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GROUNDWATER SAMPLES DO NOT REFLECT BACTERIAL DENSITIES AND ACTIVITY IN SUBSURFACE SYSTEMS

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Abstract—Total cell numbers, abundance of respiring bacteria and [^{3}H]thymidine and [^{14}C]leucine incorporation rates were investigated in four groundwater wells of low nutrient content. Total cell numbers in the pumped groundwater were low (14×10^3 to 279×10^3 cells mL^{-1}), and [^{3}H]thymidine and [^{14}C]leucine incorporation rates were, with one exception, below the detection limit. Therefore we exposed sediments *in situ* for 2 months which allowed us to determine bacterial numbers and incorporation rates of labeled substrates by bacteria attached to sediment particles. The two habitats differed considerably in all bacterial parameters both in magnitude and seasonal trends. Total bacterial numbers of sandy sediments ($52.1 \pm 21.3 \times 10^6$ cells cm^{-3}) corresponded in average to 663 cm^3 of pumped groundwater ($78.5 \pm 61.5 \times 10^6$ cells L^{-1}). For the fraction of respiring bacteria this ratio was on average 3032 cm^3 (sediments: $10.3 \pm 5.4 \times 10^6$ respiring cells cm^{-3}; groundwater: $3.39 \pm 6.01 \times 10^6$ respiring cells L^{-1}). The percentage of respiring bacteria in sandy sediments was between 6.0 and 41.4% (average 19.8) compared to 1.0 to 24.9% (average 5.23) in the pumped groundwater. Our results stress the importance of studying the microbial communities attached to sediment, as pumped groundwater samples may not be representative for the real structure and dynamics of microbial assemblages in subsurface environments.
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Key words—bacteria, groundwater, exposed sediments, INT, thymidine, leucine

INTRODUCTION

In recent years, the role of microorganisms in groundwater ecosystems has been the subject of an increasing number of investigations. Although there have been several methodological improvements in the measurement of bacterial biomass and activity, our knowledge on energy flow, food web and community structures of different subsurface environments is still small compared to the huge amount of data available for surface waters.

Any study of the groundwater microbiota in the water-saturated zone should include both sediment and the groundwater, because substantial differences have been found between the microbial communities of these two habitats (Kölbel-Boelke and Hirsch, 1989). Samples collected from the subsurface sediments and the aquifer water should be undisturbed and uncontaminated. However, it is very difficult to obtain representative samples of sediments, because many of the commonly used sampling methods cause drastic changes of the groundwater microflora (Rusterholtz and Mallory, 1994) and can lead to artifacts. Sediments and groundwater from wells differ in their microbial community structure (Hirsch *et al.*, 1992). The data of groundwater microbiological investigations commonly originate from the water present in the relatively large, open pores between sand-sized mineral grains. Water in these large pores is subject to less surface tension force than water in small pores. Therefore it represents the main part of the pumped sample, in addition to microrganisms which may be attached to smaller particles that were pumped up. The large-pore habitat, which is predominantly populated by free-living rather than attached bacteria, may, however, exhibit much less community diversity and activity than the sediment as a whole. The lower density and diversity in groundwater relative to sediments might thus be the result of a sampling artifact (Chapelle, 1993). To overcome these problems some attempts have been made to study the possible colonization of sediments, e.g. by exposing sterilized sediments into groundwater wells (Hirsch and Rhades-Rohkohl, 1990; Marxsen, 1982).

As part of a large program with the aim to investigate the influence of gravel-pit lakes on groundwater systems (Sampl *et al.*, 1995), the specific objectives of this study were to compare the number and activity of microorganisms from sandy deposits and well water from different sites in Austria during an annual period. We also wanted to test the validity

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of using groundwater to monitor the microbiological parameters of subsurface aquifers. Multiple assay comparisons were performed to achieve a more accurate characterization of the subsurface environment.

MATERIALS AND METHODS

Study sites and sampling

The study sites were located near the cities of Klagenfurt (Baggersee Weizelsdorf) and Graz (Schwarzlseen) in the southern part of Austria. Samples were drawn and incubated twomonthly from May 1992 to March 1993 from four wells: W1 and W2 are situated in a groundwater investigation field in the surrounding of the gravel-pit lake "Baggersee Weizelsdorf" and S1 and S2 are located at the northern and eastern part of the gravel-pit lakes "Schwarzlseen". The sampling stations W2 and S2 (outflowing stations) were influenced from surface waters by the closely situated lakes (Fig. 1). Details of the investigation area, sampling methods and determination of chemical parameters are published elsewhere (Sampl *et al.*, 1995). Prior to sampling the wells were flushed by pumping at least five well volumes of water until pH and conductivity were stable. For the estimation of bacterial abundances, samples of 100 mL were fixed with formaldehyde (2% final conc.). To measure bacterial activity, growth and production rates, well water was filled into sterile 1 L bottles, placed on ice and processed immediately after sampling.

