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Investigations into the Number of Respiring Bacteria in Groundwater from Sandy and Gravelly Deposits

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Abstract. Samples were collected from organically polluted and unpolluted groundwater of sandy and gravelly deposits. After filtration onto polycarbonate filters (0.2 μm pore size) the number of respiring bacteria was recorded by microscopically counting cells containing red INT-formazan spots, which characterize respiring bacteria. The total number of bacteria was simultaneously recorded by epifluorescence microscopy after staining with acridine orange. The number of respiring bacteria in the groundwater samples (55–490 $\times$ 10^3/cm^3) is within the range of values for other aquatic biotopes, but as the total number of bacteria in groundwater was usually higher, the proportion of respiring groundwater bacteria (0.66–7.4%) was lower. Mainly larger bacteria, rods, and bacteria on particles could be identified as being active, whereas hardly any respiratory activity could be detected among small cocci and free interstitial bacteria. If the supply of dissolved organic matter (DOM) is adequate, the biomass of respiring bacteria correlates well with oxygen concentration, but there is no direct correlation between DOM concentration in groundwater and active bacterial biomass. Nor could any relationship be observed between the biomass of total and respiring bacteria, or between the quantity of respiring bacteria and heterotrophic bacterial activity.

Introduction

In recent years an increasing number of publications on the ecology of bacteria in groundwater habitats have appeared. Thus, we know that the numbers of viable microorganisms (CFU) determined on different media are several orders of magnitude lower than the bacterial numbers determined by epifluorescence microscopy [3, 6–8, 10].

Certainly not all microscopically observed bacteria can be regarded as "active," but the number of active bacteria is probably higher than the number of CFU, because plate count methods only estimate those bacteria that are able to grow under special, artificial conditions. The measurement of heterotrophic bacterial activity in groundwater samples using ^{14}C-labeled substrates [6, 7] has shown considerable uptake in slightly polluted and unpolluted groundwater, although uptake was lower than in nearby streams and rivers.

Respiring bacteria with an active electron transport system reduce 2-(4-iodophenyl)-3(4-nitrophenyl)-5-phenyl tetrazolium chloride (INT) to INT-formazan, which is insoluble in water and deposited in respiring cells as dark red spots. Thus, bacteria with an active electron transport system can be counted microscopically [4] and also assessed in relation to total bacterial numbers [12].

This method (counting respiring and total bacteria) from Zimmerman et al. [12] was applied to groundwater samples from unpolluted and slightly polluted sites. Further comparison was made with the number of CFU on three media, at different organic carbon concentrations.

Materials and Methods

The investigations were carried out with groundwater from sandy and gravelly deposits, which were unpolluted to slightly organically polluted. The water was pumped up through wells, from 3.5 to 5.1 m below the surface, and 0.5 m above a water-impervious clay layer, in a groundwater investigation field in the valley of the River Fulda, near Fulda (FRG). Details of the investigation area, sampling methods, determination of chemical parameters, epifluorescence microscopical counts of bacteria, and uptake kinetic investigations were published earlier [6, 7]. Chemical oxygen demand (COD) was determined using a modified permanganate method.

Numbers and volumes of bacterial cells were determined after staining with acridine orange on polycarbonate membranes (0.2 μm pore size, Nuclepore Corp.) prestained with Sudan Black B (Merck). The filter pieces were mounted in a small drop of water on a microscope slide for counting. The cover slips were surrounded with nail varnish to prevent drying out.

Determination of the numbers of respiring cells closely followed the method of Zimmerman et al. [12]. One milliliter of a 0.2% aqueous solution of INT (Serva) was added to 10 ml water samples. The samples were kept in a shaking water bath at 7.5°C in the dark, as the natural water temperatures were 7–8°C. Because temperature was rather low, the incubation time was extended to 40 min (from 20 min). The reaction was halted by adding 0.5 ml of 37% formaldehyde.

