Subsurface landfill leachate - home to complex and dynamic eukaryotic communities

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Cover: aquifer sediments from Banisveld and groundwater fauna that we firstly looked for and we never found
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"…*to know* or *not to know*, that is the question!

Not to know means: superstitions, blind egoism, untamed competition, disagreement, enmity, war, starvation, disaster.
To know means for humanity: solid organization, rational activity, cooperation, solidarity, peaceful evolution.
To know means for human being living the time *to be* with satisfaction, and waiting for the moment *not to be* with serenity"

Emil G. Racoviţă, 1927

dedicated to my parents

Contents

Chapter 1

General introduction

Background on importance of groundwater

After glaciers, which enclose approximately 24 million km^3 of frozen freshwater, the subsurface comprises 97% of all freshwater in the world (Gibert et al., 1994), which makes it the largest and the most important reservoir of available freshwater on Earth (Shiklomanov, 1993). The remaining 3% of freshwater exists as surface waters (i.e. lakes, rivers and wetlands). As a comparison, the volume of water in the subsurface (10 million km^3) is approximately 100 times larger than that in freshwater lakes (91.000 km^3), and 5.000 times larger than the water in rivers (2.000 km^3) (Shiklomanov, 1993).

Groundwater ecosystems reside in all water reserves stockpiled in the subsurface. Groundwater may be captured in large karstic aquifers, it accompanies and flows under rivers forming a hyporheic environment (i.e. the water saturated sediments along and underneath rivers) (Orghidan, 1959), or flows in phreatic regime through permanently saturated voids in any type of rock (Fig. 1). Groundwater is an important component of the natural water cycle; its hydrogeochemistry depends largely on, and is directly influenced by the quality and recharge of the percolating surface waters. Pristine groundwater is generally oligotrophic (Gounot, 1994), and its chemistry is influenced by the type of rock and minerals it passes and dissolves (Drever, 1997). The environmental characteristics of groundwater, such as temperature, pH or dissolved oxygen, remain relatively constant during all seasons.

While groundwater was long imagined as an inexhaustible resource, today the availability of groundwater is compromised in many parts of the world, as human society depends largely on this resource as drinking water supply (Vörösmarty et al., 2000). For example, about 75% of the inhabitants of the European Union depend on groundwater for their water supply (Gibert, 2001). Although the importance and the

great necessity of groundwater for human society and ecosystem health are now largely recognized, aquifers are heavily subjected to pollution wherever human activity exists. The depletion of clean groundwater reserves is due to various causes (Fig. 1) such as the world general population increase, causing an increase in water demand, agricultural practices and landscape alteration, increase in urban area and demand for public drinking water, industrial activities including electricity production and mining, tourism and climate change (Danielopol et al., 2003). The understanding manifested in this respect by society appears insufficient since, all over the world, groundwater is still a main sink for hazardous contaminants such as fertilizers, pesticides, herbicides, oil products, chlorinated solvents, and heavy metals.

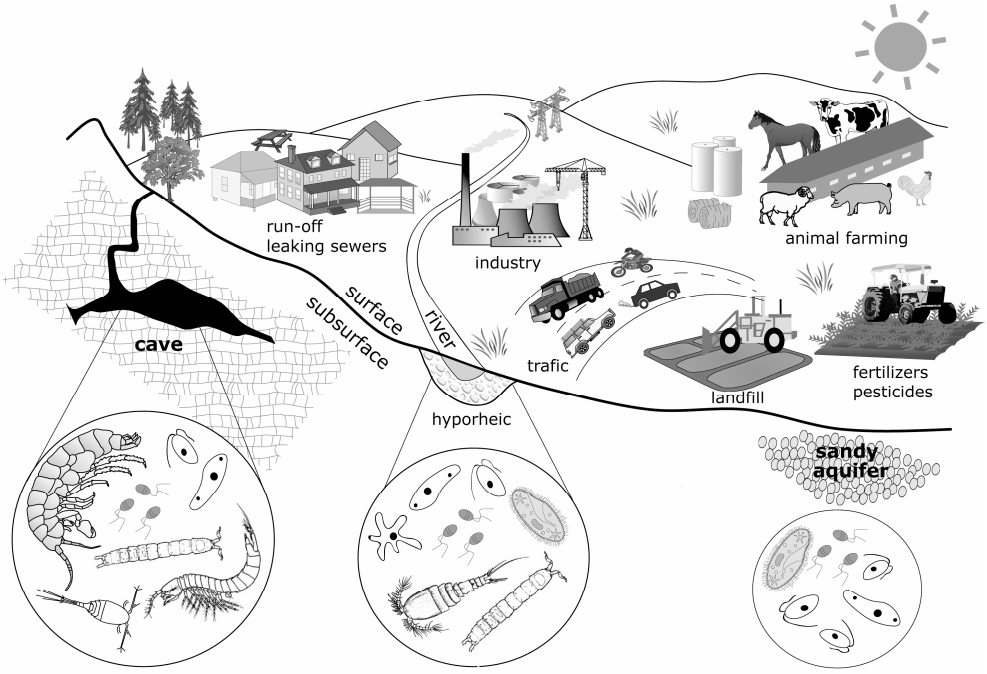

FIG. 1. Schematic view upon various types of groundwater ecosystems with possible sources of groundwater pollution. Groundwater biota are presented for each subsurface aquatic environment.

This thesis regards the threatening effects of a particular source of groundwater contamination, which is represented by landfills. Percolating rainwater carries the contaminants present in municipal or industrial waste disposals into nearby aquifers with consequences for the quality of groundwater. While in solution, the pollutants in groundwater may become even more hazardous as they can be carried away, polluting also other places (e.g. phreatic aquifers, cave ecosystems), and can be taken up easier by subsurface organisms. Landfills consist primarily of organic materials, but hazardous contaminants such as monoaromatic hydrocarbons of the BTEX complex (benzene, toluene, ethylbenzene and xylene), chlorinated compounds or heavy metals, can also be present (Christensen et al., 2001; Kjeldsen et al., 2002). Most of the landfills, especially those constructed in the past (up to 4.000 old landfills in The Netherlands alone) (Röling et al., 2000b), were not sealed with liners preventing the leaching of pollutants. Although landfilling operations are discontinued in many countries, discharge of contaminants into the environment continues, and usually large changes occur in the hydrogeochemistry and microbiology of the adjacent aquifers (Christensen et al., 1994; Kjeldsen et al., 2002). Pollution plumes generally form and migrate large distances, sometimes towards residential areas and other places of public interest, polluting drinking water supplies and the adjacent surface aquatic ecosystems such as rivers, lakes, or wetlands (Cozzarelli et al., 2000; Grossman et al., 2002; Ludvigsen et al., 1998; Ludvigsen et al., 1999).

Natural attenuation in groundwater ecosystems

The awareness arisen in the 1980s and manifested in recent years of the reduced quality of groundwater led lately to the development of remedial measures. The first strategies were based on physical removal of pollution by soil excavation (National Research Council, 2004) in combination with treatment and controlled storage, pumping and cleaning the contaminated groundwater, or techniques to physically retain the pollution by, for example, filtration or particle settling. These techniques are sometimes hard or even impossible to apply due to the high costs, or they have limited effect (Swett and Rapaport, 1998). Research performed in the last decade suggests that the subsurface has

an intrinsic potential to attenuate the contaminants. Usually, pollutant concentrations decrease with the distance from the source due to physicochemical processes such as dilution, sorption, precipitation or dispersion (Christensen et al., 1994). Natural attenuation, which is believed to be the least expensive means of bioremediation (Röling and Van Verseveld, 2002), relies especially upon the activity of naturally occurring microorganisms (Christensen et al., 2001, Lovley, 2001). Unlike other processes involved in natural attenuation (e.g. dilution, sorption, dispersion), microbial biodegradation is the only process truly decreasing the mass, concentration and/or toxicity of pollutants (Röling and Van Verseveld, 2002).

Policies have been designed to rely on monitored natural attenuation for the management of landfill leachate plumes (Rügner et al., 2006; United States Environmental Protection Agency, Office of Solid Waste and Emergency Response, 1999). This strategy requires information on whether the degrading microorganisms are present in the polluted environment, whether they really transform or mineralize pollutants *in situ*, and, if so, they do this at a significant rate, and how the process will behave over time (Haack and Bekins, 2000). Thus the potential for natural attenuation of an aquifer must be firstly evaluated. Furthermore, information on how the polluted environment selects certain types of microorganisms and how microbial communities can affect their environment may help in the development of strategies and tools needed for monitoring natural attenuation. Long-term monitoring of the activity and evolution of microbial communities in polluted aquifers, combined with information on how environmental conditions (e.g. pollution loads, availability of electron acceptors, physical shifts of pollution plumes) change over time, could then lead to models useful in the prediction of natural attenuation in aquifers.

Objectives of the thesis

Natural attenuation-related studies have primarily addressed the presence and capacity of degrading bacteria to diminish the mass and toxicity of pollutants. The presence and activity of eukaryotic microorganisms (e.g. protozoa), as factors affecting bacterial

biodegradation abilities, has often been neglected. The present thesis places an emphasis on the characterization of microeukaryotic communities present in a polluted aquifer downgradient of the Banisveld landfill (Boxtel, province of Noord-Brabant, The Netherlands). The structure and composition of microeukaryotic communities are regarded in relation to bacterial communities and environmental factors, especially those that are associated with pollution by landfill leachate. For identifying the factors that contribute to structuring of microbial communities in the aquifer, the spatial and temporal dynamics in microbial community structure, in relation to aquifer hydrochemistry, was examined over a period of six years. The contribution and roles of eukaryotic microorganisms (protozoa) in biodegradation of organic matter was studied in groundwater-related systems with different nutrient limitations. The information obtained and presented in this thesis can be useful in bioremediation of polluted environments, especially of organically contaminated aquifers, and those polluted by landfill leachate in particular, where growth-limiting nutrients, such as nitrogen and phosphorus, needed by the degrading microorganisms, are present in limited amounts, and can be recycled via protozoan predation.

Eukaryotic life in groundwater

Unicellular and multicellular eukaryotic organisms are ubiquitous and important parts in food webs of both pristine and polluted groundwater ecosystems. Despite the lack of light and scarcity of food, the groundwater domain is inhabited by various types of micro-, meio- and macro-organisms (Botosaneanu, 1986; Chapelle, 2001; Wilkens et al., 2000). These organisms play important roles in groundwater, recycling and transferring matter and energy. The food webs in groundwater (Fig. 2A) are simpler compared to those in surface habitats such as lakes (Fig. 2B) or rivers (Fig. 2C) (Hancock et al., 2005) and consist of chemoautotrophs and heterotrophic components. Energy losses from one food web component to another are minimal, and high ecological efficiency is ensured (Gibert et al., 1994).

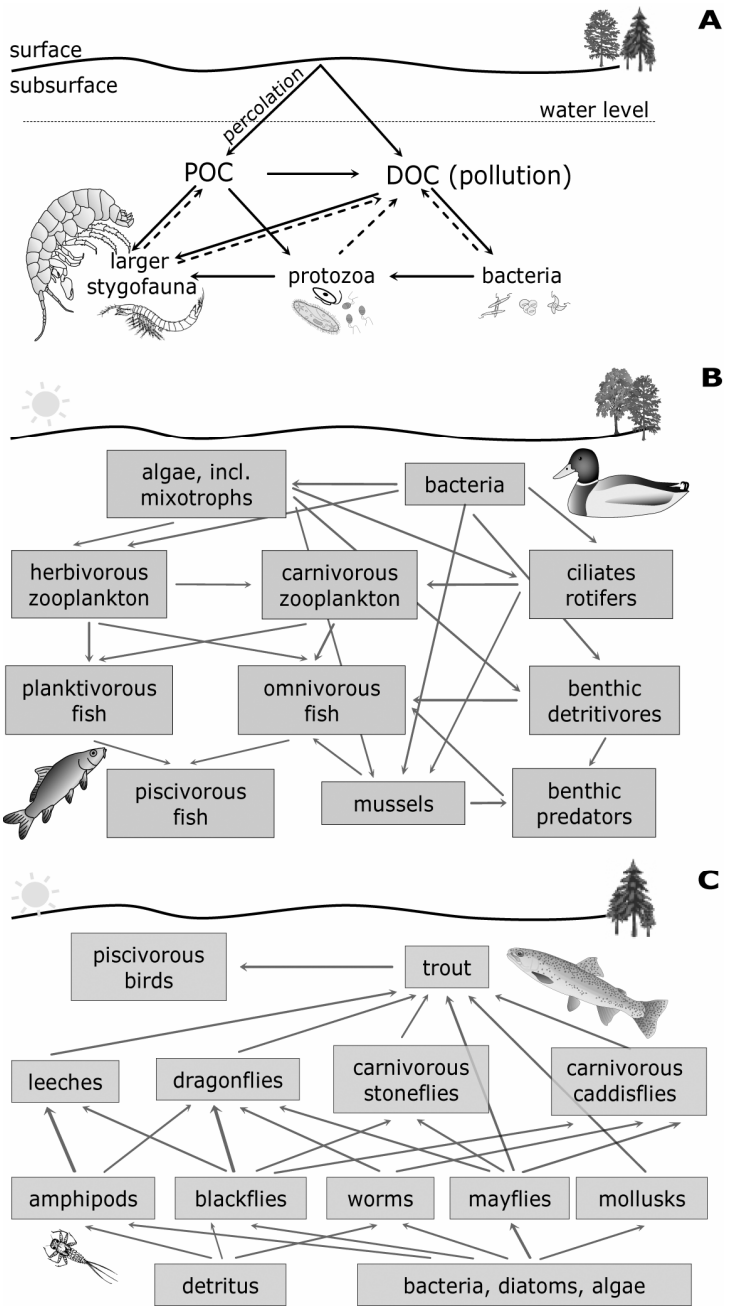

FIG. 2. Comparison of a groundwater food web (A) with more complex food webs in above ground aquatic ecosystems [i.e. in lakes (B) and rivers (C)]. POC - particulate organic carbon, DOC - dissolved organic carbon (modified from Hancock et al., 2005).

Unicellular groundwater microbiota
Emphasis on eukaryotes

Due to the absence of light and green plants, the base of the groundwater food web is represented by heterotrophic microorganisms, namely Bacteria and Archaea. These organisms generate energy via redox reactions (e.g. nitrate-, sulfate- or iron reduction) and consume and transform organic compounds (Gibert et al., 1994). Prokaryotic microorganisms can be consumed by protozoa, which may themselves be consumed by groundwater invertebrates or other protozoa. Prokaryotic microorganisms are documented in groundwater since 1926 when anaerobic sulfate-reducing bacteria were detected in an aquifer in California (Chapelle, 2001). Later, Gurevich (1962) associated groundwater quality to microbial quality, and Köbel-Boelke and coworkers (1988) established the importance and roles of microorganisms in determining water chemistry. Microorganisms of various types inhabit the subsurface aquatic domain from very near to the surface to thousands of meters deep (Ghiorse, 1997; Sinclair and Ghiorse, 1989). The subsurface harbors almost the same biomass, or an even larger biomass compared to that of surface ecosystems (Whitman et al., 1998). Most bacteria (90-99%) are associated with sediment surfaces, forming colonies and biofilms, while only a small fraction are found suspended in pore water (Albrechtsen and Winding, 1992; Griebler, 2001; Holm et al., 1992).

Eukaryotic microorganisms are represented in aquifers by protozoa (i.e. flagellates, ciliates and amoebae), unicellular fungi and algae. Protists, the main consumers of bacteria, can play important roles in groundwater food webs, making bacterial biomass available to higher trophic levels (groundwater metazoa) (Novarino et al., 1997). By feeding on bacteria, protists can reduce soil aggregation (Kota et al., 1999). Abundance and diversity of protists generally increase upon pollution (Madsen et al., 1991; Novarino et al., 1997). Organic pollution in aquifers, and subsequent biodegradation, often results in the development of anaerobic conditions. Protists are also encountered in anaerobic aquifers; they affect the abundance and diversity of bacteria (Kinner et al., 1997; Kinner et al., 1998; Kinner et al., 2002),

influencing indirectly contaminant biodegradation. By feeding on bacteria, protists can reduce degraders populations, and so slow down the process of biodegradation (Kota et al., 1999; Travis and Rosenberg, 1997). However, protists can also contribute positively to organic contaminant biodegradation by recycling limiting nutrients to pollutant-degrading bacteria (Mattison and Harayama, 2001; Mattison and Harayama, 2005; Ratsak et al., 1996). Protists can also enhance bioremediation by maintaining hydraulic conductivity of aquifers (Sinclair et al., 1993), as a result of reduced bacterial clogging (Mattison et al., 2002).

Unicellular phototrophs, such as green algae, have been encountered in aquifers (Beloin et al., 1988; Sargent and Fliermans, 1989; Sinclair and Ghiorse, 1989), but their functioning in the subsurface remains largely unknown. Algae are most likely introduced in aquifers from the surface by percolating rainwater, or from surface water recharges (e.g. lakes or streams) (Sinclair and Ghiorse, 1989). Many algae are mixotrophic and can survive in the absence of light by feeding on bacteria (Jones, 2000). The occurrence of unicellular fungi in low numbers have been described for a few pristine and polluted aquifers (Madsen and Ghiorse, 1993; Madsen et al., 1991; Sinclair and Ghiorse, 1989), but their identity and activities have hardly been investigated. Fungal signatures derived from phospholipid fatty acids were detected in an aquifer contaminated by a landfill in Denmark (Ludvigsen et al., 1999). Luo and coworkers (2005) identified ascomycetous and basidiomycetous fungal DNA in a clone library generated from sediments of a hydrocarbon contaminated aquifer.

Groundwater fauna

The groundwater ecosystem harbors a large number of animal species (more than 7000 reported in 1986) that are known exclusively from subterranean waters (referred to as *stygobites*) (Botosaneanu, 1986). These animals especially inhabit caves and flooded rock fissures, but also coarser sediments along rivers. These species are proposed to have a surface origin (or may have derived from already-present and adapted groundwater ancestors) either freshwater or marine, and to have

colonized the groundwater realms in different periods by active migration or passive isolation (Danielopol and Rouch, 2005; Holsinger, 2000; Notenboom, 1991). The groundwater fauna is dominated by crustacean species belonging to the orders *Amphipoda*, *Isopoda*, *Copepoda*, *Decapoda*, *Bathynellacea*, *Thermosbaenacea*, *Remipedia*, *Mictacea* and *Spaeleogriphacea* (Sket, 1999). Stygobites have developed strategies of adaptation to the energy-poor environment by reducing their body size and metabolism. The diet of groundwater invertebrates is not specialized; most of them are polyphagous.

Due to the lack of light in their environment, groundwater animals have lost their pigmentation and visual function, or sometimes even the whole visual apparatus. They have instead acquired a better sensitivity of the sensory organs located on longer appendages compared to those of their surface relatives (Danielopol and Rouch, 1991). Stygobites typically show delayed maturity, greater longevity, fewer and larger eggs and longer ontogenetic development compared to those of their relatives at the surface (Gibert et al., 1994). The groundwater species are vulnerable to disappearance because of disturbance of any kind. The invertebrates living in groundwater are stenobiontic, they live in relatively stable environments (Leys et al., 2003), and abrupt hydrogeochemical changes, especially brought about various anthropogenic activities, may threaten the groundwater fauna existence. Therefore the subterranean animal assemblages provide information on the health and functional state of the groundwater ecosystem (Malard et al., 1996). For example, pollution from sewage (Malard et al., 1994), heavy metals (Plénet, 1995), inorganic chemicals (Mösslacher, 2000) and pesticides (Notenboom et al., 1994) have led to extinction of species in various stygobiontic communities.

Groundwater fauna play important roles in aquifers. Like protists, they maintain the interstitial voids clear, preventing bacterial clogging in sediment pores (Rockhold et al., 2002), modifying the redox gradients, or promoting biofilm activity (Gibert and Deharveng, 2002; Humphreys, 2002). Most groundwater invertebrates ingest sediment particles coated with microbes, they digest the microbes and other organic materials and excrete nutrients and grazed sediment particles. This stimulates further bacterial growth by providing cleared sediment particles for

recolonization by other bacteria (Hakenkamp and Palmer, 2002), and by recycling of nutrients.

