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Microbial biodiversity in groundwater ecosystems

C. GRIEBLER AND T. LUEDERS
Helmholtz Zentrum München – German Research Center for Environmental Health, Institute of Groundwater Ecology, Neuherberg, Germany

SUMMARY

1. Groundwater ecosystems offer vast and complex habitats for diverse microbial communities. Here we review the current status of groundwater microbial biodiversity research with a focus on Bacteria and Archaea and on the prospects of modern techniques for enhancing our understanding of microbial biodiversity patterns and their relation to environmental conditions.
2. The enormous volume of the saturated terrestrial underground forms the largest habitat for microorganisms on earth. Up to 40% of prokaryotic biomass on earth is hidden within this terrestrial subsurface. Besides representing a globally important pool of carbon and nutrients in organisms, these communities harbour a degree of microbial diversity only marginally explored to date.
3. Although first observations of groundwater microbiota date back to Antonie van Leeuwenhoek in 1677, the systematic investigation of groundwater microbial biodiversity has gained momentum only within the last few decades. These investigations were initiated by an increasing awareness of the importance of aquifer microbiota for ecosystem services and functioning, including the provision of drinking water and the degradation of contaminants.
4. The development of sampling techniques suitable for microbiological investigations as well as the application of both cultivation-based and molecular methods has yielded substantial insights into microbial communities in contaminated aquifers, whereas knowledge of microbial biodiversity in pristine habitats is still poor at present.
5. Several novel phylogenetic lineages have been described from groundwater habitats, but to date no clearly 'endemic' subsurface microbial phyla have been identified. The future will show if the rather low diversity generally found in pristine oligotrophic aquifers is a fact or just a result of low abundances and insufficient resolution of today's methods. Refined approaches complemented by statistically rigorous applications of biodiversity estimates are urgently needed.
6. Factors identified to control microbial diversity in aquifers include spatial heterogeneity, temporal variability and disturbances such as pollution with chemical anthropogenic contaminants. Although first insights into the importance of individual biogeochemical processes may be obtained from surveys of microbial diversity within functional groups, direct links to groundwater ecosystem functioning have rarely been established so far.

Keywords: aquifer contamination, ecological concepts, functional diversity, microbial biodiversity, spatial and temporal heterogeneity

Correspondence: Christian Griebler, Helmholtz Center Munich – German Research Center for Environmental Health, Institute of Groundwater Ecology, Ingolstädter Landstrasse 1, 85764 Neuherberg, Germany. E-mail: christian.griebler@helmholtz-muenchen.de

Introduction

The greatest diversity of living organisms on our planet is found within the microbes. They are ubiquitous and abundant. Invisible to the human eye, microbes are generally responsible for processes of global relevance and the turnover of energy and matter. Their distribution on earth is limited by only a few factors, *i.e.* extreme temperature (>121 °C), extremely acidic or alkaline pH (<0.5 and >12.5), and water availability (Hendry, 2006). Microbes thus inhabit almost every habitat on this planet, and are found even in hot springs, glacier ice, the atmosphere, the terrestrial subsurface and groundwater ecosystems, which will be the focus of this review.

Today's understanding of microbial diversity and distribution patterns is still in an early stage and extremely patchy, especially for groundwater ecosystems. Three decades ago, the idea that the distribution of active microbes is limited to top soil and rhizosphere environments or, at best, to the top 100 m of the earth's crust, was a widespread belief among microbiologists (Alexander, 1971). In the 1970s, the extraction of oil and drinking water from the subsurface and the corrosion and clogging of pipes and wells triggered a closer microbiological investigation of saturated subsurface environments (e.g. Ehrlich *et al*., 1983). Large programs, such as the U.S Department of Energy (DOE) Subsurface Science Program (SSP) (Balkwill *et al*., 1997), or a priority program of the German Research Foundation (DFG) focussing on shallow groundwater systems (Hirsch, 1992b) provided first systematic insights into subsurface microbiology. Today we know that (i) the entire earth's underground (within the above limits of life) is colonised by microbes, (ii) microbial communities in the subsurface consist mainly of Bacteria and Archaea, but also of Protozoa and Fungi and (iii) these microbial communities are active and relevant for biogeochemical processes.

In this review, we give an overview of the current understanding of microbial diversity in groundwater ecosystems, with emphasis on the shallow (<50 m below surface) subsurface. The terms ground water, aquifer, and groundwater ecosystem are used synonymously rather than as distinct terms to delineate the liquid phase from the solid matrix. We focus on prokaryotes and interpret observed diversity patterns in an ecological conceptual context.

Basic features of groundwater ecosystems

Groundwater ecosystems are generally devoid of photosynthesis and lack inputs of fresh, easily available organic carbon. These factors are among the most important distinctions to surface soil and aquatic microbial communities (Table 1; Gibert, 1994) Microbial communities within aquifers are thus expected to consist largely of heterotrophs well adapted to the nutrient-poor and oligotrophic groundwater environment (Ghiorse & Wilson, 1988; Madsen & Ghiorse, 1993). Lithoautotrophs, which fix carbon dioxide and meet their energy requirements by oxidising inorganic electron donors, are another important component of groundwater microbial communities. However, significance has been attributed to this group only recently, and especially for deeper subsurface habitats (Gold, 1992; Stevens & McKinley, 1995; Kotelnikova & Pedersen, 1997; Stevens, 1997).

Groundwater habitats are characterised by hydrological, chemical and geological heterogeneity (Madsen & Ghiorse, 1993). The vertical layering of strata is unique in every given aquifer and may be very complex. However, within different zones, environmental conditions can be very stable. Because of the lack of light, low availability of organic carbon and nutrients, and comparably low constant temperatures in surface-near aquifers, such systems have been considered as 'extreme' habitats (Danielopol, Pospisil & Rouch, 2000). However, groundwater microbes are well adapted to these conditions, and for them, strong environmental fluctuations are more likely to be an 'extreme' challenge.

Groundwater ecosystems vary in size and structural complexity ranging from small systems (a few kilometres), such as alluvial aquifers along streams, to large regional aquifers (hundreds of kilometres). They can cover a small water body in a cave or whole karstic systems with complex connectivities within mountains (Gibert, 1994; Gibert *et al*., 2000; Danielopol *et al*., 2003). Many subsurface environments are hydrologically connected and thus can be considered a continuum of ecosystems (Fig. 1; Wall Freckman *et al*., 1997). The transition zones (ecotones) between soils, vadose zones, aquatic sediments, and the saturated subsurface are considered to harbour a greater diversity and activity of biota, which regulate the transfer of nutrients, particles, organisms and energy between compartments (Gibert *et al*., 1990). However, transfer rates

Table 1 Comparison of characteristics of surface and subsurface habitats from a microbial perspective (compiled from Madsen & Ghiorse, 1993; Gibert, 1994; Whitman *et al.*, 1998)

Habitat	Water residence time	Prokaryotic biomass		Organic matter availability	Community	Mode of metabolism	Habitat stratification	Habitat structure	Environmental dynamics	System productivity
		(kg)	(%)							
Ground water	2 weeks to 100 000 000 years	$22–215 \times 10^{12}$	6–40	POM excluded by filtration, low concentrations of DOM	Mainly Prokaryota, protists, fungi, low abundances of fauna	Heterotrophy and chemoautotrophy	Geological, chemical and hydrological stratification	Sediment of varying grain sizes and porewater space, rock and fissure water, caves	Constant, predictable	Primarily oligotrophic
Surface water	2 weeks to 10 years	0.25×10^{12} (incl. sed.)	0.04–0.06	DOM and POM abundant but variably distributed	Higher animals, protists, fungi, prokaryotes	Photoautotrophy, heterotrophy and rarely chemoautotrophy	Hydrological and chemical stratification, geologic stratification in sediments	Open water column, hyporheic zone, fine-grained lake sediment	Diel and seasonal dynamics, unpredictable	Oligotrophic to eutrophic
Marine systems	4000 years	305×10^{12} (including sediment)	56–86	DOM and POM abundant but variably distributed	Higher animals, protists, fungi, prokaryotes	Photoautotrophy, heterotrophy and rarely chemoautotrophy	Hydrological and chemical stratification, geologic stratification in sediments	Open water column and fine grained subseafloor sediment	Diel and seasonal dynamics, moderate predictability	Oligotrophic to eutrophic
Soils	Days to centuries	26×10^{12}	5–7	DOM and POM abundant	Prokaryotes, fungi, protists, higher animals	Heterotrophy and rarely chemoautotrophy	Hydrological and chemical stratification, geological stratification	POM (humus) and sediment of varying grain sizes	Diel and seasonal dynamics, unpredictable	Oligotrophic to eutrophic

POM, particulate organic matter; DOM, dissolved organic matter.

