RESEARCH ARTICLE

Functional microbial community response to nutrient pulses by artificial groundwater recharge practice in surface soils and subsoils

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Abstract

Subsurface microorganisms are essential constituents of the soil purification processes associated with groundwater quality. In particular, soil enzyme activity determines the biodegradation of organic compounds passing through the soil profile. Transects from surface soil to a depth of 3.5 m were investigated for microbial and chemical soil characteristics at two groundwater recharge sites and one control site. The functional diversity of the microbial community was analyzed via the activity of eight enzymes. Acid phosphomonoesterase was dominant across sites and depths, followed by L-leucine aminopeptidase and β-glucosidase. Structural [e.g. phospholipid fatty acid (PLFA) pattern] and functional microbial diversities were linked to each other at the nonwatered site, whereas amendment with nutrients (DOC, NO_3^-) by flooding uncoupled this relationship. Microbial biomass did not differ between sites, whereas microbial respiration was the highest at the watered sites. Hence, excess nutrients available due to artificial groundwater recharge could not compensate for the limitation by others (e.g. phosphorus as assigned by acid phosphomonoesterase activity). Instead, at a similar microbial biomass, waste respiration via overflow metabolism occurred. In summary, ample supply of carbon by flooding led to a separation of decomposition and microbial growth, which may play an important role in regulating purification processes during groundwater recharge.

Introduction

Subsurface microbial, chemical and physical soil processes directly influence groundwater chemistry by the biodegradation, adsorption and dilution of organic and inorganic matter. These mechanisms are essential for purification processes associated with drinking water production. The latter is performed by artificial groundwater recharge near Basel (Switzerland) in a former floodplain area called 'Lange Erlen' where embanked forest sites are periodically (maximum 10 days) flooded with water from the river Rhine. In contrast to the commonly used unvegetated, slow sand filter systems (Duncan, 1988; Weber-Shirk & Dick, 1997; Peters *et al.*, 1998), the recharge area in the 'Lange Erlen' comprises no biofilm, but shows constant and satisfactory purification capacities since the system was established in 1912 (Rüetschi, 2004). This calls for studies to assess the function of subsoil microorganisms in the transformation and degradation of both natural and xenobiotic compounds passing through this unique recharge system.

Kandeler *et al.* (1996) hypothesized that the microbial community composition of a soil determines its potential for substrate catalysis, because most of these processes are driven by extracellular or intracellular enzymes. Soil enzyme activity is therefore a measure of the metabolic requirements and available nutrients for soil microbial communities. Soil enzymes are produced by microorganisms to decompose low-molecular-weight compounds and complex insoluble polymers (e.g. cellulose, chitin, protein) to dissolved organic matter. This enzyme-driven breakdown predicts a flow of low-molecular-weight substrates [dissolved organic carbon (DOC), dissolved organic nitrogen (DON)] suitable for

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microbial uptake (Schimel & Weintraub, 2003). Free enzymes are short-lived; however, a considerable proportion may be bound to clay and humic colloids in soil, presenting an accumulated enzyme fraction, which persists even under harsh conditions, where microbial activity is inhibited (Burns, 1982; Sarkar *et al.*, 1989; Nannipieri *et al.*, 2002; Marx *et al.*, 2005).

Flooding in groundwater recharge areas and the load of organic material herewith affect mineralization and degradation by microorganisms. During the initial phase (1–7 days), Burns & Ryder (2001) reported strong temporal peaks in *absolute* enzyme activities, followed by a general decline (day 7–21), likely caused by substrate limitation or inhibition by end products of hydrolysis. During constant flood treatments in wet prairie macrocosms, Mentzer *et al.* (2006) showed increased *specific* enzyme activity (*absolute* enzyme activity divided by microbial biomass), whereas *absolute* enzyme activity was affected only slightly. They suggested that an increase in either the microbial activity or the mobility of extant soil enzymes was responsible for these effects. Overall, the strong positive correlation between DOC concentration and β-glucosidase activity (Burns & Ryder, 2001) provides evidence that DOC is a suitable substrate in groundwater recharge systems.

Despite their importance for the biodegradation of organic matter in water purification processes, studies on enzymes at soil depth are scarce (e.g. Taylor *et al.*, 2002; Venkatesan & Senthurpandian, 2006). To the best of our knowledge, no investigation combining the impact of water recharge with soil depth on soil enzyme activities exists. The present study, with soil samplings in autumn 2005, assesses the microbial communities from the surface soil to the vadose zone (approximately 3.5 m depth) at two groundwater recharge sites and one nonwatered site. Additionally, physical and chemical soil parameters were analyzed. Microbial biomass was estimated using substrate-induced respiration (SIR) and microbial respiration by the O_2 consumption rate. The functional diversity of microbial communities was investigated by the activity of hydrolytic enzymes involved in the carbon (C), nitrogen (N) and phosphorus (P) cycle using a fluorimetric microplate assay. Moreover, as previous work revealed an altered microbial community structure due to periodic flooding (Schütz *et al.*, 2009), this study relates structural and functional diversity. We hypothesize that enzyme activities are higher at the groundwater recharge sites due to substrate supply, which prompts their production and/or the corresponding growth of the microbial population. Physicochemical differences in the soil environment due to flooding likely modify these processes by the activation of enzymes immobilized in soil. Hence, water recharge practice is expected to result in distinct enzyme patterns along the soil depth transect that mirror the degradation of organic matter as well as purification processes.

Materials and methods

Study sites

The study area 'Lange Erlen' has served as a groundwater recharge area for the city of Basel (Switzerland) since 1912. Today, approximately half of the drinking water for the city ($15 \times 10^6\ m^3\ ha^{-1}\ year^{-1}$) is obtained from this site. The floodplain area ($\sim 3\ km^2$) extends along the river Wiese, a straightened tributary of the river Rhine, descending from the Black Forest, Germany. The semi-natural forest sites are classified as *Galio–Carpinetum* (i.e. oak–hornbeam) forests (Burnand & Hasspacher, 1999). Along with water recharge processes, the floodplain area has been modified by human activities, predominantly by landfill and afforestation (*Populus canadensis*, *Fraxinus excelsior*, *Alnus nigra*, *Salix* spp.).