For the preparation of artificial sandy deposits, sediments from the sampling sites were fractioned, and sediment of 250–500 μm particle size was washed twice with distilled water, sterilized by autoclave and dried at 100°C. Additionally, we exposed sterilized sandy sediments with a particle size $>125\ \mu$m and $<250\ \mu$m in the groundwater wells W1 and W2 in September. We used containers of stainless steel nets (Marxsen, 1982) for the exposure of the sandy sediments. The inner sides of the containers were covered with a 125 μm net. The containers were attached to a string and immersed into the well-water. Exposure depth was 2 m beneath the well water surface. After 2 months, when the initially sterile sediments were expected to be in a balanced state of microbial colonization (Marxsen, 1982), the vessels were transferred into sterile 1-L glass beakers, transported to the laboratory in a refrigerator and processed immediately.

Bacterial activity

Radiotracer uptake rates in the exposed sandy sediments were estimated by using altered protocols of sediment extraction techniques for thymidine (Findlay *et al.*, 1984; Thorn and Ventullo, 1988). In contrast to other methods the samples of this study were dual labeled with [^{3}H]thymidine and [^{14}C]leucine. One gram of the exposed sediments and 1 mL of sterile filtrated groundwater were well mixed in centrifuge tubes (three parallel samples and one control). Controls were prepared with 1 g sterilized sediment mixed with 1 mL of 0.6 N NaOH. After addition of [^{3}H]thymidine (20 nmol final concentration) and [^{14}C]leucine (80 nmol final concentration) the samples were incubated for 2 h on a shaker at *in situ* temperatures. The reaction was stopped by the addition of 1 mL of extraction reagent (0.3 M NaOH, 25 mM disodium EDTA, 0.1 SDS% w/v). Macromolecules were extracted and RNA hydrolysed at 25°C for 12 h. After centrifugation at 5000 *g* for 10 min the supernatant was transferred to test tubes. The pellet was washed with 1 mL of extraction reagent, centrifuged again for 10 min at 5000 *g* and all supernatants in the test tubes were neutralized with 3 N HCl. Ice cold 10% trichloracetic acid (TCA) at amounts equal to the sample volume was added and the solution was chilled on ice for 45 min. Samples were filtered onto cellulose nitrate filters (Sartorius, 0.2 μm) and the tubes and filters were rinsed with 5 mL of ice cold 5% (w/v)

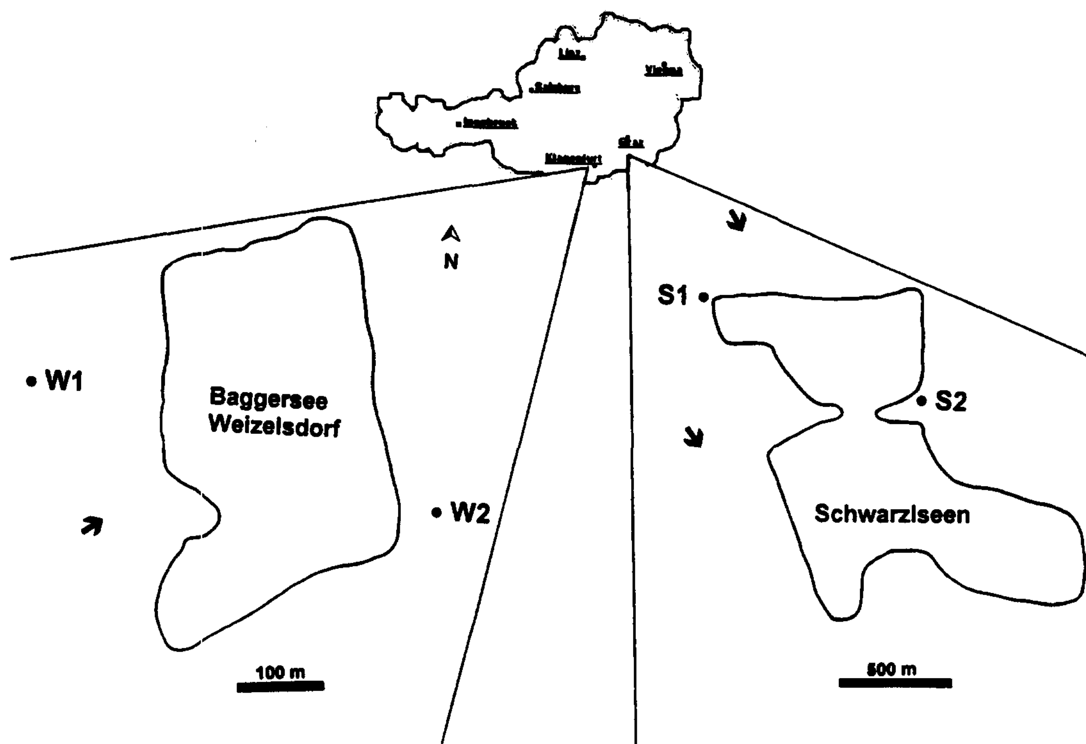

Fig. 1. Locations of sampling sites Baggersee Weizelsdorf and Schwarzlseen; arrows indicate groundwater flow.