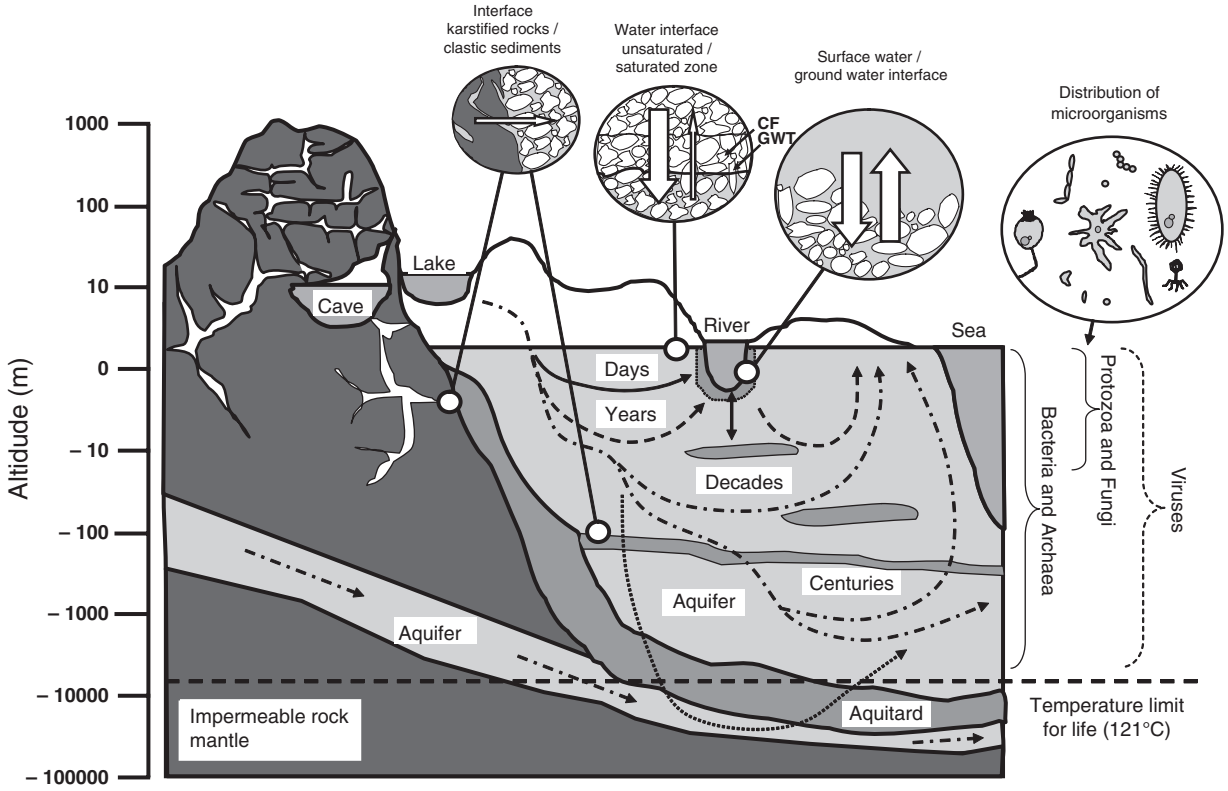

Fig. 1 Schematic view of aquatic surface and subsurface habitats. Arrows depict the flow of water carrying energy and matter through the subsurface, with boxes next to arrows indicating typical groundwater residence times. Circles highlight transition zones between habitat types. Curly braces indicate the distribution of different microbial groups in the subsurface. CF, capillary fringe; GWT, groundwater table.

may differ dramatically and may be so slow that isolation of deep subsurface habitats in particular creates opportunities for allopatric evolution even for microorganisms (Fig. 1; Fredrickson & Onstott, 2001).

Our current understanding of key biogeochemical processes and diversity of the microbes involved in and responsible for ecosystem functioning is still insufficient for most groundwater ecosystems. Sound predictions of how changes in environmental conditions, especially as a result of anthropogenic impacts, may affect microbial diversity and how changes in biodiversity will affect biogeochemical processes and the flow of energy and matter in individual habitats are urgently required.

Microbiology of the subsurface

A brief historical account

Early microbiological investigations of ground water date back to the time of Van Leeuwenhoek (1677), who described the first bacteria-like particles in well water. In the mid 19th century, Hassall (1850) carried out microscopic investigations of groundwater samples from the London water works and Cohn (1853) identified the first iron- and manganese-oxidising bacteria in groundwater wells. At the beginning of the 20th century, evidence of the existence of microorganisms in the deep subsurface was provided for brines of a Polish salt mine (Namyslowski, 1913), Californian oil-field waters (Rogers, 1917; Bastin, 1926), and Russian oil reservoirs (Ginsburg-Karagitscheva, 1933).

In 1956, the first systematic taxonomic assessment of Bacteria from a shallow aquifer in Germany suggested a distinct indigenous microbial community compared to surface waters (Wolters & Schwartz, 1956), which spawned several similar studies (see references in Hirsch, 1992b). Nevertheless, the real birth of subsurface microbiology has to be linked to the development of aseptic sampling techniques via drilling and coring in the beginning of the 1980s

(Dunlap *et al*., 1977; Wilson *et al*., 1983; Phelps *et al*., 1989). These studies clearly demonstrated the ubiquitous presence of indigenous microbes in subsurface environments. Since then, numerous programmes, conferences (e.g. the regular International Symposium for Subsurface Microbiology) and special issues in scientific journals [e.g. *Microbial Ecology* Vol. 16 (1988); *Water Science and Technology* Vol. 20 (1988); *Geomicrobiology Journal* Vol. 7 (1989); *Journal of Microbiological Methods* Vol. 21 (1995); *FEMS Microbiology Reviews* Vol. 20 (1997); *Hydrogeology Journal* Vol. 8 (2000); *FEMS Microbiology Ecology* Vol. 49 (2004)] have reflected a growing interest in subsurface microbial populations and processes.

Microorganisms in groundwater ecosystems

The total number of Bacteria found in groundwater ecosystems may vary by several orders of magnitude between 10^2 and 10^6 cells per cm^3 of ground water and between 10^4 and 10^8 cells per cm^3 of sediment (Table 2 and references therein). Comparing prokaryotic biomass estimates within important habitats of our biosphere, Whitman, Coleman & Wiebe (1998) indicated that between 6% and 40% of all prokaryotes on our planet may be hidden within the terrestrial subsurface (Table 1). Groups of microorganisms detected in aquatic subterranean systems include Bacteria, Archaea, Protozoa and representatives of yeasts and other Fungi. The distribution of some of these organisms, mainly the microeukaryotes, appears to be restricted to shallow, surface-near ground waters (Fig. 1; Madsen & Ghiorse, 1993). As this review focuses mainly on prokaryotic diversity, we refer the reader to existing reviews and original articles on subsurface microeukaryotes (Hirsch *et al*., 1992; Madsen & Ghiorse, 1993; Novarino *et al*., 1997; Haack & Bekins, 2000; Kinner *et al*., 2002; Ekendahl *et al*., 2003; Krauss, Sridhar & Bärlocher, 2005).

It has been frequently demonstrated that most prokaryotes in aquifers are attached to sediment particles, rock surfaces, and detritus, forming microcolonies or thin biofilms (Wolters & Schwartz, 1956; Pedersen & Ekendahl, 1990; Hazen *et al*., 1991; Ekendahl *et al*., 1994; Alfreider, Krössbacher & Psenner, 1997; Griebler *et al*., 2002). This attached mode of life is advantageous in carbon- and nutrient-poor environments, and sediment surfaces are geochemically

Table 2 Abundance of microbial groups in different subsurface habitats

Group	Habitat	Contamination	Abundance (cells cm^{-3})	Reference
Prokaryota				
Bacteria	Water from karst and cave systems	No	10^2–10^5	Gounot (1994); Farnleitner *et al.* (2005)
	Sediment from cave waters	No	10^4–10^8	Gounot (1994); Rusterholtz & Mallory (1994)
	Water from granite and basalt systems	No	10^2–10^5	Stevens & McKinley (1995); Pedersen (1997)
	Ground water	No	10^3–10^6	Ghiorse & Wilson (1988); Madsen & Ghiorse (1993); Pedersen (2000); Griebler (2001)
		Yes	10^3–10^7	
	Groundwater-saturated porous sediment	No	10^5–10^8	
		Yes	up to 10^{10}	
	Vadose zone sediment	No	10^4–10^8	Brockman *et al.* (1992); Kieft *et al.* (1993)
Archaea		Yes or no	up to 20% of total cell counts	Detmers *et al.* (2004)
Protozoa				
Heterotrophic Flagellata	Ground water	No	10^0–10^2	Hirsch *et al.* (1992); Madsen & Ghiorse (1993); Novarino *et al.* (1997)
		Yes	up to 10^5	Novarino *et al.* (1997); Zarda *et al.* (1998)
	Groundwater-saturated porous sediment	No	10^3–10^5	Novarino *et al.* (1997)
		Yes	up to 10^8	Novarino *et al.* (1997)
Amoebae	Ground water	Yes or no	10^{-1}–10^1	Hirsch *et al.* (1992); Madsen & Ghiorse (1993); Novarino *et al.* (1997)
Ciliata	Ground water (near surface)	Yes or no	10^{-1}–10^0	
Heliozoa	Ground water (near surface)	No	10^{-1}–10^0	

more diverse and offer more ecological niches than ground water itself (Bouwer & McCarty, 1984). The ratio of suspended to attached microbes in aquifers depends largely on the availability of DOC and nutrients, the sediment grain-size distribution, and the mineralogy of the sediments (Bengtsson, 1989; Kölbel-Boelke & Hirsch, 1989; Griebler, Mindl & Slezak, 2001; Lehman *et al.*, 2001). This ratio may span several orders of magnitude from 0.2 (Wolters & Schwartz, 1956; Harvey, Smith & George, 1984) to 10^{-4} (Alfreider *et al.*, 1997; Griebler *et al.*, 2002). Only a handful of studies have addressed the difference in microbial diversity of suspended versus attached communities, although early works based on physiological and morphological characterisation of isolates already reported significant differences (Wilson *et al.*, 1983; Kölbel-Boelke & Hirsch, 1989; Hazen *et al.*, 1991; Hirsch, 1992a; Kölbel-Boelke & Nehrkorn, 1992).

To appropriately characterise microbial diversity in ground water, both spatial and temporal patterns must be adequately captured by sampling. The relation between effective habitat size required by a microbe and sampling resolution is an important consideration (Torsvik, Øvreas & Thingstad, 2002; Horner-Devine, Carney & Bohannan, 2004a). Prokaryotic diversity in 100 cm^3 sediment sample can be similar to the regional diversity of animals (Godfray & Lawton, 2001). Brockman & Murray (1997a,b) provide a valuable discussion on how sample size may bias the resolution of various microbial patterns in subsurface environments.

