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Review: Microbial biocenoses in pristine aquifers and an assessment of investigative methods

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Abstract The current knowledge of microbial biocenoses (communities) in pristine aquifers is presented in a review, which also discusses their relevance for questions of groundwater protection. Aquifers are heterogeneous on all scales and structured in a variety of habitats. The void spaces in many aquifers are small. The biocenoses are thus predominantly composed of microorganisms and, often, microinvertebrates. Larger voids and macroorganisms occur in karst cavities. Due to the absence of light, the biocenoses depend on chemical energy resources, which are, however, scarce in non-contaminated groundwater. The microorganisms thus show small cell sizes, low population densities and reduced activity; they developed specific strategies to survive oligotrophic conditions. The review also discusses the impact of contamination on the biocenoses, and the potential use of the biocenoses or specific organisms as indicators for groundwater quality, and the limits of this approach. Bacteria are either planktonic or attached to aquifer material, which requires both fluid and solid phase sampling. Most groundwater bacteria are viable but non-culturable. Consequently, cultivation techniques give an incomplete picture of the biocenoses, while methods from molecular microbiology provide genetic fingerprints of the entire community. Different analytical methods are available to count microorganisms, identify species, characterise microbial diversity, and measure activity.

Résumé Cette revue expose l'état actuel des connaissances concernant les biocénoses microbiennes présentes dans les aquifères oligotrophes. L'impact d'une contamination sur les biocénoses est discuté, ainsi que le potentiel que représentent les communautés ou un organisme spécifique, en tant qu'indicateur de qualité des eaux souterraines. En dernier lieu les méthodes à disposition en microbiologie sont examinées.

Les aquifères sont hétérogènes à de nombreuses échelles et sont structurés en une grande variété d'habitats. Les espaces vides sont très souvent de petite taille. De ce fait, les biocénoses sont composées de manière prédominante par des microorganismes et parfois quelques microinvertébrés. Les espaces plus larges, notamment les cavités karstiques, sont peuplés de macro-organismes également.

En l'absence de toute forme d'énergie lumineuse, les biocénoses dépendent de sources d'énergie chimiques, présentes en faible quantité dans les aquifères non contaminés. Les microorganismes développent ainsi de petites tailles, une densité de population faible et une activité réduite. La physiologie des organismes est adaptée à la survie en conditions oligotrophes.

Les bactéries sont planctoniques ou attachées aux matériaux de l'aquifère, ce qui demande un échantillonnage à la fois de l'eau et du substrat. De nombreuses méthodes sont aujourd'hui disponibles pour le comptage, l'identification et la caractérisation de la diversité, ainsi que la mesure des activités des organismes des aquifères. Comme la grande majorité des bactéries est viable mais non cultivable, les techniques de cultures actuelles ne donnent qu'une image incomplète des communautés, alors que les méthodes moléculaires développées récemment offrent la possibilité d'obtenir un profil de la communauté plus complet.

Resumen Se presentauna reseña crítica del conocimiento actual de biocenosis microbiana (comunidades) en acuíferos prístinos la cual también discute su relevancia en términos de protección de aguas subterráneas. Los acuíferos son heterogéneos en todas las escalas y estructurados en una variedad de habitats. Los espacios vacíos en muchos acuíferos son pequeños. La biocenosis está por lo tanto compuesta predominantemente por microorganismos y, frecuentemente, microinvertebrados. Espacios más grandes y macroorganismos ocurren en cavidades kársticas. Debido a la ausencia de luz la biocenosis depende de recursos energéticos químicos los cuales, sin embargo, son escasos en agua subterránea no contaminada. Los microorganismos muestran entonces tamaños de células

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pequeñas, bajas densidades de población y actividad reducida por lo que desarrollan estrategias específicas para sobrevivir en condiciones oligotróficas. Esta reseña crítica también discute el impacto de la contaminación en la biocenosis y el uso potencial de la biocenosis o de organismos específicos como indicadores de la calidad del agua subterránea, así como los límites de este enfoque. Las bacterias se encuentran ya sea en forma planctónica o ligadas al material acuífero lo cual requiere muestreo de la fase sólida y la fase fluida. La mayoría de bacterias de agua subterránea son viables pero no cultivables. Por lo tanto, las técnicas de cultivo aportan un cuadro incompleto de la biocenosis mientras que los métodos de microbiología molecular aportan señales genéticas de toda la comunidad. Existen diferentes métodos analíticos para contar microorganismos, identificar especies, caracterizar diversidad microbiana, y medir actividad.

Keywords Non-contaminated aquifer · Groundwater protection · Environmental microbiology · Micro-ecology · Microbial community

Introduction

During the past decades, an increasing number of studies have revealed that aquifers are inhabited by a large diversity of microorganisms. Small invertebrates are also present in most aquifers; larger animals can be observed in karst cavities and other specific environments. In the past, these organisms were often considered separately. Today, they are recognised to share complex biological interactions and to impact a wide range of biogeochemical processes. Aquifers are thus more and more considered as ecosystems with specifically adapted biocenoses (Danielopol et al. 2003; Hancock et al. 2005; Marmonier et al. 1993; Preuss and Schminke 2004).

This change in view is reflected in progressive national and supranational groundwater protection legislations. The Swiss Water Protection Ordinance (GSchV 1998) not only defines water quality standards but also ecological goals: "the biocenosis in groundwater should be in a natural state adapted to the habitat and characteristic of water that is not or only slightly polluted". The European Water Framework Directive (EC 2000) also uses an ecological approach, although aquifers are not directly considered as ecosystems: "the status of a body of groundwater may have an impact on the ecological quality of surface waters and terrestrial ecosystems associated with that groundwater body". Previously, aquifers were mainly considered as drinking water resources. Describing aquifers as ecosystems and their inhabitants as biocenoses thus represents a new philosophy in groundwater protection.

Ecological knowledge on aquifer biocenoses seems to be most advanced for two types of environments: the hyporheic zone, i.e. the contact fringe between surface water and groundwater, and karst aquifers. These habitats are characterised by larger voids, often accessible for sampling, and intensive contact with surface ecosystems. The hyporheic zone provides living space for a variety of organisms, including fish larvae. Biological activity in this zone contributes to the natural attenuation of contaminated surface waters infiltrating into the aquifer (Boulton et al. 1998; Brunke and Gonser 1997; Gayraud et al. 2002; Malard et al. 2002; Ward and Palmer 1994). Karst aquifers are characterised by solutionally enlarged fissures and caves. A variety of animals, including mammals, live in the cavities of the unsaturated zone. Fish, amphibians, molluscs and other aquatic animals live in water-filled caves. Microorganisms in karst are involved in a variety of geochemical processes, e.g. the dissolution and precipitation of minerals (Culver et al. 2004; Malard et al. 1994; Mathieu et al. 1992; Northup and Lavoie 2001; Rouch and Danielopol 1997; Schmitter-Soto et al. 2002; Sket 1999; Smith and Wood 2002; Vervier and Gibert 1991; Wood et al. 2002). Considerable research has also been done on microbial communities in extreme hydrogeological environments, such as hydrothermal fluids and deep aquifers (Ekendahl et al. 1994; Olson et al. 1981; Schulze-Makuch and Kennedy 2000).

