1991), who found biodegradation of hydrocarbons to be best in the presence of NO_3^-.

PLFAs are essential components of microbial membranes and therefore, changes in PLFA compositions directly reflect changes in the active microbial community to altered soil conditions (Frostegård *et al.*, 1993a, b; Zelles, 1999). Biomarker PLFAs of selected members of soil microbial communities have long been recognized and employed in chemical taxonomy (White *et al.*, 1996; Morgan & Winstanley, 1997; Lechevalier & Lechevalier, 1998). However, despite the proposed primary origin, some of these fatty acids may be found in smaller amounts in lipids of other organisms, too. The microbial community structure, determined by PLFA patterns, differed between fields, predominantly between watered and nonwatered sites in the vadose zone. Either translocation of microorganisms by flooding or river water ingredients may have supported distinct microbial populations in watered sites.

In contrast to common slow sand filter plants at the investigated recharge site no biofilm matrix is present, as the shallow soil layer consists of loess loam, with litterfall and root exudates enhancing DOC concentrations (Rüetschi, 2004). Thus, the microbial community living below this uppermost soil compartment likely is responsible for biodegradation and water purification. Rüetschi (2004) showed highest purification rates in the vadose zone assessed by breakthrough curves of DOC and SAK254 (spectral adsorption coefficient at 254 nm). The importance of vadose zone microorganisms in these purification processes is strongly supported by our findings.

With increasing depth, the proportion of the bacterial PLFAs 16:1ω5, 16:1ω7, cy17:0 and 18:1ω9t, and the long chained PLFAs 22:1ω9 and 24:1ω9 became more prominent in the flooded sites, whereas the proportions of the branched, saturated PLFAs i15:0, a15:0, i16:0 and a17:0, as representatives for Gram-positive bacteria, were more dominant in the nonwatered site. Bossio & Scow (1998) reported PLFA patterns of a rice field soil but contradicting to our results they observed reduced monoenoic PLFAs and increased branched PLFAs as respond to flooding and organic matter input. However, their studies are limited to the top soil layer, and therefore not directly comparable to our findings in the vadose zone. On the other hand, water stress, as investigated by Williams (2007) in irrigated and drought-prone prairie soils (0–10 cm depth), led to an increase in 16:1ω5 and 18:1ω9t, and a decrease in 15:0 and a17:0, which corresponds to our results. The two long chained PLFAs 22:1ω9 and 24:1ω9, which increased in the vadose zone at the watered sites, are usually not found in bacteria. Moreover, they are both scarcely described and their occurrence at depth remains obscure.

Relative to Gram-negative bacteria, Gram-positive bacteria become more abundant with depth (Blume *et al.*, 2002;

Fierer *et al.*, 2003). This is particularly evident in the vadose zone as determined by microscopic (Balkwill & Ghiorse, 1985) and plate count studies (Colwell, 1989). These findings are in line with the composition of the microbial community at the nonwatered site but contrast that of the watered sites. Generally, Gram-negative bacteria show a positive correlation with moisture (Kieft & Brockman, 2001). Moreover, they are considered to be fast growing, to utilize a variety of C sources, and to adapt quickly to changing environments (Ponder & Tadros, 2002; Hinojosa *et al.*, 2005). Environmental conditions, e.g. nutrients and moisture, regularly alternate with flooding, which likely fostered Gram-negative bacteria, most pronounced in the upper vadose zone. Presumably, this is the main water purification zone within the depth transect.