Most studies have revealed the presence of at least partially active microbes in aquifers (e.g. Ringelberg, Sutton & White, 1997). As Kieft & Phelps (1997) stated in their review on microbial activities in the subsurface: 'Even if one knows the percent of cells capable of metabolic activity, one would ideally like to know the *in situ* rate of that metabolic activity'. Ten years later, this is still not routine. Most activity measurements remain based on incubations of freshly collected material on site or in the laboratory. However, data from microcosms and enrichments generally tend to overestimate actual activities. Bacterial carbon assimilation rates based on ^{14}C-substrate incorporation, revealed bacterial doubling times between 1 and 320 days, and thousands of days were estimated using ^{3}H-labelled substrates for groundwater sediments from 200 to 450 m depth (Phelps *et al.*, 1994). Doubling times inferred by modelling microbial activities based on groundwater chemistry, advection and mass balance calculations were in the range of centuries (Phelps *et al.*, 1994). In general, differences in microbial activities between subsurface and surface environments can reach up to 10 orders of magnitude (Kieft & Phelps, 1997). In the future, techniques such as stable isotope tracing and activity-based molecular methods may help to determine more precisely *in situ* metabolic rates of groundwater microbes (e.g. Griebler *et al.*, 2004; Chang *et al.*, 2005).

Further details on general aspects of subsurface microbiology can be found in a number of other reviews (Ghiorse & Wilson, 1988; Madsen & Ghiorse, 1993; Amy & Haldeman, 1997; Bachofen, Ferloni & Flynn, 1998; Chapelle, 2001; Fredrickson & Fletcher, 2001; Goldscheider, Hunkeler & Rossi, 2006). Here we concentrate on diversity patterns of microbial species, phylogenetic lineages, and functional groups in groundwater ecosystems.

Microbial species concept and subsurface biodiversity

With few exceptions, microorganisms cannot be identified by morphological criteria. Physiological characterisation, on the other hand, requires isolation and cultivation, and is usually successful only for a small percentage of microbes present in a sample (Rappé & Giovannoni, 2003). Recent estimates indicate millions of extant species within the domains Bacteria and Archaea (Curtis, Sloan & Scannell, 2002; Torsvik *et al.*, 2002). Prokaryotic biodiversity estimates are further complicated by the fact that the definition of a bacterial species itself is not trivial (Rossello-Mora & Amann, 2001). Recent investigations of microbial diversity (species richness or functional diversity) in environmental samples often rely exclusively on genetic descriptors. Torsvik, Goksoyr & Daae (1990) estimated 7000 prokaryote species in a soil sample based on the diversity of retrieved bacterial genomes. Sandaa *et al.* (1999), using a similar approach, estimated about 10 000 species in 10 g of soil. Re-analysis of these data by means of abundance models that assume species are unevenly distributed revealed even up to 10^7 distinct genomes (Gans, Wolinsky & Dunbar, 2005). However, all of these investigations are from soil, and it has not been systematically addressed to date whether the generally lower microbial biomass in groundwater systems

corresponds to a reduced, similar or even higher microbial diversity.

Microbial diversity in ground water

Early studies of groundwater microbial populations mostly applied cultivation-based techniques, such as enrichments and most probable number (MPN) counts, and interpretations were largely restricted to the comparative assessment of total cell numbers and general activity estimates (see Ghiorse & Wilson, 1988; Madsen & Ghiorse, 1993; Balkwill *et al.*, 1997). These cultivation-based screenings of both shallow and deep ground water revealed only limited diversity of bacteria, and typical isolates were mostly close relatives of well-known surface heterotrophs, such as members of the Proteobacteria, Bacteroidetes, Actinobacteria and Firmicutes (Hirsch *et al.*, 1992; Boivin-Jahns *et al.*, 1995; Stim, 1995; Zlatkin *et al.*, 1996). The affiliation of representative genera frequently isolated or of species originating from groundwater habitats is shown in Figs 2 & 3. Madsen & Ghiorse (1993) concluded in their review that Bacteria with simple life cycles appear to be most abundant and most widely distributed in the subsurface, while filamentous, spore-forming, and cyst-forming microbes appear to be generally absent or restricted to the first metres below groundwater table. This simplified picture does not hold true anymore in view of more recent data.

With the realisation in the mid-1990s of the potential of cultivation-independent techniques for analysing natural microbial communities (Amann, Ludwig & Schleifer, 1995), the question whether aquifers harbour as-yet unperceived microbial diversity could finally be tackled. As for other environments, the direct extraction, cloning and phylogenetic analysis of nucleic acids has proven most valuable to describe microbial biodiversity in groundwater ecosystems. A number of novel microbial lineages have been observed as substantial components of indigenous microbial communities in diverse groundwater systems (Boivin-Jahns *et al.*, 1996; Pedersen *et al.*, 1996; Dojka *et al.*, 1998). More recent efforts aim towards enhancing our understanding of the role of microbial biodiversity in ground water, especially as drivers of biogeochemical processes and for the resistance and resilience of aquifers against anthropogenic perturbations. In view of the limited availability and comparability of commonly used diversity descriptors for groundwater microorganisms (e.g. Shannon-Wiener index; see Martin, 2002; Bohannan & Hughes, 2003; Hill *et al.*, 2003) biodiversity in this review refers mostly to microbial taxon richness, although functional diversity is also discussed.

Pristine shallow porous aquifers

Microbial communities of pristine shallow aquifers, which provide drinking water to many municipalities, have been intensively studied. Wolters & Schwartz (1956) screened 265 bacterial isolates from a porous, alluvial sand and gravel aquifer (5–50 m below surface) and distinguished 40 strains within the Proteobacteria, Actinobacteria and Bacteroidetes. More than 30 sediment cores from 10 to 90 m depth across the unsaturated and saturated zone of an aquifer were analyzed by Hoos & Schweisfurth (1982) for the presence of different functional groups. These included aerobic organoheterotrophs and nitrifying, manganese-oxidising, sulphur-oxidising, iron-reducing and sulphate-reducing bacteria. All functional groups were detected at several depths, and local occurrence was correlated with local sediment structure. No significant correlation with depth was found for any of these functional groups. Other early studies in pristine shallow aquifers showed microbial communities distinct from those of the overlying surface soil and that total diversity was lower (Balkwill & Ghiorse, 1985; Bone & Balkwill, 1988). Genus-level identification revealed members of the α-Proteobacteria and β-Proteobacteria, Bacteroidetes, Actinobacteria and also Bacilli (Hirsch & Rades-Rohkohl, 1983, 1992; Hirsch *et al.*, 1992). Isolate diversity varied among boreholes of the same aquifer as well as with depth, depending on physico-chemical conditions (Kölbel-Boelke & Nehrkorn, 1992). All reports mentioned above support the idea that indigenous groundwater microbial communities are distinct from those found in surface environments.

Only a small number of more recent studies using molecular methods exist for uncontaminated aquifers. However, microbial communities in pristine aquifers have been repeatedly compared to those in adjacent contaminated aquifers (see sections below). The communities described in pristine porous aquifers with

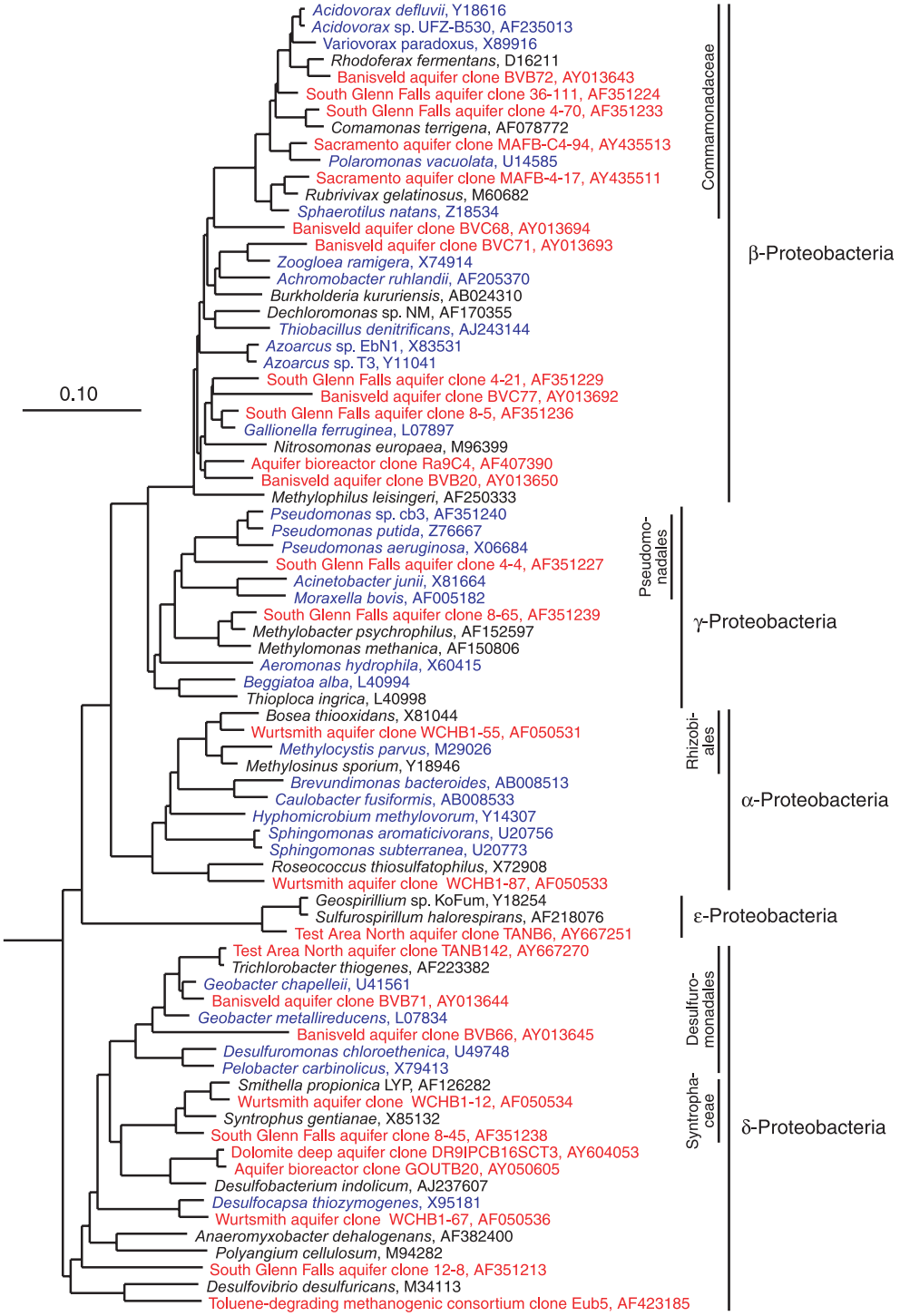

Fig. 2 Phylogenetic tree of groundwater Proteobacteria showing the affiliation of genera frequently isolated or strains actually originating from groundwater environments (marked in blue) and of environmental bacterial 16S rRNA gene sequences retrieved from aquifer samples (marked in red). The tree was reconstructed for almost full-length 16S rRNA gene sequences with the ARB software environment (Ludwig *et al.*, 2004) using maximum likelihood algorithms and a 50% base frequency conservation filter deduced for the lineages shown. Scale bar represents 10% sequence difference. Accession numbers of reference sequences are indicated. Selected non-proteobacterial bacteria were used as outgroup (see Fig 3).