The first investigation site 'Hintere Stellimatten' (W1) started operating for groundwater recharge after afforestation in 1977. Because of excess infiltration rates ($11\ m^3\ m^{-2}\ day^{-1}$), an artificial landfill (loess loam) and bulldozing were performed in 1981. The site is mainly covered by alder swamp forest (*P. canadensis*, *A. nigra*, *Salix* spp.) and the herb layer is dominated by *Rubus caesius* and *Urtica dioica*. Sparsely vegetated sites comprise some spots of *Poa trivialis*, *Potentilla reptans*, *Agropyron repens* and *Geum urbanum*. The second investigation site, 'Verbindungsweg' (W2), was established as a flooding area after afforestation and artificial landfill (loess loam) in 1970. 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*, *Rubus caesius/fruticosus*, *Duchesnea indica*, *Iris preudacorus*, *Seneco aquaticus* and *Cardamine pratensis*. The shady northern part is dominated by poplars and the soil bare of vegetation. The nonwatered control site 'Bachtelenweg' (NonW), a grassland under extensive management practice, is situated between these two flooding sites.

Soil parameters

The investigated field sites differed in the soil texture predominantly in the upper soil layers. Landfill loess loam expanded to about 160 cm depth at W1, whereas it constituted only the top soil layer (0–40 cm) at W2 and NonW. Subsequently, sand and gravel followed at all sites down to 400 cm depth. Interspersed silt and clay lenses were equally present at W1 in 160–280 cm depth and scattered clay parts were present at W2 in 280–340 cm depth. At NonW clay lenses were primarily observed below 160 cm depth. Partial Mn depositions and rust spots were detected in deeper soil layers at all sites, indicating periodical water logging and anoxic conditions. The pH values ($CaCl_2$) at watered sites ranged between 7.4 and 7.2 and between 7.1 and 6.6 at the nonwatered site. The average pH value of the river Rhine water used for flooding was 8.2. The total soil C content

[% dry weight (DW) soil] ranged from 2.14 to 0.25 and decreased with depth to about 88% and 82% in the watered and nonwatered sites, respectively. The soil N content (% DW soil) ranged from 0.23 to 0.03 and decreased with depth by about 80% at all fields.

Infiltration system

The forested groundwater recharge sites (11 sites, total area 22 ha) are periodically flooded with prefiltered water from the river Rhine. Prefiltration is performed by a rapid sand filter (80-cm quartz sand layer), where approximately 95% of complex particulate matter is eliminated. The prefiltered water contains 1.7 mg DOC L^{-1}, 6.0 mg NO_3^- L^{-1} and 0.12 mg P L^{-1} (Rüetschi, 2004). Watering cycles usually consist of 10 days of flooding and 20 days of desiccation and regeneration, but longer interruptions due to revisions occur. Depending on the soil surface structure, the water fills to variable heights (maximum 50 cm) and drains at a speed of 1–2 m day^{-1} through the soil surface. After flooding for 2–3 days, the groundwater table rises from 4–5 to 2–3 m soil depth. The groundwater flows horizontally in the aquifer for 200–800 m. After 10–30 days, purified water is pumped out of groundwater wells, collected and, after a brief chemical treatment with ClO_2, delivered to consumers.

Soil sampling

Three vertical core drillings (diameter 25 cm) from the soil surface down to a maximum of 3.5 m depth were conducted at each watered site (W1, W2) in November 2005. Four weeks before drilling, the flooding regime was stopped to lower the groundwater level. Soil cores were excavated at 0.5-m intervals and placed carefully into soil core boxes, representing the full soil profile along the depth transect. Soil samples (100 g) were taken at 30-cm distances using 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, W1 and W2. During drain installations at NonW, three horizontal soil samplings through the inner part of the trench down to 3.5 m depth were performed in the same time period. Three depth transects were randomly chosen and 34 soil samples were directly taken from the inner trench wall and frozen (− 20 °C) until analysis. Before all analyses, the required soil substrate was thawed at 8 °C for 24 h. Visible organic material (fine roots and leaves) and stones were removed, and soil was sieved (5 mm).

Soil analysis

Chemical and physical soil characteristics

Soil samples were analyzed for soil pH, moisture, C, N, DOC, nitrate (NO_3^-) and sulfate (SO_4^{2-}) content. 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 after sieving by the mass difference before and after drying at 105 °C for 24 h. The total C and N contents were determined from oven-dried and pulverized (swing mill, Retsch MM 200) aliquots of the bulk soil using an element analyzer (CHN 1000, Leco). Results are expressed as % per soil dry weight.

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 200–350 cm depths. A soil salt (0.01 M $CaCl_2$) solution was prepared (1 : 4 w/v), shaken for 1 h (2.24 g) and centrifuged for 15 min (358 ***g***). The supernatant was filtered through sterile 0.45-μm membrane filters (Millex HA) and stored at 8 °C until analysis (maximum 1 h). The DOC content was analyzed after acidification and air-purging (N55, O45, Carbagas, CH) using a TOC analyzer (TOC-5000 A, Shimadzu) in quintuplicate. NO_3^- and SO_4^{2-} were measured in an ion chromatograph (IC-690, Metrohm). All results are expressed as mg g^{-1} DW soil.