In other hydrogeological environments, namely in unconsolidated sand and gravel aquifers, microbiological research most often focuses on three aspects:

- Fate and transport of microbial pathogens. Experimental works dealing with this topic often use harmless model substances to simulate the transport of pathogens, e.g. bacteriophages to simulate viruses, and microspheres to simulate bacteria and protozoans (e.g. Auckenthaler et al. 2002; Craun et al. 2002; DeBorde et al. 1998; Edberg et al. 1997; Flanigan and Rodgers 2003; Flynn 2003; Golas et al. 2002; Herwaldt et al. 1992; Lillis and Bissonnette 2001; Lisle and Rose 1995; Mahler et al. 2000; Nasser et al. 1993; Rossi et al. 1998; Schaffter and Parriaux 2002; Szewzyk et al. 2000).
- Natural attenuation processes (e.g. Bloom et al. 2000; Jean et al. 2002; Ruiz-Aguilar et al. 2002; Smets et al. 2002).
- Bioremediation of contaminated sites (e.g. Anderson and Lovley 1997; Hohener et al. 1998; Holliger et al. 1995; Hunkeler et al. 1999; Puhakka et al. 2000; Thomas and Ward 1992).

In contrast, the knowledge of microbial communities in pristine aquifers is still insufficient. The term 'pristine aquifers' is used to describe aquifers that are not contaminated or only slightly polluted. These aquifers are most often oligotrophic, i.e. characterised by limited carbon, energy and nutrient resources. This shortage is likely to induce reduced microbial activities, cell sizes and cell numbers, which in turn could be influenced by a change in the chemical water composition due to human activities. Microbial biocenoses in pristine aquifers are composed of bacteria, archea (formerly archaeabacteria), viruses and protozoans; the invertebrate fauna includes crustaceans, nematodes, oligochaetes, mites and others (Griebler and Mösslacher 2003).

There are several motives for studying biocenoses in pristine aquifers: The "hidden biodiversity in groundwater" (Danielopol and Pospisil 2001) might be considered

as something valuable that has to be protected from human impacts. Studying the microbial biocenoses and their activity provides information on biogeochemical processes and the natural attenuation capacity of the aquifer. Biocenoses also could be used for the monitoring of groundwater quality in an integrative way. Changes in the biocenoses might indicate contamination or other disturbances resulting from human activities.

The goal of this study is to present the current knowledge on microbial biocenoses in pristine aquifers and to discuss their relevance for questions related to groundwater protection.

This review deals mainly with bacteria, i.e. eubacteria (true bacteria) and archaea, because of their predominance in most aquifers and importance in biogeochemical processes. The focus is on freshwater aquifers that are used or could be used as drinking water resources. Highly mineralised and/or thermal groundwaters are not discussed in detail. The first section describes microbial biocenoses in pristine aquifers, their abundance, metabolic activity and ecological interactions, and how different types of contaminants impact the biocenoses. The second section presents an assessment of sampling techniques and analytical methods to characterise microbial communities and their activity.

As the literature on microbial communities in pristine aquifers is relatively scarce, studies on other types of environments (e.g. contaminated aquifers, surface freshwaters, soils, hydrothermal fluids) were also evaluated for this review. Some terms, concepts and observations derived from such environments can partly be applied to pristine aquifers.

Microbial biocenoses in pristine aquifers

Introduction to microbial life in pristine aquifers

Pristine aquifers (as defined in the introduction) represent extreme environments for life. The living spaces in most unconsolidated sand and gravel aquifers and fissured aquifers are small (μm to mm), and the biocenoses are thus mainly composed of microorganisms and small invertebrates. Larger void spaces and macroorganisms occur in karst aquifers, the hyporheic zone and other specific groundwater habitats. Due to the total absence of light, groundwater ecosystems depend entirely on chemical energy sources. However, chemical energy and carbon sources are scarce in most oligotrophic groundwater. Substances that are easily taken up by microorganisms are generally oxidised very quickly, and are often completely removed within the soil and unsaturated zone before reaching the primary aquifer.

All aquifers, except for fossil groundwater resources, participate at the global hydrologic cycle by recharge and discharge processes. Aquifers thus interact with other ecosystems, and are exchanging water, energy, substances and organisms with those. Groundwater recharge may transport substances and microorganisms from surface ecosystems into the aquifer (Balkwill et al. 1998); discharge operates in the opposite direction (Boissier et al. 1996). The living conditions (e.g. temperature, pH, water chemistry, flow velocity) in many aquifers are nearly stable, and the biocenoses are more or less shielded against direct influences from the surface. Karst conduits, the hyporheic zone and other specific habitats often show stronger variations in response to surface processes.

The microorganisms present in aquifers can be divided into different categories, such as autochthonous vs. allochthonous and planktonic vs. benthic.

Autochthonous microorganisms are those that permanently thrive inside the aquifer. Allochthonous species originate from other environments, such as the soil zone and surface waters, and are passively transported into the aquifer, most often together with recharge. Some of them might adapt to the living conditions in the aquifer and thus become part of the autochthonous biocenosis. Microbial pathogens in groundwater are most often, but not always, allochthonous (Auckenthaler and Huggenberger 2003).

Planktonic microorganisms are either free-floating or associated with suspended particles in the water. Benthic microorganisms live attached to solid aquifer material, i.e. on the surfaces of mineral grains and on rock surfaces. They can further be subdivided into benthic vagile and sessile (Griebler et al. 2002; Holm et al. 1992; Lehman 2001; Lehman et al. 2001; Pedersen et al. 1996). Since the report of Harvey et al. (1984), most researchers have concluded that attached bacteria dominate oligotrophic subsurface environments in terms of biomass and activity, and that most planktonic cells are inactive subsets of benthic organisms. Most microbial cells are not permanently benthic or planktonic. Laboratory studies indicate that there is equilibrium between attachment and detachment processes (Ahn and Lee 2003).

Aquifer heterogeneity and microbial habitats

Aquifer heterogeneity can be observed on different scales. On a macro scale, the entire aquifer is heterogeneous, as it may consist of different types of geological material, e.g. sand layers with gravel channels and clay lenses. The different sizes and types of mineral grains and the resulting variability of pore sizes illustrate heterogeneity on a micro scale.

Different types of parameters are heterogeneously distributed within the aquifer: lithological-mineralogical parameters, such as grain-size distribution, rock type and mineral spectrum; geometric parameters, such as fissure aperture, pore size and karst conduit diameter; hydraulic parameters, such as porosity and hydraulic conductivity; and hydrogeochemical parameters, such as pH, oxygen partial pressure and concentrations of dissolved solids.