TCA. The filters were washed three times with 3 mL of ice cold 5% TCA, placed in scintillation vials and allowed to dry at room temperature. After addition of 10 mL of scintillation cocktail (Packard) samples were counted in dual label modus with internal quench correction in a Beckman LS 6000IC liquid scintillation counter for 2 × 10 min until 2% standard deviation was reached.

Time-courses from 0.5 to 3 h were followed twice in W2 and S1. They were always characterized by a linear incorporation of [^{3}H]thymidine and [^{14}C]leucine. Saturating concentrations of [^{3}H]thymidine and [^{14}C]leucine were determined experimentally by measuring incorporation rates at various concentrations of radiotracers.

The relative efficiency of DNA and protein extraction from sediment was estimated by a method modified from Fallon *et al.* (1983). Two hundred milligrams of glucose and 40 mg of yeast extract were added to 500 mL of groundwater. The suspension was incubated on a rotary shaker at 20°C until turbidity was visible. After addition of [^{3}H]thymidine (10 nM final conc.) and [^{14}C]leucine (40 nM final conc.) the suspension was incubated for 4 h at 20°C. Afterwards cells were isolated by centrifugation and the pellet was washed three times with filter-sterilized autoclaved groundwater. To test extraction efficiency the pellet was resuspended in 20 mL of filter-sterilized autoclaved groundwater and 1 mL of the suspension was added to 1 g of sediment (5 parallel samples, 2 controls) or artificial groundwater. Aqueous samples were immediately processed according to the method of Fuhrman and Azam (1980). The extraction of macromolecules from sediment suspensions was performed as described above. For the calculation of the extraction efficiency, the recovery from the aqueous samples was considered to be 100%.

[^{3}H]thymidine and [^{14}C]leucine incorporation in the well water were measured with a dual labeling method modified from Chin-Leo and Kirchmann (1988): [^{3}H]thymidine and [^{14}C]leucine were added to three autoclaved polyethylene vials containing 10 mL of well water to a final concentration of 5 nmol and 20 nmol, respectively. The vials were incubated at *in situ* temperatures for 1–4 h. Abiotic absorption of the labeled substrate was estimated by measuring the incorporation of radiolabeled substrate in samples previously fixed with formaldehyde (2% final conc.). Samples were mixed with 10 mL of ice-cold TCA and after 10 min filtered onto cellulose nitrate filter (Sartorius, 0.2 μm). The filters were washed three times with ice cold 5% TCA and placed in a scintillation vial with 10 mL of Packard scintillation cocktail. The measurement of samples and calculations were performed as described above.

Total and metabolically active bacteria

Actively respiring bacteria were enumerated by incubation with 2-(*p*-iodophenyl)-3*p*-(nitrophenyl)-5 phenyltetrazolium chloride (INT) modified from Zimmerman *et al.* (1978). Approximately 1 g of mixed fresh sediment from sandy exposures was weighted in flasks and 10 mL particle-free distilled water (PFWD) were added. After sonication for 30 s (Bandelin Sonopuls HD 60; pulse modus with 50% maximum intensity), 0.5 mL of the suspension was put in a glass tube containing 6 mL of PFWD. One mililiter of a 0.2% aqueous solution of INT was added and mixed well. After incubation in a water bath for 1 h at 20°C, we added formaldehyde (2% final conc.) to stop the reaction. At this stage it was possible to store the sample overnight.

To count total bacterial numbers, INT samples were additionally stained with 4′,6′,-diamidino phenylindole (DAPI). DAPI was added to a final concentration of 5 μg mL^{-1} (Schallenberg *et al.*, 1989) and incubated in the dark at 4°C for 5–10 min. One mililiter of the suspension was mixed well, filtered through a 0.2 μm black membrane filter (low pressure) and washed with 5 mL of PFWD. Filters were placed on a glass slide with a drop of glycerine oil on the filter and covered with a cover slip. DAPI stained bacteria were counted at a magnification of 1600× with a Zeiss Axioplan microscope under UV-epifluorescence excitation (filter combination 365/395/397 nm). Enumeration of all active bacteria was carried out without changing the counting field of the filter under bright-field microscopy.

For determination of total number of bacteria in pumped water samples (well water), 100 mL were fixed with formaldehyde (2% final conc.) and 10–20 mL stained with the fluorochrome DAPI (final conc. 1 μg mL^{-1}) according to Porter and Feig (1980). At least 400 bacteria were counted on black membrane filters (Poretics; 0.2 μm) at 1600× magnification (with a Zeiss Axioplan microscope).

To determine the number of respiring cells, 20–50 mL of well-water samples were incubated for 1 h at *in situ* temperature after the addition of INT (200 mg L^{-1} final conc.). The reaction was stopped with formalin (final conc. 2% vol/vol), bacteria were stained with DAPI (1 μg mL^{-1} final concentration) and filtered on 0.2 μm Cellulose nitrate filters (Sartorius). Samples were counted as described above.

RESULTS AND DISCUSSION

Well water

The total number of bacteria pumped from groundwater wells ranged from 13.9 to 278.7 × 10^{6} L^{-1} (Fig. 2). In both study sites bacterial numbers were higher in the outflowing groundwater (W2 and S2) and maximum abundance was reached in May (W2).