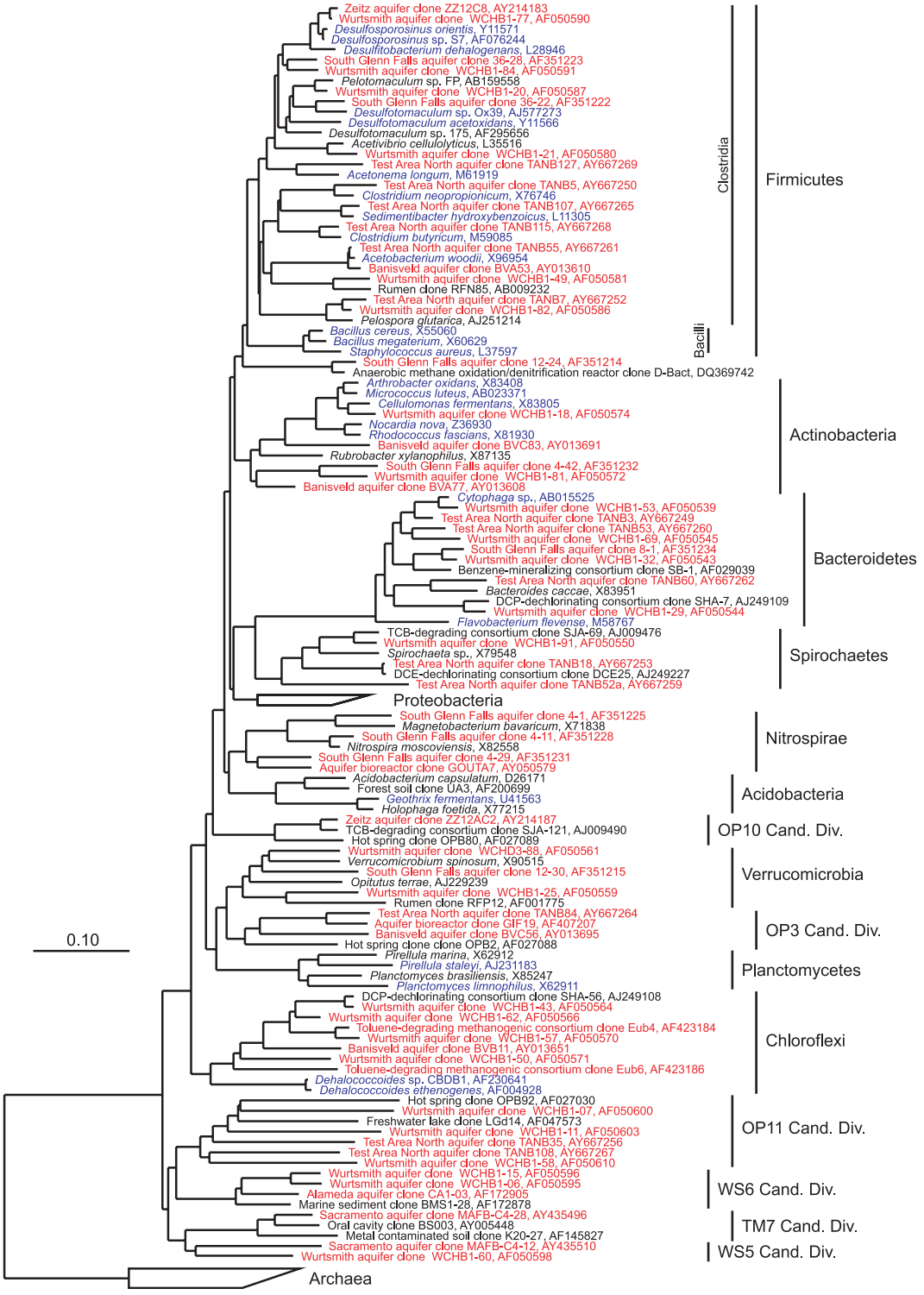

Fig. 3 Phylogenetic tree of groundwater Bacteria other than Proteobacteria, showing the affiliation of genera frequently isolated or strains originating from groundwater environments (marked in blue) and of environmental bacterial 16S rRNA gene sequences retrieved from aquifer samples (marked in red). The tree was generated as indicated in Fig 2. Designations for uncultured lineages follow Rheims *et al.* (1996), Dojka *et al.* (1998) and Hugenholtz *et al.* (1998).

molecular methods are essentially dominated by the lineages detected by cultivation, i.e. by members of different Proteobacteria, the Firmicutes, Actinobacteria and Bacteroidetes (Figs 2 & 3). However, an 'endemic' groundwater microbiota has not been described. This apparent lack of endemism may be one important distinction between the biodiversity of microbes and that of fauna in groundwater ecosystems (e.g. Marmonier *et al*., 1993). Extensive research is still needed to understand the main factors controlling microbial biodiversity in pristine ground waters. Potentially important factors include local hydrogeochemistry, availability of oxidisable substrates, dispersal of microbes from overlying unsaturated zones, and the simple food-web structure in aquifers.

The deep subsurface

The Subsurface Science Program of the US Department of Energy, initiated in 1985, promoted intense research on indigenous microbes up to several thousands of metres below surface. Early studies documented the presence of a number of morphologically and physiologically interesting microorganisms, which were stated to be unique for oligotrophic aquifers at the time (e.g. Hazen *et al*., 1991). Most isolates showed a high potential for utilising different electron donors and acceptors (e.g. Fredrickson *et al*., 1991), or could metabolise xenobiotics not normally occurring in their original environment (e.g. Ventullo & Larson, 1985). In an initiative to archive subsurface microbial biodiversity and promote general access to this genetic resource, the US DOE founded the Subsurface Microbial Culture Collection (SMCC) in 1986 (Balkwill, 1993). Today, the SMCC contains a vast array of isolates (>10 000), but also a high ratio of redundant genera within the Proteobacteria, Bacteroidetes, Actinobacteria and Firmicutes (Balkwill *et al*., 1997). Further extensive research has been conducted on microbial communities in deep, mostly fractured basalt biospheres, both ground water and marine, in the quest for surface-independent ecosystems based on hydrogen-driven lithoautotrophic microbial communities (Stevens & McKinley, 1995). Here, we focus on relatively shallow aquifers, since a number of excellent reviews on deep subsurface microbial systems is available (Zlatkin *et al*., 1996; Pedersen, 1997, 2000; Stevens, 1997; Nealson, Inagaki & Takai, 2005).

Karstic systems and caves

Much less information is available on the microbiology of shallow fractured rock or karstic groundwater systems, despite the fact that these are often utilised as a source of drinking water. Farnleitner *et al.* (2005) monitored seasonal changes in microbial community composition in ground water from two alpine karst springs, one from a dolomite karst with constant water flow and high residence time, and one from a limestone karst with short residence time and strongly fluctuating discharge. Cell counts were always higher in the limestone karst than in the dolomite karst (6.3 versus 1.5×10^4 mL^{-1}, respectively). Distinct but temporally stable microbial community fingerprints were recorded year-round not only in the dolomite, but also in the limestone spring. Sequence analysis revealed diverse cultured and uncultured members of the Proteobacteria, and showed Nitrospirae and Bacteroidetes to dominate the communities. Community composition of the suspended microbes was unchanged over time, even during a heavy rainfall event that dramatically increased discharge of the limestone spring for 10–20 h. This observation suggests that a characteristic and temporally very stable autochthonous microbial community exists in such karst systems.

O'Connell *et al.* (2003) investigated the archaeal community of a fractured basalt aquifer in an area impacted by a mixed waste plume. Ground water from five wells was analyzed with molecular methods and showed low diversity and highly similar pattern of archaeal diversity. The detected Eury- and Crenarchaeota grouped exclusively within uncultured lineages previously detected in many other environments (see Schleper, Jürgens & Jonuscheit, 2005; for an overview). Given its high detection frequency, a 'Snake River Clade' was even proposed within Group I.1a of uncultured Crenarchaeota. In a follow-up study, the metabolic and phylogenetic diversity of bacteria was investigated in two wells at the same site (Lehman *et al*., 2004). Much higher bacterial 'most probable numbers' (MPN) were found close to the former mixed-waste injection well, and a diverse community dominated by Bacteroidetes, Firmicutes, Actinobacteria and diverse Proteobacteria was described. A large portion of these sequences had no close match in public databases.