Consequently, and from an ecological point of view, aquifers can be considered as a heterogeneous assemblage of discrete macro- and micro-scale habitats, providing a variety of living conditions (Figs. 1 and 2). Aquifer heterogeneity results in a heterogeneous distribution of the microbial communities and their activity (Brockman and Murray 1997; Hakenkamp et al. 1994; Murphy et al. 1997; Russell et al. 1994). Aquifer heterogeneity also has a major impact

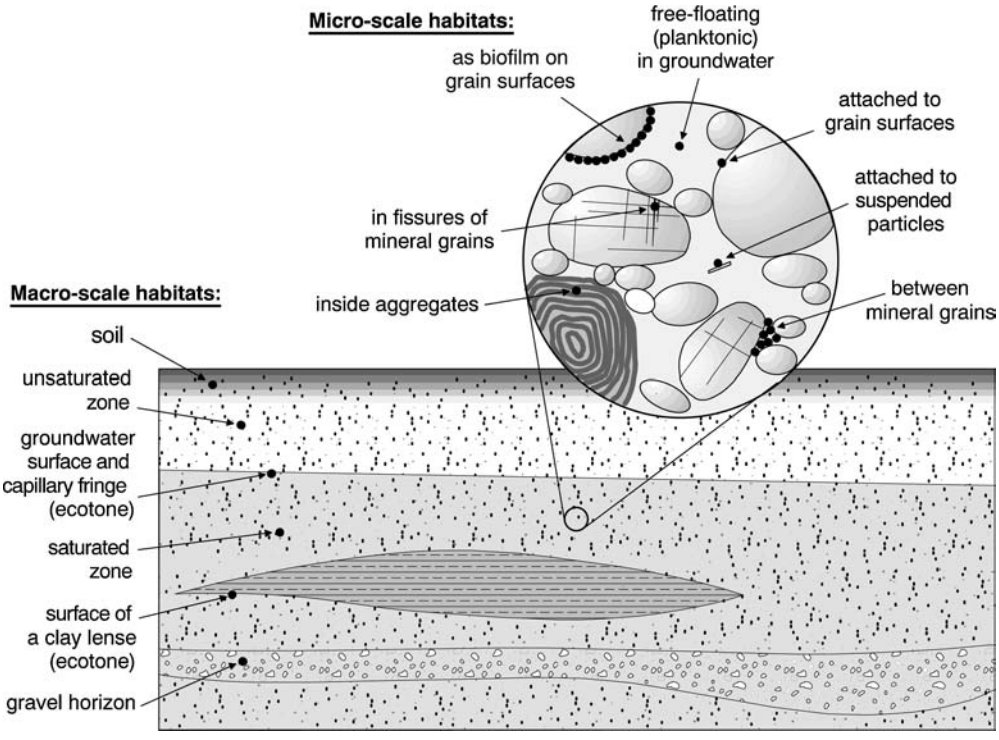

Fig. 1 Schematic illustration of ecological macro- and micro-scale habitats for microorganisms in a heterogeneous sand and gravel aquifer; the wider pore-spaces are also accessible for small invertebrates. Preferential transport may occur within the gravel horizon

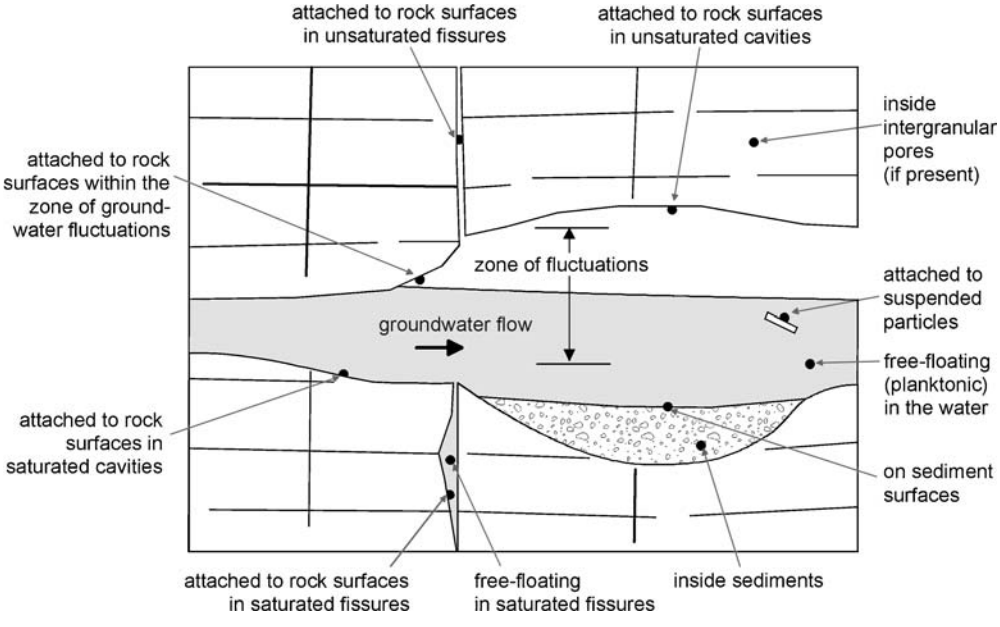

Fig. 2 Schematic illustration of habitats for microorganisms and microinvertebrates in a karst aquifer. Wider cavities are also accessible for larger invertebrates and higher animals. The cave sediments may include similar micro-scale habitats as the aquifer shown in Fig. 1

on the transport of microorganisms within the aquifer. Microorganisms, including pathogenic bacteria, viruses and protozoans, may rapidly be transported along preferential flow paths within the aquifer, e.g. in a thin but highly conductive gravel horizon within a low permeable sedimentary sequence (Flynn 2003; Li et al. 1996).

The liquid phase, i.e. the groundwater, is generally less heterogeneously distributed than the solid aquifer material. Some groundwater parameters, such as temperature, may be nearly homogeneously distributed in space and nearly constant in time. Other parameters may appear homogeneous on a macro-scale but may still show significant heterogeneities on a micro-scale. For instance, within a generally aerobic groundwater body, a sharp decrease of the oxygen partial pressure may be found on a micro-scale within mineralogical aggregates associated with organic matter. In aquifers and other environments, microscopic anaerobic habitats play an important role in the microbial community structure, increasing the richness of the biological activities (Capuano et al. 1995; Ghiorse et al. 1996; Santegoeds et al. 1999).