Microbial biomass and respiration

Microbial biomass (C_{mic}) and respiration (O_2) were determined after incubating soil for 3 days at room temperature (18 °C) using an automated electrolytic O_2 microcompensation apparatus (Scheu, 1992). For the 0–40 cm depths, 2 g, and for all other depths 4 g soil (DW) was taken to compensate for the decrease in microbial biomass with depth. Basal soil respiration was determined as the average O_2 consumption rate (μL O_2 g^{-1} h^{-1}) at 22 °C, with readings every 30 min after equilibration at 10–20 h. Microbial biomass was determined by SIR. Soil samples were supplemented with 8 mg glucose g^{-1} DW, which was added as an aqueous solution, adjusting the water content to 80% of the water-holding capacity. The mean of the four lowest measurements was taken as the maximum initial respiratory response (MIRR) and microbial biomass C (μg C_{mic} g^{-1} DW) was calculated as 38 × MIRR (μL O_2 g^{-1} h^{-1}) (Beck *et al.*, 1997).

Enzyme analyses

A range of hydrolytic enzymes involved in C, N and P processes in soil were investigated using a fluorimetric microplate assay (Marx *et al.*, 2001). The activities of α-D-glucosidase, β-D-glucosidase, *N*-acetyl-β-glucosaminidase, β-xylosidase, β-cellobiohydrolase, acid phosphomonoesterase, L-leucine-aminopeptidase and L-tyrosine-aminopeptidase were measured using 4-methylumbelliferon (MUB)- and 7-amido-4-methyl-coumarin (AMC)-labeled substrates. Enzyme activities were determined as the release rate of fluorescent MUB and AMC from the MUB- or AMC-labeled substrates, respectively.

Aqueous soil suspensions (fresh weight soil : water = 1 : 50 at 0–40 cm depth and 1 : 25 at deeper layers, to compensate for biomass decrease with depth) were prepared from each soil sample. Of each, 50 μL was transferred in triplicate into the vials of a 96-well microplate and 50 μL of buffer (carbohydrases, acid phosphomonoesterase: 100 mM MES acid buffer, pH 6.1, aminopeptidase: 50 mM Trizma buffer, pH 7.8) and 100 μL substrate were added, yielding a final substrate concentration of 500 μM. Microplates were incubated at 30 °C and the release of MUF and AMC was measured after 0, 30, 60, 120 and 180 min using an automated luminescence spectrophotometer (Biolumine™, 960 Molecular Dynamics; emission 446 nm, excitation 377 nm). The fluorescence produced was converted into an amount of MUB/AMC according to a soil-specific standard calibration. This consisted of increasing amounts of substrates (0, 10, 20, 50, 80 and 120 μL) and constant amounts of soil solution (50 μL), and corrects for the possible quenching effects on the fluorescence intensity of MUB/AMC. Enzyme activities were reported as nmol MUB/AMC g^{-1} h^{-1} DW soil. The *specific* enzyme activity was calculated by dividing the measured *absolute* enzyme activity by the microbial biomass of the respective sample and is reported as nmol MUF/AMC g^{-1} h^{-1} C_{mic} DW soil.

Statistical analyses

For statistical analyses, data of single soil layers were merged into six depths to represent the major soil strata: 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 and 280–340 cm. Statistical analysis was performed on log transformed data. Soil nutrients, microbial biomass and respiration, and enzyme activities were analyzed for site and depth differences using repeated ANOVA procedures. A pairwise comparison of means was performed using Tukey's honestly significance difference (HSD) test. Repeated measure ANOVA and Tukey's HSD were calculated using SAS (SAS Institute, Cary, NC). Site differences are statistically confirmed (three replicates per site), treatment, i.e. flooding differences are based on a pseudoreplicated design (2 × 3 replicates for watered sites and three replicates for the nonwatered site) and were concluded from the results.

Site differences were analyzed further using the Monte Carlo permutation test (MCPT). Interactions between the structural and the functional diversity of the microbial communities at each site were analyzed by MCPT. Soil phospholipid fatty acid (PLFA) patterns (% nmol, data in Schütz *et al.*, 2009) were used as a measure of structural diversity and were related to the *specific* enzyme activity. MCPT was calculated using CANOCO (CANOCO 4.5, Lepš & Šmilauer, 2003).

Results

Soil nutrient status

The total soil C content, DOC, NO_3^- and SO_4^{2-} decreased significantly with soil depth at all sites (Table 1) and significant site and watering effects were detected (Fig. 1). The DOC content at NonW was the highest throughout the entire soil profile, with an average difference of 15-fold compared with watered sites. DOC ranged from 2.2 mg g^{-1} DW (W1) to 42.1 mg g^{-1} DW (NonW) in the top soil layer, and from 0.2 mg g^{-1} DW (W1) to 6.0 mg g^{-1} DW (NonW) at the 280–340 cm depth. This corresponds to reduction rates between 85% and 87% at all sites along the depth transect. The amount of NO_3^- in the 40–220 cm depth and of SO_4^{2-} in the 40–340 cm depth at both watered sites exceeded that of NonW. The NO_3^- concentrations in the top soil layer were 2.3 mg g^{-1} DW (W1), 3.1 mg g^{-1} DW (W2) and 0.5 mg g^{-1} DW (NonW). The initial NO_3^- concentrations in the top soil layer were reduced by about 97% in the lower vadose zone at W1 and W2, whereas the decrease at NonW was less (71%). SO_4^{2-} concentrations in the top soil layer were 2.7, 2.5 and 0.8 mg g^{-1} DW, and decreased with depth by 34%, 47% and 21% in W1, W2 and NonW, respectively.

