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[1] Limnologische Fluss-Station des Max-Planck-Instituts für Limnologie, Damenweg 1, D-36110 Schlitz, Germany and [2] Institut für Wasserforschung GmbH, Zum Kellerbach 46, D-58239 Schwerte, Germany

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Abstract—Activities of the extracellular enzymes β-glucosidase and phosphatase and bacterial densities were investigated during the filtration process at several sites in a groundwater recharge plant at the Ruhr river (Hengsen recharge plant in Schwerte, Germany). Low numbers of microorganisms and low levels of activity in this type of habitat, compared to most surface waters, caused methodological problems when determining microbial activity. In this study, fluorigenic model substrates, which enable hydrolytic rates as low as 1 nmol $(L \times h)^{-1}$ to be measured, were used to determine extracellular enzyme activities. Highest activities were determined in surface water (107 nmol $(L \times h)^{-1}$ for β-glucosidase and 252 nmol $(L \times h)^{-1}$ for phosphatase), which decreased during the filtration process in the gravel prefilter and the main sand filter until the end of subsurface flow (1.6 nmol $(L \times h)^{-1}$ and 6.8 nmol $(L \times h)^{-1}$, respectively). Similarly, bacterial numbers decreased from 3.4×10^6 to 0.29×10^6 cells mL^{-1}. These data showed that microbial activity within the prefilter and the shallow layers of the sand filter had the greatest impact on water quality. In addition to its involvement in the continuous purification of surface water, the microbial community in the sand filter probably acts as a biological buffer against ephemeral increases in the loads of organic matter and nutrients in the recharge plant. © 2001 Elsevier Science Ltd. All rights reserved

Key words—extracellular enzyme activity, fluorigenic model substrates, slow sand filtration, groundwater recharge, β-glucosidase, phosphatase

INTRODUCTION

Artificial groundwater recharge has been used as a method for securing a potable water supply since the 19th century. This process, in which surface water recharges groundwater by slow sand or bank filtration, was initially used to eliminate pathogens, but later it was also used to eliminate chemical impurities (Schmidt, 1996).

Microbial activity is one of the most important factors in the purification processes associated with groundwater. This activity involves the degradation of organic matter and the reduction of electron acceptors such as oxygen, nitrate and sulphate (Ghiorse and Wilson, 1988). In addition, the transport, adsorption and mobilisation processes of organic and inorganic matters are also influenced by microbial activity. The quality of groundwater recharge is therefore dependent on the structure and physiology of the microbial community.

Low numbers of microorganisms and low levels of microbial activity cause several methodological problems when determining microbiological parameters in oligotrophic groundwater habitats. Hence, despite its important role, little is known about microbial activity in this type of habitat (Madsen and Ghiorse, 1993).

Low-molecular-weight organic matter constitutes less than 20% of the total organic matter in subsurface aquatic habitats, so heterotrophic microorganisms enhance their access to the organic matter pool by producing extracellular enzymes (Marxsen and Fiebig, 1993). These enzymes hydrolyse macromolecular organic matter, inducing the release of low-molecular-weight organic compounds. This hydrolysis is a crucial, initial step in the microbially mediated degradation of organic matter, and is also important in the regeneration of inorganic nutrients, such as of phosphate, from macromolecular compounds (e.g. Chróst, 1990; Münster, 1992; Marxsen and Schmidt, 1993).

The aim of the current study was to evaluate spatial variability in microbial activity within a groundwater recharge plant in terms of extracellular phosphatase and β-glucosidase activities. We determined these extracellular enzyme activities using 4-methylumbelliferyl substrates, which are more sensitive than chromogenic substrates and are there-

*Author to whom all correspondence should be addressed. Tel.: +49-6642-9603-0; fax: +49-6642-6724; e-mail: jmarxsen@mpil-schlitz.mpg.de

fore more suitable in oligotrophic aquatic systems (Hoppe, 1983). The applicability of these fluorigenic substrates in groundwater with low-level extracellular enzyme activity had been shown previously (Hendel and Marxsen, 1997). Only water samples could be considered although the larger fraction of bacterial cells must be assumed to be sessile (Marxsen, 1982). However, underground sediments are most difficult to sample, and interstitial water is not only traditionally used for characterising the development of microbial activity during underground passage, but it is the object of purification. Because of this, the development of microbial activity in the free water phase is regarded as important for characterising the development of water quality during the purification process.