Research on groundwater fauna in The Netherlands is limited to the work of Notenboom and collaborators (Notenboom, 1982; Notenboom et al., 1992; Notenboom and De Boom, 1990; Notenboom and De Winter, 1983; Notenboom et al., 1996). These studies have especially suggested that groundwater fauna is restricted to aquifers associated with the large rivers passing through The Netherlands. Along the larger rivers, such as Meuse and Rhine, subsurface sediments are coarser, and confer larger and better aerated living environments for groundwater fauna, compared to the fine-grained sediments present in most of the country.

Description and research history of the Banisveld landfill

The presence and importance of eukaryotes in landfill-leachate contaminated aquifers and the influence of this type of polluted environments on microeukaryotic communities were investigated in an aquifer underlying one of the 4.000 old landfills in the Netherlands, the Banisveld landfill. This landfill with harmful consequences upon the surrounding groundwater ecosystems is located 5 km southwest of Boxtel (Province of Noord-Brabant) at 51°33'20" northern latitude, and 05°16'57" eastern longitude (Fig. 3). The landfill was formed in an excavated sand pit, it has a surface area of approximately 6 ha, and the volume of waste dumped therein is estimated to be 400.000 m^3. Most of the wastes are present below the water table. The landfill was operational between 1965 and 1977 as a dumping site for household refuse and industrial waste. After 1977, the landfilling operations were discontinued, the landfill was covered with soil, and vegetation composed of various types of grasses developed. The landfill was not sealed by natural or artificial liners, and therefore a plume of pollution formed. It migrates with a velocity of 4-10 m/year towards a nature reserve nearby (Kampina) (Van Breukelen et al., 2003). Geologically, the phreatic aquifer neighboring the landfill consists of a 7-9 m thick layer of fine unconsolidated clayey sands. Below these relatively permeable sediments, alternations of clay, peat and sand layers are present. The sediments are of a fluvio-eolian origin and were deposited

during the Pleistocene under periglacial conditions (Van Breukelen et al., 2003).

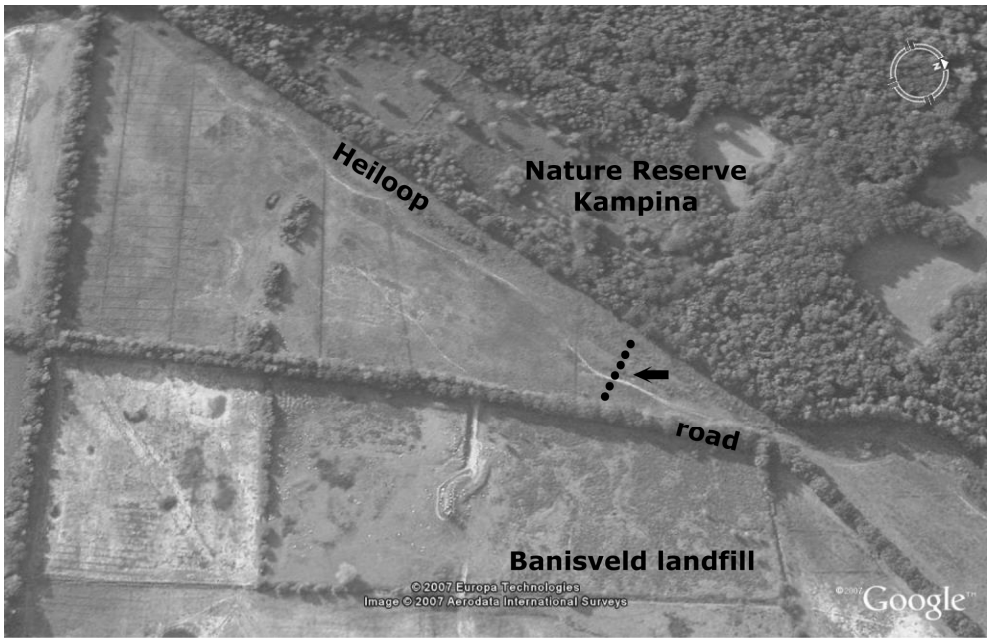

FIG. 3. Aerial photograph (Google Earth) of the Banisveld landfill and surroundings, with indication (arrow) of the piezometric transect downgradient of the landfill.

Since 1998, the site has been the scene of hydrogeochemical and microbiological research. The chemical composition of the pristine groundwater and leachate was determined, the biogeochemical processes governing the attenuation of landfill leachate, and the redox processes coupled with the oxidation of organic materials, the main pollutants in the aquifer, were identified (Van Breukelen, 2003; Van Breukelen et al., 2003; Van Breukelen and Griffioen, 2004; Van Breukelen et al., 2004). In general, the aquifer pollution plume is an anaerobic iron-reducing environment. Iron reduction occurs also below the pollution plume, while above the plume the dominant redox process is denitrification (Van Breukelen et al., 2003). The pristine groundwater is slightly acid (pH 4-6), has low alkalinity (0.2-6.6 mmol/l) and low concentrations of methane (0.0-0.56 mmol/l), sulfide (0-9 µmol/l), and Fe^{2+} (0.002-0.47 mmol/l) (Van Breukelen et al., 2003). The leachate

has a neutral pH, high alkalinity (51.3-56.8 mmol/l), and it contains high concentrations of dissolved organic carbon (9.0-10.3 mmol C/l), ammonium (19.2-19.8 mmol/l) and iron (0.20-1.24 mmol/l) (Van Breukelen et al., 2003). A decrease in the concentration of organic micropollutants (benzene, ethylbenzene, xylene and naphthalene) with distance from the landfill was observed. Close to the landfill the concentrations of benzene, ethylbenzene, xylene and naphthalene were 28, 53, 120 and 19 µg/l, respectively; further downgradient of the landfill these concentrations reduce close to 0 (Van Breukelen et al., 2003). This decrease was due to microbial biodegradation, as the high concentration of chloride (used as conservative tracer) remained relatively constant along the flow path. Dilution of organic pollutants could be thus excluded (Röling et al., 2001; Van Breukelen et al., 2003).

The landfill leachate appears to have a strong influence on the microbial communities present in the aquifer, selecting for specific groups of microorganisms. A specific *Geobacteraceae* phylotype was dominantly present in the part of the polluted aquifer closest to the landfill, where the organic biodegradation occurred at a relatively high rate (Lin et al., 2005). This *Geobacter* phylotype appeared to be involved in BTX (benzene, toluene and xylene) degradation in anaerobic microcosms experiments (Botton, 2007). The functional diversity is higher in the contaminant plume compared to that outside the plume (Röling et al., 2000a). The bacterial and archaeal populations were related to the hydrochemistry of the plume (Mouser et al., 2005) and were different inside the plume compared to those outside (Lin et al., 2005; Röling et al., 2001).

General methodology

Microorganisms, either prokaryotic or eukaryotic, but also the larger invertebrate fauna, manifest distinct physiological and morphological traits that help in their identification. However, classical morphology-based identification of these organisms is difficult, time consuming, and involves the presence and efforts of many specialists for each group. Culture independent molecular analyses revolutionized the field of microbial ecology for the last two decades. For groundwater in general,

and for polluted aquifers in particular, the focus had especially been directed to bacterial and archaeal communities (Botton, 2007; Lin et al., 2005; Ludvigsen et al., 1999; Mouser et al., 2005; Röling et al., 2000a; Röling et al., 2000b; Röling et al., 2001). Less attention has been paid to eukaryotic microorganisms in polluted sediments and groundwater.

The research described in this thesis is mainly based on culture-independent molecular analysis of the 16S rRNA and 18S rRNA genes retrieved from environmental groundwater and sediment samples (Fig. 4). 16S rRNA and 18S rRNA genes are present in all prokaryotic and eukaryotic organisms, respectively. These genes contain evolutionary well-conserved regions (Head et al., 1998), as well as regions specific to certain taxonomic groups, and have proven to be excellent phylogenetic markers. Amplification of these genes by polymerase chain reaction (PCR) and subsequent fingerprinting by various methods (e.g. denaturing or thermal gradient gel electrophoresis – D/TGGE) provides an impression of the diversity of these communities. The purified PCR-amplified rRNA gene fragments can be introduced into cloning vectors, and competent *Escherichia coli* cells can be transformed with the environmental DNA fragments (Röling and Head, 2005). Several methods of screening clone libraries (e.g. amplified ribosomal DNA restriction analysis - ARDRA, or DGGE) diminish the number of clones selected for sequencing. Representative rRNA-clone types can then be sequenced, and detailed phylogenetic analysis can be performed. Experimental and mathematical modeling approaches were used in Chapter 5 of this thesis for identifying the indirect roles and contribution of protists to organic matter biodegradation by predating on bacteria and recycling of limiting nutrients.

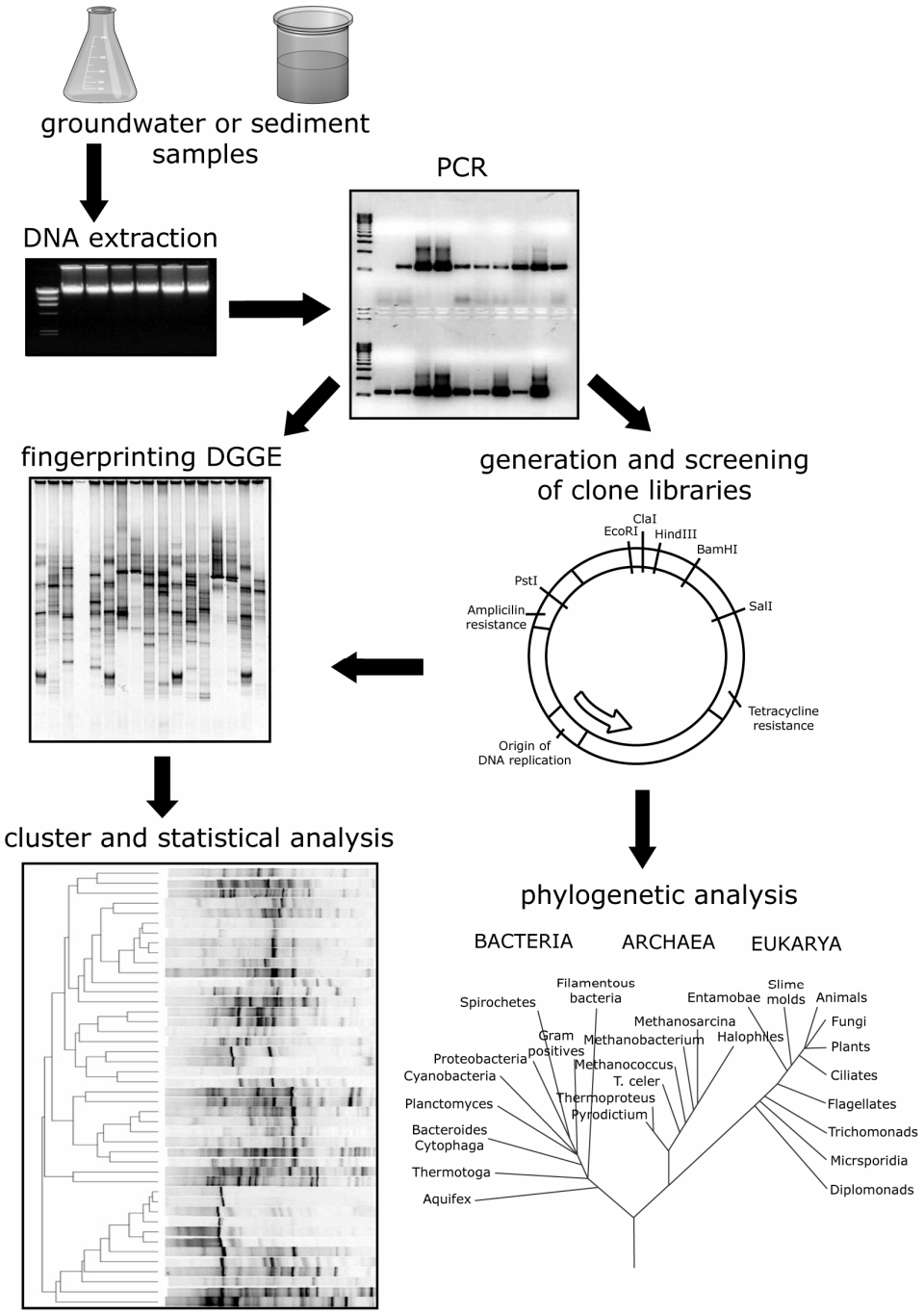

FIG. 4. Flow-scheme of culture-independent molecular techniques employed in the present thesis.

Thesis outline

The building blocks of this thesis consist of four major research chapters (chapters 2 to 5). Chapter 2 presents the structure of bacteria and eukaryotic communities in 48 sediment samples obtained at different distances from the Banisveld landfill, and different depths in the aquifer. The heterogeneity in microbial community structure was remarkable, and appeared to relate to the large heterogeneity in the abiotic characteristics. Chapter 3 continues on Chapter 2, and consists of detailed phylogenetic analysis of some of the samples fingerprinted in Chapter 2. A diverse eukaryotic community composed of heterotrophic nanoflagellates, yeast-like fungi and green algae is revealed by cloning and sequencing approaches. The abilities and importance of eukaryotes in relation to the process of natural attenuation are discussed. Chapter 4 looks upon the spatial and temporal dynamics of the landfill-leachate plume in the aquifer based on hydrochemical measurements, and the spatiotemporal changes in bacteria and the eukaryotic communities over a period of six years. The contribution of eukaryotes to organic matter biodegradation is regarded in Chapter 5 of this thesis. Chapter 5 is a combination of mathematical modeling validated by experimental culturing of organic matter degrading bacteria in the presence of predating flagellate protozoa and nutrient limitations. The results obtained in the four research chapters (chapters 2 to 5) are evaluated in Chapter 6, the general discussion.

Chapter 2

Spatial heterogeneity in sediment-associated bacterial and eukaryotic communities in a landfill leachate-contaminated aquifer*

Abstract

The heterogeneity in eukaryotic and bacteria community structure in surface and subsurface sediment samples downgradient of the Banisveld landfill (The Netherlands) was studied using a culturing-independent molecular approach. Along a transect covering the most polluted part of the aquifer, sediment was sampled at 1-m intervals, until a depth of 5.5 m, at four distances from the landfill. At two distances, replicate sediment sampling was performed. Two drillings were placed in a nearby clean area as reference. Denaturing Gradient Gel Electrophoresis banding patterns revealed high bacterial and eukaryotic diversity and complex community structures. Bacteria and eukaryotic community profiles in polluted samples grouped distinct from those in clean samples. Bacteria community profiles in surface samples clustered together and separately from subsurface community profiles. Belowground bacteria profiles clustered location-specific. Eukaryotic community structure did not significantly relate to distance from the landfill or depth. Eukaryotic 18S rRNA gene diversity decreased with depth, and was significantly lower in polluted samples than in clean samples. No significant spatial autocorrelation of bacteria or eukaryotic communities was observed over 1-m depth intervals per sampling location. Spatial heterogeneity in sediment-associated microbial community appears much larger than in groundwater. We discuss how on the one hand spatial heterogeneity may complicate the assessment of microbial community structure and functioning, while on the other hand it may provide better opportunities for natural attenuation.

Keywords

heterogeneity, landfill leachate, eukaryotic community structure, aquifer microbiology, Banisveld

*Submitted as Brad, T., B. M. van Breukelen, M. Braster, N. M. van Straalen, and W. F. M. Röling

Introduction

Groundwater is an important drinking water resource all over the world, but is polluted in many places. A particular threat to groundwater is the large number of unlined, old landfills in many countries. Percolating rainwater transports complex mixtures of pollutants in landfill waste into the underlying aquifer, leading to the formation of anaerobic plumes, which migrate with the groundwater flow (Christensen et al., 2000; Christensen et al., 2001). Natural attenuation processes, especially biodegradation, can diminish the toxicity of many components in landfill leachate (Christensen et al., 2000). Research on natural attenuation of leachate components has mainly focused on the hydrogeochemistry and on the composition and activity of prokaryotic communities (Christensen et al., 2000; Christensen et al., 2001; Ludvigsen et al., 1997; Ludvigsen et al., 1998; Röling et al., 2000a; Röling et al., 2000b; Röling et al., 2001; Van Breukelen et al., 2003; Van Breukelen et al., 2004; Van Breukelen and Griffioen, 2004).

While the role of bacteria in the degradation of pollutants is subject of extensive research, the presence and activity of eukaryotic organisms in polluted sediments and groundwater, including aquifers contaminated by landfill leachate, has received relatively minor attention. Microbial eukaryotes and mesofauna have important roles in groundwater food webs, making bacterial productivity available to higher trophic levels (Novarino et al., 1997). Eukaryotes can reduce bacterial clogging (Mattison et al., 2002) and soil aggregation (Kota et al., 1999). Especially protists can influence the process of biodegradation in aquifers by their feeding on bacteria. Predation either negatively influences biodegradation by reducing degraders populations, or positively by recycling of limiting nutrients to pollutant-degrading bacteria (Mattison and Harayama, 2001; Mattison and Harayama, 2005; Ratsak et al., 1996).

A second factor that so far received minor attention and is of importance to sampling design and monitoring natural attenuation, is the spatial heterogeneity in sediment-attached microbial communities. Prokaryotic communities in polluted groundwater from the aquifer downgradient of the Banisveld landfill grouped together and differently from those in the unpolluted groundwater (Röling et al., 2001). Groundwater communities in the plume were autocorrelated over distances between 40 and 50 meters (Mouser et al., 2005). However, the composition of prokaryotic communities in groundwater differs from sediment-attached communities (Röling et al., 2000b; Röling et al., 2001). The density of sediment-bound microorganisms and their metabolic potential are also higher than in corresponding groundwater (Albrechtsen and Winding, 1992; Holm et al., 1992). Thus, knowledge on the spatial distribution of sediment-associated microorganisms, and the factors contributing to their distribution, is important to improve conceptual and predictive understanding of natural attenuation (Brockman and Murray, 1997).

We report here the bacteria and eukaryotic community structure for a set of 48 sediment samples taken from the aquifer downgradient of the Banisveld landfill (The Netherlands). Denaturing Gradient Gel Electrophoresis (DGGE)-based community profiles were numerically compared and related to the level of pollution, sampling depth and distance from the landfill. This work extends on previous work in which we, based on a limited set of five sediment samples, could not detect a relation between bacterial community structure and environmental conditions (e.g. level of pollution) (Röling et al., 2001). In addition, we characterized eukaryotic community structure in detail.

Material and methods

Site description

The Banisveld landfill is located 5 km southwest of Boxtel (The Netherlands) at 51°33'22" N and 05°16'55" E. The landfill has a surface area of approximately 6 ha, and the volume of waste dumped therein is estimated to be 400.000 m^3. The site functioned between 1965 and 1977 as a waste disposal for household refuse and industrial wastes. After 1977 landfilling operations were discontinued and the landfill was covered. Liners preventing the migration of the leachate were absent, and a plume of leachate formed in the underlying aquifer. The lithology of this aquifer consists of a 7-9 m thick layer of fine to coarse-grained unconsolidated clayey sands. Anaerobic iron reduction is the major process in the plume, while also above and below the plume anaerobic conditions prevail (Van Breukelen et al., 2003). The groundwater velocity is 4-10 m/year towards a nature reserve (Van Breukelen et al., 2003). More site characteristics are found in Van Breukelen et al., (2003).