Table 1. Repeated measure ANOVA table [*F*-values and degrees of freedom (d.f.)] on the effect of site (watered: W1, W2; nonwatered: NonW) and soil depth (0–40, 40–100, 100–160, 160–220, 220–280 and 280–340 cm) for soil nutrients, microorganisms and enzyme activity

	Within-subjects effects		Between-subjects effects
	Depth (d.f. 5)	Depth × site (d.f. 10)	Site (d.f. 2)
Soil nutrients			
C (%C)	24.66***	0.75	2.61
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***
Microorganisms			
C_{mic} (μg g^{-1} DW)	34.24***	1.82	2.65
O_2 (μL g^{-1} h^{-1} DW)	16.66***	1.17	7.73*
Enzyme activity (nmol h^{-1} g^{-1} DW)			
α-D-Glucosidase	18.02***	1.88	1.74
β-D-Glucosidase	50.3***	3.33***	11.78***
Cellobiohydrolase	76.76***	4.98***	5.2*
N-acetyl-β-glucosaminidase	86.78***	4.43***	9.07*
β-Xylosidase	86.96***	4.02***	4.13
Acid phosphomonoesterase	27.25***	2.71*	7.35*
L-Leucine-aminopeptidase	63.47***	1.24	4.12
L-Tyrosine-aminopeptidase	68.02***	1.83	3.86

*,***with $P < 0.05$, 0.001, respectively.

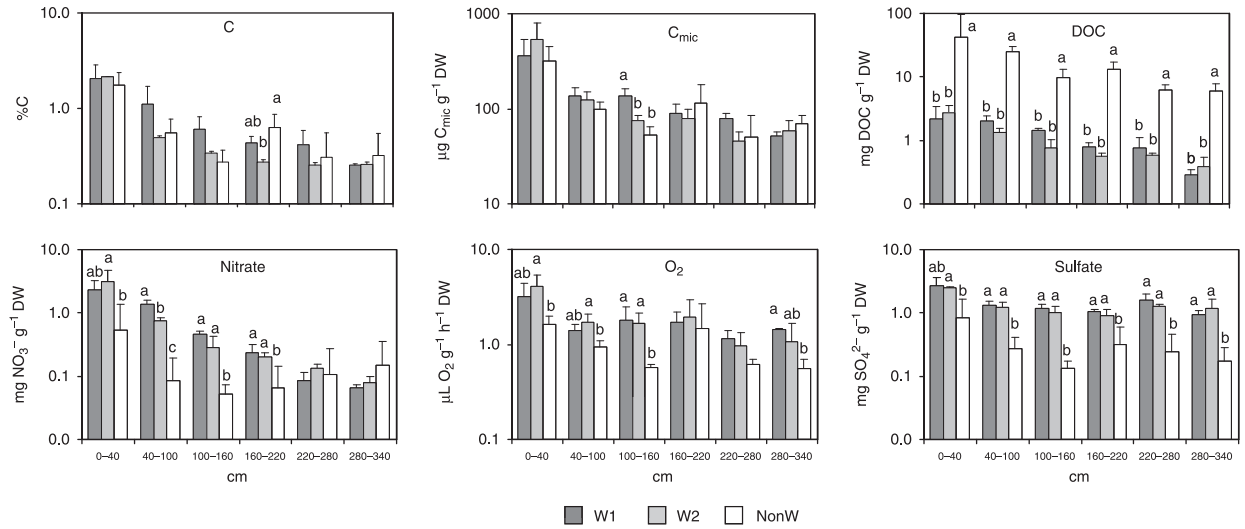

Fig. 1. Soil nutrients (C in %, DOC, NO_3^- and SO_4^{2-} in mg g^{-1} DW) and microbial community status (C_{mic} in µg g^{-1} DW and O_2 in µL g^{-1} h^{-1} DW) in six soil depths (0–40, 40–100, 100–160, 160–220, 220–280, and 280–340 cm) at the watered sites W1 and W2, and the nonwatered site NonW. Bars of the same depth sharing the same or no letter are not significantly different (Tukey's HSD, $P < 0.05$).

Biomass and activity of soil microorganisms

The microbial biomass ranged from 52 to 359, 58 to 538 and 70 to 320 µg g^{-1} DW soil at W1, W2 and NonW, respectively, and significantly declined with soil depth by 78–89% (Fig. 1, Table 1). The surface soil layer (0–40 cm) comprised an average of 42–58% of the total microbial biomass; hence, the second half of the microbial biomass (again 42–58%) was located in the 40–340 cm depth. Differences between sites were detected only in the 100–160-cm layer where the microbial biomass of W1 surpassed W2 and NonW (Fig. 1).

Microbial respiration, measured as the O_2 consumption rate, ranged between 3.2 and 1.4, 4.1 and 1.1 and 1.6 and 0.6 µL g^{-1} h^{-1} DW at W1, W2 and NonW, respectively (Fig. 1). Respiration rates were significantly lower in deeper soil layers (W1, − 55%; W2, − 74%; and NonW, − 65%), and generally higher in watered sites (Table 1). This was most pronounced in the top soil layer and the upper vadose zone, but effects were also present at the 280–340 cm depth (Fig. 1).

Enzyme activity

The activities of the following eight enzymes of the soil C, N and P cycle were analyzed: α-D-glucosidase, β-D-glucosidase, cellobiohydrolase, β-xylosidase, *N*-acetyl-β-glucosaminidase, L-leucine-aminopeptidase, L-tyrosine-aminopeptidase and acid phosphomonoesterase. The highest enzyme activities were found in the 0–40 cm depth, whereas only minor or no activity was measured in the 280–340 cm depth (Table 2). This corresponds to a decrease of almost 100% from the top soil to the groundwater table, and is reflected in significant depth gradients for all enzymes (Table 1). Significant overall field differences were detected most strongly for β-D-glucosidase, and further for cellobiohydrolase, *N*-acetyl-β-glucosaminidase and acid phosphomonoesterase. Distinct significant depth *x* site interactions occurred for β-D-glucosidase, cellobiohydrolase, *N*-acetyl-β-glucosaminidase and β-xylosidase, and less pronounced interactions were found for acid phosphomonoesterase. Below 220 cm, aminopeptidase and acid phosphomonoesterase were the most active enzymes, but no differences occurred between sites.