The filters were observed using epifluorescence microscopy (Zeiss Photomikroskop, HBO 50 W, BP 450–490, FT 510, LP 520, immersion objective Planapo 63/1.40, magnification × 1,260). The numbers of INT-formazan-containing bacteria and total bacteria were counted and classified on the basis of their shape and size by alternating epifluorescence and transmitted illumination and by simultaneous illumination, respectively. Red spots of INT-formazan, which characterize respiring cells, were counted only if they occurred in a cell recognized by acridine orange staining.

The numbers of CFU were determined on a beef-extract-peptone medium (10 g beef extract, and 10 g peptone per liter) according to the Standard Methods for the Examination of Water and Waste Water (1971), after filtration onto membrane filters (Sartorius standard nutrient pad set, SM 140 64-050N), PYGV medium (0.25 g Bacto-Peptone, 0.25 g Bacto-Yeast Extract, 15 g Bacto-Agar, all Difco, 0.25 g glucose, 20 ml of Hutner mineral salts solution, as modified by Cohen-Bazire, and 5 ml double-concentrated vitamin solution in 1 liter of distilled water; [11]), and PYGV 5% medium (as PYGV, but only 0.0125 g Bacto-Peptone, 0.0125 g Bacto-Yeast Extract, and 0.0125 g glucose per liter). For further details on the preparation of PYGV and PYGV 5% media see [1] and [2]. Incubation was at 20°C in the dark. CFU were counted after 4 weeks.

The heterotrophic bacterial activity was measured by determining the uptake of ^{14}C-labeled glucose at one concentration (600 μg glucose/liter = 3.33 μmol/liter, incubation at 7.5°C for 1 hour). The relative uptake potential v_r (measured at saturation concentration) corresponds to the maximum uptake velocity V_{max}, as shown earlier [6]. There were no diffusion problems.

Results and Discussion

The number of respiring bacteria in the groundwater samples is in the range of values from other aquatic biotopes in Central Europe [12]. However, the

Table 1. Total numbers of bacteria and numbers of respiring bacteria in groundwater and other aquatic environments

Sample source	No. of samples	Total no. of bacteria (10^6/cm^3)	No. of respiring bacteria (10^6/cm^3)	Proportion of resp. bacteria (%)
Groundwater (this study)	11	2.0–30	0.055–0.49	0.66–7.4
Lakes and ponds in N. Germany[a]	4	1.1–4.2	0.1–1.5	5–36
Baltic Sea[a]	3	0.1–2.4	0.1–0.3	6–12
Lough Neagh, N. Ireland (eutrophic)[b]	4	64–110	13–32	21–29

[a] From [12]; [b] from [9]

Table 2. Comparison of data (from April and June 1983) from microscopic analysis of total and respiring bacteria

	Mean cell volume (μm^3)	Rod-shaped cells (% of total)	Cells >1 μm (% of total)	Cells on particles (% of total)
Total bacteria	0.072–0.090	46–70	13–26	35–81
Respiring bacteria	0.11–0.27	91–98	34–90	67–95

total number of bacteria is usually higher so that the proportion of respiring bacteria in groundwater is usually less than that found in those environments (see Table 1). It must be emphasized that the groundwater samples contain less than 10% of the total bacteria of the groundwater-bearing sandy and gravelly deposits [8].

Table 2 shows that mainly the larger bacteria are respiring; in particular, hardly any active cells could be observed among the small cocci. The percentage of respiring bacteria on particles is much higher than the proportion of free interstitial cells which show respiratory activity. This observation corresponds with earlier reported results [8] that the bacteria on sand particles are on average larger and possess a higher proportion of rods than the interstitial bacteria. This probably reflects the better nutrient supply at particle surfaces.

The number and the volume of respiring bacteria correlate well with oxygen concentration if there is a sufficient supply of organic substances (Fig. 1a). The single sampling station whose values clearly deviate from this relationship is no. 28, which is the only site with nearly natural conditions. The correlation coefficients for oxygen concentration versus number of respiring cells are $r = 0.79^*$ (significant at 5% level) when sampling station 28 is excluded, and $r = 0.36$ (n.s.) when 28 is included, and, for oxygen concentration versus volume of respiring cells, $r = 0.85^*$ and $r = 0.30$ (n.s.), respectively.