In macro-ecology, the contact zones between different habitats or different ecosystems are referred to as ecotones, which are often characterised by a high biodiversity and

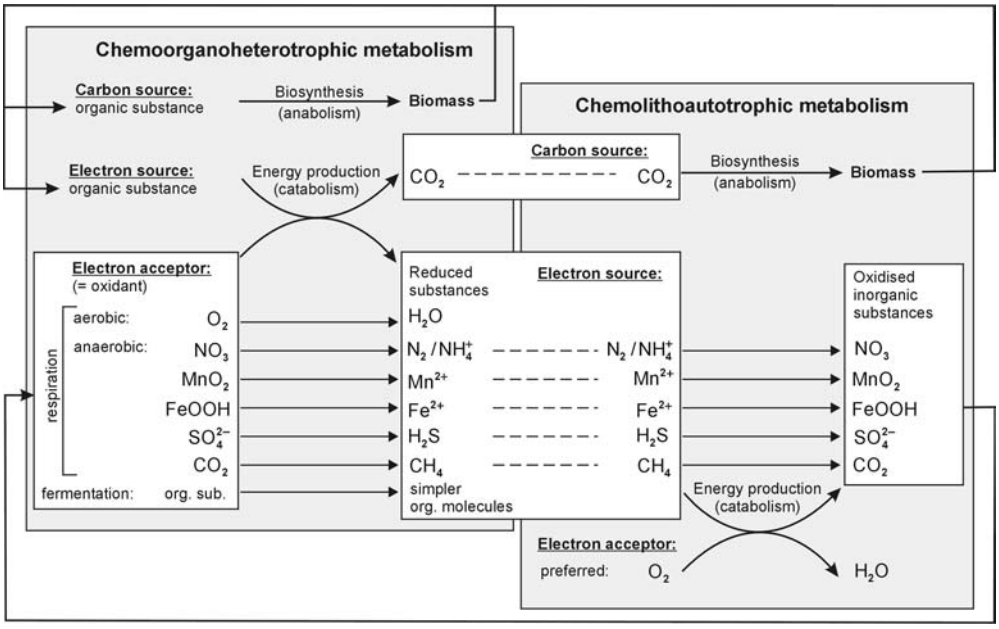

Fig. 3 Simplified illustration of the metabolic pathways of microorganisms in aquifers

biological activity. The ecotone concept can be applied to the complex habitat structures found in aquifers. The most important ecotone in an aquifer is the groundwater table with the capillary fringe, i.e. the contact zone between the unsaturated and saturated zones. Other important ecotones can be found at the contact surfaces between different lithological units, e.g. clay and sand, or the contact zones between surface waters and groundwater, i.e. the hyporheic zone. However, most available studies dealing with groundwater ecotones focus on the invertebrate and higher fauna (Galassi 2001; Plenet and Gibert 1995; Vervier et al. 1992; Williams 1993), while the concept has rarely been applied to microbial biocenoses.

Carbon and energy sources for microbial life in aquifers

Although some aquifers contain remnants of fossil organic matter (McMahon and Chapelle 1991), fresh organic carbon (OC) from the soil and surface waters is the major carbon source for most groundwater bacteria. In natural groundwaters, dissolved organic carbon (DOC) often makes up more than 90% of the total organic carbon (TOC) (Batiot et al. 2003). DOC concentrations in soil waters often range between 20 and 200 mg/L. A large proportion of the DOC is degraded in the soil zone, while only a small, mostly recalcitrant fraction, reaches the groundwater. Inside the aquifer, DOC concentrations decrease with increasing depth and travel time, while the proportion of recalcitrant DOC increases. DOC concentrations in young and shallow groundwater often range between 0.5 and 2 mg/L; deep and old groundwater is often free of DOC (Drever 1997; Neff and Asner 2001; Pabich et al. 2001).

Oxygen is a key parameter controlling microbial life. Its contents in groundwater vary between 0 and 100% saturation (11 mg/L at 10 °C). Oligotrophic aquifers with low DOC and nutrient concentrations are often aerobic. The O_2 partial pressure also depends on hydrogeological factors. The highest values can be observed in shallow and unconfined aquifers, turbulent groundwater, and aquifers directly connected to aerobic surface waters (Gavrieli et al. 2002; Malard and Hervant 1999).

Some essential elements for microbial growth are present in sufficient quantities in most pristine aquifer (e.g. Ca, Mg, K), while the availability of others might be a limiting factor (e.g. N, S, P, Fe). Microorganisms contribute actively to mineral weathering, thereby releasing the elements that are essential for their metabolism (Rogers and Bennett 2004)

Metabolic interactions between microbial populations

Organisms in aquifers use two main types of metabolic pathways, as illustrated in Fig. 3.

Chemoorganoheterotrophic organisms generate energy by the oxidation of organic substances (catabolism) and also depend on OC for the biosynthesis of their cellular material (anabolism). Most bacteria, all protozoans, animals and fungi belong to this type. There are two types of heterotrophic catabolism: respiration and fermentation. Respiration is the complete oxidation of organic matter to CO_2. Different oxidants can be used as an electron acceptor, either O_2 (aerobic) or oxidised inorganic substances (anaerobic). Fermentation is the anaerobic, energy-yielding transformation of organic molecules, such as glucose, into CO_2 and simpler organic molecules, such as ethanol (Madigan et al. 2000).

Chemolithoautotrophic organisms use CO_2 as a carbon source for biosynthesis and gain energy from the oxidation of inorganic components. Several eubacteria and archaea belong to this metabolic type. These organisms are able to live in water that is free of OC, e.g. in old, fossil, deep, thermal or mineral water (Dewettinck et al. 2001; Vachee et al. 1997).

Figure 3 also illustrates the metabolic interactions between different microorganisms. In surface freshwater ecosystems, interactions between populations have been studied in detail, such as symbiosis, synergism, commensalism, competition, antagonism, parasitism, and predator–prey relations (Martin 2002; Schubert 1991). Some of these terms and concepts can also be applied to microbial communities (Gobat et al. 2004). In aquifers, the type of interaction between microbial populations depends both on the metabolic pathways and on the groundwater flow direction. For example, aerobic bacteria create the living conditions for anaerobic bacteria further downgradient, while there is no influence in the opposite direction (commensalism). Synergism means that all populations benefit, which is the case in biofilms (Doig et al. 1995; Ross et al. 2001). Competition for the scarce OC and energy sources has to be expected in oligotrophic aquifers. Bacteriophages (bacterial viruses) are parasitic to groundwater bacteria (Ackermann and DuBow 1987). Protozoans and invertebrates act as predators; bacteria are their prey (DeLeo and Baveye 1997; Sinclair and Alexander 1989).

Bacterial abundance and activity in aquifers

Microorganisms living in oligotrophic aquifers developed special living strategies; they are able to use extremely small energy gradients and to take up the substances they need at very low concentration levels. The cell-sizes are smaller and the growth rates are slower than in nutrient-rich environments. Balkwill and Ghiorse (1985) assessed the morphological diversity of microbial biocenoses in pristine aquifers and found cells with dimensions of only 0.4 to 0.9 μm, suggesting that the bacteria were in a state of starvation and reduced activity.