Generally, increasing *trans/cis* or cyclo/precursor ratios of unsaturated PLFAs are used as indicators for stress in microorganisms (Guckert *et al.*, 1986; Heipieper *et al.*, 1992; Kieft *et al.*, 1994; White *et al.*, 1996). Thereby the term 'stress' is used in a broad sense and comprising exposure to toxins, starvation or desiccation. At the watered study sites the *trans/cis* ratio of 18:1ω9 exceeded the nonwatered site below 100 cm. Firstly, considerable amounts of bacteria can be transported from soil to groundwater (John & Rose, 2005). Microorganisms translocated by water recharge will be exposed to altered environmental conditions and to enhanced competition with established species, resulting in the higher stress values observed (Balkwill *et al.*, 1998). Secondly, the residing microbial community at depth may be affected by alterations in oxygen availability or ion concentrations. In contrast, at the nonwatered site bacterial movement through macropores is limited and environmental conditions are more stable. Thus, transport and related changes in environmental conditions appear to be the main factors for increasing or constantly higher stress in the vadose zone. This is in line with findings of Bossio & Scow (1998) where flooding with river water did not affect stress indicators (*trans/cis* and cyclo/precursor ratios) in the shallow soil layer.Changes comparable to the *trans/cis* ratio were observed for the iso/anteiso ratio of 15:0 and 17:0. Increasing iso/anteiso ratios were reported at higher temperatures, due to the maintenance of membrane fluidity (Kaneda, 1991; Petersen & Klug, 1994). In the watered sites, soil temperature is periodically determined by the infiltrating Rhine water. Flooding leads to enhanced (+8 °C compared with air temperature in November) and more homogenous temperatures within the soil profile throughout a year (Rüetschi, 2004). However, it should be noted that some Gram-negative, anaerobe SO_4^{2-} reducing bacteria (e.g. *Desulfovibrio, Desulfobacter*, *Desulfobulbus*) also contain *iso* and *anteiso* PLFAs, with a predominance of iso series (Parkes & Taylor, 1983; Taylor & Parkes, 1983; Kaneda, 1991). Higher iso/anteiso ratios, therefore, could also be caused by the presence of SO_4^{2-} reducers due to periodically anaerobic conditions during flooding but the persistence of electron acceptors such as NO_3^- and SO_4^{2-} at depth points against the existence of anaerobic microniches.

The UI showed significantly higher ratios in the upper vadose zone of the watered sites. A low UI indicates C limitation, or more generally, nutrient limiting conditions in soil (Zelles *et al.*, 1994; Kieft *et al.*, 1997; Bossio & Scow, 1998). This supports our hypothesis of higher nutrient input and turnover in this biologically active layer of the watered sites.

Conclusions

Groundwater recharge performed in the 'Lange Erlen' distinctly affected community structure, physiological status and metabolic activity of the soil-inhabiting microorganisms. These changes likely derive from enhanced resource availability and altered environmental conditions due to flooding with river Rhine water. Differences between watered and nonwatered sites were most pronounced in the upper vadose zone (100–220 cm), but still apparent in the lower vadose zone (220–340 cm). This indicates, that a biologically active layer has developed below the rooted zone, likely responsible for water purification. Besides nutrient input, flooding may cause passive transport of microbial cells as well as changes in temperature and aerobic/anaerobic conditions. Hence, the competitive ability of each microorganism determines the microbial community, which led to the establishment of groups well adapted to the specific resources and environment.

Microbial biomass was not significantly enhanced throughout the soil profile at the watered sites, indicating the community at the 'purifications sites' in the upper vadose zone to be highly active. Nutrient limitations (e.g. nitrogen) was reversed at watered sites and allowed a specialized microbial community to effectively degrade organic compounds. The slightly increased NO_3^- and SO_4^{2-} concentrations in the groundwater of the recharge site may be a hint that the inhabiting microbial population is working under full capacity. This gives evidence, that the system may well function for another century or longer.

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Supporting Information

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

Table S1. Average dissimilarity and contribution of single pospholipid fatty acids PLFAs (%) to dissimilarities between study sites in six depths (0–40 40–100, 100–160, 160–220, 220–280 and 280–340 cm).

Fig. S1. Phospholipid fatty acid pattern (PLFA in % ± SD, proportion > 1%) in six different depths (0–40, 40–100, 100–160, 160–220, 220–280 and 280–340 cm) at the study sites.

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