A relationship between oxygen and total number of bacteria (and total volume) could not be observed (Fig. 1b). From earlier, more extensive investigations it is known that bacterial biomass is small at oxygen concentrations below 1 mg/liter, but that at higher concentrations small-to-high biomass values can be found (depending on, for example, the concentration of organic substances).

The good correlation between oxygen concentration and the proportion of

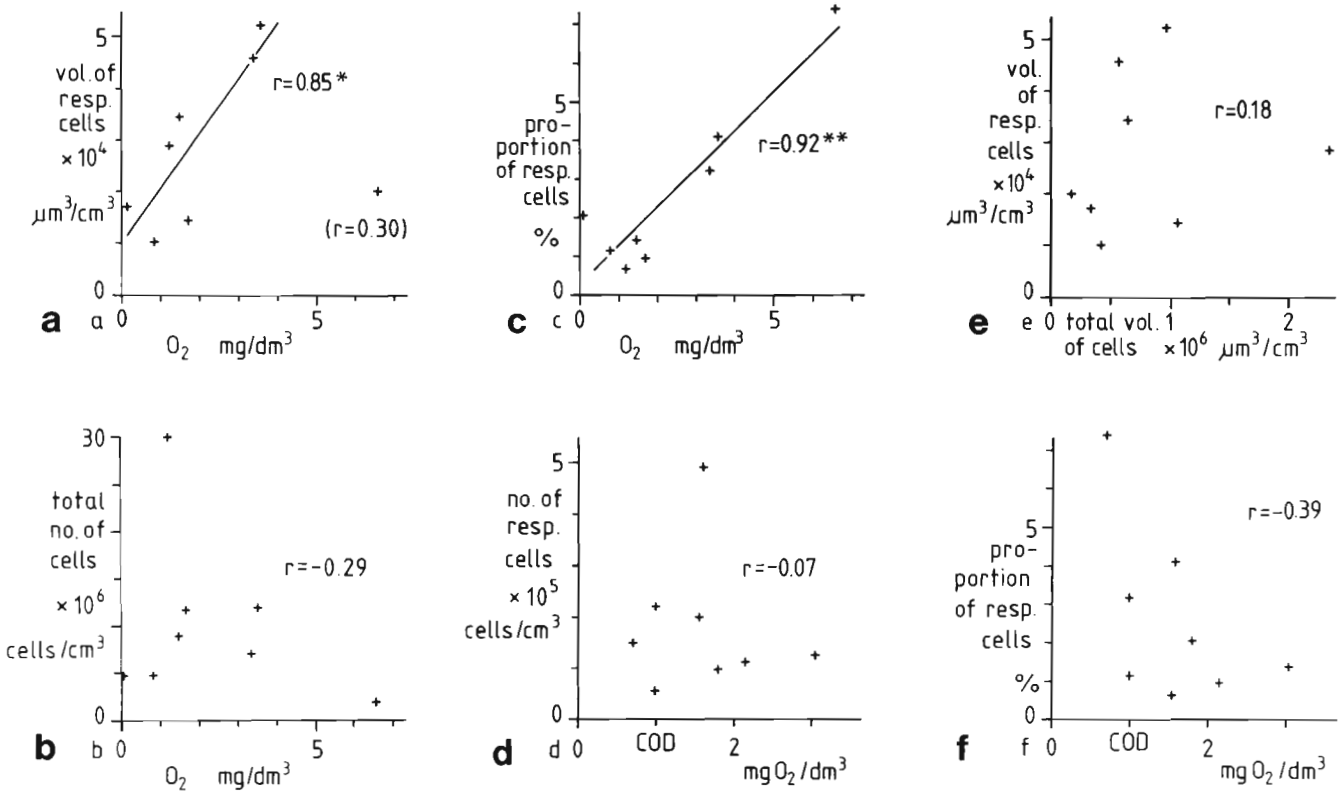

Fig. 1. Relationships between several parameters from the groundwater investigation in April 1983. Correlation coefficients were calculated using samples from all stations. An exception is the relationship between volume of respiring cells and O_2 concentration **a** where the value in brackets ($r = 0.30$) was calculated on the basis of all samples but the unbracketed value ($r = 0.85$) without the deviating result from station 28 (see Fig. 2).
* significant at 5% level.
** significant at 1% level.