Acid phosphomonoesterase showed the highest activities at all sites throughout the soil depth transect (except W1, 0–40 cm; W2, 160–220 cm; NonW, 100–160 cm; Table 2). Generally, L-leucine-aminopeptidase was the second and β-D-glucosidase the third most active soil enzyme across soil depth transects and sites. At 40–100 cm depth, the activity of all enzymes at W1 exceeded those at W2 and NonW. With the exception of α-D-glucosidase and L-tyrosine-aminopeptidase, this pattern continued in the 100–160 cm depth. At the 160–220 cm depth, the enzyme activity of β-D-glucosidase was lower at W2 than at W1 and NonW, and lower than that of acid phosphomonoesterase at NonW.

Enzyme activity and soil nutrient status

Using a MCPT, soil nutrients and microbial parameters were related to the pattern of enzyme activities for the summed and separated sites (Table 3). MCPT for all sites showed significant correlations between microbial biomass (C_{mic}) and respiration (O_2) and the enzyme activity pattern (Table 3). Separate MCPT for each site revealed highly significant

Table 2. Enzyme activity (nmol g^{-1} h^{-1} DW soil, SD in parentheses) in six soil depths at watered (W1, W2) and nonwatered (NonW) sites

Enzyme activity (nmol g^{-1} h^{-1} DW)	Depth (cm)	W1	W2	NonW
α-D-Glucosidase	0–40	9.84 (7.63)	7.96 (5.53)	4.70 (1.89)
	40–100	2.81 (0.45)**a**	0.70 (0.66)**b**	0.64 (0.31)**b**
	100–160	0.90 (0.76)	0.02 (0.02)	0.00 (0.00)
	160–220	0.21 (0.35)	0.00 (0.00)	2.82 (2.27)
	220–280	0.35 (0.61)	0.24 (0.29)	0.94 (1.63)
	280–340	0.03 (0.04)	0.05 (0.08)	0.94 (1.62)
β-D-Glucosidase	0–40	**289 (166)**	**237 (164)**	**197 (132)**
	40–100	**116 (17.3)a**	**10.2 (7.34)b**	**20.3 (9.71)b**
	100–160	**20.8 (5.79)a**	1.40 (1.55)**b**	1.57 (1.36)**b**
	160–220	**10.9 (12.1)a**	0.38 (0.65)**b**	**10.7 (1.12)a**
	220–280	0.96 (0.67)	0.74 (0.18)	**7.90 (13.7)**
	280–340	0.53 (0.68)	1.27 (0.95)	**3.38 (5.32)**
Cellobiohydrolase	0–40	42.3 (23.6)	56.4 (61.1)	39.4 (29.3)
	40–100	20.7 (1.62)**a**	0.79 (0.58)**b**	1.90 (1.65)**b**
	100–160	2.94 (1.01)**a**	0.07 (0.12)**b**	0.00 (0.00)**b**
	160–220	0.85 (1.11)	0.00 (0.00)	1.01 (0.78)
	220–280	0.13 (0.22)	0.17 (0.15)	0.16 (0.28)
	280–340	0.00 (0.00)	0.00 (0.00)	0.40 (0.68)
N-acetyl-β-glucosaminidase	0–40	101 (53.0)	96.4 (68.2)	79.0 (54.2)
	40–100	55.7 (12.1)**a**	6.01 (5.41)**b**	8.55 (3.69)**b**
	100–160	10.6 (3.35)**a**	0.87 (1.04)**b**	0.05 (0.07)**b**
	160–220	7.34 (9.88)	0.16 (0.28)	2.25 (1.69)
	220–280	0.52 (0.21)	0.47 (0.34)	0.47 (0.81)
	280–340	0.13 (0.22)	0.19 (0.26)	0.65 (1.12)
β-Xylosidase	0–40	37.3 (14.2)	44.6 (35.2)	41.4 (28.5)
	40–100	21.5 (2.96)**a**	3.25 (2.78)**b**	4.15 (2.84)**b**
	100–160	4.40 (2.19)**a**	0.23 (0.34)**b**	0.06 (0.11)**b**
	160–220	1.29 (1.54)	0.01 (0.02)	1.40 (1.12)
	220–280	0.19 (0.33)	0.16 (0.18)	0.35 (0.60)
	280–340	0.00 (0.00)	0.08 (0.12)	0.58 (1.00)
Acid phosphomonoesterase	0–40	**260 (40.5)**	**323 (202)**	**299 (221)**
	40–100	**181 (12.1)a**	**44.5 (23.2)b**	**46.1 (33.7)b**
	100–160	**48.8 (21.0)a**	**6.91 (6.60)b**	**2.67 (1.38)b**
	160–220	**16.1 (7.22)ab**	**1.83 (2.85)a**	**47.5 (34.8)b**
	220–280	**8.89 (2.99)**	**3.95 (1.65)**	**14.7 (25.5)**
	280–340	**3.97 (0.17)**	**5.88 (0.98)**	**13.0 (20.4)**
L-Leucine-aminopeptidase	0–40	**280 (193)**	**206 (96.6)**	**271 (222)**
	40–100	**71.6 (23.1)a**	**23.4 (5.58)b**	**28.8 (10.2)b**
	100–160	**17.3 (4.39)a**	**5.76 (3.53)b**	**5.19 (1.69)b**
	160–220	**7.93 (4.14)**	**4.13 (2.48)**	**11.32 (5.10)**
	220–280	**3.30 (1.64)**	**2.71 (1.51)**	**3.95 (5.67)**
	280–340	**2.37 (0.47)**	**3.47 (0.20)**	**5.44 (4.84)**
L-Tyrosine-aminopeptidase	0–40	81.2 (48.2)	91.7 (65.5)	174 (129)
	40–100	39.4 (13.7)**a**	7.96 (2.33)**b**	15.3 (6.05)**b**
	100–160	7.93 (0.97)**a**	2.73 (0.91)**ab**	2.49 (1.68)**b**
	160–220	4.48 (2.12)	2.09 (0.75)	4.15 (2.39)
	220–280	**2.53 (1.79)**	**1.54 (0.28)**	1.36 (1.83)
	280–340	**1.10 (0.26)**	**1.63 (0.17)**	1.55 (1.96)