The number of respiring bacteria in pumped groundwater showed minimal values of 0.8 × 10^{6} cells L^{-1} in March in W1 and a maximum of 31.3 × 10^{6} cells L^{-1} in July in S2. Total numbers and the numbers of respiring bacteria were very low and corresponded to the low nutrient levels of the well water (Table 1). The percentage of active, i.e. respiring, bacteria expressed as % of total bacteria was usually higher in the inflowing groundwater wells. None of the bacterial parameters indicated a characteristic seasonal trend and all showed considerable fluctuations between sampling dates and sites.

Bacterial [^{3}H]thymidine and [^{14}C]leucine incorporation rates could not be measured in most cases, because the incorporation rates were below the detection limit (counts from fixed controls were as high as from samples). To increase the sensitivity of the method we tried to use tritium labeled leucine but no statistically reliable differences between samples and controls were detectable (data not shown). Longer incubation times, which could be used to achieve higher counts per minute are problematic, because incorporation rates were not linear and a decrease in the uptake of labeled THY and LEU was observed in most cases (data not shown). Only at S2 in July, when number and frequency of INT-reducing bacteria were very high, did incorporation rates of THY and LEU give results similar to the measurements in the closely located gravel-pit lake (Alfreider *et al.*, in prep.). These results indicate that the oligotrophic status of groundwater may, as in our

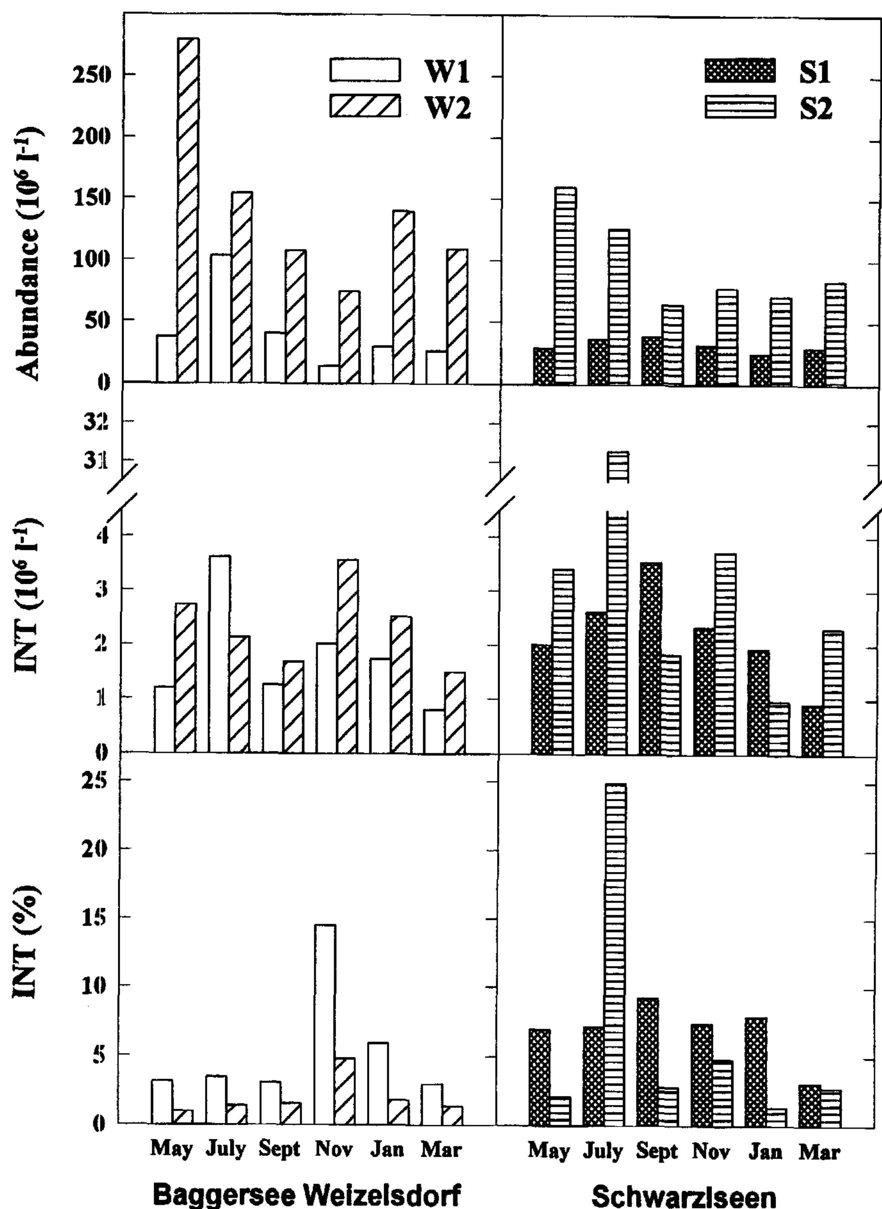

Fig. 2. Numbers of total (abundance 10^6 L^{-1}) and active (INT 10^6 L^{-1}) bacteria and frequencies of active bacteria (INT %) from groundwater of the study sites Baggersee Weizelsdorf (W1, W2) and Schwarzlseen (S1, S2) between May 1992 and March 1993.

case, not allow the application of common radioisotope methods for the estimation of bacterial activity.