MATERIALS AND METHODS

Sampling site

Water was sampled from the Hengsen recharge plant (Fig. 1), near the river Ruhr in Schwerte (Germany), in which groundwater is recharged by slow sand filtration. The river water is dammed to an artificial lake (Lake Hengsen). After passing through gravel prefilters, the water discharges through the main sand filters and is treated after a 50 m underground passage with chlorine dioxide and sodium hydrochloride before being fed into the public water supply.

Sampling

Three litre-samples were taken at seven sampling sites representing various stages of the artificial groundwater recharge process (Fig. 1). Sample 1 was taken from Lake Hengsen directly before the prefilter. Sample 2 was taken after the prefilter. Samples 3–5 were taken from a group of observation wells at various depths in the main sand filter (sample 3: 1.9 m; sample 4: 2.9 m; sample 5: 4.1 m). Sample 6 was taken at the end of the 50 m underground passage, and sample 7 was taken in the recharge plant directly before chlorination. Water from the observation wells was pumped to the surface through plastic tubes using a battery-powered pump. The pump rate was $4\,L\,min^{-1}$, and a minimum of 20 L was pumped through the tubes before taking the samples. All samples were returned to the laboratory in a cooled, insulated container, and processed within 1 h. These samples were used for the enzyme assays and bacterial counts.

Enzyme assays

Extracellular β-D-glucosidase activity was measured using methylumbelliferyl-β-D-glucoside (MUF-glc) as substrate, and phosphatase activity was measured using methylumbelliferyl phosphate (MUF-P), as described in detail by Hendel and Marxsen (1997).

Briefly, the method was as follows. Concentrations of stock solutions were $300\,\mu mol\,L^{-1}$ for methylumbelliferone (MUF) and $10\,mmol\,L^{-1}$ for MUF-glc and MUF-P (all MUF-chemicals were obtained from Sigma). Experiments were performed with 100 mL final water volume in each of nine Erlenmeyer flasks. Three of these flasks were used for calibration (by adding pure MUF), two served as controls, and four were used for determining enzyme activity. The final substrate concentration was $250\,\mu mol\,L^{-1}$. β-glucosidase assays were carried out at enzyme saturation, which had been determined previously. Phosphatase did not reach enzyme saturation at MUF-P concentrations of between 10 and $500\,\mu mol\,L^{-1}$, but the same concentration was used as for β-glucosidase. All samples were incubated at 25°C in a shaking waterbath. Preliminary tests showed that enzyme conformation does not change at this temperature. During the tests, 3 mL-subsamples were taken from the flasks every 60 min, with the minimum time being 300 min. β-glucosidase subsamples were boiled immediately for three minutes and then cooled before addition of 300 µL of ammonium glycine buffer at pH 10.5, whereas with phosphatase samples, the buffer was added prior to boiling. Fluorescence was measured at 365 nm excitation and 450 nm emission wavelengths with a Kontron SFM 25 fluorimeter. Enzyme activities were calculated from linear regression of increases in MUF concentration. All statistical calculations were done after Sachs (1984).

Total numbers of bacteria

Bacterial numbers were determined by epifluorescence microscopy after staining with acridine orange. The water samples (200 mL each) were fixed with 2% (w:v) formaldehyde (final concentration) immediately after sampling. For surface water samples, 1 mL sample and 0.1 mL of an aqueous and particle-free 0.01% (w:v) acridine orange solution were processed in a filtration tower as described by Preuß and Hupfer (1998) (black polycarbonate filter of 0.2 µm pore size and 13 mm diameter, Nuclepore; staining