Sediment sampling

Sediment samples were obtained in November 2002 from two locations and in June 2003 from eight locations (Fig. 1). Four of the locations (P_1, P_2, P_4 and P_6) sampled in June 2003 followed the main direction of the groundwater flow in the most polluted part of the aquifer (Van Breukelen et al., 2003) and were located 21, 30, 48 and 68 m downgradient of the landfill (Fig. 1). For determining the heterogeneity over short distances, at two locations (P_1 and P_4), replicate drillings (P_1' and P_4') were placed within a distance of 4 m. Two reference locations (C_1 and C_2) were chosen in a clean part of the aquifer, approximately 200 m west of the polluted part of the aquifer. Samples were cored at intervals of 1 m, until a depth of 5.5 m. Sample codes represent the depths; for example "1m" means a sample from the depth between 1.0 and 1.5 m; "5m" means a sample from 5.0 to 5.5 m deep. For analyzing the reproducibility of the DNA extraction, three sediment samples, coded A, B and C, were taken in November 2002 (Fig. 1). Their locations and

sampling depth of these samples closely corresponded to samples P_1-1m, P_1-3m and C_1-5m, taken in June 2003.

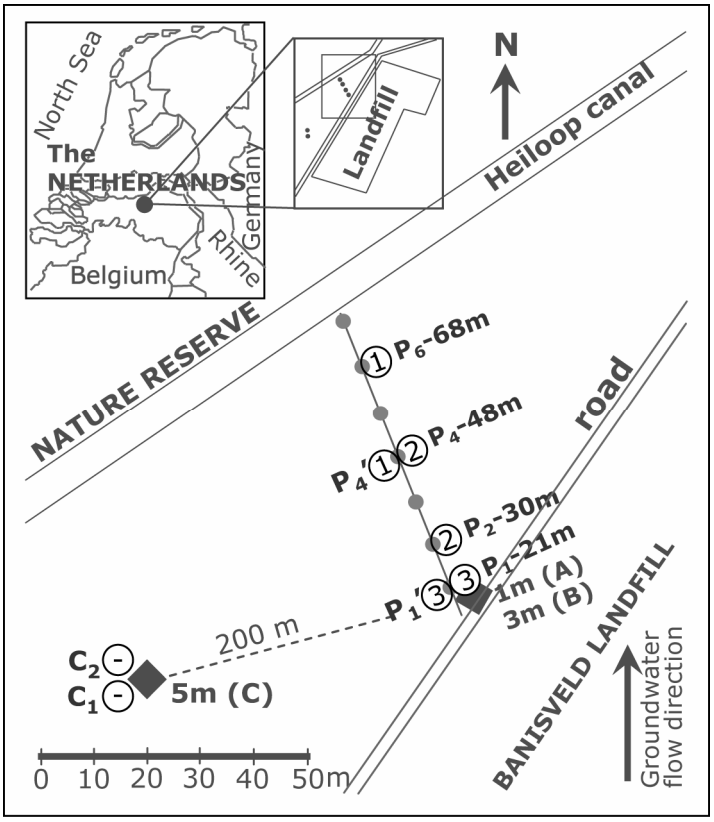

FIG. 1. Schematic view of the borehole locations from which sediment was sampled in November 2002 (◆) and June 2003 (○) at the research area downgradient of the Banisveld landfill. The June 2003 sampling comprised four sampling locations along the main direction of the groundwater flow [coded P_1, P_2, P_4 and P_6, with distance (in m) to landfill indicated], with two replications at 4 m distance (P_1' and P_4'). Two reference locations were in a clean part of the aquifer (C_1 and C_2); their positions relative to the P-boreholes are not shown to scale. The numbers in the circles indicate the depth (in m) at which landfill leachate was encountered. The text next to the November 2002 boreholes indicate the depths where the samples used for testing the reproducibility of the DNA extraction were obtained, and their codes. ● indicate groundwater monitoring wells installed in June 1998 (Van Breukelen et al., 2003).

Sediments were cored using a manual drilling device, which did not require the use of drilling fluid. The first 1.0-1.5 m were dug using an open clay auger ("ram horn") made of steel in a form similar to a corkscrew. A polyvinyl chloride (PVC) casing was then inserted into the borehole to prevent the hole from collapsing. A bailer drilling device (Eijkelkamp B.V., The Netherlands) was inserted through the casing and used for drilling in water-saturated sediments. A bailer is a hollow tube with a check valve at the bottom. The bailer is attached to a metallic bar, which can be extended for reaching larger depths. Drilling is done by alternating ramming and lifting the bailer. The sediment enters the bailer through the check valve when rammed on the bottom of the borehole, and remains in the bailer as the check valve closes during lifting. During drilling the PVC casing was gradually sank into the borehole and occasionally extended to reach greater depths with additional casing elements.

When the desired depth was reached, an undisturbed sample was taken by pressing onto the aquifer sediments using an extensible core pusher mounted with a 50-cm long PVC core holder with 6 cm inner diameter. A PVC piston was installed into the core holder and a core catcher was attached at the end of the core holder. A core catcher is a conic device made of steel with sharpen rim for hewing through the sediment. On the inner part, the core catcher was endowed with flexible fingers that allowed the sediment core to pass through the core holder, but not slide back out. As the corer penetrated the sediments, the piston remained just above the sample. The piston was gradually pulled up by using a cord for helping the sediment sample drawing into the core holder, and the steel core catcher retained the sample into the core holder. After retrieval, the sediment cores were capped, tightly sealed with tape, kept at 4^{o}C and processed within 24 h. The outer ends of the cores (3-5 cm) were removed in the laboratory, as they were shortly exposed to oxygen and might be contaminated on the outside. The remaining sediment was homogenized aseptically by manual mixing.

Physicochemical measurements

Pore water was extracted in the field directly from the sediment cores or in the laboratory by centrifugation of 100 g fresh sediment for 50 minutes at 4500 rpm. Electrical conductivity (EC) and ammonium concentrations were determined to evaluate the landfill leachate contamination of the aquifer, as these measurements were previously found to be good indicators of pollution for the aquifer investigated here (Röling et al., 2000b). EC was measured using a GMH 3410 CE digital conductivity meter (Greisinger Electronic, Regenstauf, Germany). Ammonium concentration was determined by spectrophotometry using a Skalar SA-40 autoanalyser (Skalar BV, Breda, The Netherlands) (Krom, 1980).

Community profiling

Indirect DNA extractions were performed: microorganisms were released from 50 g of fresh, homogenized sediment, by extracting for one hour in an orbital shaker at 150 rpm with 100 ml 0.1% sodium pyrophosphate. The mixture was then left to stand for 60 seconds in order to allow heavy sediment particles to settle. The liquid phase containing microbial cells was filtered through 0.22 µm mesh-sized cellulose-membrane filters (Sartorius AG, Göttingen, Germany) using a vacuum pump. The filters were frozen at -80°C until DNA extraction. DNA was isolated using the FastDNA SPIN Kit (BIO 101 Systems, Irvine, California) according to the instructions of the manufacturer, after filters were aseptically cut into small pieces. In order to test the suitability and reproducibility of the DNA isolation method, three sediment samples obtained in November 2002 were subjected to two replicate indirect DNA extractions on 50 g sediment and two replicate DNA isolations directly on 0.5-g sediment samples, using the FastDNA SPIN Kit.

Bacteria-specific PCR was performed in a 25 µl (total volume) mixture containing 0.4 µM forward primer F357-GC (Muyzer et al., 1993), 0.4 µM reverse primer R518 (Muyzer et al., 1993), 0.4 mM of each deoxynucleoside triphosphate (dNTP), 10 µg of bovine serum albumin (BSA), PCR buffer, 2 U of Taq polymerase, and 1 µl of undiluted DNA template. Amplification was performed in a Perkin-Elmer DNA Thermo Cycler using an initial denaturation of 94°C for 4 min, followed by 35

cycles of 94°C for 30 s, 54°C for 1 min, and 72°C for 1 min, and a final elongation at 72°C for 5 min.

For Eukarya, we initially employed a test on the suitability of two sets of primers (set A - primers Euk1A and Euk516r-GC and set B - primers Euk1209f-GC and Uni1392r) described by Díez et al. (2001). Like observed by Diez et al. (2001), the A-set, which amplifies a 560 bp fragment of the 18S rRNA gene, generated a higher number of bands in DGGE profiles compared to the B-set which amplifies 210 bp of the 18S rRNA gene (data not shown). Therefore, the A-set of primers was employed in this study for Eukarya community profiling. PCR amplification was performed in a 25 µl (total volume) mixture containing 0.4 µM forward primer Euk1A (Díez et al., 2001), 0.4 µM reverse primer Euk516r-GC (Díez et al., 2001), 0.4 mM of each deoxynucleoside triphosphate (dNTP), 10 µg of bovine serum albumin (BSA), PCR buffer, 2 U of Taq polymerase, and 1 µl of undiluted DNA template. Amplification consisted of an initial denaturation at 94°C for 130 s, followed by 40 cycles of 94°C for 30 s, 56°C for 45 s, and 72°C for 130 s, and a final elongation at 72°C for 7 min.

Denaturing Gradient Gel Electrophoresis (DGGE) was performed with the BioRad DCode™ (Hercules, California) system. PCR products were loaded onto 1-mm-thick 8% (w/v) polyacrylamide (ratio of acrylamide to bisacrylamide, 37.5:1) gels. A 30 to 55% linear denaturing gradient was used for Bacteria-specific PCR products and a 20 to 35% linear denaturing gradient for Eukarya-specific PCR products. The 100% denaturant was defined as 7 M urea and 40% (v/v) formamide. Electrophoresis was performed in 1×TAE buffer (40 mM Tris-acetate, 1 mM Na-EDTA; pH 8.0) at 200 V and 60°C for 4 hours. The gels were stained in 1×TAE buffer containing 1 µg/ml ethidium bromide and were recorded with a charge-coupled device camera system (The Imager, Appligen, Illkirch, France). To aid in later conversion and normalization of gels, a marker was added on the outsides of the gels, as well as after every four samples. The outer two lanes of each gel were not used.

Statistical analysis

DGGE images were converted, normalized, and analyzed with the GelCompar II software package (Applied Maths, Kortrijk, Belgium). For band-based analysis only bands with an intensity corresponding to at least 1% of the total intensity of the DGGE track were taken into account. The number of DGGE bands, and their intensities, were used to calculate the Shannon-Weaver index according to: $H'=-\Sigma P_i \log P_{i,}$ where $P_{i=}n_i/N$; n_i is the surface of band i in a densitometric curve and N is the sum of all band surfaces. Shannon-Weaver indices were correlated to the depth below surface, distance from the landfill, and EC values (Systat 7.0, SPSS Inc., Chicago, Illinois).

Similarities between tracks were calculated in GelCompar II by using the Pearson product-moment correlation coefficient (whole densitometric curve based) and visualized using the unweighted pair group clustering method with arithmetic averages (UPGMA). Cluster analysis of similarity matrices does not allow for testing the statistical significance of differences between clusters or sets of samples. Therefore, similarity coefficients were classified into discrete groups, which were subsequently tested to determine whether their averages were significantly different (Van Verseveld and Röling, 2004). As the data were not normally distributed, a non-parametric analysis (Mann-Whitney U test) was performed using Systat 7.0 (SPSS Inc., Chicago, Illinois). In general, the similarities among samples sharing a particular characteristic were assigned to group 1, while similarities between samples that differed in this characteristic were assigned to group 2. The characteristic could be a particular sampling location, depth or degree of pollution (clean, polluted). As an example, to test whether bacterial communities at location P_6 grouped significantly better to each other than to those at other locations, we assigned to group 1 those similarity coefficients comparing among the P_6 samples, while the similarity coefficients comparing P_6 samples to samples from another locations were assigned to group 2. This approach was also used to determine whether the bacteria and eukaryotic community profiles in the replicated locations (P_1 and P_1'; P_4 and P_4' and C_1 and C_2) were significantly more different from each other than from those obtained at the other locations. Correlation between similarity matrices derived from

bacteria and eukaryotic community profiles was determined using a Mantel test (Legendre and Legendre, 1998).

Multivariate Mantel correlograms (Legendre and Legendre, 1998) were used to analyze the vertical spatial structure in microbial communities, per borehole. Normalized Mantel statistic values were calculated for five distance classes ranging from 1 m to 5 m and tested for significant autocorrelation by a permutation test with 1000 iterations. Calculations were made using matrices containing community dissimilarity coefficients and geographic distance (sampling distance in vertical direction). Dissimilarity was calculated as one minus Pearson correlation coefficient (Franklin et al., 2002). A program for performing the Mantel correlograms based on the input of similarity matrices from GelCompar II was written in Maple 7.0 (Waterloo Maple Inc.).

Results

Reproducibility of DNA extraction

Reproducibility of DNA extraction is needed to ensure a representative fingerprint of the microbial community for a particular sample. In preliminary experiments, replicate direct DNA extractions of 0.5 g samples sometimes revealed considerable variation (<85% similarity) in their subsequent DGGE profiles. Therefore, we developed an indirect DNA isolation method based on cells separated from 50 g sediment. This method was compared to the direct DNA extraction method using 0.5 g samples. Reproducibility of DNA extraction was tested by replicate DNA extraction on three sediment samples, taken in November 2002 (Fig. 1). DGGE profiling after Eukarya and Bacteria specific amplification of rRNA genes revealed that reproducibility with the indirect extraction of 50 g sediment was in general better than the direct extraction of 0.5 g sediment (Table 1), and was always above 85% similarity. Therefore, the indirect extraction was used for detailed characterization of the spatial heterogeneity in the sediment-associated microbial communities in the aquifer downgradient of the Banisveld landfill, in June 2003 (Fig. 1).

TABLE 1.

Reproducibility of DNA extraction methods**.** Similarities (%) between DGGE profiles of Bacteria or Eukarya communities derived from duplicate DNA extractions on three sediment samples (see Fig. 1 for sampling locations), using two different extraction methods; an indirect method using 50 g sediment and a direct DNA extraction on 0.5 g-sediment.

	Bacteria		Eukarya	
Sample	**Direct**	**Indirect**	**Direct**	**Indirect**
A	95	99	94	91
B	60	98	98	94
C	85	99	52	89

Physicochemical measurements

Measurements of electrical conductivity (EC) and ammonium concentrations, indicators of the presence of landfill leachate at the Banisveld research site (Röling et al., 2001), revealed that leachate was present 3 m below surface in June 2003 close to the landfill (P_4 and P_4'). However, pollution was detected at only 1 to 2 m below the surface at distances over 30 m from the landfill (Fig. 1). EC ranged from 793 to 2580 μS cm^{-1}, while ammonium concentrations were between 66.0 and 130.7 mg/l. Previous hydrochemical analysis on groundwater samples had shown that the aquifer had been clean up to a depth of 4 m over the period 1998-1999 (Van Breukelen et al., 2003). In line with Van Breukelen and Griffioen (2004), the pollution plume appears to have extended to the surface since then. Locations P_1 and P_4 were replicated at 4-m distance, labeled P_1' and P_4' respectively. Despite the small horizontal distance between the sampling locations, large differences (up to 66%) in EC were observed at comparable depths, indicating considerable hydrochemical heterogeneity. Locations C_1 and C_2 were selected in a clean part of the aquifer, as reference locations. Their EC values ranged from 60 to 578 μS cm^{-1} and ammonia concentrations were between 2.6 to 3.6 mg/l. In order to test for the effect of pollution on the eukaryotic and bacteria community structure and biodiversity, samples with EC values over 750 μS cm^{-1} (threshold also marking the distinction between clean and polluted in Van Breukelen et al., 2003) were considered polluted, while sediments with lower EC were defined as clean.

Bacteria community structure

DGGE-based bacteria community profiles were complex; the number of bands per profile ranged from 10 to 36 (21 bands on average), and cluster analysis showed large differences between samples (Fig. 2). However, bacteria community profiles in polluted samples (EC>750 µS cm^{-1}) were significantly more related to each other ($P<0.001$; average similarity among polluted samples was 33.3%) than to those in clean samples (average similarity between groups of 25.5%). With the exception of P_6-0m, the bacteria community profiles from surface samples formed a cluster at a low similarity of 15% (Fig. 2). These profiles were significantly more related to each other than to those of subsurface samples (Mann-Whitney U test, $P<0.001$). The average similarity among surface communities was 29.5%, while the average similarity between surface communities on the one hand and subsurface samples on the other hand was 8.2%.

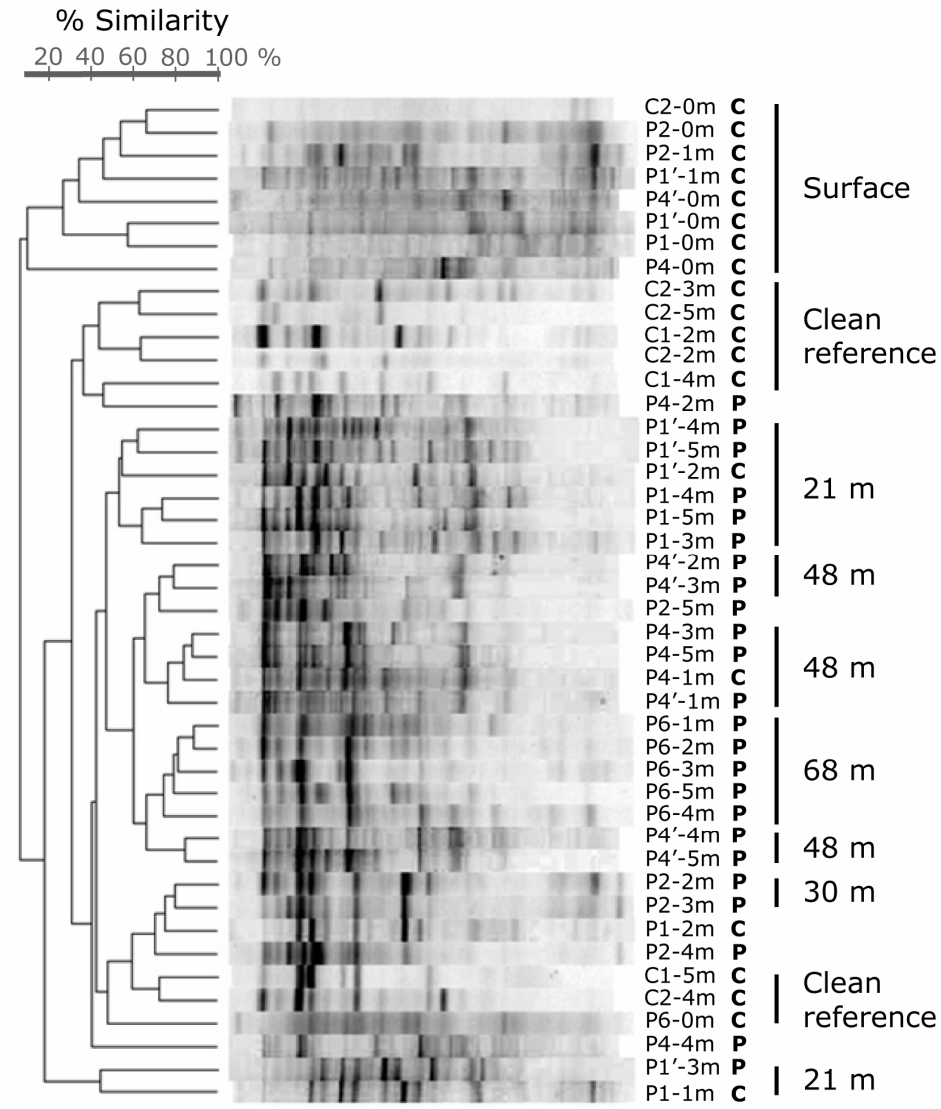

FIG. 2. UPGMA-based clustering of 16S rRNA gene-based DGGE profiles of Bacteria (30-55% denaturant gradient) in sediment samples downgradient from the Banisveld landfill, after Pearson correlation. For each sample, the location and depth designation (see Fig. 1) and pollution level (P for polluted and C for clean) are shown.