Values in bold are the three most active enzymes within the respective soil layer across all sites. Means within a row with the same or no letter are not significantly different (Tukey's HSD, $P < 0.05$).

correlations between NO_3^- and enzyme activity patterns at both watered sites (explaining variabilities 89% and 92% for W1 and W2, respectively), whereas the enzyme activity pattern at NonW was significantly linked to the soil C content (explaining variability 81%). Additionally, DOC correlated with the enzyme activity pattern at W1, and

Table 3. MCPT results of explained variability (%) and *F*-values on the basis of soil nutrients, microorganisms and enzyme activity at all sites and separated in watered (W1, W2) and nonwatered (NonW) sites

	All sites		W1		W2		NonW	
	Explained variability (%)	*F*	Explained variability (%)	*F*	Explained variability (%)	*F*	Explained variability (%)	*F*
C (%C)	80	225.27**	1	1.96	1	0.56	81	67.38**
N (%N)	1	2.71	1	1.77	0	1.67	0	0.42
DOC (mg g^{-1} DW)	0	1.49	1	3.39*	0	1.27	2	1.93
Nitrate (mg g^{-1} DW)	0	2.42	89	147.32**	92	232.20**	3	2.46
Sulfate (mg g^{-1} DW)	1	0.87	1	2.32	2	4.18*	1	0.19
C_{mic} (μg g^{-1} DW)	4	14.08**	1	1.72	0	0.95	1	0.97
O_2 (μL g^{-1} h^{-1} DW)	1	3.66*	1	2.37	1	2.80*	0	0.44
Σ Explained variability (%)	87		95		96		88	

*, **with $P < 0.05$, 0.01, respectively.

Table 4. MCPT results of explained variability (%) and *F*-values on the basis of the PLFA pattern (% nmol) and *specific* enzyme activities (log) in all sites and separated in W1, W2 and NonW

	All sites		W1		W2		NonW	
	Explained variability (%)	*F*	Explained variability (%)	*F*	Explained variability (%)	*F*	Explained variability (%)	*F*
α-D-Glucosidase	5	3.55**	2	0.47	5	1.25	11	3.74*
β-D-Glucosidase	7	4.5**	8	2.07	2	0.51	9	3.29*
Cellobiohydrolase	0	0.49	6	1.33	3	0.48	1	0.26
N-acetyl-β-glucosaminidase	5	2.94*	8	1.88	12	2.99*	0	0.41
β-Xylosidase	3	1.7	2	0.6	5	1.22	21	5.79***
Acid phosphomonoesterase	4	3.07*	2	0.39	15	3.35*	25	5.41**
L-Leucine-aminopeptidase	2	0.93	3	0.65	3	0.69	8	3.92*
L-Tyrosine-aminopeptidase	1	1.09	14	3.03*	3	0.85	6	2.84*
Σ Explained variability (%)	27		45		48		81	

*, **, *** with $P < 0.05$, 0.01 and 0.001, respectively.

SO_4^{2-} and respiration (O_2) with the enzyme activity pattern at W2.

Relation between structural and functional diversity

The structural diversity of the microbial community at the study sites was assessed as PLFA patterns in Schütz *et al.* (2009). The enzyme activities given in this study represent the functional diversity of microorganisms (Kandeler *et al.*, 2000; Waldrop *et al.*, 2000; Mentzer *et al.*, 2006). The overall MCPT revealed strong relations between the enzyme activity pattern and the microbial biomass (Table 3, first column), reflecting the common decline with depth. Therefore, *specific* enzyme activity (i.e. enzyme activity divided by C_{mic}) was used as a functional diversity measure, as this normalizes across effects of the depth gradient (Waldrop *et al.*, 2000; Mentzer *et al.*, 2006) and is thus more closely related to the community composition. MCPT of the PLFA pattern and the *specific* enzyme activities revealed connectivity between functional and structural diversity (Table 4). Significant relations at NonW were detected for α-D-glucosidase, β-D-glucosidase, β-xylosidase, L-leucine-aminopeptidase, L-tyrosine-aminopeptidase and acid phosphomonoesterase. In contrast, at water recharge sites, only L-tyrosine-aminopeptidase (W1), and *N*-acetyl-β-glucosaminidase and acid phosphomonoesterase (W2) showed significant correlations to the PLFA pattern. In sum, the variability explained by the individual enzymes reached 81% for NonW, whereas only 45% and 48% were explained in W1 and W2, respectively.

Discussion

Effects of depth, flooding and soil nutrients on microorganisms

Pore size, nutrient limitations, availability of electron acceptors and large surface areas for attachment all have major effects on microbial abundance and activity in the vadose zone and aquifer (Ghiorse & Wilson, 1988). These environmental parameters likely contributed to the decline in

microbial biomass from top soil to the groundwater table at the investigated sites. However, about half of the total microbial biomass (58%, 42% and 55% in W1, W2 and NonW, respectively) was located at the 40–340 cm soil depth. This contradicts the widespread opinion that subsurface microbial biomass can be neglected and points to a significant impact on soil processes. Of the few studies available for subsoil microorganisms, Fierer *et al.* (2003) reported approximately 35% of the total biomass below 25 cm in a 2-m deep soil profile. This supports our findings and highlights a corresponding capability of the vadose zone microorganisms for biodegradation.