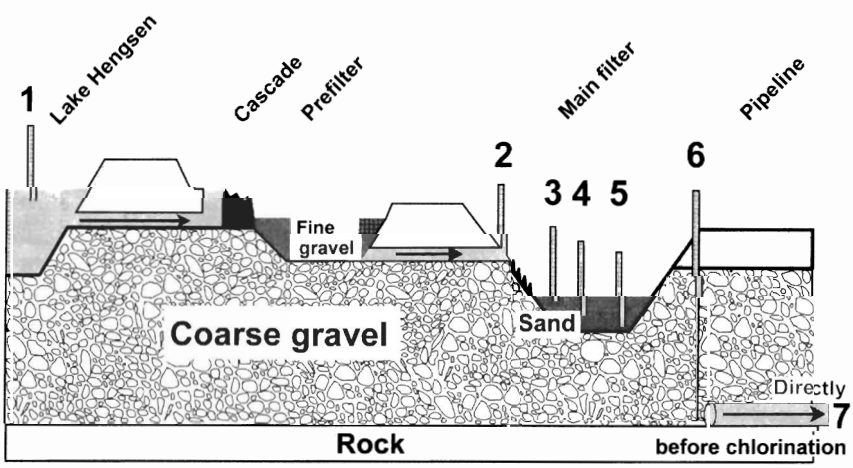

Fig. 1. Sampling sites in the Hengsen recharge plant (numbering of sampling sites is explained in the text).

time 3 min). For subsurface water, 10 mL sample and 1 mL acridine orange solution were used. After drying in the dark, bacteria fluorescing green or red were counted using an epifluorescence microscope (Leitz Ortholux II, filters BP 470-490 and LP 515) and a fluorescence-free immersion oil (Cargille Type A). Total bacterial numbers in each sample were calculated from the evaluation of 20 fields of view using the equation

$$\text{Total number of bacteria/mL} = (N_m F_f)/(VF)$$

where N_m is the mean number of bacteria per field, F_f the surface area of filter (mm^2), F_g the surface area of filter per field of view (mm^2), and V the sample volume filtered (mL). Typical standard deviation of the method is 11% as determined previously by treating 10 subsamples of one reference sample.

RESULTS

Increases in MUF concentrations during sample incubations were always less than 5% of the substrate concentration. Linearity in the increase in product concentration during incubation was generally most obvious in samples with higher rates of substrate hydrolysis (sites 1 and 2).

Extracellular enzyme activity was detected in all samples (Fig. 2). The highest rate of phosphatase activity was measured at site 1 (252 nmol $(L \times h)^{-1}$). The phosphatase activity at site 2 was much lower (87.3 nmol $(L \times h)^{-1}$), and had decreased further at sites 3–7 (6.8–13.6 nmol $(L \times h)^{-1}$). There was no relevant decrease in phosphatase activity while water passed through the main sand filter (sites 3–5). A similar trend was observed with β-glucosidase activity apart from a significant decrease during the initial passage through the main sand filter. The β-glucosidase activity was lower than that of phosphatase activity, but as with phosphatase, the highest activity was measured at site 1 (107 nmol $(L \times h)^{-1}$). The activity was lower at site 2 (21.4 nmol $(L \times h)^{-1}$), and had decreased further at sites 3–7 (1.2–3.6 nmol $(L \times h)^{-1}$).

During pre-filtration of the surface water, bacterial densities decreased from 3.4×10^6 to 5.4×10^5 cells mL^{-1} (Fig. 2). After slow sand filtration and subsurface discharge, the densities were 1.8×10^5 and 2.9×10^5 bacteria mL^{-1}, respectively. Bacterial densities and β-glucosidase and phosphatase activities were positively correlated (bacteria vs. β-glucosidase: $r^2 = 0.963$***; bacteria vs. phosphatase: $r^2 = 0.903$***).

DISCUSSION

Within the water of the Hengsen recharge plant phosphatase activities were found to be higher than β-glucosidase activities. This trend has also been observed in previous studies conducted in a range of aquatic habitats (Table 1). High extracellular enzyme activities reflect a high substrate availability and are characteristic of untreated water (Chróst, 1990), as observed in Lake Hengsen. Reductions in enzyme activities, as observed during the passage of water, therefore reflect an improvement in its chemical quality, indicating that the recharge plant fulfilled its function. Similarly, the decrease in bacterial densities demonstrates that the recharge plant also improved the microbiological quality of the water discharged through it.