The microbial communities in pools and streams of caves, which are also considered ground water, can be very rich. The first microbiological investigations of caves date back to the early 20th century, mainly focussing on microscopically visible components of microbial communities such as Protozoa (Schreiber, 1929; Dudich, 1930). The very large and deep Lechuguilla Cave in New Mexico has been particularly well studied. This gypsum- and sulphur-bearing cave receives only small amounts of allochthonous organic material, but is characterised by extensive microbial colonisation. Almost 100 species of Fungi and a number of unspecified chemoheterotrophic and chemoautotrophic bacteria have been isolated (Cunningham *et al.*, 1995). Similarly, largely uncultivated bacterial sequences within the Proteobacteria, Actinobacteria, Cytophagales and Firmicutes have been found in the oligotrophic Fairy Cave system in Colorado. (Barton, Taylor & Pace, 2004). Other cave systems even harbour microbial food webs based entirely on primary production by lithoautotrophs. Examples are the Movile Cave in Romania (Sarbu, Kane & Kinkle, 1996) and Lower Kane Cave in Wyoming (Engel *et al.*, 2003), where biotic assemblages are energetically driven by methane and sulphide oxidation. Microbes identified in Movile Cave and Lower Kane Cave microbial mats include the *Thiobacillus*-related strain LV43 (Vlasceanu, Popa & Kinkle, 1997) and the ϵ-proteobacterial 'Lower Kane Cave Clusters' (Engel *et al.*, 2003), respectively, whose sequences are closely related to those retrieved from other sulphide-oxidising habitats. More information on the microbiology of selected caves can be found in Brigmon *et al.* (1994), Rusterholtz & Mallory (1994), Sarbu (2000), Simon & Benfield (2001) and Simon *et al.* (2001). However, as cave systems are strongly influenced by surface waters (e.g. precipitation, sinking streams) and the terrestrial surface (e.g. by fauna migrating via the cave entrance), definition of typical stygobite microbiota has not been possible to date (Gounot, 1994).

The hyporheic zone

The transition zone between surface running waters and the adjacent groundwater systems, *i.e.* the hyporheic zone, plays an essential role in biogeochemical cycling and the biodiversity of both ecosystems (Gibert *et al.*, 1990). Although information on the hydrology, geochemistry, and invertebrate diversity of hyporheic habitats is abundant, microbial communities have been less commonly characterised. Available studies mainly focus on the abundance, activity and spatial distribution of bacteria in river bed sediments (Fischer & Pusch, 1996; Claret, Marmonier & Bravard, 1998; Ellis, Stanford & Ward, 1998), rather than on microbial diversity and community composition. Feris *et al.* (2003) investigated microbial community structures in hyporheic sediments of three gravel streams with focus on seasonal changes and differences between the rivers. Denaturing gradient gel electrophoresis (DGGE) analysis showed clear seasonal patterns, similar community composition in different riffles within a given stream, and different communities among systems. However, several genera occurred in all three streams. These included *Pseudomonas*, *Aquabacterium*, *Rhodoferax*, *Hyphomicrobium* and *Pirellula* spp. Each taxon showed pronounced seasonal trends in abundance, with peaks occurring in autumn. Clear seasonal trends in hyporheic sediment microbial communities from three temperate streams were also presented by Hullar, Kaplan & Stahl (2006). Summer communities were dominated by β-Proteobacteria and γ-Proteobacteria and a *Bacillus* sp., while the communities in winter and spring were characterised by members of the Firmicutes, γ-Proteobacteria and Nitrospirae. These examples suggest that microbial communities of the hyporheic groundwater ecotones are more dynamic than those in aquifers, for which seasonal dynamics have not been reported. More details on the microbial ecology of hyporheic zones are given in Pusch *et al.* (1998), Mermillod-Blondin *et al.* (2000) and Mauclaire & Gibert (2001).

Factors controlling microbial diversity in ground water

Numerous abiotic and biotic parameters may directly or indirectly control microbial diversity in ecosystems. Here we give an overview of factors controlling microbial diversity in general, and provide supporting or contradicting data from groundwater environments where possible.

Evolutionary aspects

The large population sizes and genetic flexibility of prokaryotes are among the important reasons why the

microbial diversity in most ecosystems is high (Table 1). Unlike most eukaryotic species, prokaryotic populations represent a mixture of genetically diverging clones on which natural selection can act (Torsvik *et al*., 2002). Because of often short generation times, prokaryotes can evolve faster than higher organisms. Groundwater habitats, especially deep ones, are also frequently isolated in terms of organismic exchange. For example, sequence analysis of the 16S rRNA and *recA* genes of 39 *Arthrobacter* strains from heterogeneous deep subsurface sediments revealed that isolates from strata with low permeability were monophyletic, while isolates from a highly permeable gravel-rich layer were from several closely related lineages (Van Waasbergen *et al*., 2000). The authors suggested selection for specific dominant genotypes by locally uniform conditions over long periods to be responsible for the low biodiversity in isolated layers, while environmental fluctuations and microbial movement appeared to be important driving forces in the highly permeable layer.

Microbial evolution can be expected even in shallow aquifers. Herrick *et al*. (1997) showed that horizontal transfer of the naphthalene dioxygenase (*nahAc*) gene occurred quite recently at a site contaminated by coal-tar waste. The *nahAc* gene was exclusively located on the chromosome in isolates from an upstream sample, while in all downstream isolates the gene was plasmid-borne, with one isolate carrying the gene on both the chromosome and a plasmid. Furthermore, horizontal transfer of two marker genes for the degradation of 3-chlorobenzoate from the introduced *Pseudomonas* sp. strain B13 (γ-Proteobacteria) to two indigenous aquifer microbes closely related to *Alcaligenes* spp. (β-Proteobacteria) was observed by Zhou & Tiedje (1995). Molecular analysis of chlorobenzene and non-chlorobenzene degraders in groundwater samples from an air force base revealed that a new chlorobenzene degradation gene had evolved, originating from former non-degradative genes within bacteria indigenous to the aquifer (Van der Meer *et al*., 1998). Such genetic mechanisms may allow microbial populations to adapt rapidly to changing environmental conditions, such as contamination by xenobiotics.

Biogeography

The existence of biogeographical patterns in Bacteria has been controversial for much of the last century (Hedlund & Staley, 2003). In general, three important processes contribute to the establishment of biogeographical distribution patterns: speciation, dispersal and extinction. Speciation rates increase with population size and growth rate, as well as with fluctuating environmental conditions (Cohan, 2002). Due to smaller population sizes, low activity levels and constant environmental conditions, evolution may be less rapid in groundwater habitats than in other environments. Compared to higher organisms, extinction is less likely for microbes because of huge local population sizes and usually high dispersal. For prokaryotes it may be true that the rate of dispersal and speciation is faster than the rate of local extinction (Dykhuizen, 1998).

Precise data on the rates of these processes acting on microbial populations are still lacking for groundwater ecosystems (and most other habitats). However, there are several arguments in favour of a geographically distinct distribution of microbes, especially in subsurface environments. First, there are areas that have obviously been isolated from the surface for several thousands or millions of years, enough time for microbial populations to evolve (Table 1; Fredrickson & Onstott, 2001). Furthermore, some microbes have narrow ecological niches that reflect extreme environmental conditions. In such cases ubiquitous distribution and exchange is unlikely. This contention is supported by recent studies on hyperthermophilic Archaea (Whitaker, Grogan & Taylor, 2003) and hot spring Cyanobacteria (Papke *et al*., 2003), suggesting that geographical isolation and marked distribution patterns can indeed be observed for some microbial taxa. However, our understanding of the biogeography of microbial populations in ground water is still poor and requires further research.

Habitat size

The relationship between species richness and habitat size is one of the most consistent of all ecological patterns and appears to be one of the few universal laws in ecology (Lawton, 1999). Data supporting this taxa-area relationship are available for Bacteria in surface waters (Reche *et al*., 2005) and salt-marsh sediments (Horner-Devine *et al*., 2004b) but not for groundwater ecosystems. Structural complexity of habitats and the diversity of ecological niches tend

to increase with habitat size and may be the most important factors controlling taxa-area relationships. Future studies targeted to evaluate these relationships for microbes in groundwater ecosystems should investigate either aquifer units of varying size or samples of similar sediments taken at varying distance to each other.

Spatial heterogeneity

Spatial heterogeneity is another major factor influencing microbial diversity (Brockman & Murray, 1997a,b). Groundwater habitats are physically and chemically heterogeneous in various dimensions, from pore to ecosystem scale. A strong relationship of microbial diversity and activity patterns can be expected with sediment mineralogy and organic content, hydraulic conductivity and sediment grain size. Only few studies have addressed the distribution of bacterial populations and functional groups in relation to habitat heterogeneity, but a pioneering study found significant variation in bacterial diversity with depth in single boreholes and among adjacent boreholes in a shallow homogeneous sandy aquifer (Kölbel-Boelke, Anders & Nehrkorn, 1988). Diversity differences were attributed to small-scale hydraulic and sediment heterogeneities (Kölbel-Boelke & Nehrkorn, 1992). Thus, while diversity in a given sample may be low, spatial heterogeneity can greatly increases overall microbial diversity in shallow aquifers. In unsaturated sediments, diversity might be higher than in otherwise similar but saturated sediments, because hydrological connectivity reduces habitat patchiness (Zhou *et al.*, 2002).

For deep subsurface environments, correlations between heterogeneity and diversity have also been reported. Fliermans (1989) screened 1100 strains that had been isolated between zero and about 300 m below surface. The great majority (85%) of these isolates were unique to a single depth, while only 3% were present at four or more depths. Similarly, Takai *et al.* (2003) demonstrated a relationship between lithological and geochemical variation and the diversity of archaeal communities in Cretaceous rock. They exposed quartzite sediment in a multi-level sampler at the interface between Cretaceous shale (rich in organic carbon, low sulphate content, high methane concentration) and sandstone (poor in organic carbon, high sulphate content, low methane concentration) in 10-cm intervals. Although total microbial diversity remained relatively constant over the depth intervals, significant community shifts were observed in archaeal populations, mainly methanogens, especially at the geochemical interface.

Temporal variability and disturbance

The temporal dynamics of subsurface microbial communities is not well studied. It has been suggested that microbial diversity in natural systems may peak at intermediate intensities or frequencies of small-scale disturbances (Ibekwe *et al.*, 2002; Fierer, Schimel & Holden, 2003). Temporal changes in subsurface habitats may be attributed to hydraulic events such as precipitation, ice melting (especially in karst systems) and flooding, natural or anthropogenic temperature fluctuations, and, more important, the introduction of contaminations caused by anthropogenic activities (see below).