Several studies assessed the number of bacterial cells in the soil, unsaturated zone, aquifer material and groundwater. Most studies confirmed that less than 10% of the cells in pristine groundwater are culturable under laboratory conditions, sometimes less than 0.1%. Cultivation techniques thus provide an incomplete picture of the biocenoses, although higher numbers can be obtained using low-nutrient (oligotrophic) cultivation media (Beloin et al. 1988; Konopka and Turco 1991; Marxsen 1988). Recent developments focus on using specific signal molecules (e.g. cyclic adenosine monophosphate (cAMP)) in order to increase the cultivation efficiency for heterotrophic bacteria from aquatic (marine) environments (Bruns et al. 2002). In some cases, however, the number of culturable counts was nearly equal to the number of direct visual counts, indicating that most cells were in an active state (Sinclair et al. 1990).

The highest cell numbers were found in the soil, about 10^9 g^{-1} dry weight. Below the soil, cell numbers are 10 to 1000 times lower, ranging between 10^6 and 10^8 g^{-1}. There is often no significant difference between the unsaturated and saturated zone, only a slight decrease with depth. Higher numbers were usually found in coarse grained, highly permeable layers, while there was not always a clear correlation between OC and cell number (Balkwill and Ghiorse 1985; Bone and Balkwill 1988; Konopka and Turco 1991; Sinclair et al. 1990). In groundwater from different types of aquifers, planktonic cell numbers often range between 10^4 and 10^6 cells mL^{-1}, about two orders of magnitude lower than the number of benthic cells. The culturable counts are much lower, often less than 10 cells mL^{-1} (Farnleitner et al. 2005; Haveman and Pedersen 2002; Marxsen 1988; Ultee et al. 2004). These studies show that the number of attached (benthic) cells most often exceeds the number of suspended (planktonic) cells, and that most of the bacteria are non culturable and in a state of reduced activity.

A detailed study on the abundance and activity of aerobic bacteria in pristine aquifers was carried out in two coastal plain sites, Oyster and Abbott Pitt, in Virginia, USA (Zhang et al. 1998). In drilling cores of up to 6.5 m length, grain size distribution (GSD), TOC, and microbial abundance and activity were measured in depth intervals between 5 cm and 1 m. Activity was measured by means of acetate incorporation (at Oyster) and glucose mineralisation (at Abbott Pitt). TOC and GSD (0.12–0.25 mm) in the Oyster site are nearly homogeneous. Viable bacteria counts decrease from 10^7 g^{-1} in the soil to 400 g^{-1} near the water table at a depth of 2 m. Within the saturated zone, both cell numbers and microbial activity show little variation, and no clear correlation with GSD. The Abbott Pitt site is more heterogeneous, with a coarse grained layer at a depth of 4–5 m. More than 100-fold higher activities were measured there, indicating that the transport of nutrients, organic matter and O_2 with flowing groundwater is an important factor for microbial activity. Increased activities were also measured near the water table, where the groundwater is in contact with the air in the pores of the unsaturated zone (ecotone). Apart from these zones, there is a decrease of TOC and microbial activity with depth.

Impact of contaminations on biocenoses in pristine aquifers

The water legislation cited in the introduction demands that "the biocenoses in groundwater should be characteristic of water that is not contaminated or only slightly polluted". This raises the question, how different contaminants influence the biocenoses. Several studies investigated the impact of contaminants on the invertebrate fauna (Plenet et al. 1996; Richardson and Kiffney 2000). Unlike most invertebrates, many microorganisms can rapidly adapt to changing environmental conditions due to their metabolic flexibility and ability to exchange genetic information (Chapelle 2001; Dröge et al. 1999; Lawrence 2002).

Generally speaking, the impact of a contamination on the biocenoses depends on the quantity and quality of the contaminant. For organic contaminants, it is important to distinguish between substances that are degradable by many different species, contaminants that can only be degraded by specialists, and recalcitrant substances that are not degradable at all. It is important to distinguish two types of inorganic contaminants: on one hand, there are inorganic contaminants that some or most bacteria can use for their

metabolism (ie. they can "eat," destroy, degrade or transform the contaminant), for example nitrate. On the other hand, there are inorganic contaminants that bacteria cannot use for their metabolism. For both organic and inorganic contaminants, toxicity effects also have to be considered; these may affect specific organisms only or harm the entire biocenoses. As groundwater organisms share complex ecological interactions, a direct impact on one species is likely to induce indirect effects on other species. The transport behaviour of the contaminant in the aquifer dictates the geometry of the plume and thus the spatial extension of possible effects on the biocenoses.

As pristine aquifers are carbon-limited environments, an input of biodegradable organic contaminants is likely to stimulate microbial activity and O_2 consumption. A sequence of redox zones with corresponding microbial communities can be differentiated within contaminant plumes, and the aquifer may locally transform into an oxygen-limited system (Haack and Bekins 2000). Franklin et al. (2000) studied the impact of buried vegetable rubbish on groundwater bacteria. Statistical evaluation of the data proved clear separation of the microbial communities in the pristine (aerobic) and contaminated (anaerobic) zones of the aquifer. Higher cell numbers and microbial diversity were found in the contaminated zones.

In contrast, inorganic and organic contaminants that cannot be used for bacterial metabolism have less influence on the microbial biocenoses, e.g. chromium and many heavy metals, and recalcitrant organic contaminants. Zhou et al. (2002) studied the microbial communities in different types of contaminated and pristine soils and aquifers, and found that even extremely high levels of Cr-III did not significantly influence microbial diversity. Similarly, at another site with aerobic groundwater contaminated by trichlorethylene (TCE) at low concentrations (0–3 ppm), no effect on the microbial community structure was observed. TCE is recalcitrant under aerobic conditions.

Some substances may be problematic for human health even at extremely low concentration levels, e.g. pesticides. de Lipthay et al. (2004) investigated the effect of an herbicide mixture at low concentrations on the bacterial communities in a sandy aquifer. The bacterial communities were then studied in solid phase samples under pesticide influence and in control samples. The cell numbers were similar in all samples. However, the bacteria from the influenced samples were easier to cultivate and showed a higher capacity to degrade herbicides (adaptation). Differences were also observed in the microbial community structures. Even low concentrations of contaminants may thus have an impact on the microbial community structure and activity, although this impact may be difficult to detect because of the high natural aquifer heterogeneity.

Bioindicators for groundwater quality

Although the impact of different types of contaminants on the aquifer biocenoses are far from being completely understood, there are several approaches that use specific micro- and macroorganisms or the entire biocenosis as indicators for groundwater quality.

Bacteria of faecal origin, such as *Escherichia coli*, are well-established indicators for hygienic groundwater quality (Auckenthaler and Huggenberger 2003; Lehloesa and Muyima 2000). Most faecal bacteria are quite harmless but their presence in a water sample indicates the possible presence of microbial pathogens, which are much more difficult to detect. Faecal bacteria may also occur in pristine ground and surface waters that are not impacted by human activity; they can be attributed to wild animals (Niemi and Niemi 1991). Recent research revealed that small numbers of microbial pathogens, such as *Legionella*, might be detectable in pristine waters without any discernible contamination source, and without the presence of faecal indicator bacteria (Brooks et al. 2004; Schaffter et al. 2004). In some cases, microbial pathogens thus seem to be part of the "natural" biocenosis in groundwater.