In general, a sampling location-specific grouping of subsurface bacteria communities was observed (Fig. 2), although with its exceptions. As an example, some of the bacteria community profiles of subsurface samples obtained at 21 m distance from the landfill (P_1-2m and P_1'-3m) clustered outside the P_1 group. Bacteria community profiles from subsurface samples at the clean reference locations (C_1 and C_2) clustered separately from those from the sampling transect downgradient from the landfill (P locations; Fig. 1), at 37% similarity. However, two DGGE profiles of the reference samples (C_1-5m and C_2-4m) clustered with DGGE profiles obtained from polluted samples. Statistical analysis confirmed the visual interpretation of the cluster analysis (Fig. 2); overall similarity among profiles from subsurface samples from the same location (52.1% average similarity) was significantly higher ($P<0.001$) than between profiles from different locations (36.3% similarity). A subsequent pair-wise comparison of locations revealed significant differences in bacterial community structure between most locations (Table 2). However, no significant differences between the replicated locations (C_1 vs. C_2, P_1 vs. P_1', P4 vs. P_4') were observed. Also a comparison of depth-specific similarities in bacteria community structure among replicated locations with their similarities to community profiles derived from the same depth at the other locations indicated that the replicated C_1 vs. C_2, and P_1 vs. P_1' were significantly ($P<0.001$) more similar to each other than to other locations. This was not observed for the replicated P_4 vs. P_4' ($P=0.12$).

Despite location-specific clustering of subsurface bacteria community profiles, multivariate Mantel correlograms revealed that even over a vertical distance of 1 m no significant autocorrelation in community structures per sampling location was observed ($P>0.05$). The dissimilarity in bacteria community profiles was already around 45% at one meter inters-ample distance and increased slightly ($r=0.18$; $P=0.056$) with vertical inter-sample distance (Fig. 4A).

TABLE 2.
Pair-wise comparison of sampling locations (see Fig. 1 for position) in similarities of their DGGE-based bacteria community profiles. P-values are indicated, grey-highlighted values indicate significant differences in community structure between locations. Only subsurface samples were used in the comparisons.

	C_1	C_2	P_1	P_1'	P_2	P_4	P_4'
C_1							
C_2	0.95						
P_1	0.39	0.01					
P_1'	0.45	<0.01	0.19				
P_2	0.48	0.01	0.15	0.05			
P_4	0.98	0.14	0.16	0.43	0.05		
P_4'	0.01	<0.01	<0.01	<0.01	0.04	0.58	
P_6	<0.01	<0.01	<0.01	<0.01	<0.01	0.03	<0.01

Eukaryotic community structure

The eukaryotic community profiles revealed on average 14 bands, with a minimum of 4 and a maximum of 27 bands. Eukaryotic community composition varied largely over horizontal and vertical sections in the aquifer and did not cluster visually according to depth, distance from the landfill or presence of pollution (Fig. 3). Nevertheless, the eukaryotic community profiles in polluted samples were more similar to each other (P=0.02; average similarity of 34.5% among polluted samples) than to those from clean samples (average similarity between groups of 30.1%).

Several samples from larger depths (4-5 m) with low diversity (up to 6 bands) clustered together at a high similarity of 90%. However, eukaryotic community profiles obtained in polluted samples from larger depths (5 m samples) did not group significantly (P>0.05) more to each other (within 5-m group similarity average was 35.9%) than to those in samples from smaller depths (average similarity between 5-m samples and less-deep samples was 31.2%).

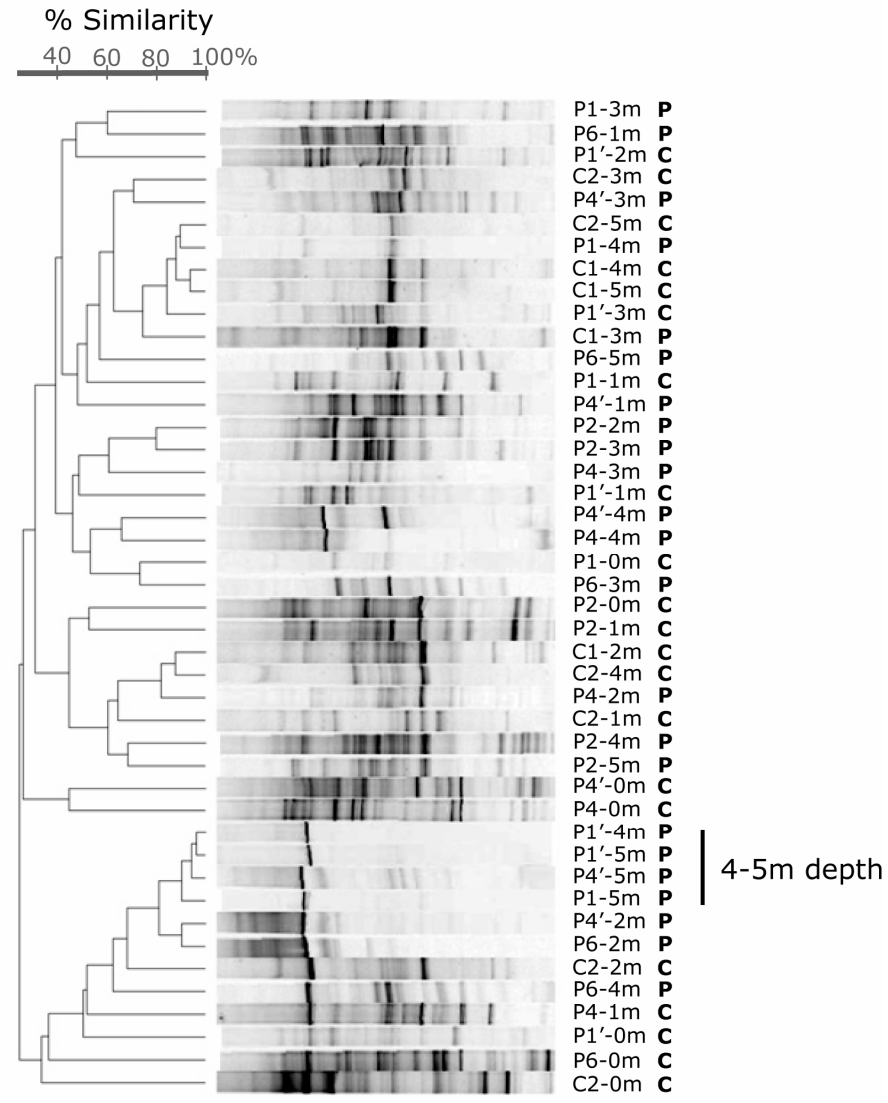

FIG. 3. UPGMA-based clustering of 18S rRNA gene-based DGGE profiles of Eukarya (20-35% denaturant gradient) in sediment samples downgradient from the Banisveld landfill, after Pearson correlation. For each sample, the location and depth designation (see Fig. 1) and pollution level (P for polluted and C for clean) are shown.

In agreement with visual interpretation of Fig. 3 and in contrast to the observations made for bacterial community profiles, no significant (P=0.15) location-specific clustering was observed. Like for the bacteria community profiles, Mantel correlograms did not reveal autocorrelation of the eukaryotic community structure with vertical distance (m) between samples, with two exceptions (distance class 2 m for locations P_4 and C_2). The dissimilarity in subsurface eukaryotic community profiles was already over 60% at one meter inter-sample distance and increased slightly, but not significantly (r=0.08; P=0.15), with vertical inter-sample distance (Fig. 4B).

A slightly higher, but significant, similarity (P=0.044) was observed in the eukaryotic community profiles (overall average similarity 34.9%) compared to the variation in the bacteria community profiles (average similarity 33.0%). A Mantel test revealed that the eukaryotic and bacteria community profiles obtained in the same sediment samples were significantly related to each other (r=0.128; P<0.001).

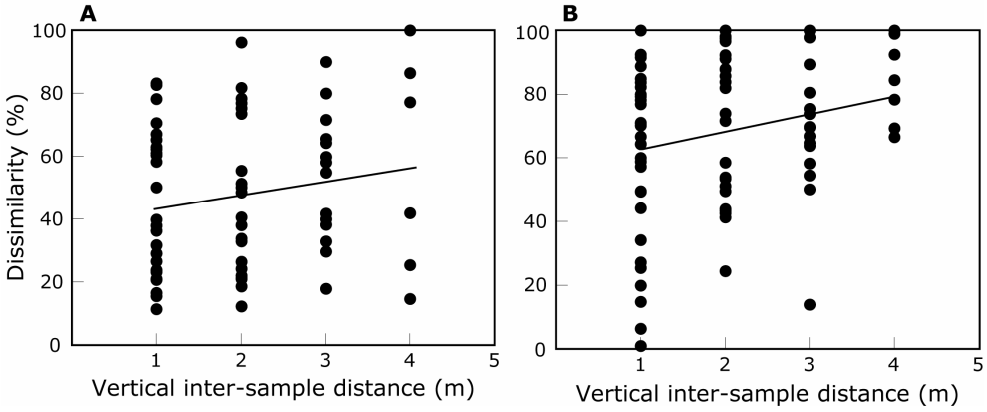

FIG. 4. Relation between dissimilarity in Bacteria (A) and Eukarya (B) community profiles and vertical inter-sample distance. Dissimilarities and inter-sample distances were determined per borehole.

Eukarya 18S rRNA gene biodiversity

Eukaryotic 18S rRNA gene diversity, based on the Shannon-Weaver index, decreased with depth both in the polluted and clean reference part of the aquifer (Fig. 5A: r=0.55 and 0.83, respectively; P<0.01). A significant negative association (r=0.54; P<0.01) was also observed between eukaryotic diversity and pollution-indicator EC (Fig. 5B). Eukaryotic communities in polluted sediments (average Shannon-Weaver index 1.94) were less diverse (P=0.007) compared to those in clean sediments (average Shannon-Weaver index 2.43). No correlation (P>0.05) was observed between eukaryotic diversity and the distance from the landfill. No relationship (P>0.05) was detected between the bacterial 16S rRNA gene diversity and the depth below the surface, distance from the source of pollution, or EC values. Bacterial 16S rRNA gene diversity in polluted samples (average Shannon-Weaver index of 2.65) was also not significantly different (P=0.79) from that in clean sediments (average Shannon-Weaver index of 2.66). The eukaryotic 18S rRNA gene diversity was significantly lower (ANOVA, P<0.05) compared to that of the bacteria in both polluted and clean sediments. The eukaryotic 18S rRNA gene diversity was not significantly (r=0.103, P>0.05) related to that of the bacteria.

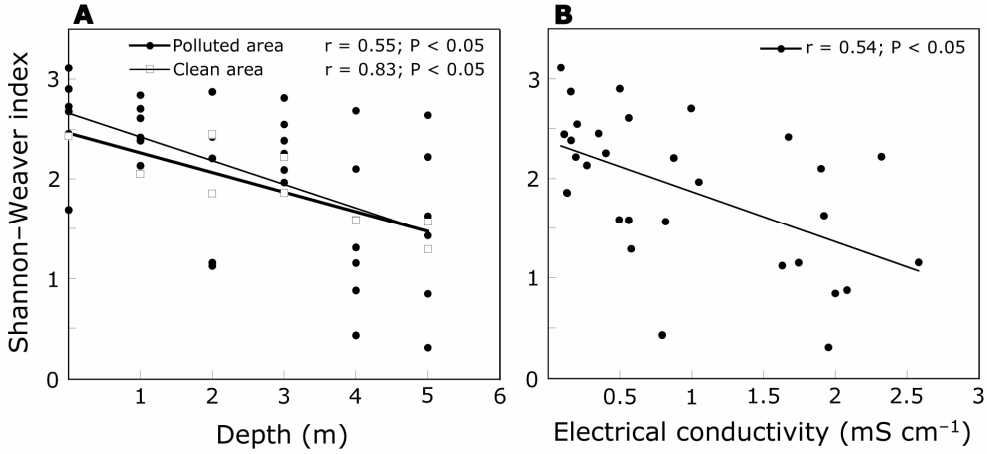

FIG. 5. Trends in eukaryotic diversity (Shannon-Weaver index) against sampling depth (A) and against electrical conductivity, a measure of the degree of pollution (B).

Discussion

Complex sediment-associated eukaryotic and bacteria community structures, revealing considerable spatial heterogeneity, were obtained after cultivation-independent community profiling of amplified rRNA gene fragments. The different steps in this molecular approach can present pitfalls such as inadequate cell lysis during nucleic acid extraction and PCR bias (Von Wintzingerode et al., 1997). We optimized our DNA extraction procedure in order to achieve high reproducibility in community profiling, therefore the variation in community profiles is not due to experimental artifacts. Since all samples were treated in a similar manner, procedural pitfalls can be considered to be the same for all samples, allowing between-sample comparisons and comparison of community profiles to environmental characteristics. Many eukaryotes have multiple copies of the 18S rRNA gene (Fredslund and Mills, 2003). Therefore, care should be taken when interpreting the eukaryotic biodiversity from DGGE patterns, and we interpret a band as a sequence type rather than a species.

We observed a significant relationship between sediment-associated community structure and pollution level for the aquifer polluted by the Banisveld landfill. Using phospholipids fatty acid (PFLA) analysis to profile sediment-associated microbial communities, Ludvigsen et al. (1999) made a similar observation for an aquifer polluted by the Danish Grindsted landfill. DGGE analysis, as we applied, allows for higher resolution of microbial communities than PFLA analysis.

The correlation between pollution and sediment-associated community structure is in line with previous observations on groundwater community structure at the same research location (Lin et al., 2005; Mouser et al., 2005; Röling et al., 2000a; Röling et al., 2001). Detailed studies on aquifer sediments are more ecologically relevant compared to studies on groundwater samples, especially when evaluating the potential for natural attenuation. Biodegradation potential in sediment can be higher than in groundwater (Holm et al., 1992). Even though groundwater is present in sediment samples, the community structure of sediment-associated microorganisms is different from that in adjacent

groundwater in landfill-leachate polluted aquifers (Röling et al., 2000b; Röling et al., 2001) as water constitutes a minor fraction of sediment. Also, the density of sediment-attached microorganisms, in numbers per unit volume, is in general one order of magnitude higher than in groundwater (Albrechtsen and Winding, 1992; Holm et al., 1992). Thus, sampling of sediment will provide a more complete view on microbial community structure and functioning than sampling groundwater.

Drawbacks to sediment sampling are time-consuming, expensive sampling and the potential impact of location-specific chemical and physical characteristics on the development of sediment-associated communities which may complicate interpretation of community profiles. Indeed, in a previous study we did not observe a relation between community structure and pollution when analyzing a small set of five sediment samples (Röling et al., 2001). The large set of samples analyzed here revealed a relation of community structure to level of pollution. Despite this relation, we also observed that bacteria community profiles clustered location specific, while eukaryotic community profiles did not show any clear tendency of grouping based on distance from the landfill or depth. Furthermore, even over small distances (1 m) large differences in community structure and lack of spatial autocorrelation were noted. In contrast, microbial communities were autocorrelated over 40 m horizontal distance in groundwater from the same aquifer (Mouser et al., 2005). Previous geochemical research on sediments from the aquifer at Banisveld revealed large spatial heterogeneity in sediment properties, such as organic carbon (0.054 to 0.255% w/w) and clay content (2.22 to 6.99% w/w) (Van Breukelen et al., 2003). These factors may have contributed to the large heterogeneity in sediment-associated community structure and lack of autocorrelation over short distances. Heterogeneity in environmental characteristics such as sediment-attached resources and granulometry, together with spatial isolation of microorganisms, are key parameters that influence microbial abundance and the structure of microbial communities in sandy aquifers with low groundwater flow (Albrechtsen, 1994; Ludvigsen et al., 1997; Ludvigsen et al., 1999; Zhang et al., 1998; Zhou et al., 2002; Zhou et al., 2004), such as that investigated here. Spatial heterogeneity can have an important impact on small-scale microbe-mediated biogeochemical reactions (Cozzarelli et al., 1999;

Davis et al., 2003; Zhang et al., 1998). The lack of autocorrelation in community structure even over one meter depth intervals suggests that sediment sampling needs to be carried out at smaller scales, if one aims at more fully characterizing microbial communities and functioning at the Banisveld location**.** On the other hand the lack of autocorrelation allowed for the use of classical statistical procedures that assume independence of observations.

As discussed above, sediment sampling will provide a more complete view on community structure and functioning than groundwater sampling. However, the observed large spatial heterogeneity can hamper the design of adequate, cost-effective monitoring strategies and cause difficulties in interpreting the data as function of space (Franklin and Mills, 2003; Mouser et al., 2005). On the other hand, the spatial heterogeneity in microbial community composition of sandy polluted aquifers might be a trait favorable for natural attenuation. During its transport through an aquifer, a contaminant and its degradation products may pass more types of bacteria if the environment is more heterogeneous, and thus may have a higher chance of becoming degraded.

The heterogeneous bacterial community structures may also be determined, to an unknown extent, by eukaryotes such as bacteria-predating protozoa, and/or vice versa. A significant relation between bacterial community structure and eukaryotic community structure was observed. Eukaryotes were present throughout the aquifer. Eukaryotes have previously been encountered in many contaminated aquifers (Kinner et al., 1997; Kinner et al., 1998; Kota et al., 1999; Madsen et al., 1991; Novarino et al., 1994; Novarino et al., 1997; Sinclair et al., 1993; Snyder et al., 2000; Thomas et al., 1997; Zarda et al., 1998, including anaerobic aquifers (Kinner et al., 2002; Ludvigsen et al., 1997). PFLA biomarkers of microalgae and fungi were detected in most investigated sediment samples from an anaerobic Danish aquifer polluted by landfill leachate, and the occurrence of these markers was correlated with the presence of leachate (Ludvigsen et al., 1997). Targeting 18S rRNA genes allows for larger resolution of community structure than PFLA analysis. Using DGGE analysis to fingerprint eukaryotic communities, we also observed that eukaryotic community

structure related to pollution level. The eukaryotic 18S rRNA gene diversity in polluted sediment was lower than in clean sediment. In the accompanying manuscript (Chapter 3) we will report the occurrence of bacteria-predating protozoa besides the presence of algae and fungi, on the basis of phylogenetic analysis and culturing.

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Chapter 3

Eukaryotic diversity in an anaerobic aquifer polluted with landfill leachate*

Abstract

Eukaryotes may influence degradation processes in groundwater ecosystems by activities such as predation on bacteria and recycling of nutrients. Culture-independent phylogenetic analysis of 18S rRNA gene fragments and culturing were employed to obtain insight in the eukaryotic community composition in an anaerobic sandy aquifer polluted with landfill leachate (Banisveld, The Netherlands). The microeukaryotic community in five sediment samples obtained at 3 to 5 m depth along a transect downgradient (21-68 m) from the landfill, and from one clean reference location, was diverse. Fungal sequences, especially belonging to yeasts of the *Basidiomycota*, dominated most clone libraries. Sequences of green algae (*Chlorophyta*) were detected in parts of the aquifer close (<30 m) to the landfill. The bacteria-predating nanoflagellate *Heteromita globosa* (*Cercozoa*) was retrieved in enrichments and its sequences dominated the clone library derived from the polluted aquifer at 5 m depth, and 21 m downgradient of the landfill. The number of culturable eukaryotes ranged from 10^2 to 10^3 cells/g sediment. Culture-independent quantification revealed slightly higher numbers. Groundwater mesofauna was not detected. We conclude that the food chain in this polluted aquifer is short and consists of prokaryotes and fungi as decomposers of organic matter and protists as primary consumers of the prokaryotes.

Keywords

18S rRNA, biodiversity, landfill leachate, groundwater, Banisveld, natural attenuation

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Introduction

Food webs in aquifers comprise besides bacteria and archaea also eukaryotes. Groundwater eukaryotes may range from single-celled heterotrophic nanoflagellates (*Protozoa*) and fungi to amphipod crustaceans, each with important roles in the functioning of the groundwater ecosystem (Danielopol et al., 2003). The occurrence of fungi in low numbers has been described for a few pristine and polluted aquifers, but their identity and activities have to our knowledge hardly been investigated (Madsen et al., 1991; Madsen and Ghiorse, 1993; Sinclair and Ghiorse, 1989). Protozoa, especially nanoflagellates, selectively graze on the biomass of the bacteria community (Novarino et al., 1997), and recycle nutrients (Ratsak et al., 1996). Phototrophs, such as green algae, have been encountered in aquifers (Sinclair and Ghiorse, 1989). These phototrophs are believed to be introduced in aquifers by means of surface recharge (Sinclair and Ghiorse, 1989). Many algae are mixotrophic and can survive in the absence of light by feeding on bacteria (Jones, 2000).