Temporal dynamics due to hydrological events may affect the distribution of individual redox zones in shallow aquifers (Vroblesky & Chapelle, 1994). Haack *et al.* (2004) analyzed the microbial community structure in a contaminated shallow aquifer in relation to geochemical conditions and recharge. Molecular community profiling demonstrated that after extended lack of recharge communities at the upper aquifer margins differed in composition and diversity from communities at the same depths at other times, when the water table was higher. These results illustrate the significant impact of hydrologic change on groundwater microbial communities, but more extensive research is needed to better understand microbial responses.

Contamination

Compared to the few studies on pristine aquifers, immense efforts have been devoted to understanding microbial communities in contaminated aquifers (Anderson & Lovley, 1997; Haack & Bekins, 2000; Franzmann *et al.*, 2002). In most cases, organic pollutants introduced into oligotrophic aquifers shifted or increased local microbial diversity (Cho & Kim, 2000; Baker *et al.*, 2001; Röling *et al.*, 2001; Feris *et al.*, 2004b; Johnson *et al.*, 2004). However, such diversity increases can partially be attributed to high concentrations of microbes introduced with the contaminant

(Cho & Kim, 2000). Contamination of an aquifer in many cases may cause a pronounced decrease in biodiversity in cases of toxic compounds (e.g. heavy metals, organic solvents). Moreover, the type of contamination, *i.e.* point-source versus diffuse contamination, has distinct impacts on aquifer microbial communities. In the following, we review current microbial community research in a perspective of the different classes of contaminants that frequently affect aquifer ecosystems.

Petroleum hydrocarbons

One of the first systematic studies on prokaryotic diversity in polluted shallow aquifers is that of Kämpfer, Steiof & Dott (1991), who isolated more than 1300 strains from sediment and water samples of a fuel oil contaminated site. Sediment samples were characterised by a high proportion of Gram-positive isolates (*Arthrobacter*, *Nocardia*, and *Bacillus* spp.). In contrast, groundwater samples were dominated by Gram-negative *Pseudomonas*, *Comamonas* and *Flavobacterium* spp.

In a pioneering study using molecular methods, Dojka *et al.* (1998) constructed clone libraries to assess prokaryotic community composition in an aquifer contaminated mainly with jet fuel and chlorinated solvents. In a depth transect through three distinct redox zones of the contaminant plume, a highly diverse bacterial community was described. Of over 800 clones screened from six libraries, about 50% were unique. The clones fell into no less than 20 of the currently about 52 recognised phyla (Rappé & Giovannoni, 2003) of the domain Bacteria, of which six (candidate divisions WS1 to WS6) were first described in the study (Figs 2 & 3). Numerous clones related to the δ-proteobacterial benzoate-utilising *Syntrophus gentianae* (Wallrabenstein *et al*., 1995) and euryarchaeotal *Methanosaeta* spp. were detected in the methanogenic, highly contaminated plume core. This provided circumstantial evidence for these microbes to be involved in the syntrophic degradation of hydrocarbons *in situ*. Rooney-Varga *et al.* (1999) showed that microbial community patterns varied markedly within uncontaminated, Fe^{3+}-reducing and methanogenic zones of a petroleum-contaminated aquifer. Most notably, uncultured Geobacteriaceae were particularly common within the zone of Fe^{3+}-reduction and anaerobic benzene oxidation.

Further fuel components of concern in ground water are oxygenates, such as methyl tert-butyl ether (MTBE), which are widely used as additives to enhance the fuel octane index. Together with fuel from leaking storage tanks, MTBE often escapes into ground water, where it proves very recalcitrant, especially under anoxic conditions. MTBE can be degraded co-metabolically or be used as sole growth substrate by a variety of aerobes (Fayolle, Vandecasteele & Monot, 2001). However, so far only a limited number of species (e.g. β-proteobacterial *Rubrivivax* relatives; Kane *et al*., 2001) have been linked directly to MTBE degradation in aquifers.

Similar levels of bacterial species richness and diversity were observed in both uncontaminated and contaminated regions of a BTEX- (benzene, toluene, ethylbenzene and xylenes) and MTBE-impacted aquifer (Feris *et al*., 2004b). Diverse members of the Proteobacteria, Firmicutes, Bacteroidetes, Verrucomicrobia and Chloroflexi were found in different zones of the plume. However, the contamination did not cause community changes at the phylum level; rather closely related species appeared to be selected for within distinct zones. Bakermans & Madsen (2002) found an equally high diversity in ground water sampled along a contamination gradient in a aquifer impacted by coal-tar waste. Sequences related to aerobic bacteria (e.g. *Nitrospira*, *Methylomonas*, and *Gallionella*) predominated a pristine reference well, whereas sequences related to facultative and obligate anaerobes and contaminant degraders (e.g. *Azoarcus*, *Syntrophus*, and *Desulfotomaculum*) were found in the contaminated wells (Figs 2 & 3). Interestingly, this study also revealed the presence of a previously undescribed phylum within this aquifer, representatives of which are involved in nitrate-dependent oxidation of methane (Raghoebarsing *et al*., 2006), demonstrating that such processes also may occur in aquifers.

One of the few cultured members of the ubiquitous *Acidobacteria* phylum, *Geothrix fermentans*, was isolated from a petroleum-contaminated aquifer (Coates *et al*., 1999). This novel Fe^{3+}reducer is capable of utilising organic acids like acetate and lactate. It also reduces alternative electron acceptors like nitrate and humic acids and is capable of fermentative growth. Thus, this *Acidobacterium* can be characterised as a versatile anaerobe adapted to the environmental conditions prevailing in contaminated aquifers, and close relatives

have indeed been detected at other contaminated sites (Rooney-Varga *et al*., 1999).

Halogenated hydrocarbons

In contrast to our preliminary understanding of microbial diversity at petroleum and MTBE contaminated aquifers, more information is available on the diversity of microbes important for the bioremediation of halogenated hydrocarbons. Chlorinated compounds, such as di-, tri and polychlorinated ethenes (DCE, TCE, PCE), can be aerobically oxidised to CO_2, co-metabolically utilised by some anaerobes such as Clostridia and homoacetogens (Terzenbach & Blaut, 1994), or they can be anaerobically reduced, via various intermediates, to ethene. Various substrates, including H_2 and acetate, serve as electron donor, and the contaminant is respired only when more attractive electron acceptors are lacking. By isolation and cultivation, it has been possible to link these degradation processes to specific aquifer microbes within diverse bacterial phyla, such as the δ-proteobacterial *Desulfuromonas michiganensis* (Sung *et al*., 2003) and clostridial *Desulfitobacterium* spp. (Finneran *et al*., 2002). Furthermore, distinct microbes within the deeply-branching Chloroflexi, *i.e.* several *Dehalococcoides* spp., are specialised in respiring halogenated hydrocarbons (Maymo-Gatell *et al*., 1997; He *et al*., 2003). Such organism have proven useful for cleaning-up contaminated subsurface environments (e.g. Major *et al*., 2002).

In an aquifer contaminated with chlorinated ethenes, putative Clostridia related to *Desulfitobacterium* and *Dehalobacter* spp. and capable of utilising halogenated organic compounds (halorespirers) were detected as well as homoacetogens related to *Acetobacterium*. This finding indicates both respiratory and co-metabolic removal of PCE at the site (Davis *et al*., 2002). Similarly, Macbeth *et al.* (2004) observed a high abundance of homoacetogenic *Acetobacterium* spp in the indigenous, TCE-degrading microbial community stimulated by lactate injection in a fractured basalt aquifer. Furthermore, a diverse community, including Clostridia, Sphingobacteria, other Bacteroidetes, Proteobacteria, Spirochaetes and members of the OP11 candidate division, were observed (Figs 2 & 3) while *Dehalococcoides*-like organisms were only detected with genus-specific primers. Finally, Connon *et al.* (2005) reported stimulation of (co-metabolic) removal of chlorinated ethene by sparging a contaminated aquifer with a propane/air mixture. The increased removal activity could be correlated with an increased abundance of the yet-uncultured TM7 candidate division (Fig. 3), originally described from a peat bog (Rheims, Rainey & Stackebrandt, 1996). These results point towards strong site-specific differences among microbial communities removing chlorinated ethene and underscore the need to understand connections between specific microbial community members and biodegradation processes in aquifers.

Landfill and wastewater leachates

Other anthropogenic perturbations that may seriously alter aquifer hydrogeochemistry and microbial biodiversity are landfill leachates. Complex mixtures of a range of toxicants and inorganic nutrients enter the ground water, and a precise understanding of effects is required to monitor occurring natural attenuation. Röling *et al.* (2001) showed that within a landfill leachate plume, contaminant degradation occurred under iron-reducing conditions, while the dominant process in surrounding ground water was nitrate reduction. Microbial community fingerprints from groundwater samples were distinct within and outside the plume, and a clear relationship was detected between the dominant redox processes and the identified Bacteria. Upstream of the landfill, β-Proteobacteria like *Gallionella* and *Azoarcus* spp. dominated the community, while *Acetobacterium* and *Geobacter* spp. were found directly below the landfill (Figs 2 & 3). Geobacteraceae were highly diverse in the aquifer, and the occurrence of specific members of this lineage, including a so-called 'plume cluster', was closely correlated with the pollution level and ongoing biodegradation processes (Lin *et al*., 2005). These results illustrate that the combined assessment of hydrogeochemical conditions and microbial community composition can be valuable for long-term monitoring programmes and to identify spatial and temporal patterns of complex contaminant mixtures resulting from landfill leachates (Mouser *et al*., 2005).

The effects of a similarly complex groundwater contamination with livestock wastewater have been investigated by Cho & Kim (2000). Wells were sampled several 100 m up- and downstream of a zone where wastewater from an intensive stock

farming infiltrated the aquifer. The wastewater input roughly doubled the diversity of microbial communities and caused a profound community shift. In the uncontaminated ground water, diverse β-Proteobacteria and the so-called 'WJG group 1', which is affiliated to the TM7 candidate division (Fig. 3), dominated the community. Clostridia and Bacteroidetes dramatically increased in the contaminated wells downstream, where the 'WJG group 1' was nearly undetectable. This documents a loss of indigenous microbial biodiversity within an aquifer through anthropogenic perturbation.