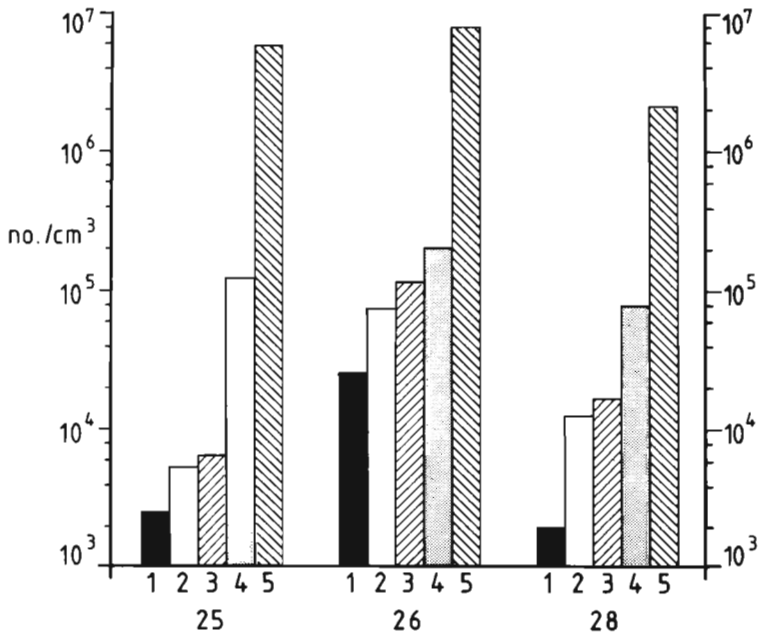

Fig. 2. Numbers of total and respiring bacteria and CFU in June 1983 at three contrasting sampling stations. Station 25: medium polluted (oxygen concentration: 0.1 mg/dm^3 O_2, COD: 3.8 mg/dm^3 O_2), Station 26: slightly polluted (ox. conc.: 3.6 mg/dm^3 O_2, COD: 1.6 mg/dm^3 O_2), Station 28: unpolluted (ox. conc.: 7.0 mg/dm^3 O_2, COD: 0.4 mg/dm^3 O_2). Histogram bars: 1, CFU on beef-extract-peptone medium; 2, CFU on PYGV medium; 3, CFU on PYGV 5% medium, 4, number of respiring bacteria; 5, total number of bacteria.

Table 3. Ranges of data from glucose uptake investigations (April 1983)

Relative uptake potential (nmol/dm^3/h)	1.0–6.6
Specific relative uptake potential (fmol/h/cell)	0.10–1.4

respiring bacteria, including values from sampling station 28, should be noted (Fig. 1c).

There was no relationship between the concentration of dissolved organic substances (measured as COD) in the groundwater and the quantity of respiring bacteria (Fig. 1d). A slight positive correlation was observed between dissolved organic matter content and the total number of bacteria in earlier studies, but with medium-to-high organic matter concentrations, low values for bacterial biomass also occur.

No clear relationship could be seen between the biomass values of total and respiring bacteria (Fig. 1e). The fraction of respiring cells seems to be rather variable, depending on the particular environmental conditions, as can be seen, for example, from the positive correlation with oxygen concentration. A calculated negative relationship with COD is not significant (Fig. 1f).