The abundance and diversity of protists generally increase upon pollution (Madsen and Ghiorse, 1993; Novarino et al., 1997). Biodegradation of organic pollution often results in the development of anaerobic conditions. Also under these conditions protists are present and affect the abundance and diversity of bacteria (Kinner et al., 2002). Protists can indirectly affect contaminant biodegradation. By feeding on bacteria, protists can reduce degrader populations, influencing negatively the process of biodegradation (Kota et al., 1999; Travis and Rosenberg, 1997). However, protists can also positively contribute to organic contaminant degradation by recycling limiting nutrients to pollutant-degrading bacteria (Ratsak et al., 1996), stimulating the activity per bacterium (Mattison and Harayama, 2001; Mattison et al., 2002), or sustaining bioremediation by maintaining hydraulic conductivity of the aquifer as a result of reduced bacterial clogging (Mattison and Harayama, 2005; Sinclair et al., 1993).

Landfill leachate is an important polluter of groundwater. Studies on biodiversity in anaerobic landfill-leachate polluted groundwater, and polluted groundwater in general, have mainly focused on prokaryotes (e.g. Christensen et al., 2001; Röling et al., 2001). Despite the potential contribution of eukaryotes to ecosystem functioning and to biodegradation, the occurrence and diversity of eukaryotes in anaerobic aquifers has hardly been addressed. Phospholipid fatty acids (PLFA) biomarkers of fungi and algae were detected in an anaerobic aquifer polluted with leachate from a landfill in Denmark (Ludvigsen et al., 1999). Fungal and algal PLFA concentrations were highest close to this landfill and decreased with the distance from the source. PLFAs of protists were not detected nor could protists be cultured (Ludvigsen et al., 1999). In the accompanying paper (Chapter 2) we describe the eukaryotic community structure in the anaerobic, sandy aquifer situated downgradient of the Banisveld landfill (The Netherlands), using 18S rRNA gene based denaturing gradient gel electrophoresis (DGGE). General fingerprinting methods such as DGGE, however, do not indicate which types of eukaryotes are present.

In order to enhance insight into the types of eukaryotes in anaerobic landfill-leachate polluted aquifers, culture-independent phylogenetic analysis was combined with sampling of groundwater metazoa and culturing of microeukaryotes. Phylogenetic analysis was performed on clone libraries derived from five sediment samples taken along a transect downgradient from the Banisveld landfill and one clean reference sample. Culture-dependent and culture-independent estimations of the most probable number (MPN) were performed for the quantification of eukaryotes.

Material and Methods

Site description

The Banisveld landfill is located 5 km southeast of Boxtel, The Netherlands, at 51°33'22" N and 05°16'55" E. The landfill has a surface area of approximately 6 ha, and an estimated 400.000 m^3 volume of waste was dumped there between 1965 and 1977. Landfilling occurred in a dug sand pit, and most of the waste is present below the water table. Liners preventing the migration of the leachate were not installed and an anaerobic iron-reducing plume of pollution formed, while also above and below the plume anaerobic conditions prevail. The lithology of the aquifer consists of a 7-9 m thick layer of fine to coarse-grained unconsolidated clayey sands. More site characteristics are found in Van Breukelen et al. (2003).

Groundwater mesofauna sampling

Between December 2001 and November 2002, 11 groundwater wells, which were installed downgradient of the Banisveld landfill in June 1998 (Van Breukelen et al., 2003), and 28 groundwater monitoring wells in the surrounding Kampina nature reserve were sampled for the occurrence of groundwater fauna. Each groundwater well downgradient of the Banisveld landfill contained usually 3 screens, one above, one within and one below the plume of pollution. 200-3000 liters of groundwater were pumped per filter using a Honda Water Pump WX10 and filtered through 55 µm mesh-sized planktonic nets. The dross in the nets was collected in plastic jars, fixed in a solution of 4% formaldehyde and colored with Bengal Rose. These samples were analyzed for the presence of groundwater invertebrates using a stereomicroscope. The applied method was successfully validated at a location (St. Agatha, Noord Brabant, The Netherlands) where groundwater fauna was previously encountered (Schminke and Notenboom, 1990).

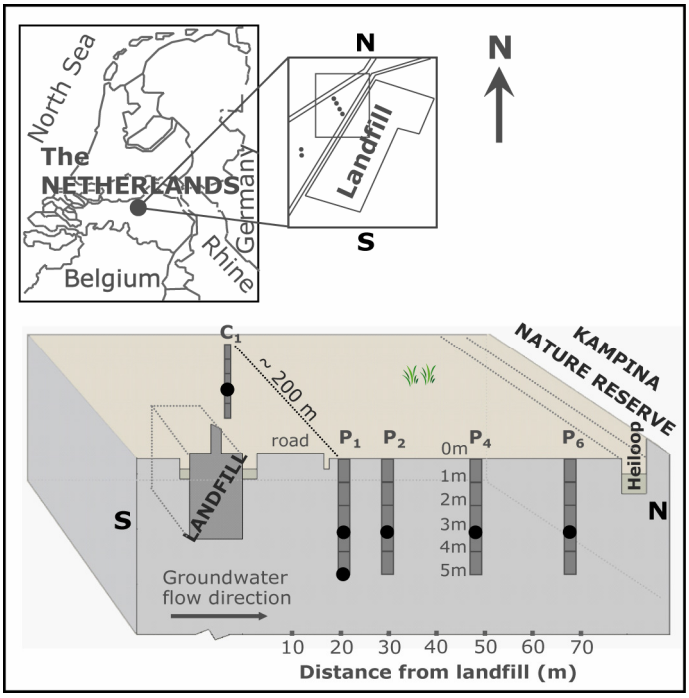

FIG. 1. Map of the Banisveld landfill research area with a longitudinal section through the aquifer located downgradient of the landfill. Circles represent locations of sediment samples used for construction of clone libraries.

Culture-independent phylogenetic analysis of 18S rRNA gene fragments

Sediment samples for phylogenetic analysis and culturing were obtained in June 2003 from the locations depicted in Fig. 1, and were also used in the study described in Chapter 2. A selection of the DNA extracts obtained in that study was also used in the current paper. Four sediment samples (P_1-3m, P_2-3m, P_4-3m and P_6-3m) obtained from the plume of pollution at a depth of 3 m, with increasing distances from the landfill (21, 30, 48 and 68 m, respectively), were selected for detailed phylogenetic analysis. For the location closest to the landfill (P_1) also a sample taken at 5 m depth was selected, because the DGGE analysis revealed a single dominant band that also occurred in samples taken further downgradient at the same depth. DNA extracted from a

sediment sample taken from an anaerobic unpolluted location approximately 200 m west of the polluted part of the aquifer (Fig. 1) at 3 m depth was used to construct a clone library corresponding to a clean reference location.

The Shannon-Weaver index of biodiversity (H') was calculated for each clone library as $- \Sigma p_i \ln p_i$. p_i is the number of clones belonging to ARDRA (sequence) type i divided by the total number of clones in a library.

Representatives of ARDRA types that occurred more than once, and all ARDRA types occurring only once in a library, were subjected to DGGE, and their profiles were compared to the environmental DGGE profiles of the samples from which the clones had been derived. PCR with eukaryotic primers Euk1A and Euk516r-GC, and subsequent DGGE, were performed as described in the accompanying paper (Chapter 2).

Both strands of the 18S rRNA gene (*Saccharomyces cerevisiae* 18S rRNA gene positions 4 to 1438) were sequenced. The obtained sequences were compared to sequences deposited in the GenBank DNA database by using the BLAST algorithm to obtain the most closely related sequences (Altschul et al., 1990). Chimera checks of the 18S rRNA gene sequences were performed via the Chimera-Check-program from RDP (Maidak et al., 1999), and chimerical sequences were removed from further phylogenetic analysis. Sequence alignment was performed by ClustalW. Distance analysis on unambiguously aligned sequences using the correction of Jukes and Cantor (1969) and bootstrap resampling (100 times) were done with the TREECON package (Van de Peer and Wachter, 1994), and the distance matrix was used to construct the tree via the neighbor-joining method (Saitou and Nei, 1987).

Most Probable Number (MPN) - PCR quantification and profiling of eukaryotes

Serial tenfold dilution of the DNA extracts were made in sterile water in triplicate and used as templates in a PCR with the eukaryotic universal primers Euk1A and Euk516r (Díez et al., 2001). Dilutions that showed products were scored as positive, and used to calculate the MPN. Positive PCR products were reamplified with the same primers, except that the Euk516r primer contained a GC clamp (Euk1A and Euk516r-GC). Products were run onto DGGE gels to determine whether the dominant bands in the diluted DNA templates corresponded to the dominant bands in the undiluted DNA templates.

Enrichment and culture-based quantification of protozoa

Protozoa were enriched from eight sediment samples (P_1-0m to P_1-5m, P_4-3m and C_1-3m). For each sample, 10 g of fresh sediment was transferred to a 100-ml glass bottle containing 50 ml of 0.03% Tryptone Soy Broth (TSB) medium. In addition, protists were aerobically and anaerobically enumerated by a microtiter plate-based twenty four-wells Most Probable Number (MPN) method as described by Darbyshire et al. (1974) and modified by Rønn et al. (1995), for three of the sediment samples selected for cloning (P_1-3m, P_4-3m and C_1-3m). The medium used was 0.03% TSB. A tenfold serial dilution was made up to a dilution of 10^{-6} in fourfold. Cultivation was initiated within six hours of sampling. Bottles and microtiter plates were incubated in the dark at 20°C and were screened for the presence of protists with an inverted microscope at 600× magnification after 1, 3 and 7 days of incubation. For anaerobic incubations, anoxic conditions were ensured by incubating under an anaerobic atmosphere consisting of 5% CO_2 and 95% nitrogen gas, while aliquot samples for microscopic inspection were removed in an anaerobic glovebox. Enrichments in which protists were observed were maintained by successive transfers of 10 ml from the respective culture to 50 ml fresh TSB medium in sterile 100-ml bottles.

While during sediment coring for most samples no attempt was made to ensure anaerobic conditions during drilling and post-drilling processing (Chapter 2), three sediment samples (P_1-3m, P_4-3m and C_1-3m) were taken anaerobically for subsequent anaerobic culturing. Immediately after coring, the sediment cores were capped in the field and transferred to cylindrical containers made of steel, which were tightly sealed and flushed with nitrogen gas. In the laboratory, these samples were transferred to an anaerobic glovebox, the ends of each core (3-5 cm) were removed and the remaining sediment was homogenized by manual mixing.

Molecular screening of enrichments

DNA isolation on concentrated 2 ml aliquots of the enrichments (5 min centrifugation, 14.000 rpm) was performed with the FastDNA SPIN Kit (BIO 101 Systems, Irvine, California) according to instructions of the manufacturer. PCR was conducted with the eukaryotic primers Euk1A and Euk516rGC (Díez et al., 2001), the PCR products were loaded onto DGGE gels and the obtained profiles for enrichments were related to the environmental DGGE profiles. In order to determine whether the enriched protists were related to the dominant sequences in the sediment sample P_1-5m, 560 bp-long PCR products were sequenced.

Nucleotide sequence accession number

Nucleotide sequences have been deposited in the GenBank DNA database under accession numbers EU091827 to EU091878.

Results

Representation of eukaryotic diversity in 18S rRNA gene clone libraries

Four clone libraries (P_1, P_2, P_4 and P_6) were constructed from anaerobic leachate-contaminated sediment samples obtained from a depth of 3 m at increasing distances downgradient from the Banisveld landfill (21, 30, 48 and 68 m, respectively). For location P_1, an additional clone library was constructed from a sample taken at 5 m depth, because DGGE analysis revealed a dominant band that also occurred in samples taken further downgradient at the same depth (Chapter 2). A reference clone library was constructed from a sample taken at 3 m depth in a part of the aquifer that was not influenced by the leachate (NH_4^+ range 2.7 - 3.7 mg/l) (Fig. 1). Coverages, based on ARDRA screening of the clones, ranged from 66% (for P_1-3m) to 98% (for P_6-3m) (Table 1).

The Shannon-Weaver index revealed the lowest eukaryotic diversity for sample P_1-5m (index value 0.62), while the largest value of the Shannon-Weaver index (2.50) was detected for the sample P_1-3m (Table 1). The values obtained in the present study are slightly smaller compared to the DGGE-based Shannon-Weaver index values obtained in the accompanying paper (Chapter 2), where all bands in DGGE profiles were considered for biodiversity calculations.

DGGE screening of each unique ARDRA type revealed that the most intense DGGE bands in the environmental profiles were in general represented by the clone libraries (Fig. 2). The banding patterns of most clones corresponded to bands visible in DGGE profiles of the environmental samples. Thus, overall the diversity in the environmental samples was well covered by the relatively small clone libraries (32 to 43 clones per library).

TABLE 1.
Characteristics of eukaryotic communities in six sediment samples obtained from the aquifer polluted by the Banisveld landfill (see Fig. 1 for locations). Numbers of clones and different ARDRA types, Coverage (C) and Shannon-Weaver index of biodiversity (H') based on sequences contribution to clone libraries were obtained from clone libraries. DGGE-based Shannon-Weaver index values were directly derived from DNA extracts (as in Chapter 2). MPN-estimates were obtained by culturing (eukaryotes/g sediment) and PCR-based culture-independent MPN (18S rRNA gene copies/g sediment).

Sample	Distance from landfill (m)	No. clones analyzed	No. ARDRA types	C (%)	H' Cloning	H' DGGE	Aerobic MPN	Anaerobic MPN	MPN PCR
P_1-3m	21	33	17	66	2.50	2.57	450	558	1860
P_1-5m	21	36	4	97	0.62	0.87	ND*	ND	760
P_2-3m	30	43	13	81	1.81	2.40	ND	ND	3000
P_4-3m	48	37	9	95	1.92	2.16	1260	558	86
P_6-3m	68	42	4	98	0.77	2.08	ND	ND	1860
C_1-3m	Non-polluted	32	4	97	1.12	2.48	138	138	860

* ND = not determined

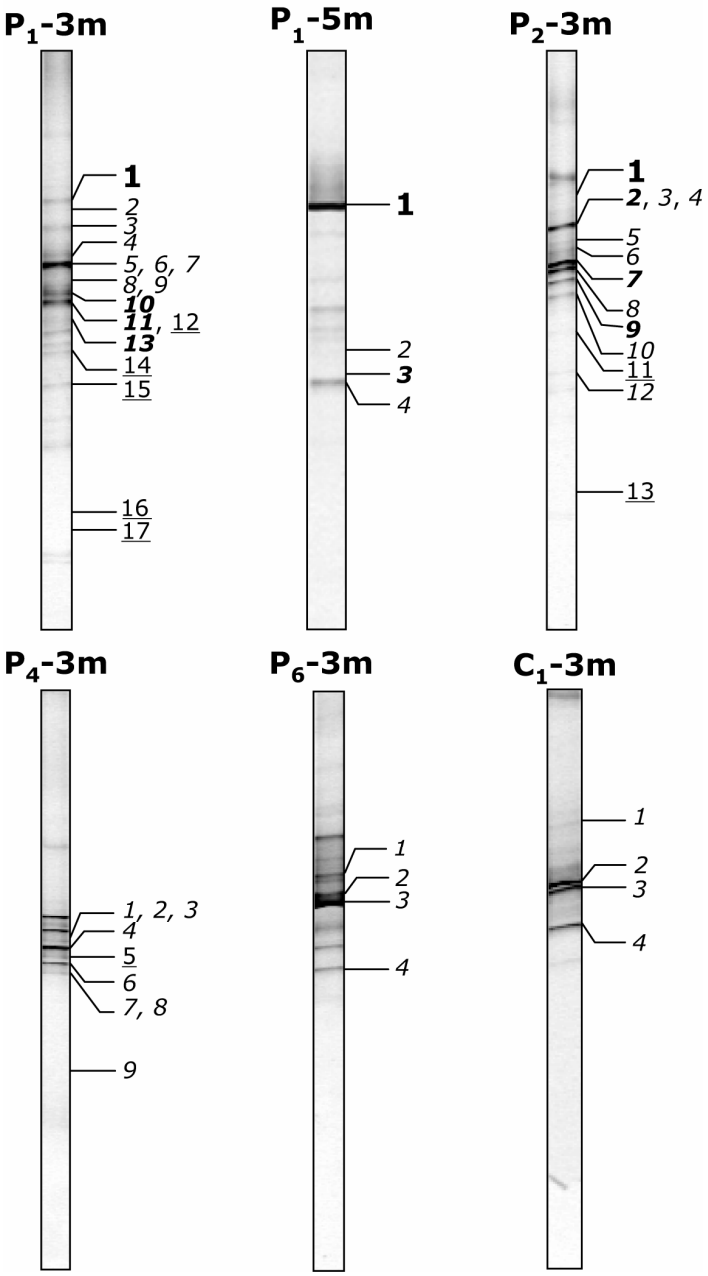

FIG. 2. Linking of eukaryotic clone identities to environmental DGGE profiles (20-35% denaturant gradient). The identities of the numbered bands are given in Table 2. Bold-faced numbers refer to *Cercozoa*-like clones, bold italic-faced numbers to *Chlorophyta*-like *clones*, italic numbers to *Fungi*-like clones and underlined numbers represent other eukaryotes.

Phylogenetic diversity in microeukaryotes

18S rRNA gene fragments corresponding to *Saccharomyces cerevisiae* 18S rRNA 4 to 1438 positions were sequenced for representatives of fifty-one clone types, revealing unique ARDRA and DGGE profiles (Table 2). Fungi were the most abundant group encountered (Fig. 3) and dominated the clone libraries generated for samples from 3-m depths. At locations closer to the landfill (P_1-3m, P_1-5m and P_2-3m) *Fungi* were less represented (Fig. 3; 8-81% of total clones) compared to locations at larger distances from the landfill and the clean reference sample (Fig. 3; 95-100% contribution to clone libraries). Sequences most closely related to protozoa (Table 2: the cercozoan flagellate *Heteromita globosa*) were only identified in polluted sediments obtained close to the landfill (P_1 and P_2), and dominated (83% contribution) the clone library generated for the sample obtained from 5 m depth at 21 m distance from the landfill (Fig. 3). Likewise, sequences most closely related to *Chlorophyta* (green algae) were also only identified close to the landfill (P_1 and P_2) and constituted 8 to 12% of the clone libraries (Fig. 3).

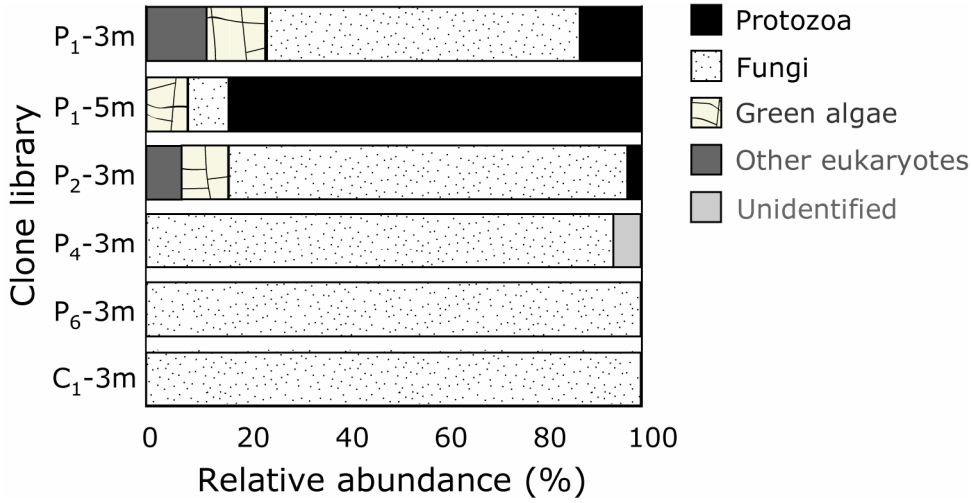

FIG. 3. Relative abundance of various phylogenetic groups of eukaryotes in clone libraries generated from aquifer sediment samples.