Radioactive metals

Data are also available on the effects of heavy metal pollution on groundwater microbial diversity. Contamination with highly soluble and radioactive uranium (VI) species is of particular concern, as they rapidly spread with groundwater flow. Fortunately, many microbes capable of reducing other metallic electron acceptors are capable of reducing U(VI) to insoluble U(IV) and thus immobilise the element (Lovley, 1997). Consequently, a promising strategy for reducing the spread of groundwater contamination by radioactive uranium is to stimulate microbial metal reduction.

In a laboratory microcosm study, Holmes *et al.* (2002) thus amended sediments from a uranium-contaminated aquifer with acetate to stimulate U(VI)-reduction. Dramatic increases in $Fe^{3+}$and concurrent U(VI)-reduction were accompanied by a significant increase in the abundances of members of the Geobacteriaceae. Similarly, Vrionis *et al.* (2005) studied bacterial diversity along a geochemical gradient in a uranium-contaminated aquifer after two seasons of acetate-stimulated bioremediation. Clone libraries were generated from DNA extracts of groundwater and sediment samples taken at various distances from the injection well and at different depths. Results indicated that clones related to sulphate reducers within the δ-proteobacterial *Desulfobacter* and *Desulfocapsa* spp. were more abundant near the injection well and in deeper zones, where $Fe^{3+}$was depleted and sulphide concentration was high. Further downstream, where iron was reduced, higher relative abundances of *Desulfuromonas*, *Pelobacter* and *Geobacter* spp. were associated with a very efficient removal of U(VI). Thus, again, local microbial community composition co-varied with the presence of contaminants and contaminant conversion.

Pesticides

The input of pesticides and their degradation products to ground waters results mainly from diffuse agricultural sources. This discriminates these contaminants from the compounds discussed above, which mostly originate from point sources. Furthermore, the concentrations of diffuse contaminants such as pesticides are usually very low (a few $\mu g\ L^{-1}$), making effects on microbial communities much more difficult to demonstrate. Nevertheless, a strong increase in pesticide degradation activity *in situ* was found in an aquifer after exposure to phenoxy acid herbicides (De Lipthay *et al.*, 2003). Pesticide degradation activity was strongly correlated with MPN counts of *Pseudomonas* spp. and the abundance of genes coding for enzymes involved in both phenoxy acid, and 2,4-dichlorophenoxyacetic acid degradation. Members of the genus *Pseudomonas* are known in general to metabolise a wide range of xenobiotics (Sayler *et al.*, 1990), but only a small fraction of the *Pseudomonas* isolates from the site actually showed 2,4-D degradation capacity. In a subsequent study at the same site., neither isolate-profiling nor direct genetic fingerprinting detected a significant effect of herbicide exposure on the structure of the total microbial community (De Lipthay *et al.*, 2004). In contrast, Johnson *et al.* (2004) observed a clear increase in species richness and diversity of cultivable groundwater bacteria from aquifers impacted by isoproturon. Isoproturon degradation rates were associated with the occurrence of genera like *Pseudomonas* and *Brevundimonas* spp. However, effects differed among sites, and further research is needed to pinpoint the conditions in which groundwater microbial communities are altered in response to diffuse pesticide contaminations.

Diversity within functional groups

The analysis of microbial diversity in environmental samples based on ribosomal marker genes is only of limited value when functional groups of microorganisms are to be investigated. This is especially true for clades distributed among distinct phyla, when close relatives do not share the same physiology of interest, or when a functional affiliation of environmental

sequences is not possible due to lack of cultured relatives. Therefore, the analysis of functional marker genes has long been a preferred approach to screen environmental samples. Typically, conserved key enzymes of characteristic catabolic pathways or dissimilatory electron transfer reactions are targeted. Here we summarise recent results obtained for specific functional groups in groundwater environments.

Methane- and ammonia-oxidisers

A classical functional group accessible with functional marker genes are the methanotrophs, which are distributed within different proteobacterial lineages and can be targeted via genes encoding the particulate or soluble methane monooxygenases (pMMO, sMMO) (Murrell & Radajewski, 2000). The diversity and distribution of methane oxidisers in aquifers is of interest especially at aerobic sites contaminated with halogenated solvents, since they are well-known to co-metabolise TCE (Bowman *et al.*, 1993; see also above). Microbes containing sMMO degrade diverse chlorinated compounds at high rates. In a survey accompanying the stimulated bioremediation of a TCE-contaminated aquifer, methane injection increased both the detectability and diversity of pMMO genes (Baker *et al.*, 2001) . Although communities were dominated by methanotrophs related to *Methylobacter* and *Methylomonas* spp., which express only minimal TCE-degradation potential in pure culture, unidentified methanotrophs distantly related to *Methylosinus trichosporium* were also detected. The detectability of sMMO, however, remained low. These observations are likely to explain the failure of the methane-injection trial to substantially enhance co-metabolic degradation of TCE at the site.

Similar motivation spawned the study by Newby *et al.* (2004), who screened a basalt aquifer for methanotroph diversity, again in the context of TCE-contamination. Stable-isotope analyses suggested methane oxidation and a wide diversity of methanotrophs was detected, including also sequences closely related to known TCE degraders. Interestingly, the well with the highest dissolved methane concentration (about 300 nM) was the only well where methanotrophs were directly detectable with specific 16S rDNA primers, while a nested PCR approach was necessary for all other wells. Detectable sMMO was mostly related to *Methylocystis* strains, where a putatively novel cluster of sequences was detected also on 16S rDNA level. An uncontaminated part of the aquifer was also screened for its diversity of methane and ammonium oxidisers. The latter can be targeted via the gene encoding ammonium mono-oxygenase (AMO), which is used to gain energy for an autotrophic lifestyle. AMO displays both structural and functional similarities to the pMMO of methanotrophs (Holmes *et al.*, 1995) and also catalyses the oxidation of TCE, facilitated by substrate similarities (Hyman *et al.*, 1995). A vast diversity of AMO, pMMO, and sMMO genes resides within the microbial community of the investigated basalt aquifer. In addition to well-known representatives of the different clades, yet-unaffiliated genes encoding enzymes that are involved in catabolic cellular pathways were also commonly detected.

A synthesis of microbiological observations before molecular methods were available, molecular identification, and physiological profiling has recently expanded the known diversity of methanotrophs in ground water by re-discovering the enigmatic *Crenothrix* bacterium (Stoecker *et al.*, 2006), originally observed in drinking water wells over a century ago (Cohn, 1870). This filamentous microbe is a member of the γ-Proteobacteria and contains an unusual copy of the pMMO gene (Stoecker *et al.*, 2006). This explains why this microbe preferentially occurs at interfaces where methane-rich anoxic ground water meets oxygen-containing water. The anaerobic oxidation of methane, however, by electron acceptors such as NO_3^- or SO_4^{2-} (Boetius *et al.*, 2000; Valentine & Reeburgh, 2000; Raghoebarsing *et al.*, 2006) remains almost uninvestigated for groundwater ecosystems.

Lithoautotrophs

The above-mentioned ammonia oxidising bacteria are lithoautotrophic, capable of building their entire biomass carbon from assimilated CO_2. Lithoautotrophic life styles may be very relevant in deeper groundwater habitats, where oxygen is absent and inorganic electron donors are much more abundant than readily-degradable organic carbon (Gold, 1992; Kotelnikova & Pedersen, 1997; Pedersen, 1997; Stevens, 1997). Here, the assimilation of inorganic carbon is thought to be fuelled by hydrogen or methane as electron donors, coupled to S_0, SO_4^{2-}, and/or Fe^{3+}as electron acceptors. There are also indications that

autotrophic methanogens and acetogens are present and active in deep subsurface environments (Stevens & McKinley, 1995; Kotelnikova & Pedersen, 1997).

Lithoautotrophy may well be important also in shallow aquifers, as suggested by the diversity of genes for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) in various anaerobic and microaerobic samples from a BTEX-contaminated aquifer (Alfreider *et al*., 2003). RubisCO is the key enzyme for CO_2 fixation via the Calvin-Benson-Bassham cycle, and is generally found in aerobic lithoautotrophs and nitrate-reducing *Thiobacillus* spp. (Kinkle & Kane, 2000). The observed diversity of RubisCO genes indicated the presence of various obligate and facultative lithoautotrophic Proteobacteria, as well as different *Thiobacillus* and *Halothiobacillus* spp. Although this observation indicates a potential role of such autotrophs in the re-oxidation of reduced sulphur species at the site (Alfreider *et al*., 2003), *in-situ* activity of such processes remains to be substantiated. The diversity and distribution of genes involved in any other of the five presently known pathways of CO_2 fixation (Thauer, 2007) have not been investigated within groundwater systems so far.

Denitrifiers

Denitrifiers are phylogenetically diverse and therefore also unsuited for a targeted detection by means of ribosomal markers. However, functional marker assays based on dissimilatory key enzymes, *i.e.* nitrite reductases (the copper-containing NirK and cytochrome *cd*1 NirS), have been developed (Braker, Fesefeldt & Witzel, 1998). Santoro, Boehm & Francis (2006) used these assays to screen the diversity of denitrifying microbial communities along a salinity gradient within a coastal aquifer where nitrate-rich ground water is discharged into the ocean. The *nirK* and *nirS* gene pools in this aquifer were remarkably diverse and almost exclusively composed of unaffiliated and novel sequence types, including two very deeply-branching unidentified *nirK* lineages. Furthermore, a distinct assembly of *nirK* and *nirS* gene pools was apparent along the salinity gradient.