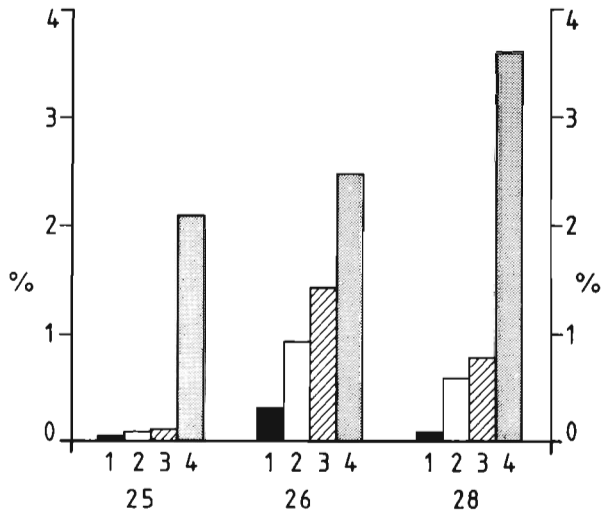

Fig. 3. CFU and respiring bacteria as % of total number of bacteria. For explanation of histogram bars, see Fig. 2.

No correlation was observed between the quantity of respiring biomass and the heterotrophic bacterial activity, measured as glucose uptake potential (Table 3) (nor between total biomass and heterotrophic activity).

From the comparison of the numbers of CFU on different media of respiring and of total bacteria at three stations with different conditions (Fig. 2), it can be seen that the number of CFU at station 26 (with medium concentration of oxygen and DOM) reaches 1.4% of the total cell number (Fig. 3) and about 60% of the number of microscopically observed respiring cells (Fig. 4). Under the almost natural conditions of station 28, the corresponding values are 0.78 and about 20%, whereas at station 25, with hardly any oxygen but rather high DOM concentration, they are only 0.11 and about 5%.

The proportions of heterotrophic bacteria growing on the three media were highest on that medium that was poorest in organic carbon (Fig. 2). The CFU numbers would probably have been higher if a lower temperature (8°C) and longer incubation time had been provided. The fractions of cells that formed colonies on the same medium at different sampling stations were quite different. This is a well-known effect.

The numbers of CFU were surprisingly high in relation to the numbers of active cells, especially at station 26. Here the CFU number reached about 60% of the microscopically determined respiring cells. One reason might be that some bacteria, which were "inactive" in the groundwater at the time of sampling, had grown on the plates, especially on the PYGV 5% medium with the lowest organic carbon content. Another point is that the red spots which characterize respiring cells under the microscope, were often only recognized with difficulty. This means that the respiring cell numbers were perhaps always underestimated to a certain, but similar, degree.

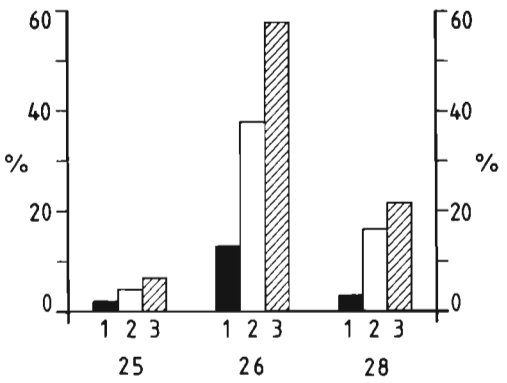

Fig. 4. CFU as % of the number of respiring bacteria. For explanation of histogram bars, see Fig. 2.

Therefore, it seems that most "active" bacterial populations occurred in groundwater habitats with medium concentrations of oxygen and organic matter, in this case the slightly polluted areas. This agrees well with earlier results [6, 7] which showed the highest heterotrophic bacterial activity in the same areas.

According to results obtained with the INT method, up to approximately 30% active cells were found in the eutrophic Lough Neagh (N. Ireland) [9]. These values correspond roughly to parallel measurements using the nalidixic acid method [5]. Some limitations of the INT method (i.e., problems in the detectability of cells under the microscope; not all "active" cells reduce INT) might have contributed to the observation of only a small proportion of respiring cells, compared with the total number of cells in the groundwater samples. Nevertheless, it seems possible that the rather low percentage of respiring cells detected in the subterranean biotope compared with surface aquatic biotopes [9, 12] results from the different environmental conditions.

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