Sandy deposits

After exposing of sediments for two months *in situ*, a dense microbial flora had developed, and 17.4 to 88.0 × 10^6 bacterial cells per gram dry weight (gdw^{-1}) were found. The number of INT reducing bacteria varied between 1.4 to 20.5 × 10^6 cells gdw^{-1}, and the ratio of active bacteria fluctuated between 6.0% and 41.4% (Fig. 3).

Radiotracer recovery efficiencies reached an average value of 80% and all measured [^{3}H]thymidine and [^{14}C]leucine incorporation rates were corrected accordingly. Bacterial [^{3}H]thymidine and [^{14}C]leucine incorporation rates (Fig. 4) showed lowest seasonal rates in July for both study sites with minimal [^{3}H]thymidine incorporation rates of 4.0 × 10^{-15} mol gdw^{-1} h^{-1} and minimal [^{14}C]leucine incorporation rates of 0.5 × 10^{-12} mol gdw^{-1} h^{-1} in W2. These values did not correspond to the lowest bacterial abundance and number of respiring bacteria, which were measured in September (Fig. 3). In S1 (not influenced by the lake) the highest [^{3}H]thymidine incorporation rates were measured in March with 38.8 × 10^{-15} mol gdw^{-1} h^{-1}, whereas maximal

Table 1. Physical and chemical description of the well-water samples

Parameter	S1 Min.	S1 Avg.	S1 Max.	S2 Min.	S2 Avg.	S2 Max.	W1 Min.	W1 Avg.	W1 Max.	W2 Min.	W2 Avg.	W2 Max.
Temperature (°C)	9.3	10.5	11.2	7.4	11.7	15.7	7.3	8.8	11.1	4.1	11.4	20.7
pH	7.1	7.2	7.3	7.4	7.5	7.6	7.1	7.5	8.0	7.1	7.7	8.0
Oxygen (mg L^{-1})	8.5	8.7	9.5	3.6	6.5	8.3	8.0	8.8	9.5	2.3	6.1	9.7
TOC (mg L^{-1})	1.5	1.8	2.5	1.1	1.9	2.9	0.9	1.1	1.4	1.1	2.0	3.1
Total phosphorus (μg L^{-1})	0	10	10	0	10	40	2	3	11	1	2	3
NO_3-N (mg L^{-1})	8.3	10.7	12.3	3.6	4.3	4.7	1.3	2.0	3.8	0.8	1.1	1.6

[^{14}C]leucine uptake was found in September (5.9×10^{-12} mol gdw^{-1} h^{-1}).

The effect of different particle sizes was tested by exposing sediments for 2 months from September to November. Sandy sediments from groundwater wells W1 and W2 showed that in the sediment fraction of 125–250 μm grain size the microbial abundance and activity were higher than in the sediments with a grain size between 250 and 500 μm (Table 2). With the exception of W1, also the proportion of active