Sulphate reducers

Also sulphate reducers can be assessed by means of functional marker genes (Wagner *et al*., 2005). However, in contrast to denitrifiers, sulphate reducers can also be targeted with a number of genus- or lineage-specific ribosomal probes. Using approaches that target rRNA, Detmers *et al.* (2001, 2004) found that Gram-positive, spore-forming *Desulfotomaculum* spp. constitute the dominant population of sulphate reducers at a deep (about 120 m) lignite seam in a pristine anoxic aquifer. They suggested that lignite-derived organic compounds can serve as energy source for microbial metabolism in environments otherwise devoid of organic inputs. Using both the dissimilatory sulphite reductase (Dsr) as a specific marker and rRNA probes, *Desulfotomaculum*-relatives were found to dominate about 3 km deep subsurface habitats (Baker *et al*., 2003). This further highlights the importance of these sulphate reducers in anoxic subsurface ecosystems. *Desulfotomaculum* and the closely related *Desulfosporosinus* lineages may be very important also in contaminated aquifers, where they may be directly or indirectly involved in hydrocarbon degradation (Robertson, Franzmann & Mee, 2000; Bakermans & Madsen, 2002; Morasch *et al*., 2004; Pombo *et al*., 2005). In other aquifers impacted by hydrocarbon contamination, non-clostridial sulphate reducers (e.g. *Desulfobacter*, *Desulfovibrio* and *Desulfobulbus* spp.) were also detected (Kleikemper *et al*., 2002).

Methanogens

Methanogens are a unique functional group within the Archaea. They are of interest in anaerobic aquifers where they conduct terminal respiratory reactions along anaerobic degradation pathways in the absence of alternative electron acceptors. As Archaea, methanogens are frequently targeted with archaeal 16S rRNA assays. Although a specific functional marker for methanogens, the methyl-coenzyme M reductase (Mcr) is established and has been applied to numerous other environments (Friedrich, 2005), the diversity of *mcrA* genes within methanogenic aquifers has not been explored to date.

Methanosarcina-like organisms were found in ground water from depths of 45–68 m of a Swedish granite aquifer (Kotelnikova & Pedersen, 1997). A psychrophilic relative of *Methanohalophilus* sp. and a new alkaliphilic *Methanobacterium* sp. were isolated from various depths in this study. Acetotrophic *Methanosaeta* spp. have frequently been reported from contaminated sites (Dojka *et al*., 1998; Struchtemeyer

et al., 2005). Kleikemper *et al*. (2005) investigated the diversity of methanogens in an aquifer impacted by petroleum and detected both hydrogen-utilising *Methanospirillum* sp. and acetate utilising *Methanosaeta* sp. In addition, they detected an even larger diversity of uncultured Archaea, which could not be unambiguously classified as methanogens based on ribosomal markers alone.

Degraders of specific contaminants

Catabolic marker gene assays are also a very important tool for the specific detection of specialised groups of contaminant degraders. Although frequently applied to contaminated marine and surface environments (Galvao, Mohn & de Lorenzo, 2005), these assays have rarely been used for ground water where they have always targeted microbes involved in the degradation of aromatic hydrocarbons. In a pioneering study, Beller *et al*. (2002) investigated the quantity and diversity of benzylsuccinate synthase genes (*bssA*), which code for a key enzyme of anaerobic toluene and xylene oxidation. They observed considerable changes in gene copy numbers over time, but no diversity shifts. Phylogenetically distinct *bssA* genes of largely uncultured Bacteria have been described from tar-oil contaminated aquifers characterised by sulfate reduction (Winderl, Schaefer & Lueders, 2007).

For aerobic degraders, the diversity of naphthalene-degrading *nahAc* genes was screened in a coal-tar impacted aquifer (Bakermans & Madsen, 2002). Indigenous *nahAc* gene pools were distinct among contaminated monitoring wells, more diverse than within naphthalene-degrading isolates from the same site, and absent outside the contaminated area. Similarly, the diversity of mono- and dioxygenases oxidising aromatic rings has been used to monitor the diversity of aerobic degraders in sediments and ground water impacted by BTEX, and to follow the establishment of degrader populations upon contaminant pollution (Hendrickx *et al*., 2005, 2006). Genes encoding aromatic ring-hydroxylating dioxygenases (RHDs) were also investigated in ground water at an aromatic hydrocarbon-contaminated landfill (Taylor & Janssen, 2005). Considerable diversity of unidentified dioxygenases was detected and quantitative and compositional community shifts occurred among wells differing in contamination level. A group of specific RHD sequences, designated the 'T-cluster', dominated the highly contaminated ground water, but the carriers of these unaffiliated genes remain to be identified.

Groundwater biodiversity, ecosystem functioning and perturbations

Ecosystem functioning depends on complex interactions between physico-chemical and biological factors. The processes acting in individual habitats or ecosystems are a direct result of functional-trait diversity within biotic communities. Changes in diversity therefore may be linked to changes in ecosystem processes (Humbert & Dorigo, 2005). Although microbial species in aquifers may display functional redundancies, like in other environments, each species may also possess very specialised catalytic abilities. A decrease in species diversity could therefore result in a deficiency in a physiologic potential within a given community. It has been proposed that a low level of biodiversity may allow an ecosystem to function efficiently under constant conditions, but that greater biodiversity may be called for in fluctuating environments (Humbert & Dorigo, 2005). Some of the data summarised above indicate that microbial communities characterised by constant composition and low diversity are frequently found in pristine oligotrophic aquifers. It is not clear, however, whether microbial biodiversity in unperturbed aquifers is generally low, or whether low estimates are just a result of low abundances and insufficient resolution of methods used to estimate diversity. Refined approaches and statistically rigorous applications of biodiversity estimates are urgently needed (Gans *et al*., 2005; Hong *et al*., 2006).

Diverse communities have also been suggested to be more resistant to species invasions (McGrady-Steed, Harris & Morin, 1997; Kennedy *et al*., 2002) and environmental perturbations (Botton *et al*., 2006). If this holds true also for groundwater environments, oligotrophic aquifers may be very vulnerable to anthropogenic impacts. Communities may show very limited capacities of resistance to perturbations and of resilience. An important point is, however, that anthropogenic perturbation does not necessarily cause a loss of microbial diversity in groundwater ecosystems. Rather, profound shifts in community composition at similar levels of diversity and richness are often reported (Rooney-Varga *et al*., 1999; Röling

et al., 2001; Feris *et al.*, 2004a; Vrionis *et al.*, 2005). In several cases these shifts are even accompanied by an increased biodiversity (Cho & Kim, 2000; Baker *et al.*, 2001; Johnson *et al.*, 2004). The substantial losses in microbial biodiversity reported from contaminated soils and marine sediments (Torsvik *et al.*, 1998; Gans *et al.*, 2005) cannot currently be confirmed for ground water. Two hypotheses may explain these observations: (i) the applied methods were not powerful enough to detect losses of 'rare' species below a certain abundance threshold, or (ii) microbial diversity in pristine ground waters is constantly low, such that any perturbation, which tends to be accompanied by a substrate input, is more likely to cause an increase in species abundance and biodiversity rather than a loss.

Conclusions

Although there are many unique aspects to microbial life, microbes and higher organisms also share many fundamental features (Andrews, 1991). Historically, microbial ecology has largely evolved as a subdiscipline of microbiology and is only gradually assimilating concepts and perspectives developed in ecology. However, there is an increasing awareness of the utility of applying ecological concepts to microorganisms (Horner-Devine *et al.*, 2004a; Martiny *et al.*, 2006). A theoretical framework comprising elements of both microbial and animal ecology is likely to be most useful for understanding groundwater biodiversity and ecosystem functioning (Gibert & Deharveng, 2002; Danielopol & Griebler, 2008).

Both cultivation-dependent and cultivation-independent surveys have consistently revealed communities dominated by diverse heterotrophic Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes as important components of groundwater environments. Molecular studies have also frequently detected several mostly uncultivated lineages, *i.e.* Acidobacteria, Chloroflexi, Verrucomicrobia and Nitrospirae (Dojka *et al.*, 1998; Rooney-Varga *et al.*, 1999; Bakermans & Madsen, 2002; Feris *et al.*, 2004b), as well as phyla for which cultured representatives are totally unknown (e.g. the OP, WS and TM7 candidate divisions; Dojka *et al.*, 1998; Macbeth *et al.*, 2004; Connon *et al.*, 2005; Fig. 3). However, all of the detected lineages occur also in other environments. Thus there is no evidence for an endemic groundwater microbiota, as is frequently observed for groundwater fauna (e.g. Marmonier *et al.*, 1993; Sket, 1999; Danielopol *et al.*, 2000).

Nevertheless, there are numerous indications that subsurface microbial communities are distinct from those found in soil and surface waters. These differences are not apparent on the level of phyla or genera, but rather by the specific phylogenetic composition of groundwater microbial communities and by their special physiological capabilities. Both metabolically versatile and specialised microbial genera appear to dominate, particularly in anoxic aquifers (e.g. *Desulfotomaculum*, *Geobacter*, and *Methanosaeta* spp.). However, ecophysiological interpretations are not possible for any of the often abundant uncultivated lineages (*i.e.* Chloroflexi, OP11). Elucidation of the biogeochemical role of such 'unseen' microbial diversity in both contaminated and pristine aquifers will be one of the major tasks for groundwater microbial ecology in the coming years. Although groundwater microbes clearly are critically linked to groundwater self-purification and the success of bioremediation strategies, the specific functional relevance of the considerable phylogenetic microbial diversity remains yet to be determined. Metagenomic approaches and recent technical advances towards higher throughput and lower costs for large-scale sequencing are likely to foster groundwater microbial biodiversity studies greatly, as already shown for other habitats (Venter *et al.*, 2004; Goldberg *et al.*, 2006). Thus, the search in groundwater microbes and groundwater metagenomes for novel biochemical and biotechnological applications (Uchiyama *et al.*, 2005) may open an almost untapped reservoir of biological potentials. However, for a comprehensive understanding of the coupling between aquifer microbial diversity and ecosystem functioning we should never forget that 'although details of single organisms matter and are of great interest, ecologists would profit most from uncovering underlying patterns, rules and laws' (Lawton, 1999).

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