Easily decomposable phosphorus and carbohydrate compounds are the natural substrates of the extracellular enzymes investigated in the current study. The decreases in enzyme activities within the recharge plant can be attributed to reductions in the availability of these labile compounds (cf. Table 2 for DOC), and in the case of phosphatase also to the decreased phosphorus requirements of the microbial community, the abundance and activity of which decreases on progression through the recharge plant.

Bacterial densities in lake Hengsen (3.4×10^6 mL^{-1}) are similar to values from eutrophic lakes (Overbeck, 1965). Densities decreased during

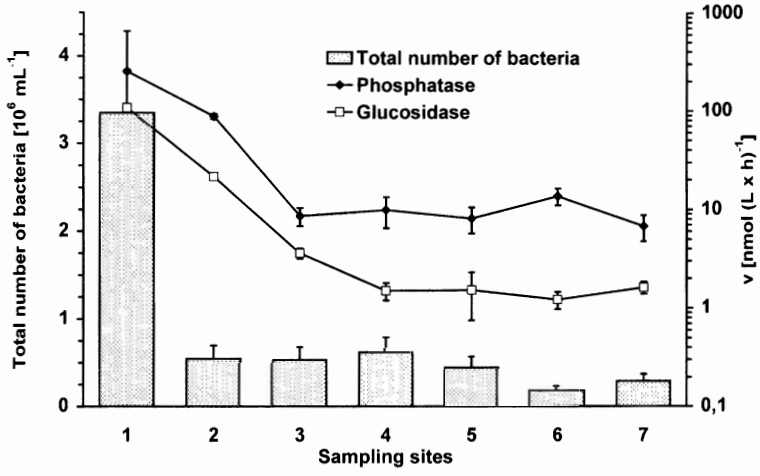

Fig. 2. Phosphatase and β-glucosidase activities ($n = 4$) and total numbers of bacteria ($n = 3$) with 95% confidence intervals at seven different sampling sites.

Table 1. β-glucosidase and phosphatase activities (nmol $(L \times h)^{-1}$) in different aquatic habitats

β-glucosidase activity (nmol $(L \times h)^{-1}$)	Phosphatase activity (nmol $(L \times h)^{-1}$)	Habitat	References
1.2–107	6.8–252	Groundwater recharge plant	This study
4.4–61	11.8–102	Lake water	Münster (1992)
3.2–72	12.8–140	River water	Chappell and Goulder (1995)
2.8–75	30–118	Stream water	Hendel and Marxsen (1997)
0.7–5.0	2.0–7.9	Freshwater reservoir	Vrba *et al.* (1992)
1–35	11–77	Water recharge plant	Miettinen *et al.* (1996)
0.36–3.0	14–27	Brackish water	Saliot *et al.* (1996)
1.1–291	5.5–320	Subsurface waters	Hendel and Marxsen (1997)

Table 2. Specific activity of β-D-glucosidase and phosphatase (with 95% confidence intervals) and typical concentrations of DOC and P_i at seven different sampling sites. DOC and P_i have been monitored previously (Schöttler and Schulte-Ebbert. 1995). Mean values from a long-term study from 1986–1988 are given. They have been shown to remain constant within the water recharge plant throughout the years

Sampling site	Specific β-glucosidase activity with 95% confidence intervals (amol $(h \times \text{cell})^{-1}$)		Specific phosphatase activity with 95% confidence intervals (amol $(h \times \text{cell})^{-1}$)		DOC (mg L^{-1})	P_i (mg L^{-1})
1	32	21–69	75	49–163	3.6	0.72
2	39	37–41	160	149–172	3.0	0.71
3	6.7	4.5–12.9	16	10–31	1.9	0.68
4	2.8	1.8–3.2	19	10–23		
5	2.6	1.6–6.6	14	11–32	1.7	0.58
6	6.7	4.1–15.4	76	46–172	1.3	0.46
7	5.6	4.1–8.2	23	15–36		

the filtration process to about $0.1 \times 10^6\,mL^{-1}$, which are similar to densities found in deep groundwater (Pedersen and Ekendahl, 1990), but are lower than those usually determined in shallow groundwater ($> 10^6\,mL^{-1}$, Marxsen, 1988).