Long-term water recharge practice was expected to foster microorganisms by the additional input of DOC by flooding because microbial biomass is generally linked to C_{org} in soil depth transects (Fierer *et al.*, 2003). In natural waters, 50% of DOC are humic acids and resistant to microbial degradation. Thirty percent are hydrophilic acids with a relatively unknown composition and 20% are suitable for microbial degradation (Thurman, 1985). In our study sites, complex organic matter was eliminated about 95% before flooding and the DOC content decreased from 2.2 mg L^{-1} (River Rhine) to 1.7 mg L^{-1} (filtrate water) to finally 0.6 mg L^{-1} (groundwater) (Rüetschi, 2004). However, microbial biomass did not differ between watered and nonwatered sites, except for a difference between W1 and W2 and NonW at the 100–160 cm depth. This was likely due to the deeper humus and fluvial silt horizon at that site. The lack of response contrasts with other studies that observed high numbers of microorganisms due to the amendment with metabolizable organic substrates by water recharge practice (Balkwill *et al.*, 1998; Kieft & Brockman, 2001). On the one hand, responses in microbial biomass might be restricted to distinct soil layers, as Schütz *et al.* (2008) revealed biomass enhancement in 0–10 cm at the watered sites. On the other hand, microbial life strategy may account for a shift in the microbial community: Gram-positive bacteria, i.e. mainly slow-growing K-strategists (Esperschütz *et al.*, 2007), were dominant in the nonwatered site, whereas Gram-negative bacteria, i.e. mainly fast-growing r-strategists, were dominant in the watered sites (Schütz *et al.*, 2009). Copiotroph r-strategists, such as *Proteobacteria* and *Bacteroides*, are characterized by a short life cycle and a dominance with easily metabolizable organic substrates (Fierer *et al.*, 2007), whereas oligotroph K-strategists have a longer life cycle and better withstand nutrient depletion. Hence, varying parts of r- and K-strategists in a microbial community led to different turnover rates, resulting in similar microbial biomass at watered and nonwatered sites (Fig. 2).

Microbial respiration (O_2 consumption rate) decreased with soil depth at all sites, and was higher at the watered sites at the 0–160 and 280–340 cm depths. This increase in respiration can either be linked to microbial stress or to

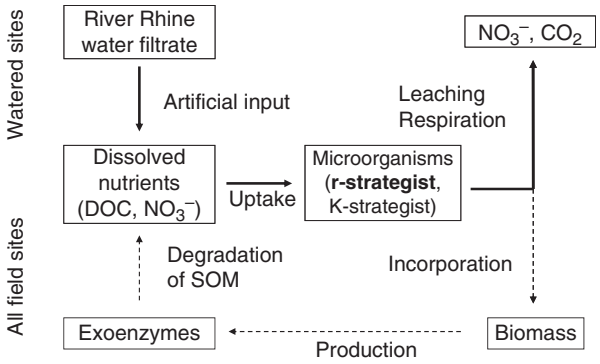

Fig. 2. Microbial nutrient cycle model for water recharge and control sites. Arrows with solid lines represent the predominant C flow at water recharge sites, i.e. in soils with specific nutrient pulses, but with the limitation of others. Arrows with dashed lines show decomposition as a function of soil enzymes, a predominant pathway in soils without such a priming effect.

substrate supply mechanisms (Anderson & Domsch, 1993; Wardle & Ghani, 1995; Xiang *et al.*, 2008). Previous investigations showed a different microbial community composition, particularly higher stress markers (PLFA *trans*/*cis* ratio), at the watered sites from the 100–340 cm depth (Schütz *et al.*, 2009). Osmotic stress can result from drying/rewetting cycles (Schimel *et al.*, 2007; Xiang *et al.*, 2008), oxygen depletion or organic solvents (Anderson & Domsch, 1990; Fliessbach *et al.*, 1994). Reduced oxygen saturation was observed at W2 during a 10-day watering period from 90% to 65% in summer, and from 100% to 85% in winter, for filtrate- and groundwater, respectively (Rüetschi, 2004). Moreover, the groundwater table at the watered sites rises from 4–5 to 2–3 m depth during flooding. The occurrence of anaerobic conditions was also indicated by higher PLFA *iso*/*anteiso* ratios at the watered sites (Schütz *et al.*, 2009).

Nutrient amendment due to flooding (DOC, NO_3^-) leads to excess C and N conditions at watered sites, which is evident from N indicator plants growing there. Additionally, Schimel *et al.* (2007) stated that repeated drying/rewetting cycles can pump C out of an ecosystem, thus enhancing the turnover rates. This is apparent at the watered sites with significantly lower DOC concentrations. Yet, this did not result in a corresponding increase in microbial biomass likely due to the fact that nutrients other than C or N became limiting. Excess C was then released by microbial overflow metabolism (Schimel & Weintraub, 2003), as signaled by the higher microbial (waste) respiration at watered sites. Excess N leached into the groundwater as NO_3^-, indicated by the considerable amount of 10.6 mg L^{-1} $year^{-1}$ in 2006 (Industrial Works of Basel, IWB, 2007), which slightly surpassed the maximum contaminant level of 10 mg L^{-1} in the United States and the World Health

Organization guideline (Spalding & Exner, 1993). Most likely, P was limited as indicated by preliminary fertilizer studies with C, N and P (data not shown) and by the enzyme activity discussed in the next paragraph.

Effects of depth on enzyme activity

Enzyme activity significantly decreased with soil depth at all sites, which corresponds to the results from Taylor *et al.* (2002) and Venkatesan & Senthurpandian (2006) at 2 and 4 m depth, respectively. This decline is mainly related to a decrease in the soil organic matter (SOM) content and microbial biomass (Speir & Ross, 2002). Freeman *et al.* (1995) showed for β-D-glucosidase, phosphomonoesterase and sulfatase that the top soil layer (0–10 cm) is the enzymatically most active horizon. In our study, β-D-glucosidase was very active in the upper soil layers, whereas acid phosphomonoesterase and L-leucine-aminopeptidase were active throughout the entire depth transect. This mirrors either the occurrence of specific substrates or the lack of nutrients (Chrost, 1991; Beck & Beck, 2000; Shackle *et al.*, 2000; Nannipieri *et al.*, 2002).