TABLE 2.

Phylogenetic association of eukaryotic 18S rRNA gene clones corresponding to unique ARDRA types in clone libraries from the aquifer polluted by the Banisveld landfill. Identity of clones as determined by nearly complete 18S rRNA gene sequencing; each sequenced clone type has a number corresponding to its designation in fig. 2 and fig. 4. For each clone type, the frequency at which occurs in a certain clone library and its closest relative in GenBank database, with accession number, percentage identity and taxonomic affiliation are given.

ARDRA type	Frequency of clones (%)	Closest relative in GenBank DNA database	Accession no.	Identity (%)	Phylogenetic affiliation
P1-3m 1	12.1	*Heteromita globosa*	AY965866	97	Cercozoa, Cercomonadida
P1-3m 2	3.0	*Candida parapsilosis*	AY055857	99	Ascomycota, Saccharomycetales
P1-3m 3	6.1	Rhizosphere zygomycete	AJ506030	96	Zygomycota
P1-3m 4	27.3	*Rhizophlyctis rosea*	AY635829	96	Chytridiomycota, Spizellomycetales
P1-3m 5	3.0	*Cryptococcus terreus*	AB032649	99	Basidiomycota, Filobasidiales
P1-3m 6	3.0	*Rhodotorula aurantiaca*	AB030354	99	Basidiomycota, Sporidiobolales
P1-3m 7	6.1	*Spizellomycete* sp.	DQ536477	96	Chytridiomycota, Spizellomycetales
P1-3m 8	3.0	*Pseudozyma flocculosa*	DQ092923	99	Basidiomycota, Ustilaginales
P1-3m 9	9.1	Uncultured fungus clone	AF372716	97	Fungi
P1-3m 10	3.0	*Chlorella ellipsoidea*	X63520	99	Chlorophyta, Chlorellales
P1-3m 11	3.0	*Pseudochlorella* sp.	AB006049	99	Chlorophyta, Chlorellales
P1-3m 12	3.0	Uncultured fungus clone	DQ244016	96	Fungi
P1-3m 13	6.1	*Stichococcus bacillaris*	AJ311637	99	Chlorophyta, Microthamniales
P1-3m 14	3.0	*Polytrichum brachymitrium*	AY126979	99	Streptophyta, Polytrichales
P1-3m 15	3.0	*Xenillus tegeocranus*	AF022042	98	Arthropoda, Oribatida
P1-3m 16	3.0	*Isotoma viridis*	AY596361	97	Arthropoda, Collembola
P1-3m 17	3.0	*Prismatolaimus dolichurus*	AY284727	99	Nematoda, Enoplida

TABLE 2.
Continued

ARDRA type	Frequency of clones (%)	Closest relative in GenBank DNA database	Accession no.	Identity (%)	Phylogenetic affiliation
P1-5m 1	83.3	*Heteromita globosa*	U42447	98	Cercozoa, Cercomonadida
P1-5m 2	2.8	*Lentinus tigrinus*	AY946269	99	Basidiomycota, Aphyllophorales
P1-5m 3	8.3	*Chlorella angustoellipsoidea*	AB006047	99	Chlorophyta, Chlorellales
P1-5m 4	5.6	*Sporidiobolus roseus*	X60181	99	Basidiomycota, Sporidiobolales
P2-3m 1	2.4	*Heteromita globosa*	AY965867	99	Cercozoa, Cercomonadida
P2-3m 2	2.4	*Chlorella saccharophila*	AB058306	97	Chlorophyta, Chlorellales
P2-3m 3	2.4	Uncultured fungus clone	DQ244016	98	Fungi
P2-3m 4	21.4	Rhizosphere zygomycete	AJ506030	96	Zygomycota
P2-3m 5	2.4	Uncultured fungus clone	AF372716	96	Fungi
P2-3m 6	4.8	*Cryptococcus skinneri*	AB032646	99	Basidiomycota, Filobasidiales
P2-3m 7	2.4	*Stichococcus deasonii*	DQ275460	99	Chlorophyta, Microthamniales
P2-3m 8	47.6	Uncultured fungus clone	DQ244018	88	Fungi
P2-3m 9	4.8	*Trebouxia jamesii*	Z68700	98	Chlorophyta, Microthamniales
P2-3m 10	2.4	*Rhodotorula glutinis*	AB021869	96	Basidiomycota, Sporidiobolales
P2-3m 11	4.8	*Fossombronia pusilla*	X78341	99	Streptophyta, Fossombroniales
P2-3m 12	2.4	Marine eukaryotic clone	AY426912	92	Eukaryota
P2-3m 13	2.4	*Gea heptagon*	AF062952	98	Arthropoda, Araneae

TABLE 2.
Continued

ARDRA type	Frequency of clones (%)	Closest relative in GenBank DNA database	Accession no.	Identity (%)	Phylogenetic affiliation
P4-3m 1	13.5	*Filobasidium elegans*	AB075545	99	Basidiomycota, Filobasidiales
P4-3m 2	13.5	*Holtermannia corniformis*	AF053718	99	Basidiomycota, Tremellales
P4-3m 3	16.2	*Cystofilobasidium bisporidii*	AB072225	99	Basidiomycota, Cystofilobasidiales
P4-3m 4	2.7	*Bullera taiwanensis*	AB072234	98	Basidiomycota, Filobasidiales
P4-3m 5	5.4	Uncultured eukaryote	AY689723	95	Eukaryota
P4-3m 6	32.4	*Rhodotorula mucilaginosa*	X84326	99	Basidiomycota, Sporidiobolales
P4-3m 7	5.4	*Rhodosporidium babjevae*	AB073270	99	Basidiomycota, Sporidiobolales
P4-3m 8	8.1	*Leucosporidium scottii*	X53499	99	Basidiomycota, Leucosporidiales
P4-3m 9	2.7	*Peniophora nuda*	AF026586	99	Basidiomycota, Aphyllophorales
P6-3m 1	76.2	*Rhodotorula mucilaginosa*	AB042787	99	Basidiomycota, Sporidiobolales
P6-3m 2	11.2	*Rhodotorula hordea*	AY657013	99	Basidiomycota, Sporidiobolales
P6-3m 3	2.9	Uncultured fungus clone	AY821991	98	Fungi
P6-3m 4	9.5	*Rhodotorula mucilaginosa*	X84326	99	Basidiomycota, Sporidiobolales
C1-3m 1	3.6	*Holtermannia corniformis*	AF053718	99	Basidiomycota, Tremellales
C1-3m 2	46.4	*Rhodotorula mucilaginosa*	AB042787	99	Basidiomycota, Sporidiobolales
C1-3m 3	14.3	*Cladosporium cladosporioides*	AJ515165	99	Ascomycota, Mycosphaerellales
C1-3m 4	35.7	*Leucosporidium scottii*	X53499	99	Basidiomycota, Leucosporidiales

The most abundant group among the *Fungi* was that of the *Basidiomycota* (50% of all analyzed clones). Solely basidiomycote sequences were retrieved from samples located further downgradient (48 - 68 m) from the landfill (clone libraries P_4-3m and P_6-3m; Table 2). Sequences closest related to *Rhodotorula mucilaginosa* (*Sporidiobolales*) were the most abundant basidiomycotes. *Rhodotorula mucilaginosa*-like sequences also dominated the clone library generated for the reference sample (C_1-3m). Other fungal sequences detected at this location (C_1-3m) were most closely related to *Tremellales* and *Leucosporidiales* of the basidiomycotes, and *Mycosphaerellales* of the ascomycotes (Table 2, Fig. 4). Closer to the landfill (P_1-3m, P_1-5m and P_2-3m), *Rhodotorula*-like sequences were less frequently encountered and a larger fungal diversity was observed. Basidiomycote fungal sequences were most closely related to *Filobasidiales*, *Ustilaginales*, *Aphyllophorales* and *Sporidiobolales*. Besides basidiomycotes also members of the *Ascomycota* (*Saccharomycetales* and *Mycosphaerellales*) and *Chytridiomycota* (*Spizellomycetales*) were present.

A number of clones (P_1-3m 3, 9 and 12; P_2-3m 3, 4, 5, 8 and 12, P_4-3m 5, and P_6-3m 3; Table 2, Fig. 4;) fell in a cluster for which no cultured representatives are yet known. These sequences related to uncultured fungi (Table 2) that were previously encountered in lakes (Lefranc et al., 2005; Slapeta and López-García, 2005; Van Hannen et al., 1999) and anoxic sediments (Dawson and Pace, 2002).

Green algae (*Chlorophyta*) were represented in clone libraries by sequences most closely related to members of the *Chlorellales* and *Microthamniales*. All clones that most closely related to the cercozoan flagellate *Heteromita globosa*, the only protozoan encountered in the six clone libraries, showed identical migration in DGGE (Fig. 2, bands coded 1 in tracks P_1-3m, P_1-5m and P_2-3m). A similar banding position was also recognized in several DGGE profiles used to describe the eukaryotic community structure in the accompanying article (Chapter 2). This band position occurred significantly more in DGGE profiles derived from polluted sediment than from clean sediment samples (Table 3; Fisher exact test $P = 0.026$).

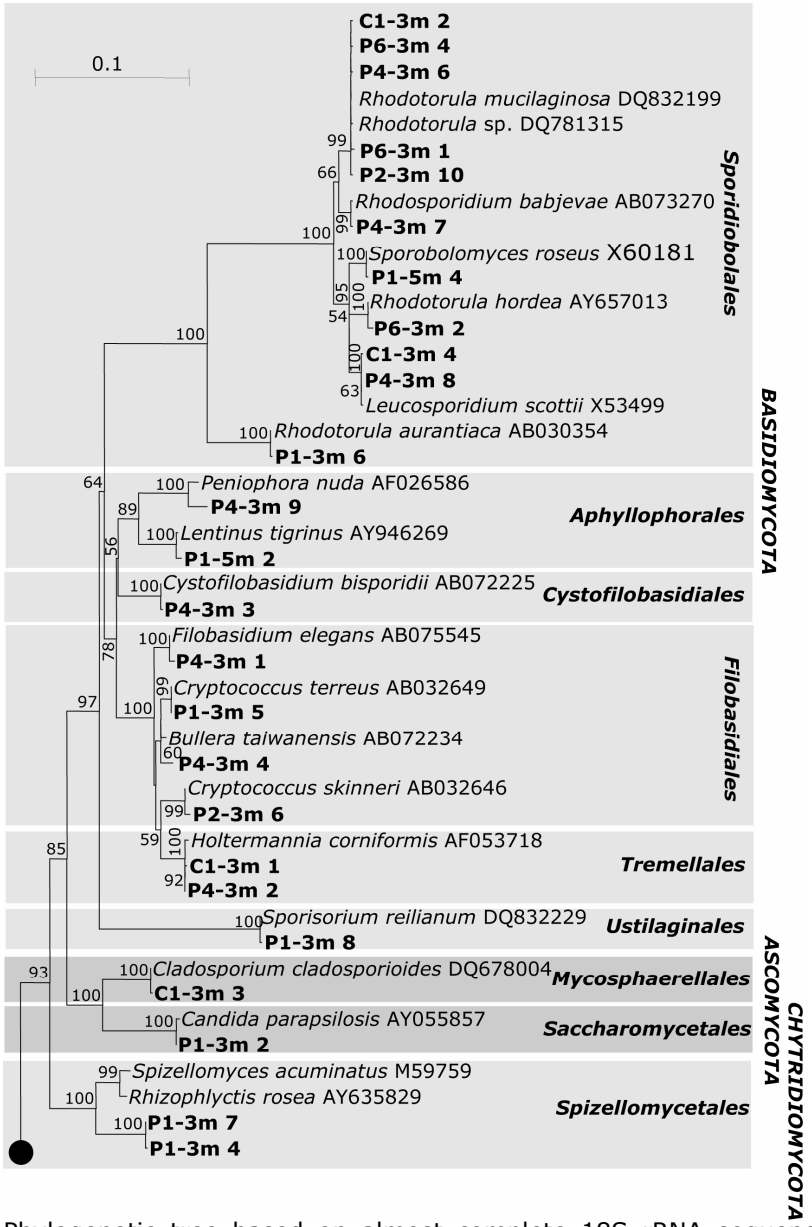

FIG. 4. Phylogenetic tree based on almost complete 18S rRNA sequences for clones (codes in bold-faced characters) in six libraries derived from the aquifer downgradient of the Banisveld landfill (The Netherlands). Each clone is named after the library designation (e.g. P_4-3m), indicating the location and depth where the clone was retrieved from (Fig. 1), followed by a number corresponding to the numbers in Fig. 2 and Table 2. Only bootstrap values above 50% are shown.

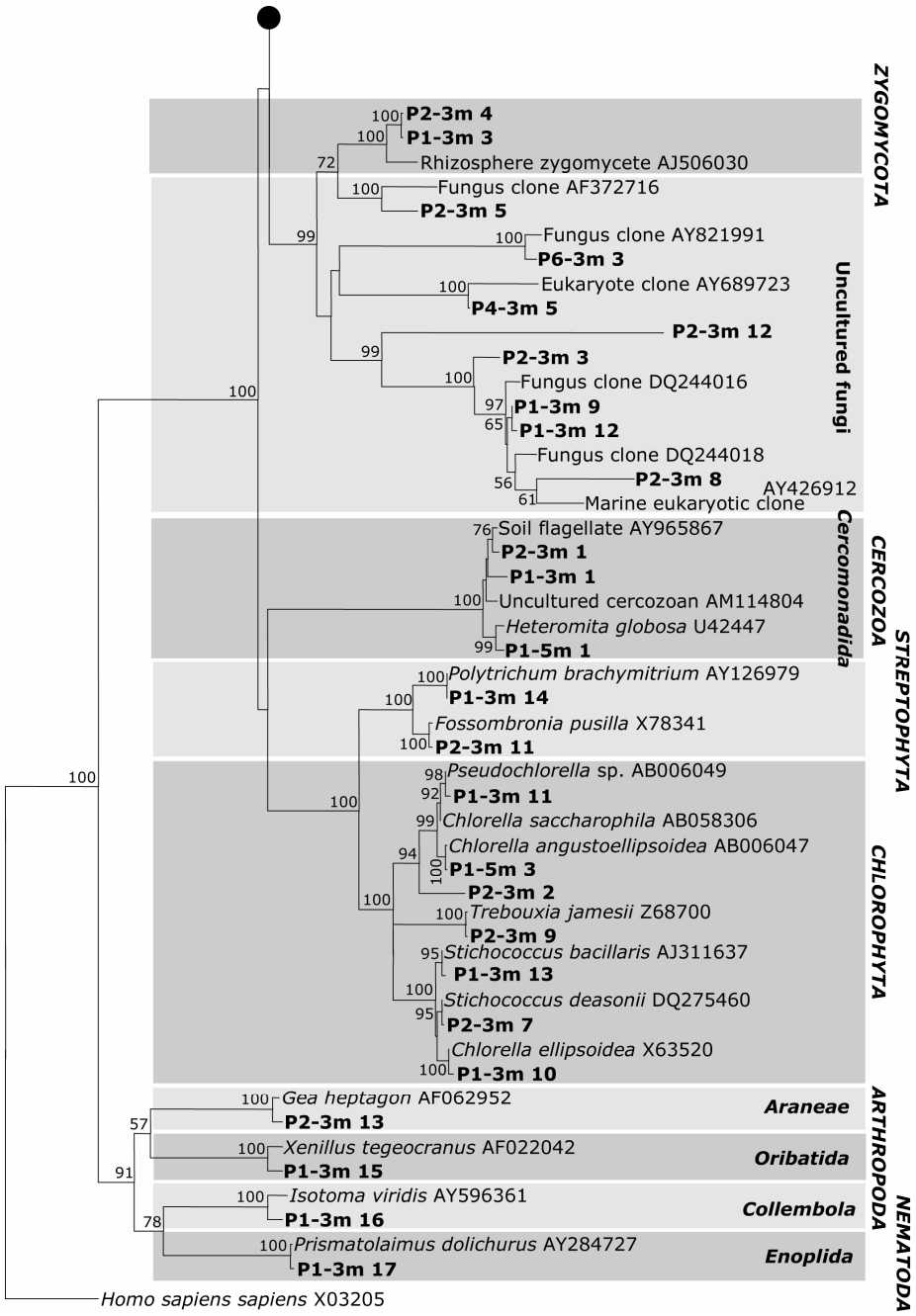

FIG. 4. Continued

TABLE 3.
Occurrence of a DGGE band, with similar banding position as clones closest related to *Heteromita globosa,* in community fingerprints of 48 sediment samples from the aquifer polluted by the Banisveld landfill (Fig. 1). Samples revealing a *H. globosa*-like banding position are marked "1", whereas absence is marked by "0". Grey-highlighted cells represent samples that were polluted with landfill leachate.

	Location							
Depth	**P_1**	**P_1'**	**P_2**	**P_4**	**P_4'**	**P_6**	**C_1**	**C_2**
0m	0	0	0	**1**	0	**1**	ND	0
1m	**1**	0	**1**	**1**	0	0	ND	0
2m	ND*	0	**1**	**1**	**1**	**1**	0	**1**
3m	**1**	0	**1**	0	0	0	0	0
4m	**1**	**1**	**1**	**1**	**1**	**1**	0	0
5m	**1**	**1**	0	**1**	**1**	0	0	**1**

* ND = not determined.

Quantification of eukaryotes by MPN-PCR and culture-dependent MPN

A culture-independent PCR-MPN approach revealed that the number of eukaryotic 18S rRNA genes ranged from 86 (for P_4-3m) to 3000 copies per gram sediment (for P_2-3m). These numbers were in general higher compared to numbers obtained by culturing (Table 1). DGGE screening revealed that the bands observed in DGGE profiles derived from the diluted DNA templates corresponded to the most intense bands observed for the undiluted DNA templates (data not shown). For the location nearest to the landfill (P_1), at both 3 and 5 m depth, a DGGE band position corresponding to that of cloned *H. globosa* sequences was recognized for all dilutions that showed positive amplification with eukaryotic primers, suggesting dominance of the nanoflagellate *H. globosa* at these locations.

The number of culturable eukaryotes ranged from 138 to 1260 cells/g sediment. With the exception of P_4-3m (48 m from landfill), where the number of eukaryotes obtained under aerobic conditions was twice as high as that obtained under anaerobic conditions, aerobic cultivation

revealed comparable numbers of eukaryotes with the anaerobic cultivation (Table 1). No obvious relation between numbers of eukaryotes and distance from the landfill or degree of pollution (clean vs. polluted) was observed.

Culturable protists

Protists were observed by microscopy in aerobic and anaerobic enrichments derived from eight sediment samples (P_1-0m to P_1-5m, P_4-3m, and C_1-3m). DGGE screening revealed that the dominant bands in profiles of enrichment cultures were related to the dominant bands in the environmental profiles. For samples P_1-3m, P_4-3m and C_1-3m, screening by DGGE revealed that the enrichments obtained from these sediments were more complex compared to those obtained from the sample P_1-5m. DGGE profiling revealed for P_1-5m the presence of a single dominant band. The migration behavior of this band corresponded to that of *H. globosa* sequence present in the clone library obtained from the same location. Sequencing revealed a 100% similarity of the cultured flagellate to the cloned *H. globosa*-like sequences. This enrichment was sixteen times successively transferred over a period of 14 months, with *H. globosa* being the only eukaryote observed under the microscope as well as in DGGE screening and sequencing. For the location P_1 (21 m distance from landfill), where aerobic enrichments of protists were performed in a vertical transect from the surface to 5 m deep, ciliates were detected in sediments from the surface and 1 m deep, while only flagellates were detected from 1 m to 5 m deep sediments (data not shown).

Groundwater fauna

Multicellular groundwater inhabitants (stygobiontic organisms) were not detected in groundwater samples taken from the polluted aquifer located downgradient of the Banisveld landfill, or in samples taken from clean references locations in its surrounding. Only non-indigenous (stygoxenic) soil-like invertebrates, such as mites, collembolans, oligochaetes, tardigrades, nematodes and stygoxenic crustaceans were observed in very low numbers (ranging from 0.03 to 2 individuals per 100 l groundwater). Among the 218 cloned 18S rRNA genes a few sequences were closest related to multicellular eukaryotes, but only to non-indigenous mosses (*Bryophyta*) and soil invertebrate metazoans (*Arachnida*, *Acarina*, *Collembola*, and *Nematoda* ; Table 2, Fig. 4).