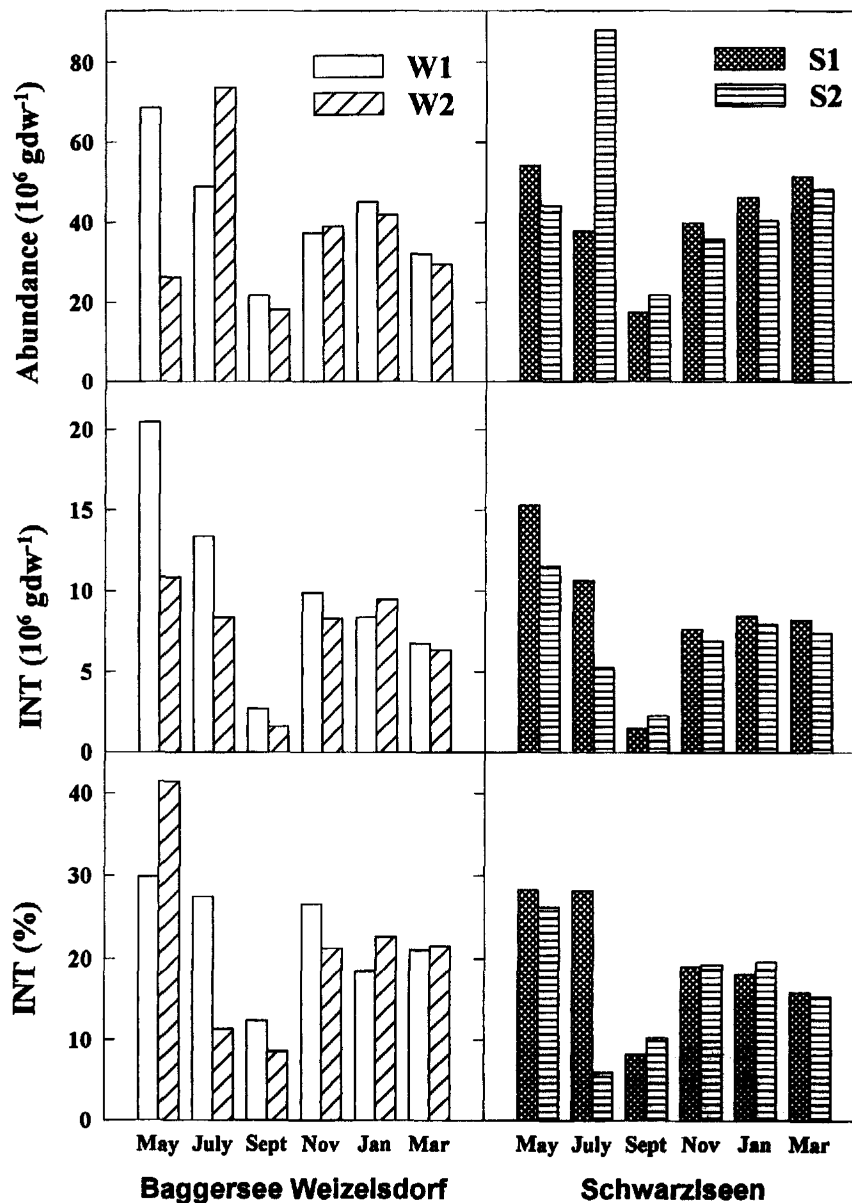

Fig. 3. Numbers of total (abundance 10^6 gdw^{-1}) and active (INT 10^6 gdw^{-1}) bacteria and frequencies of active bacteria (INT %) from exposed sediments (250–500 μm grain size) of the study sites Baggersee Weizelsdorf (W1, W2) and Schwarzlseen (S1, S2) from May 1992 to March 1993.

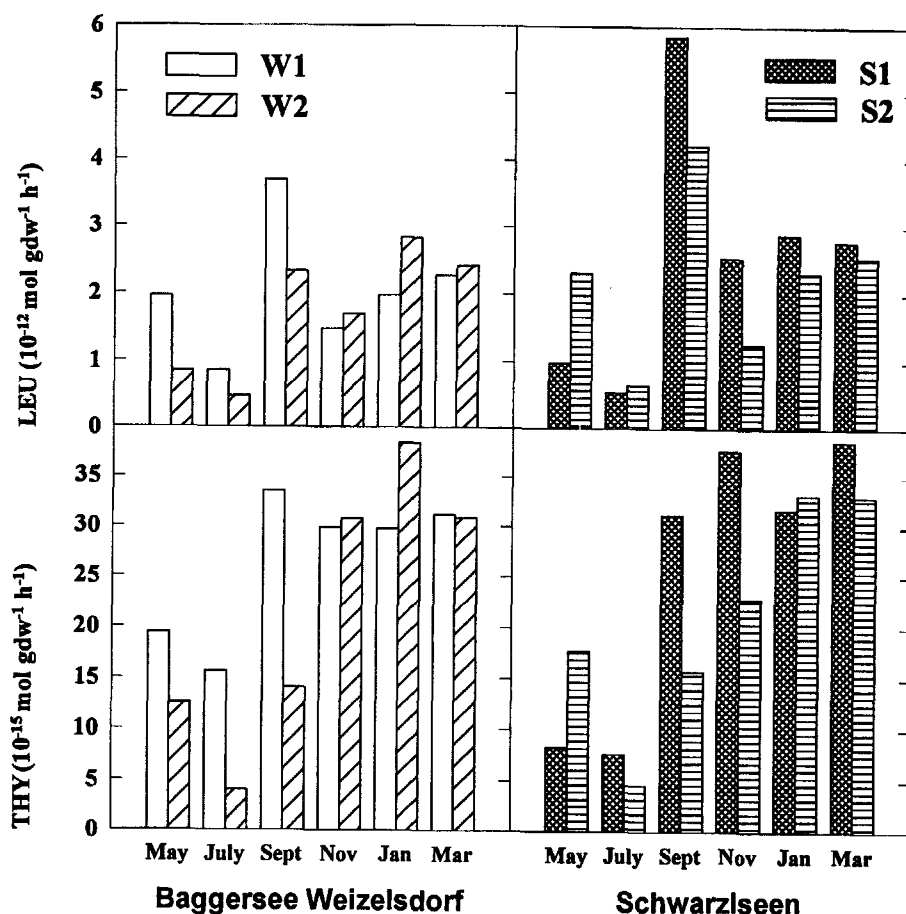

Fig. 4. Bacterial [^{14}C]leucine (LEU 10^{-12} mol gdw^{-1} h^{-1}) and [^{3}H]thymidine (THY 10^{-15} mol gdw^{-1} h^{-1}) incorporation rates from exposed sediments (250–500 μm grain size) of the study sites Baggersee Weizelsdorf (W1, W2) and Schwarzlseen (S1, S2) from May 1992 to March 1993.

bacteria was higher in the 125–250 μm fraction. In all cases, the frequency of active cells was >21%. These observations agree with the findings of Marshall (1976) who showed that with increasing surface area of sediments more bacteria and nutrients can attach. However, in our case the exposed 125–250 μm fraction did not become colonized to an extend that would be expected from the four-fold higher surface area of the sediment. A reason for the nonlinear relationship between surface area and bacterial colonization could be the reduced permeability in the fraction with smaller grain size, which also reduces fluxes of substrates and oxygen.