The invertebrate fauna has been used since 1902 to assess the trophic state and O_2 content of surface freshwater ecosystems (Baur 2003). There are promising attempts to develop similar methods for groundwater quality monitoring (Dumas et al. 2001; Hahn and Friedrich 1999; Malard et al. 1996a,b; Pipan and Brancelj 2004). The low population densities and the large number of locally restricted endemic species often limit the applicability of this approach.

In the future, it is also imaginable to use specific groundwater bacteria as indicators for chemical water quality, e.g. the increased presence and activity of bacterial species that are known to degrade pesticides could indicate pesticide contamination (see previous section). However, as shown above, the bacterial metabolism is highly flexible, and bacteria can rapidly adapt to changing environmental conditions. One bacterial species may thus use different metabolic patterns in different habitats, while different species may have a very similar metabolic pattern (Fernandez et al. 1999; Severson et al. 1991).

It is tempting to use the microbial community as a whole as an indicator of groundwater quality. The genetic fingerprint of the microbial community provides information on the presence and relative abundance of the species and thus indicates biodiversity. In macro-ecology, a high biodiversity is considered as a good sign for environmental quality, while contamination is likely to decrease the species richness (Covich et al. 2004). Contamination is also reported to decrease the biodiversity of the invertebrate fauna in groundwater (Griebler and Mösslacher 2003). The opposite effect has sometimes been described for microbial diversity: As pristine aquifers are characterised by a shortage in OC, any input of easy biodegradable organic compounds, including contaminants, is likely to stimulate bacterial activity, cell numbers and cell sizes; the apparent microbial diversity might thus increase (Cho and Kim 2000). However, this could also be a consequence of the extremely low population densities in pristine aquifers. Some bacterial species might thus simply not be detectable in a sample, although they might be present in the aquifer (Fig. 4).

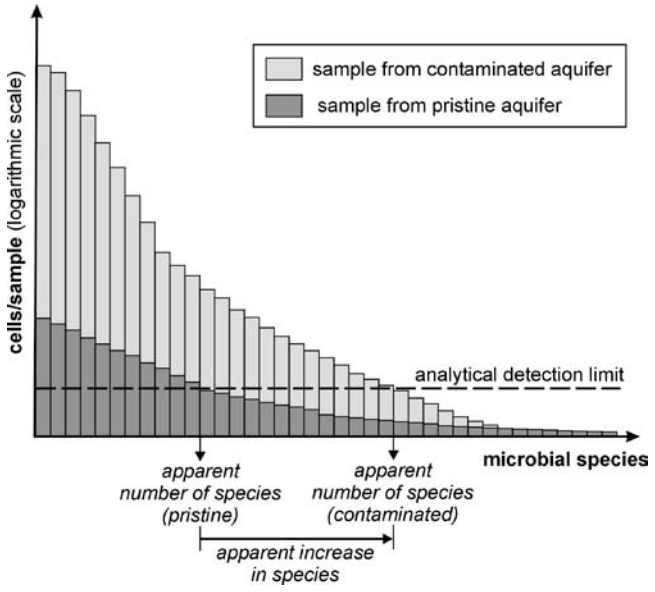

Fig. 4 Schematic illustration of the species distribution (in the order of decreasing frequency) in a pristine aquifer and an aquifer contaminated with degradable organic substances. Low numbers of microbial species and low cell numbers are often observed in samples from pristine aquifers, while the species richness might appear to increase when a contamination occurs. However, the real species number in the pristine aquifer is likely to be much higher, but most species are below the detection limit due to extremely low population densities. The species distribution below the detection limit is not known and thus purely hypothetical

Investigation methods

Introductory remark

Detailed information on methods applied in environmental microbiology can be found elsewhere (e.g. Bast 2001; Chapelle 2001; Hurst et al. 2002; Madigan et al. 2000; Maier et al. 2000; Schlegel 1993). However, there are many inherent problems when studying microbial communities in pristine aquifers: difficult accessibility, heterogeneity of the environment, low cell numbers and activities, and the sensitivity of the microbial communities to all kind of disturbances. This section consequently focuses on discussing important aspects of sampling techniques and analytical methods when applied to this specific type of environment.

Sampling techniques

Characterising microbial communities in aquifers requires both solid and fluid phase sampling, i.e. samples of the aquifer material and the groundwater (Alfreider et al. 1997; Hazen et al. 1991). Solid phase sampling needs more sophistication than water sampling. The most fundamental problem is the heterogeneous distribution of the microbial habitats in the aquifer (Figs. 1 and 2) and the resulting difficulty in obtaining representative samples. The spatial sampling strategy thus has a strong impact on the result of any microbiological investigation of aquifers (Brockman and Murray 1997; Loaiciga et al. 1992; Sievert et al. 1999). Karst aquifer systems and other hydrogeological environments additionally show strong temporal variations, which require adapted temporal sampling strategies, e.g. hourly sampling at the spring during high-flow events (Nebbache et al. 1997; Pronk et al. in press; Ryan and Meiman 1996). Another general problem is the sensitivity of microbiological biocenoses to external influences. Already the implantation of observation wells may introduce surface bacteria, O_2 or nutrients into the aquifer. The material used for sampling may also impact the microorganisms, and the number and activity of bacteria in solid and fluid phase samples may dramatically change after sampling (Brockman et al. 1998; Fredrickson et al. 1995; Haldeman et al. 1994; also C. Burn, University of Neuchâtel, unpublished diploma thesis, 2002).

Microorganismsin the solid phase of hard rock aquifers can best be analysed in rock cores from drillings (Colwell et al. 1992). As microbial contamination is likely to occur during the drilling process, laboratory analyses should be done in samples taken from the interior of the cores (Krumholz et al. 1997). Samples from unconsolidated aquifers are often homogenised and sieved; the different grain size classes are then analysed separately (Albrechtsen 1994; Hurst et al. 2002). Homogenisation of solid phase samples prior to analyses makes it possible to characterise the average composition of the microbial communities, while detailed information on their heterogeneous distribution is lost. Zhang et al. (1998) assessed the number and activity of bacteria in a sandy aquifer in samples ranging from 0.1 to 100 g. They found little influence of the sampling volume on the microbial communities, which was explained by the nearly homogeneous GSD, ranging from 0.12 to 0.25 mm.