Discussion

Food web structure in the aquifer polluted by the Banisveld landfill

Food chains in the anaerobic aquifer downgradient of the Banisveld landfill are short, consisting of nanoflagellates as top predators and prokaryotes. True groundwater invertebrates (stygofauna) were not detected in approximately 150 m^3 of groundwater pumped and filtered from the monitoring wells. The absence of groundwater fauna is probably caused by the fine sediments (Van Breukelen et al., 2003) leaving insufficient living space for groundwater invertebrates and by a lack of oxygen (Van Breukelen et al., 2003).

Both culture-dependent and culture-independent analyses revealed the presence of a cercozoan flagellate closest related to *Heteromita globosa* in landfill leachate-affected sediment. Members of the genus *Heteromita* are common and abundant heterotrophic soil flagellates (Fredslund et al., 2001). Our study is the first to show that *H. globosa* can be a dominant member of eukaryotic communities in anaerobic aquifers. The recovery of *H. globosa* in aerobic and anaerobic enrichments showed that viable cells are present and that its culture-independent detection is not due to amplification of extracellular DNA. *Heteromita* sp. had previously been encountered in landfills (Finlay and Fenchel, 1991), but its ability to grow anaerobically (Mattison et al., 2002; Mattison and Harayama, 2005) suggests that *Heteromita* sp. might be active in the aquifer itself and that its presence is not simply due to transport of cysts out of the landfill by percolating rainwater. *H. globosa* was relatively abundant in the proximity of the landfill. Here, the rate of dissolved organic carbon (DOC) degradation is highest (Van Breukelen et al., 2003), suggesting that the relatively high bacterial production is capable of supporting an additional trophic level consisting of bacteria-predating *H. globosa*.

The presence of *Heteromita* sp. is of significance for anaerobic biodegradation: laboratory studies have indicated that *H. globosa* influences toluene and alkylbenzene biodegradation (Mattison and

Harayama, 2001; Mattison et al., 2002), by enhancing the activity per bacterium. These studies were done under aerobic conditions, but possibly a similar stimulation will occur under anaerobic conditions. A *Heteromita* sp. was able to reduce bacterial clogging under denitrifying conditions (Mattison and Harayama, 2005), which will contribute to maintaining hydraulic conductivity of aquifers and sustaining biodegradation (Sinclair et al., 1993).

Also green algae (*Chlorophyta*) were encountered close to the landfill. Green algae have been previously encountered in aquifers (Beloin et al., 1988; Sargent and Fliermans, 1989; Sinclair and Ghiorse, 1989), but their functioning in the subsurface seems not to have been addressed. Algae are most likely introduced in aquifers from the surface by percolating rainwater or from surface water recharge (e.g. lakes or streams) (Sinclair and Ghiorse, 1989). Mixotrophic algae can survive by feeding on bacteria in the absence of light (Jones, 2000). However, the ability for mixotrophic growth has yet not been described for the algae species that we encountered. Sample contamination with algae during drilling seems unlikely as the same drilling water-free technology and sampling procedures were used at the other locations, and the clone libraries based on these samples did not reveal the presence of green algae.

The number of eukaryotes estimated in the present study was intermediate (i.e. 10^2-10^3 eukaryotes) to that observed at other contaminated anaerobic aquifers: 10^0-10^1 eukaryotes per g dry weight sediment for hydrocarbon-contaminated aquifers in South Dakota, US, and Germany (Thomas et al., 1997; Zarda et al., 1998), while 10^4-10^5 protists per g dry weight sediment were identified in an aquifer polluted by wastewater in Massachusetts, US (Kinner et al., 2002).

Fungal communities

A group of little studied eukaryotic organisms in (contaminated) aquifers is that of the *Fungi*. Unlike the few studies in which fungi in groundwater were just enumerated (Madsen et al., 1991; Madsen and Ghiorse, 1993; Sinclair and Ghiorse, 1989), or where fungal PLFAs were detected (Ludvigsen et al., 1999), in the present study we identified various types of fungi in sediments at different depths and distances from the Banisveld landfill. To our knowledge, is the paper by Luo and coworkers (2005) the only other study on an anaerobic aquifer, in this case polluted with hydrocarbons, in which ascomycetous and basidiomycetous fungal sequences were identified and found to dominate a clone library. While *Pezizomycetes* ascomycotes were the dominant group in the study by Luo et al. (2005), in our study *Sporidiobolales* basidiomycotes were the most abundant.

In general, sequences most closely related to the yeasts *Rhodotorula*, *Cryptococcus* and *Leucosporidium* dominated our clone libraries. Strains belonging to these genera are able to catabolize benzene compounds, and to assimilate lignin monomeric degradation products as sole carbon sources under aerobic conditions (Middelhoven et al., 1992; Middelhoven, 1993). *Cryptococcus* species are capable of utilizing humic acids as sole carbon and nitrogen source (Filip and Bielek, 2002). The ascomycetous yeast *Candida parapsilosis,* to which a clone obtained at 21 m from the landfill was closest related, degrades phenols and hydroxybenzoates aerobically (Middelhoven et al., 1992). Members of the genera *Rhodotorula*, *Cryptococcus* and *Candida* can grow anaerobically (Ekendahl et al., 2003) by fermentation and were also encountered in another anaerobic aquifer (Luo et al., 2005). Thus, the fungal species identified in our study could be quite versatile in physiological abilities. Fungi may contribute to the decomposition of organic matter, either in the landfill (from where the fungi are transported downgradient by groundwater) or in the leachate. Lignin and humic acids, compounds that are degraded by some of the cultured relatives closest related to our sequence, are abundant in landfills and landfill leachate (Christensen et al., 1998). On the downside, *Rhodotorula*, *Cryptococcus* and *Candida* species have also been implicated in various infectious diseases (Abi-Said et al., 1997; Sobel

and Vazquez, 1999) and mycoses (Alliot et al., 2000; Anaissie et al., 1989).

Fungi have also been encountered in other anaerobic settings, such as anaerobic marine environments (Edgcomb et al., 2002; Stoeck and Epstein, 2003), anaerobic marine intertidal (Dawson and Pace, 2002) and marsh sediments (Stoeck et al., 2003) where they formed just a small fraction of the total eukaryotic community. However, fungal sequences dominated clone libraries generated from anaerobic lake freshwater and brackish anoxic sediments (Dawson and Pace, 2002), as well as from anaerobic sediments in a freshwater pond and an anaerobic sewage digester (Luo et al., 2005).

In conclusion, the food web in the polluted Banisveld aquifer is simple, consisting of bacteria and fungi as potential decomposers of organic matter, and flagellate protozoans as grazers. Further information on the activity of eukaryotes in polluted aquifers, with respect to their direct and indirect contribution to contaminant biodegradation, will be of importance in assessing, monitoring and predicting natural attenuation.

Acknowledgements

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Chapter 4

Spatiotemporal dynamics in microbial community structure and hydrochemistry in a landfill leachate-contaminated aquifer*

Abstract

We examined the spatiotemporal dynamics of microbial communities in an aquifer polluted by landfill leachate (Banisveld, The Netherlands). The aim of the study was to identify whether the variation in microbial communities in space and time related to the overall spatial and temporal hydrochemical changes. The core plume of pollution was hydrochemically rather stable in time, but its upper fringe appeared to have moved up to the surface, especially at distances greater than 48 m from the landfill. Complex and spatiotemporal heterogeneous bacterial and eukaryotic communities were resolved using denaturing gradient gel electrophoresis (DGGE) of 16S and 18S rRNA gene fragments. Over the period 1998 to 2004, large fluctuations were noted in the eukaryotic communities associated with polluted and clean groundwater. The bacterial profiles of polluted samples were more similar to each other than to those in clean groundwater in 1998 and in 1999, but no longer in 2004. Unlike the eukaryotic profiles, the 1998 bacteria profiles in polluted groundwater samples were more related to each other than to those recovered from polluted samples obtained in 1999 and 2004. The temporal variation in microbial communities was greater than the spatial variation at all sampled locations in the aquifer. The pollution with landfill leachate seemed to have a smaller contribution to the distribution of microbial communities in the aquifer, in comparison with temporal fluctuations and heterogeneity in their community structure and environmental settings.

Keywords

spatiotemporal dynamics, landfill-leachate plume, hydrochemistry, bacteria community, eukaryotic community, Banisveld

*In preparation as Brad, T., C. Obergfell, B. M. van Breukelen, N. M. van Straalen, and W. F. M. Röling

Introduction

Old landfills represent a potentially substantial and detrimental source of groundwater contamination. Contaminants in landfills may migrate with the percolating rainwater into the underlying aquifers with severe consequences for the quality of groundwater. Landfill-leachate plumes are not physically and chemically static (Christensen et al., 2000; Christensen et al., 2001; Kjeldsen et al., 2002): many organic pollutants become biodegraded, and the electron acceptors are sequentially depleted, resulting in the development of spatial and temporal redox zones Christensen et al., 1994; Lovley, 1997).

Previous studies based on single time-point sampling from the aquifer polluted by the Banisveld landfill (The Netherlands) showed that functional diversity of microbial communities was higher in the contaminant plume than outside the plume (Röling et al., 2000a). Bacterial and archaeal community structures were found to vary according to the degree of pollution and the dominant redox process (Röling et al., 2001; Mouser et al., 2005). The bacteria communities outside the pollution plume showed more variation compared to the communities within the plume. Although microbial communities in the plume were clearly different from those outside the plume, even in the plume considerable differences in community composition were observed (Röling et al., 2001; Lin et al., 2005). A specific *Geobacteraceae* phylotype was dominantly present in the part of the polluted aquifer closest to the landfill, where the organic biodegradation occurred at a relatively high rate (Lin et al., 2005). Also a complex eukaryotic community consisting of fungi, algae and heterotrophic nanoflagellates was observed (Chapter 3).

The aim of the present study was to relate microbial community dynamics over time and space to the changing hydrochemistry of the Banisveld landfill leachate-contaminated aquifer. Long-term monitoring of the activity and dynamics of microbial communities in polluted aquifers, and information on how the environmental settings change in time, will assist in the development of management strategies based on natural attenuation of aquifer pollution.

Material and methods

Site description and groundwater sampling

The Banisveld landfill (Boxtel, The Netherlands) operated between 1965 and 1977 and contains approximately 400.000 m^3 of household refuse, industrial and possibly illegal and hazardous wastes, which are present below the groundwater table. The landfill has a surface area of 6 ha. Neither artificial nor natural liners sealed the landfill, and therefore a considerable plume of pollution formed which migrates towards a nearby nature reserve with a velocity of 4-10 m/year (Van Breukelen et al., 2003). Detailed site characteristics are found in Van Breukelen and coworkers (2003).

Groundwater monitoring wells were installed in June 1998 (Van Breukelen et al., 2003). One well was placed approximately 200 m upgradient of the landfill, as a reference location (coded -200), one well (coded 0) was installed in the landfill, eight wells (6, 21, 30, 39, 48, 58, 68 and 78 m downstream) were installed in a series along the main direction of the groundwater flow downgradient of the landfill, and another well (89 m downstream) was placed in front of the plume of pollution (Fig. 1). Each well comprised two or three PVC piezometers of 52 mm inner diameter with a 20-cm long screen. In general, one screen was located above the leachate plume (Fig. 1, positions *a*), one screen inside the leachate plume (positions *b*) and one screen below the leachate plume (positions *c*).

Groundwater samples for microbiological and hydrochemical analyses were taken in September 1998, October 1999, and October 2004 after three well-volumes of stagnating groundwater in each piezometer was removed. Groundwater samples were collected in sterile glass bottles using a peristaltic pump. The bottles were capped with as little headspace as possible, and transported to the laboratory within 8 h of sampling, kept at 4°C and processed within 24 h.

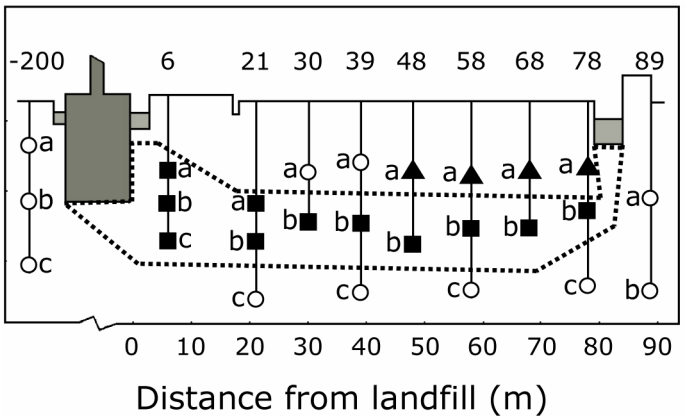

FIG. 1. Longitudinal cross-section of the research area located downgradient of the Banisveld landfill (shaded area), with representation of sampling locations. Each borehole is indicated by a number corresponding to the distance (m) from the downstream border of the landfill. Two or three screens were placed in each borehole, as indicated by *a*, *b* and *c*. ■ represent the samples that were from 1998 to 2004 polluted with landfill leachate, O designate the samples that were clean during the 6 years, ▲ are the locations that became polluted over time, and the dotted line delineates the pollution plume in 1998 (modified from Röling et al., 2001).

Samples for determination of Ca, Mg, Na, K, Fe, Mn, Si, Al, NH_4, and PO_4 were taken by connecting the sampling tube to a syringe for minimizing the contact with air. The content of the syringe was filtered through 0.45-µm filters and conserved in 50-ml polyethylene (PE) jars containing 0.4 ml concentrated HNO_3. Samples for alkalinity, Cl, Br, SO_4, and NO_3 were not filtered and kept in 50-ml PE jars. Samples for total organic carbon (TOC) were conserved in 10-ml PE tubes containing 100 µl 18% HCl.

Hydrochemical analysis

Oxygen content, pH and electrical conductivity (EC) were measured in the field using a Multi 340i/SET WTW (Weilheim, Germany), with all electrodes placed in one flow-through cell connected to the pumping device, ensuring a continuous flow of groundwater. The anions and ammonium were analyzed by spectrophotometry using a segmented flow analyzer (Skalar, Breda, The Netherlands). The cations were analyzed by inductively coupled plasma-atomic emission spectrometry (ICP-AES) on a Varian Liberty II (Varian, Palo Alto, CA). TOC was measured on a Dohrmann DC-190 TOC analyzer (Rosemount Analytical, Santa Clara, CA) after removing dissolved inorganic carbon (DIC) by adding 50 µl of 37% HCl to 1 ml of sample. Alkalinity was determined by Gran titration (Gran, 1952) in the laboratory.

PCR-DGGE profiling

Hundred ml of groundwater was vacuum filtered in the laboratory through 0.22 µm mesh-sized cellulose membrane filters (Sartorius AG, Göttingen, Germany) within 24 h of sampling. The filters were frozen and stored at -80°C until DNA extraction. The DNA was extracted from the filters obtained in 2004 by using the FastDNA SPIN Kit (Bio 101 Systems, Irvine, CA) according to instructions of the manufacturer. The bead-beat based DNA isolation using phenol-chloroform (Röling et al., 2001), which was used for samples previously taken in 1998 and 1999, was compared to the FastDNA SPIN method. Both methods yielded highly similar DGGE profiles after PCR with bacterial primers on the isolated DNA (data not shown).

Bacteria-specific PCR was performed in a 25-µl volume containing 0.4 µM forward primer F357-GC (Muyzer et al., 1993), 0.4 µM reverse primer R518 (Muyzer et al., 1993), 0.4 mM of each deoxynucleoside triphosphate (dNTP), 10 µg of bovine serum albumin (BSA), PCR buffer, 2 U of Taq polymerase, and 1 µl of undiluted DNA template. Amplification was performed in a Perkin-Elmer DNA Thermo Cycler using an initial denaturation of 94°C for 4 min, followed by 35 cycles of 94°C for 30 s, 54°C for 1 min, and 72°C for 1 min, and a final elongation at 72°C for 5 min.

For Eukarya, the PCR amplification was performed in a 25-µl volume containing 0.4 µM forward primer Euk1A (Díez et al., 2001), 0.4 µM reverse primer Euk516r-GC (Díez et al., 2001), 0.4 mM of each dNTP, 10 µg of BSA, PCR buffer, 2 U of Taq polymerase, and 1 µl of undiluted DNA template. The reaction consisted of an initial denaturation at 94°C for 130 s, followed by 40 cycles of 94°C for 30 s, 56°C for 45 s, and 72°C for 130 s, and a final elongation at 72°C for 7 min. In case insufficient PCR product was obtained, a first PCR with primers Euk1A and Uni1392r (Díez et al., 2001) was performed on the undiluted DNA. The products of approximately 1.6 kb length were diluted 50 times in sterile water and used as templates in a nested PCR with primers Euk1A and Euk516r-GC (Díez et al., 2001).

DGGE was performed with the BioRad DCode™ (Hercules, CA) system. PCR products were loaded onto 1-mm-thick 8% (w/v) polyacrylamide (ratio of acrylamide to bisacrylamide, 37.5:1) gels. A 30 to 55% linear denaturing gradient was used for *Bacteria*-specific PCR products and a 20 to 35% linear denaturing gradient for *Eukarya*-specific PCR products. The 100% denaturant was defined as 7 M urea and 40% (v/v) formamide. Electrophoresis was performed in 1×TAE buffer (40 mM Tris-acetate, 1mM Na-EDTA; pH 8.0) at 200 V and 60°C for 4 hours. The gels were stained in 1×TAE buffer containing 1 µg/ml ethidium bromide and were recorded with a charge-coupled device camera system (The Imager, Appligen, Illkirch, France). To aid in later conversion and normalization of gels, a marker was added on the outsides of the gels, as well as after every four samples. The outer two lanes of each gel were not used.

Statistical analysis

Principal component analysis (PCA) on the measured hydrogeochemical parameters was used to construct an ordination containing all sampling points. Hydrochemical dissimilarity between two samples was defined as the distance between their corresponding points in the ordination along the first principal component (PC1) axis (see also the Results section). Per sampling filter, the dissimilarities in hydrochemistry between 1998 and 1999 were compared to the dissimilarities between 1998 and 2004

using a paired *t*-test, in order to determine whether significant changes occurred in the aquifer hydrochemistry over the 6 years.

DGGE gel images were converted, normalized, and analyzed with the GelCompar II software package (Applied Maths, Kortrijk, Belgium). The similarities between tracks were calculated by using the Pearson product-moment correlation coefficient (whole densitometric curve based). These coefficients were assigned to specific groups for statistical testing (Sneath and Sokal, 1973; Van Verseveld and Röling, 2004). In general, the within-group similarity coefficients were assigned to testing variable *1* and between-group similarity coefficients to testing variable *2*. As an example, we tested whether the bacteria community profiles in polluted samples obtained in 1998 clustered significantly better to each other than to those in polluted samples in 1999. For that, we assigned to testing variable *1* those similarity coefficients comparing among 1998 samples; the similarity coefficients comparing between 1998 and 1999 samples were then assigned to testing variable *2*. As similarity data were not normally distributed, non-parametric analysis of variance (Mann-Whitney U test) was performed to test for significant differences between the two groups.

The similarities between DGGE profiles obtained from 1998 to 1999 for a particular groundwater piezometer were statistically compared (Wilcoxon matched pairs test) to those in the period 1998-2004 obtained from the same piezometer. Changes in bacteria and eukaryotic community profiles were correlated to the changes in the aquifer hydrochemistry and distance from the landfill, using Spearman correlation.

All statistical analysis was performed with Systat 7.0 (SPSS Inc., Chicago, IL).