The calculation of bacterial growth and carbon production via radiotracer techniques requires a quantitative determination of macromolecular synthesis. Numerous studies criticize that isotopic dilution and nonspecific labeling affect the accuracy of thymidine- and leucine-based estimates of bacterial growth and production in water and especially in sediments or soils. Many attempts with different methods have been made to account for most of these factors (Bååth, 1994). Kaplan *et al.* (1992) concluded from a study in stream bed sediments that the thymidine method cannot be considered as a routine assay in sediments, mainly because there is no appropriate technique for estimating isotope dilution. Carman *et al.* (1988) found that only 2% of the incorporated radiolabeled thymidine was recovered in the DNA fraction of bacteria from a coastal

Table 2. Comparison of bacterial abundance, number of active bacteria and frequency of active bacteria in exposed sediments with different grain size

	Well unit and grain size (μm)			
	W1	W2	W1	W2
Variable	125–250	125–250	250–500	250–500
Bacterial abundance (10^6 gdw^{-1})	51.45	47.33	37.32	38.99
Number of active bacteria (10^6 gdw^{-1})	11.23	13.01	9.87	8.26
Frequency of active bacteria (%)	21.83	27.49	26.45	21.18

marine sediment. Moreover, Harvey and George (1987) reported additional problems with the thymidine technique in nutrient depleted groundwater caused by non-specific labeling.

Another drawback of the application of THY and LEU incorporation assays lies in the conversion of incorporation rates to accurate rates of bacterial production by a constant factor. Moreover, it has been reported that in some environments many bacterial cells do not take up thymidine at all (Pedrós-Alió and Newell, 1989). For these reasons we used [^{14}C]leucine and [^{3}H]thymidine incorporation values directly to compare "relative bacterial activity" (Jeffrey and Paul, 1988) between similarly structured sediment samples, and incorporation rates were not extrapolated to absolute values of bacterial production or growth rates. In this case, nonspecific labeling, isotope dilution and other factors become less important (Jeffrey and Paul, 1988).

Comparison of sediment and water

A direct comparison between colonized sediment and pumped water is difficult, since the measurements in the exposed sediments refer to gram dry weight (gdw), or better to bacteria attached on surfaces, whereas well-water data refer to bacteria in suspension. To describe differences of bacterial parameters we compared equal volumes of sediment and water. One hundred and twenty-four grams of the exposed sediment (dw) corresponded to a volume of 100 cm^3. Such a comparison does not consider the structure of the habitat and thus allows only a rough estimation of the differences between sediment and water. Nevertheless, the comparison of deposits, i.e. attached bacteria, and well water, i.e. free-living groundwater bacteria, indicated significant differences: Total bacterial numbers from sandy deposits, on average $52.1 \pm 21.3 \times 10^6$ cells cm^{-3} ($n = 24$), were about three orders of magnitude higher than bacterial densities from groundwater samples ($78.5 \pm 61.5 \times 10^6$ cells L^{-1}; $n = 24$). We found on average $10.3 \pm 5.4 \times 10^6$ respiring cells cm^{-3} ($n = 24$) in the sandy deposits and $3.39 \pm 6.01 \times 10^6$ respiring cells L^{-1} ($n = 24$) in the groundwater. This difference is also reflected by the proportion of respiring bacteria which was significantly higher in the sandy deposits (avg 19.8% $\pm$ 8.28; $n = 24$) than in the groundwater samples (5.23% $\pm$ 5.26; $n = 24$).

Bacterial abundances, numbers and frequencies of respiring bacteria from all water samples do not seem to be correlated with the respective samples of exposed sediment (Fig. 5). This indicates that bacterial numbers in the pumped groundwater are not dependent on the abundance of bacteria on grain surfaces.

The introduction of sterilized sandy sediments into groundwater permits only a simple simulation of the natural environment in undisturbed groundwater sediments. However, our results suggest that the seasonal patterns of activity and numbers of bacteria