There are also several specific problems related to fluid phase sampling: The water in an observation well may differ from the groundwater in the aquifer, because the water in the well might be stagnant and exposed to atmospheric conditions. It is thus recommended to pump a large volume of water at a low pumping rate prior to sampling. The microbial communities found in water samples change dependent on the pump rate and the pumped water volume (Franklin et al. 2000). For hydraulic reasons, the water pumped from a well does not represent the groundwater around the well, but the relative contribution of a layer depends on its hydraulic conductivity. Consequently, bacteria from highly permeable layers are overrepresented in water samples (Fig. 5). Depth specific water sampling can ideally be done using multi-level observation wells. For single wells with long filter packages, different techniques were proposed, e.g. packer systems, or the simultaneous use of two pumps (Church and Granato 1996; Lerner and Teutsch 1995; Rapp et al. 1998). A proportion of the bacteria in a water sample may be attached to suspended particles. The detection of these particle-bound bacteria requires specific sample preparation (Ventullo et al. 1983).

Analytical methods

Analytical methods in environmental microbiology can be subdivided into four categories, with four corresponding objectives: count the microorganisms, identify the species,

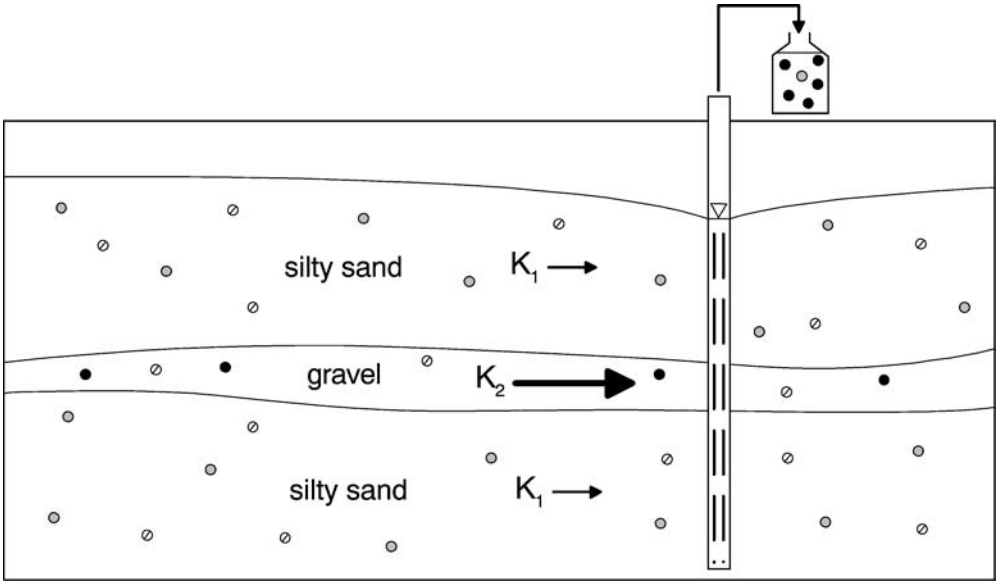

Fig. 5 Illustration of the difficulty to obtain representative water samples from an aquifer consisting of layers with different hydraulic conductivity ($K_2 > K_1$). Planktonic bacteria from highly conductive layers (*black circles*) are over-represented; bacteria from weakly conductive layers (*grey circles*) are under-represented, and bacteria that live permanently attached to aquifer material (*divided circles*) are not represented at all

characterise the diversity of microbial communities, and measure microbial activity (Table 1). Some of the methods can be used for several objectives (e.g. count and identify bacteria), and there are all types of combinations between different methods.

A well-known approach to count and identify bacteria in groundwater is the use of cultivation techniques, like heterotrophic plate count (HPC). These techniques allow the detection of faecal indicator bacteria, e.g. *Escherichia coli* (Cassidy et al. 2000; Hurst et al. 2002; Overmann 2003). Microbial cells can also be directly counted under a microscope or by means of a flow cytometer (Vives-Rego et al. 2000). In samples from pristine aquifers, the number of bacteria determined by cultivation techniques is often much lower than the number given by direct counting, because no culture medium is able to match the requirements for all bacterial species present in a sample. Within their natural habitat, the bacteria are restricted to low concentrations of substrate, which may be present under many different forms. Furthermore, the bacteria are rarely alone but interact synergistically within a complex community. A large proportion of the groundwater bacteria are thus viable but not culturable cells (VBNC) (Roszak and Colwell 1987). In many cases, less than 1% of the bacteria in oligotrophic groundwater are culturable. In some cases, culturable cells are completely absent, while the number of direct counts may still be important (Byrd et al. 1991; Cho and Kim 1999; Kell et al. 1998). Cultivation techniques consequently provide an incomplete picture of the microorganisms in oligotrophic groundwater (Hunkeler et al. 2006).

A species is defined as "the smallest diagnosable monophyletic unit with a parental pattern of ancestry and descent" (evolutionary species concept). However, bacteria reproduce by binary fission and easily exchange genetic information. Consequently, the species concept is problematic when applied to bacteria, and the terms 'operational taxonomic units' (OTU) or 'types of bacteria' are more applicable (Rosselló-Mora and Amann 2001). These terms are often used to describe and classify bacteria in groundwater (Pickup et al. 2001; Rusterholtz and Mallory 1994). The identification of bacterial species is traditionally carried out using selective growth media (i.e. cultivation techniques) and/or phenotypic analyses, which are based on morphologic, physiologic and metabolic criteria (Kent and Triplett 2002). However, different bacterial species often show similar characteristics, while different strains from one species might differ from each other. Consequently, phenotypic analyses do not always result in phylogenetic valid grouping. Techniques from molecular biology are increasingly used in the field of environmental microbiology, such as the comparison and identification of selected gene sequences. The 16S rRNA gene (ribosomal ribonucleic acid) is appropriate for that purpose, as it is present in all organisms and changed slowly during biological evolution. Comparison of the gene sequences made it possible to establish the universal phylogenetic tree of life (Woese 1987; 1998; 2000). This approach can also be used to identify bacterial species from a groundwater sample by comparing their gene sequence with information from a database of known species (Casamayor et al. 2002; Chandler et al. 1997). Specific bacteria can also be detected *in situ*, e.g. on undisturbed samples of aquifer material, using the fluorescence in situ hybridisation (FISH) method (Amann et al. 2001; Swiger and Tucker 1996; Wagner et al. 2003). It is also possible to use FISH to label specific microbial cells and then sort and count the cells by means of flow cytometry (Sekar et al. 2004).