The lower bacterial densities within the recharge plant (sites 2–7), compared to the lake (site 1), probably reflect a physical filtration effect, with a high surface area to volume ratio enhancing attachment of bacteria to the substratum. Densities of suspended bacteria were approximately halved between sites 2 and 7, i.e. as water discharged through the recharge plant. However, physical retention of suspended bacteria from the lake water was apparently most obvious in the prefilter (between sites 1 and 2). Bacterial abundance and β-glucosidase activity decreased to a similar degree between sites 1 and 2, thus resulting in similar specific β-glucosidase activity (Table 2). Specific phosphatase activity was more than doubled between these sites, but the increase was not significant.

Specific activities of both enzymes decreased between the outlet of the prefilter and the first sampling station in the main filter (sites 2 and 3), and exhibited only low fluctuations at the other downstream sites within the recharge plant. Bacterial abundance in pumped water is similar, or only slightly lower, at sites 3–7, as compared to site 2. If the relationship between suspended and particle-bound bacteria remains similar in these habitats, then also the size of the total bacterial communities in these sites can be assumed to be similar. However, extracellular enzyme activities decreased between sites 2 and 3 (for phosphatase) or between sites 2 and 4 (β-glucosidase). These bacterial communities in the downstream sites probably provide a reserve purification capacity for water, should water contamination suddenly increase.

Similar decreases in enzyme activities between lake and subsurface waters were observed during bank filtration at the Finnish lake Kallavesi (Miettinen *et al.*, 1996). However, their study could not provide a detailed insight into the different stages of the filtration process because data were obtained from too few sampling stations. Between the lake and the production wells, phosphatase activity decreased from 77 to 12 nmol $(L \times h)^{-1}$, and β-glucosidase activity decreased from 21 to 1 nmol $(L \times h)^{-1}$. While these data might appear to be comparable to those obtained in our study, much lower substrate concentrations were used by Miettinen *et al.* (1996) of about 1.9 µmol L^{-1} for MUF-P and 0.8 µmol L^{-1} for MUF-glc (compared to 250 µmol L^{-1} in this study). Thus, they cannot be compared directly to the values that we obtained. However, it can be concluded that hydrolytic rates at substrate saturation must have been higher at the Finnish site. This difference might be explained by the much higher DOC concentrations in the Finnish lake water at about 12 mg L^{-1}. This DOC was rich in humic material, and thus more refractory than the DOC from Lake Hengsen, where concentrations were only 3.6 mg L^{-1}. The lower phosphorus concentrations at the Finnish site might also explain the higher phosphatase activities.

The extracellular enzyme activities measured in our study provide a direct insight into the spatial distribution of microbial processes within the recharge plant. These data, interpreted within the

context of the DOC and phosphate concentrations, indicate that microbial activity within the prefilter and the shallow layers of the sand filter has the greatest impact on water quality. Because this impact occurs in the early stages of the recharge plant, there would appear to be a potential reserve capacity within the system to cope with any ephemeral increases in organic and inorganic loading. During the investigation period, but also in the years before (Schöttler and Schulte-Ebbert, 1995) no case of higher organic and nutrient load was observed. Thus, no measurements during an instationary loading incident could be performed to test this hypothesis.

CONCLUSIONS

1. Fluorigenic model substrates provide a useful assay for measuring the low-extracellular enzyme activities that occur during slow sand filtration in a water recharge plant, where hydrolytic rates can be as low as 1 nmol $(L \times h)^{-1}$.
2. Extracellular enzyme activity of phosphatase and β-glucosidase decreases during the purification process from lake water to drinking water over nearly two orders of magnitude. The greatest impact on this decrease is within the prefilter and the cascade between prefilter and sand filter. For β-glucosidase the greatest decrease was within the first meters of the main sand filter.
3. The microbial communities within the prefilter and the shallow layers of the sand filter, in combination with physical and chemical processes, have the greatest impact on water quality. The lower layers of the sand filter may serve as a biological buffer against ephemeral increases in the loads of organic matter and nutrients in the water entering the recharge plant from the lake.

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