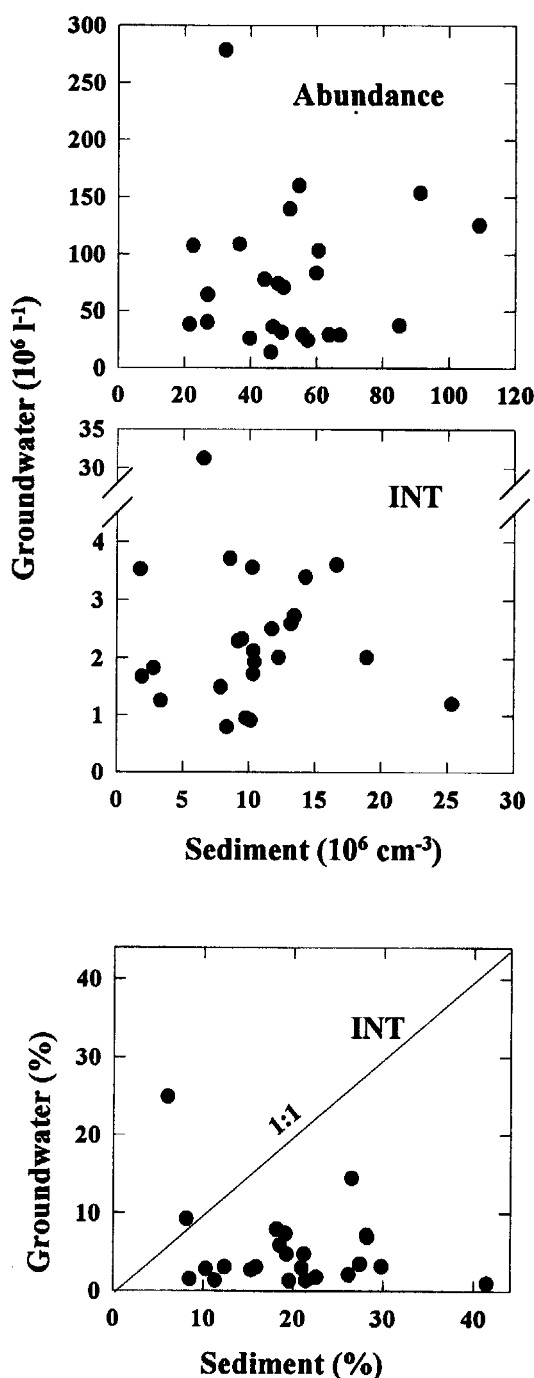

Fig. 5. Total bacterial numbers (abundance 10^6 L^{-1} and 10^6 cm^{-3}), numbers of active bacteria (INT 10^6 L^{-1} and 10^6 cm^{-3}) and frequencies of active bacteria (INT %) from exposed sediments plotted against the same parameters of the groundwater samples.

which colonized exposed aquifer sediments did not reflect the situation in the groundwater within the well, although both different habitats are influenced by the same physical and chemical parameters. The outflowing sampling station S2 was clearly influenced by the very closely situated gravel pit lake in July, and

the number of active bacteria was nearly one order of magnitude higher than at other sampling times (Fig. 2). Bacteria in the exposed sediment, however, did not follow this pattern, and activity was very low (Fig. 3).

Studies in deep subsurface systems (Hazen *et al.*, 1991) have resulted in similar conclusions: the biologically active components in aquifer ecosystems are bacteria attached to the sediment. Groundwater monitoring based on well-water samples will not accurately reflect the total microbial community or activity of bacteria in these aquifers. From our results we conclude that most bacteria in subsurface bodies are attached to sediment particles (Fig. 5). A comparison of metabolic activities of attached and free living bacteria from other reports (Marxsen, 1988; Hazen *et al.*, 1991) also shows that bacteria on particles inhabit organically rich microzones, which provide a better nutrient supply, whereas free bacteria live in a low-nutrient environment. Moreover, the diversity of microbial communities in groundwater and subsurface sediment habitats also showed considerable differences (Kölbel-Boelke *et al.*, 1988). These results, however, are based on the study of culturable populations, which represent only a small fraction of the whole bacterial assemblages. The variety between sediment and water samples may also depend on the trophic characteristics of the system. In eutrophic and shallow aquifers, no significant differences in activity were found between bacteria attached to the sediment and free living communities (Harvey and George, 1987). Hirsch and Rhades-Rohkohl (1988) suggest that most bacteria in pristine subsurface environments are attached to particles but that a large portion have motile stages. Unattached and attached communities appear to be different, but there may be a dynamic exchange of organisms between these two habitats.

CONCLUSIONS

Our study indicates that common bacteriological techniques used for activity measurements in surface waters, e.g. radioisotope methods, often yield no reasonable values in groundwater systems and are, therefore, not suitable for routine assays. By using a technique modified for exposed sediments we could show that bacteria attached to sediment particles were much more active than bacteria collected in the pumped groundwater. We assume that bacteria attached to sediment reflect microbial biomasses and activities of subsurface more properly. However, more research is required for a better understanding of the role of bacteria in the different habitats of groundwater systems. Further investigations into the microbial community structure based on molecular methods for classifying and identifying bacteria could provide results that are not biased by cultivation artifacts. A new hybridization protocol confirms the suitability of fluorescent labeled rRNA-targeted probes for the characterization of bacterial population structures even in oligotrophic systems (Alfreider *et al.*, 1996). This technique is suitable for routine applications and can be combined with well-established methods of microbial ecology that measure physiological and morphological parameters. The combination of fluorescent probes, activity-specific dyes and image analysis is a promising way to bridge the gap between taxonomic classification and physiology of individual bacterial cells.

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