Enzyme activities at W1 (40–160 cm depth) exceeded W2 and NonW by several orders of magnitude, most likely caused by artificial landfill (about 1 m of loess loam). Soil enzyme activity is strongly affected by the soil particle size distribution. In particular, silt and clay size microaggregates absorb a high percentage of the overall activity (Kandeler *et al.*, 1999; Nannipieri *et al.*, 2002). Marx *et al.* (2005) found the highest enzyme activities in the silt and clay size fractions of a loamy soil. Hence, the higher silt content at W1 had a positive impact on the enzyme activities there.

Acid phosphomonoesterase was the most important soil enzyme throughout the entire depth transects and an inverse relationship between phosphomonoesterase activity and inorganic phosphate availability has been reported (Spiers & McGill, 1979; Shackle *et al.*, 2000). The high acid phosphomonoesterase activity therefore suggests that P is limiting at all sites. Hence, the relatively low total phosphate and orthophosphate concentrations (0.05 and 0.02 mg L^{-1}, respectively; Department of Environment and Energy, AUE, 2006) in the river Rhine water used for flooding were below the demand of microorganisms.

Effects of flooding on enzyme activity

Increasing enzyme activities in top soils due to flooding were reported previously (Freeman *et al.*, 1995; Mentzer *et al.*, 2006). In contrast, the enzyme activities at our study sites did not respond to flooding and the additional input of nutrients, such as DOC and NO_3^-, herein. However, the depth gradients of both glucosidases were slightly stronger at the watered sites than at the nonwatered site, indicating a high filter quality, which is consistent with reports from other groundwater recharge plants (Hendel *et al.*, 2001). Similarly, Burns & Ryder (2001) found correlations between glucosidase activity and DOC in floodplain sediments. In the study area, flooding did not introduce complex SOM into the system due to the effective rapid sand filter as water pretreatment. The observed results are therefore in line with studies in arable, forest or wetland soils where amendment with complex C sources, such as cellulose or sewage, led to enhanced β-D-glucosidase activities (Shackle *et al.*, 2000; Dilly & Nannipieri, 2001). In sum, the lack of response of soil enzyme activity at the watered sites indicates that DOC reduction is rather a function of bacterial absorption than of enzyme activity at our sites.

Soil nutrients and microorganisms related to the enzyme activity pattern

Combining enzyme activities, i.e. the functional microbial pattern with soil nutrients and microbial parameters in one analysis (MCPT), revealed significant connectivity between the overall enzyme activity pattern and C, C_{mic} and O_2 (Table 3, all sites). Soil enzymes are produced by microorganisms to release nutrients from SOM, and thus explain these interactions. A separate analysis of the study sites indicated that at watered sites, the functional microbial pattern correlated with NO_3^-, whereas at the nonwatered site, it correlated with C. Flooding introduces DOC and NO_3^- (C/N ratio river water in 2005: 1.4), and C mineralization is favored by N availability (Mary *et al.*, 1996; Waldrop & Zak, 2006), thus explaining the correlation of the functional microbial pattern with NO_3^-. At the nonwatered site without an additional nutrient input, N likely is limited and the functional microbial pattern is therefore predominantly linked to the SOM content (Schimel & Weintraub, 2003).

Relations between structural and functional microbial community composition

Connecting the patterns of soil PLFAs and enzyme activities relates structural and functional microbial community composition (e.g. Waldrop *et al.*, 2000; Mentzer *et al.*, 2006). At the nonwatered site, *specific* enzyme activities (except β-cellobiohydrolase and *N*-acetyl-β-glucosaminidase) were significantly associated with the structural community composition, whereas at the watered sites, only three enzymes (*N*-acetyl-β-glucosaminidase, acid phosphomonoesterase and L-tyrosine-aminopeptidase) showed significant relationships. This strong correlation between structure and function at the nonwatered site reflects the lack of external nutrient input and the resulting need to gain this by degrading complex organic substrates through soil enzymes (Fig. 2). In contrast, the microbial community at the watered sites is fed with readily available nutrients, such as DOC and NO_3^-, without the necessity for the production of

a perfect matching and complex set of soil enzymes. Apparently, this led to a weak relationship between functional and structural community patterns. Hence, water recharge conditions not only uncoupled catabolism from growth (i.e. resulted in overflow metabolism), but presumably also organic matter breakdown from soil enzymes (Fig. 2).

Conclusions

Long-term water recharge practice as investigated at the 'Langen Erlen' was expected to affect the microbial community via regular nutrient (DOC, NO_3^-) pulses to the soil system (Fig. 2). However, microbial biomass was not higher due to this amendment, but the microbial community was altered. Likely, varying parts of r- and K-strategists with different turnover rates were responsible for the comparable microbial biomass. Structural and functional microbial diversities were linked to each other at the nonwatered site. Amendment with DOC and NO_3^- at watered sites uncoupled this relation and resulted in enhanced microbial respiration and NO_3^- leaching (Fig. 2). Microorganisms appear to be nutrient limited in the midst of plenty and C and N in excess is respired through overflow metabolism or leached into the groundwater (Fig. 2). Enzyme activity indicates that P is short in supply. Overall, this suggests that adding a pulse of C or N to a P-limited system leads to waste respiration and a decoupling of enzymatic decomposition and microbial growth. This highlights the need to rethink the respiratory response of soil microorganisms to added nutrients as well as the enzyme-driven breakdown of SOM in soils with considerable input of simple available C (e.g. root exudates).

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