The 16S rRNA genes can also be used to characterise the genetic diversity of microbial communities. In this case, the different 16S rRNA genes are separated using denaturing gradient gel electrophoresis (DGGE) (Boon et al. 2002; Bruggemann et al. 2000; Muyzer 1999; Muyzer and Smalla 1998) or terminal restriction fragment length polymorphism (T-RFLP) (Dollhopf et al. 2001; Moeseneder et al. 1999). The result is the genetic fingerprint of the microbial community. This technique makes it possible to

Table 1 Simplified overview of the main four objectives and the corresponding types of methods used to study microorganisms and microbial biocenoses in pristine aquifers; further explanations in the text

Objective	Type of methods	Advantage	Weakness
1. Count microorganisms	a) Cultivation techniques	Easy and robust. Ideal for the isolation of pure cultures	Culturable cells only, ineffective with oligotrophic environments. Slow growth
	b) Direct microscopic count	All cells: culturable cells and viable but non-culturable cells (VBNC)	Poor morphological information. Unable to determine species. Difficult in soil and sediment samples and in turbid water samples
	c) Flow cytometry	All cells (culturable and VBNC)	Weakness as for method 1b above, unless combined with FISH
2. Identify species	a) Phenotypic analyses	Analysis of the metabolic and catabolic activities	Sometimes ambiguous, biases induced by genomic plasticity
	b) Genetic methods (based on 16S rRNA)	No culture needed. Rapid and now reliable. Whole communities as well as specific populations can be analysed	Requires DNA/RNA extraction. Laborious with numerous samples. Sometimes numerous copies of the 16S rDNA gene within a single cell
	c) Fluorescence *in situ* hybridisation (FISH)	*In situ* detection and enumaration of known populations	Difficult to handle for solid samples. Requires information on the targetted cells beforehand. Time consuming
3. Characterise microbial genetic diversity	Fingerprinting methods (e.g. DGGE, T-RFLP)	Excellent overview of the main species present within the samples	Detection of main species only. Requires some skill
4. Measure activity	a) Measure natural bio-chemical parameters	Information on natural activity. Temporal and spatial variability. Powerful when coupled with microbial diversity measurements using non-parametric statistics	Requires direct access to the sampling locations - often difficult in aquifers. Expensive and sometimes time consuming
	b) Add specific substances	Information on potential activity	Weakness as for method 4a above. May require specific sophisticated equipment
	c) Phospholipid fatty acids (PLFA) analysis	Functional information on community structure, stress, and activities, increased significance when coupled with SIP	Databases in development. Sophisticated material
	d) Stable isotope probing (SIP)	Allow the follow-up of metabolism and co-metabolism activities, as well as the identification of main organisms involved in the processes	Difficult, sophisticated material

compare the microbial biocenoses from different samples, and to analyse their similarities using statistical methods. Non-parametric statistical analyses can be applied to combine these results with other sets of environmental data (Dewettinck et al. 2001; Farnleitner et al. 2005; Fromin et al. 2002). Microscopic analyses and flow cytometry, combined with specific coloration techniques, can be used to characterise the morphological diversity of microbial communities, e.g. the size-distribution of microbial cells within a groundwater sample (Lebaron et al. 2002).

Methods to measure microbial activity in groundwater have typically been applied in the field of contaminant hydrogeology. Some of these methods have also been used to study pristine aquifers, although activity is often extremely low in such environments. There are several approaches to measure activity. The first one is based on the measurement of natural chemical parameters, like O_2 and other oxidants, or nutrients. Microsensors are available for a variety of parameters and make it possible to measure chemical gradients on a microscopic scale (Amann and Kühl 1998;

Damgaard and Revsbech 1997; Damgaard et al. 1998; de Beer et al. 1997; Klimant et al. 1997; Santegoeds et al. 1998). Measuring isotope fractionation (e.g. $^{13}C/^{12}C$) is also a powerful tool to quantify microbial activity (Bloom et al. 2000; Hunkeler et al. 2001; Hunkeler et al. 2003). The degradation of specific substances, including isotopically labelled compounds, can be studied in different ways:

- Injecting the substance into the aquifer (push-pull test, Istok et al. 1997).
- Adding the substance to a sample of groundwater or aquifer material (Hollibaugh 1994; Kirchman et al. 1985; Marxsen 1996).
- Adding small sample volumes to a palette of different substances (Winding 1994).
- Column and microcosm studies (Baker et al. 2000; Gillham et al. 1990).

The method of microautoradiography makes it possible to detect the uptake of radiolabelled compounds (^{14}C, only for laboratory studies) into single cells (Lee et al. 1999; Nielsen and Nielsen 2002). Another group of methods consists of measuring biomolecules that indicate microbial activity, e.g. rRNA or mRNA (ribosomal/messenger ribonucleic acid) and ATP (adenosine triphosphate) (Wilson et al. 1999). It is also possible to observe the incorporation of ^{13}C labelled compounds into biomolecules, such as phospholipid fatty acids (PLFA), RNA or DNA by means of stable isotope probing (SIP) (Dumont and Murrell 2005; Wackett 2004). This approach allows the identification of microbial species involved in biodegradation, and makes it possible to follow the flow of ^{13}C along the food chain (Mauclaire et al. 2003; Pombo et al. 2002).

Conclusions

Recognising aquifers as ecosystems and their inhabitants as members of complex biocenoses represents a new philosophy in groundwater protection, which has two main aspects:

First, the biocenoses in groundwater can be considered as something valuable that has to be protected. This perspective is less aberrant than it might appear initially. Aquifers provide highly specific living conditions, and many aquifer habitats are partly isolated from other ecosystems, often since the time of sedimentation. The groundwater fauna consequently includes a large proportion of rare and endemic species, and even endemic genus's, families and orders. For example, all 160 known species of the order *Bathynellacea* (crustaceans) in Europe are restricted to aquifers (Griebler and Mösslacher 2003). Many species of the groundwater fauna are yet to be discovered. From an ecological point of view, karst aquifers and the hyporheic zone can be considered as particularly valuable, as these environments provide habitats for a broad range of invertebrates and higher animals (Hancock et al. 2005). Protecting aquifers thus helps to maintain global biodiversity (Danielopol and Pospisil 2001). This justifies the protection of aquifers independently from their actual or potential use as drinking water resources. Microorganisms are an integral part of the groundwater biocenoses but are usually not included in the concept of biodiversity protection.

Second, the biocenoses are crucial for many questions related to groundwater protection and hydrogeology. Numerous studies have demonstrated the importance of microorganisms for a wide range of geochemical processes and the natural contaminant attenuation capacity of the aquifer. These processes can only be fully understood within an ecological framework considering the complex interactions between different organisms and their environment (e.g. Röling and van Verseveld 2002). Microorganisms also influence the hydraulic properties of aquifers. For example, heterotrophic bacteria contribute to the initial karstification of carbonate rocks by generating CO_2 (Gabrovsek et al. 2000); biofilms may partly clog the pore spaces, while protozoans and microinvertebrates graze on these biofilms (DeLeo and Baveye 1997; Kota et al. 1999). To some degree, specific micro- and macroorganisms or the entire biocenoses allow to conclude on the groundwater quality. However, more research is needed to establish the correlations between different environmental parameters on one hand and the composition, diversity and activity of the microbial biocenoses on the other hand. The rapid methodological developments in the field of molecular microbiology will make it possible to measure microbial diversity and activity in aquifers more efficiently than it can be done today. In the future, the monitoring of microbial communities and their activity could become an integral part of groundwater quality monitoring.

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