Results

Hydrochemistry of the aquifer polluted by the Banisveld landfill

The redox conditions in the plume of pollution did not appear to have severely changed during the six years of investigation. Previous hydrochemical analysis showed that the anaerobic plume of pollution (depicted in figure 1) contained no nitrate and only low concentrations of sulfate (0.05 - 0.07 mmol/l) in 1998 and 1999 (Röling et al., 2001; Van Breukelen et al., 2003). Iron reduction (1.0 - 2.1 mmol/l Fe^{2+}) was instead the dominant redox process occurring in the plume. Although a decrease in the concentration of Fe^{2+} occurred from 1998 to 2004 (*t*-test = 4.42, P = 0.002), iron reduction in 2004 appeared still the dominant redox process in the plume. No significant change (*t*-test = 0.03; P = 0.97) was recorded in the sulfate concentration in plume samples during the 6 years, while nitrate was present in 2004 only in low concentrations (0.001 - 0.03 mmol/l). The concentrations of iron and sulfate did not change notably along the flow path in the plume (data not shown).

Iron reduction was also the main redox process occurring between 1998 and 2004 below the pollution plume (0.05 - 0.3 mmol/l Fe^{2+}). Below the plume, oxygen was absent and nitrate was detected during the six years period only in low concentrations (0 - 0.3 mmol/l). In general, the sulfate concentration was low below the plume of pollution during the whole study period (0.02 - 0.7 mmol/l).

In 1998 and 1999, above the plume of pollution the environment was anaerobic, nitrate was detectable in significant quantities (0.8 - 1.2 mmol/l NO_3^+) and denitrification appeared to be the dominant redox process (Röling et al., 2001; Van Breukelen et al., 2003). Some iron (0.005 - 0.3 mmol/l) and sulfate (0.1 - 0.6 mmol/l) were also present above the plume in 1998 and 1999. In 2004, the redox conditions in the above plume piezometers located within 48 m distance from landfill were comparable to those in 1998 and 1999. Here, in the above plume piezometers at 30 and 39 m distance from landfill, the concentration of nitrate in 2004 decreased considerably ranging from 0.002 to 0.003 mmol/l, the iron ranged from 0.03 to 0.2 mmol/l, and the sulfate from

0.3 to 0.6 mmol/l. The upper piezometers (coded *a* in fig. 1) located further than 48 m distance from landfill (wells coded 48, 58, 68 and 78 in fig. 1) were in 2004 no longer above the pollution plume, but inside the plume, as higher concentrations of the parameters indicative of pollution were registered at these locations, in comparison to those in preceding years. While for example 10.6 - 59.0 mg C/l (as TOC) was detected over the period 1998 - 1999, TOC was 73.5 - 128.2 mg C/l in 2004. Similarly, the ammonium concentration in the upper piezometers at more than 48 m distance from the landfill was 6.4 - 13.3 mmol/l in 2004, while over the period 1998 - 1999 it was only 0.02 - 1.8 mmol/l. Oxygen was always absent in the downgradient shallow sample locations during the 1998 - 2004 period, while nitrate concentrations reduced considerably from 1998 (0.3 - 1.2 mmol/l) to 2004 (0.001 - 0.006 mmol/l). A significant increase (*t*-test = -5.47, P = 0.012) was instead registered for the Fe^{2+} concentration in the upper piezometers located over 48 m distance from the landfill from 1998 (0.01 ± 0.007 mmol/l Fe) to 2004 (0.7 ± 0.1 mmol/l Fe), when iron reduction appeared in 2004 the dominant redox process at these locations.

Principal component analysis (PCA) of the measured hydrochemical parameters separated the sampling locations along the axis of the first principal component (PC1), which explained 65.6% of the total variance. PC1 correlated positively with parameters indicative of pollution with landfill leachate (correlation coefficients are given in parentheses): electrical conductivity (0.977), magnesium (0.970), alkalinity (0.962), chloride (0.882), ammonium (0.880), sodium (0.880), calcium (0.865), iron (0.821), potassium (0.805) and manganese (0.790). PC1 correlated negatively with silica (-0.637), and sulfate (-0.639). Thus, high scores (over 0.5) along the PC1 axis indicated the presence of pollution. The second principal component (PC2, explaining 9.7% of the variance) correlated only with manganese (0.537).

Plotting PC1 scores, as indicator of leachate pollution, against distance from the landfill revealed that for the intermediate (coded *b* in Fig. 1) piezometers, the PC1 scores in 2004 were nearly similar compared to those obtained for 1998 and 1999 samples (Fig. 2B). The lower (c) filters were in general characterized by low PC1 scores (Fig. 2C). The upper filters (coded *a* in Fig. 1) had PC1 scores that in 2004 were higher compared to those obtained in 1998 and 1999 at distances from 48 to 78 m downgradient of the landfill (Fig. 2A). This fact agrees with an upward movement of the landfill-leachate plume over time, as reported previously (Chapter 2; Van Breukelen and Griffioen, 2004). The hydrochemical similarities between the 1998 and 1999 samples obtained from the same piezometer located in the leachate plume were significantly larger than those between 1998 and 2004 samples (paired *t*-test = -2.584, P = 0.025). The samples from 1998 were thus hydrochemically more similar to those taken in 1999 than to samples from 2004.

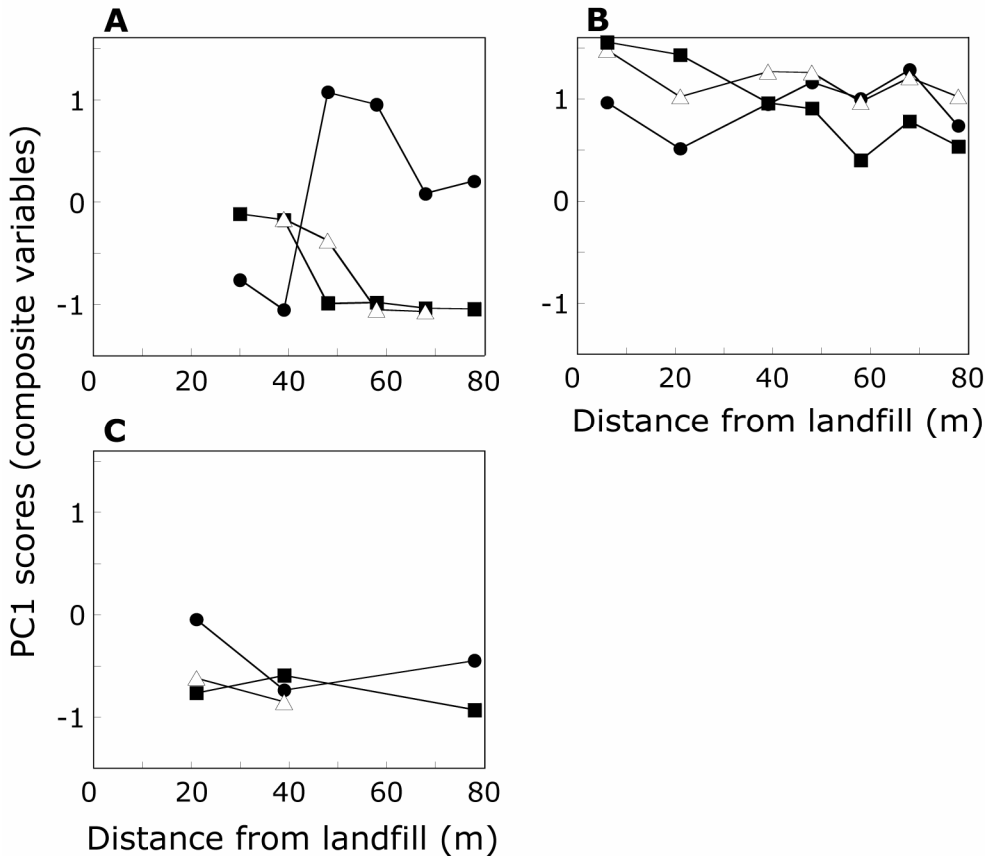

FIG. 2. Spatiotemporal dynamics in contaminant hydrochemistry in the aquifer downgradient of the Banisveld landfill. Pollution is expressed in the first principal component (PC1) value derived from principal component analysis on hydrochemical parameters (see text). PC1 values are shown for groundwater samples obtained from piezometers located in the top (A), intermediate (B) and deeper (C) part of the aquifer (designated *a*, *b* and *c*, respectively, in fig. 1). ■, 1998 samples; △, 1999 samples; and ●, 2004 samples.

Correlations between community structure and hydrochemistry from 1998 to 2004

Bacteria and eukaryotic DGGE profiles were complex and appeared to vary over time and space (data not shown). Over the whole aquifer, the eukaryotic community structure was significantly related to hydrochemistry; larger differences in hydrochemistry between two samples were accompanied by larger differences in their community fingerprints. In contrast, the bacteria community profiles were only marginally associated to hydrochemistry (Table 1). However, when plume samples were evaluated separately, hydrochemistry and bacteria community profiles were significantly related (Table 1). The similarity in bacteria profiles in polluted groundwater (Fig. 3A) also decreased with increasing distance between sampling points (Table 1). No relationship between the similarities in eukaryotic profiles and distance between sampling points was observed (Fig. 3B).

TABLE 1.
Relation between variation in microbial community and hydrochemical changes in the aquifer downgradient of the Banisveld landfill

STATISTICAL ANALYSIS		N	Spearman correlation coefficient (r)	Significance (P)
Community similarity vs. hydrochemistry (all samples)	Bacteria	528	-0.08	0.055
	Eukarya	666	-0.11	0.011
Community similarity vs. hydrochemistry (polluted samples only)	Bacteria	120	-0.32	<0.001
	Eukarya	345	-0.09	0.093
Community similarity vs. distance between sampling points (polluted samples only)	Bacteria	136	-0.30	<0.001
	Eukarya	465	-0.08	0.088
Location-specific similarities between 1998-1999 community profiles and distance from landfill (polluted samples only)	Bacteria	13	-0.72	0.006
	Eukarya	9	0.22	0.608

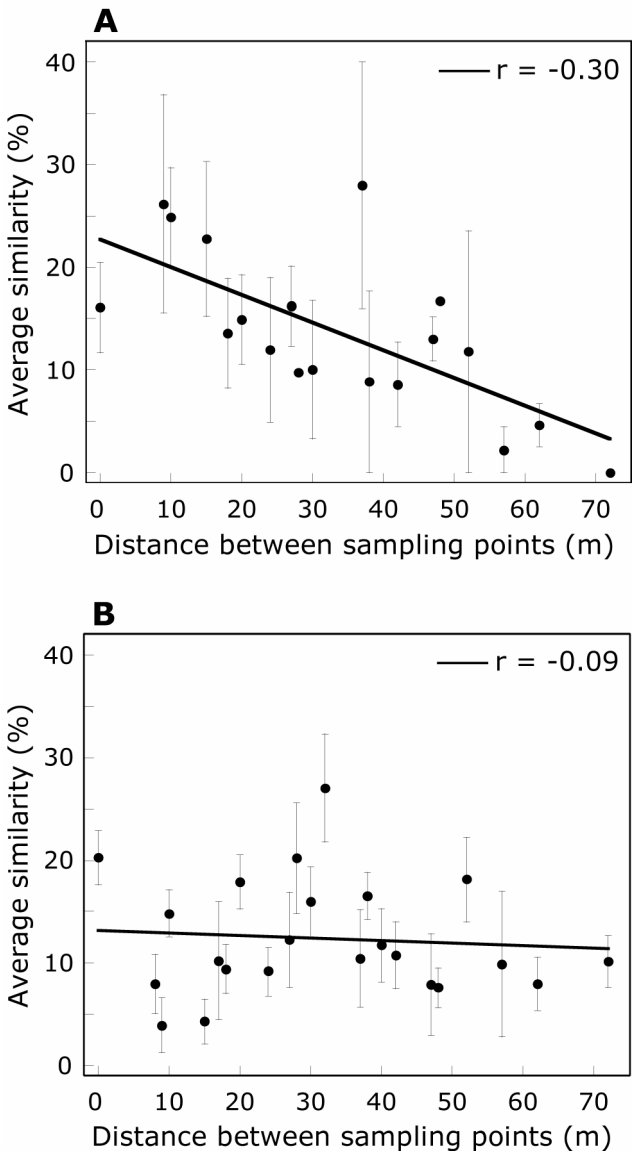

FIG. 3. Spatiotemporal variation in bacteria (A) and eukaryotic (B) community profiles against the horizontal distance between the polluted sampling locations in the aquifer at Banisveld. For each distance, average similarity with standard error was calculated taking all years together.

The bacteria communities in polluted groundwater in 1998 (Fig. 4A, column 1: similarity 52.7 ± 7.4%) were more related to each other (Mann-Whitney U test, $P < 0.05$) than to those in clean samples (average similarity coefficient between bacteria profiles in polluted and clean samples 18.3 ± 4.4%). Also in 1999 the bacteria communities associated significantly (Mann-Whitney U test, $P < 0.05$) more to each other in polluted groundwater (Fig. 4A, column 2: average similarity coefficient 52.4 ± 1.9%) than to those in clean groundwater (average similarity between bacteria profiles in polluted and clean samples 36.3 ± 1.6%). For 2004, the bacteria community profiles in polluted groundwater samples (Fig. 4A, column 3: average similarity coefficient 25.3 ± 4.8%) were no longer significantly (Mann-Whitney U test, $P > 0.05$) more related to each other than to those in clean samples (average similarity between bacteria profiles in polluted and clean samples 12.8 ± 2.08%). In contrast, in none of the years the eukaryotic community profiles in polluted samples (Fig. 4B, columns 1-3) were more related to each other than to those in clean samples (Mann-Whitney U test, $P > 0.05$). Bacteria and eukaryotic community profiles were also not significantly related to each other (Spearman correlation, $r = -0.02$, $P = 0.75$).

Temporal dynamics in community structure

The similarities among bacteria community profiles in polluted groundwater sampled in 1998 (Fig. 4A, column 1, average similarity of 52.7 ± 7.4%) and 1999 (Fig. 4A, column 2, 52.4 ± 1.9% similarity) were significantly larger (Mann-Whitney U test, $P < 0.05$) than those in 2004 (Fig. 4A, column 3, 25.3 ± 4.8% similarity). In none of the years the eukaryotic community profiles were more related to each other than to those in the other years (Fig. 4B, first 3 columns). The average similarities between the eukaryotic profiles over the period 1998 - 2004 were nearly identical and very low (10-14% similarity).

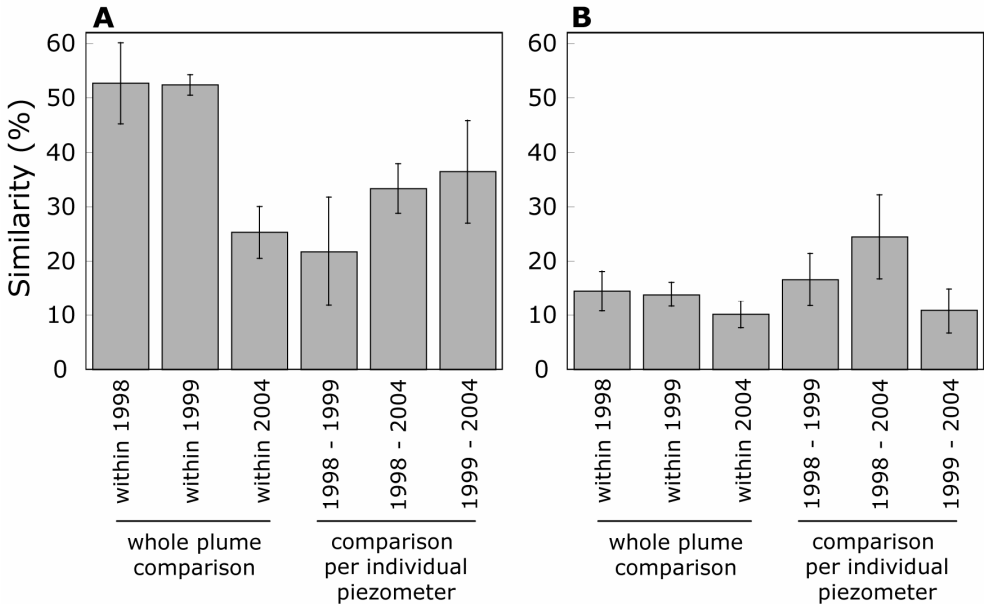

FIG. 4. Average similarities with standard errors of bacteria (A) and eukaryotic (B) communities, based on comparison of rRNA gene DGGE profiles derived from polluted groundwater samples. Average similarities are shown within each year (columns 1 to 3). Columns 4 to 6 represent the average similarities resulted after comparing the profiles obtained in a certain period of time with those in another period for the same piezometer.

The spatial variation in bacteria profiles in the plume (Fig. 4A, columns 1, 2 and 3) was significantly smaller (*t*-test, $P < 0.05$) than the temporal variation (Fig. 4A, columns 4, 5 and 6). No significant differences (*t*-test, $P > 0.05$) were detected between the spatial (Fig. 4B, columns 1, 2 and 3) and temporal variation in the eukaryotic profiles (Fig. 4B, columns 4, 5 and 6). The changes in aquifer hydrochemistry were not associated (Spearman correlation, $P > 0.05$) to variation per individual piezometer in bacteria and the eukaryotic community profiles over the periods 1998-1999, 1998-2004 and 1999-2004 (Fig. 4A and 4B, columns 4, 5 and 6, respectively). Per individual piezometer, the similarities in bacteria community profiles obtained over the period 1998-1999 (Fig. 4A: average similarity of 21.8 ± 10.0%) were not significantly different (Wilcoxon matched pairs test, $P > 0.05$) from

those over the period 1998-2004 (33.4 ± 4.5% similarity). Nor were the eukaryotic profiles obtained over the period 1998-1999 (Fig. 4B; average similarity of 16.6 ± 4.9%) significantly different from those over the interval 1998-2004 (24.5 ± 7.7% average similarity). The average similarity between profiles within a piezometer was low anyway (17.3 ± 3.4%).

The similarity in bacteria community profiles in polluted groundwater samples obtained in 1998 compared to those in 1999 per monitoring location decreased significantly with distance along the flow path (Table 1, Fig. 5).

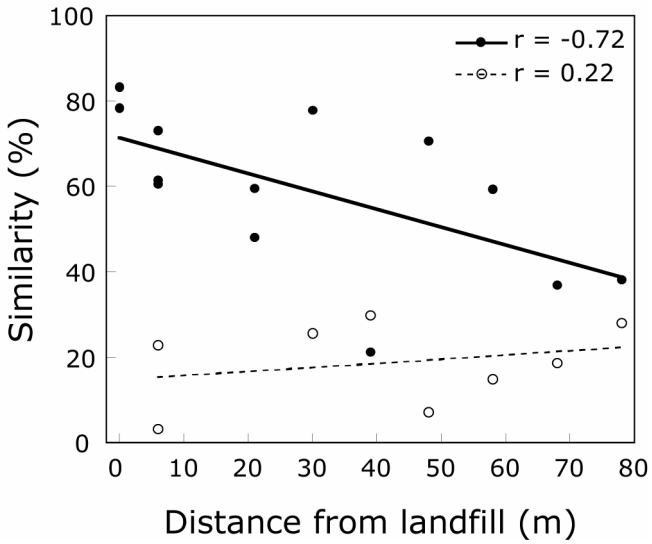

FIG. 5. Borehole-specific variation over time (1998 to 1999) in bacteria (solid line) and eukaryotic (dashed line) communities in polluted groundwater from the aquifer at Banisveld in 1998 with distance along the flow path from the Banisveld landfill. Bacteria and eukaryotic communities were based on DGGE profiling of 16S and 18S rRNA gene fragments, respectively.

The variation in bacteria community profiles in sampling points (Fig. 1: 48a, 58a, 68a and 78a) that became polluted over the period 1998-2004 (average similarity 31.8 ± 5.1%) was not significantly higher (Mann-Whitney U test, P > 0.05) than the variation in bacteria profiles in groundwater that had always been clean (average similarity 21.0 ± 6.5%) or polluted (Fig. 6A, average similarity 26.7 ± 7.3%). Neither was the variation in the eukaryotic profiles in sampling points that became polluted over the period 1998-2004 (average similarity 25.5 ± 6.7%) significantly higher (Mann-Whitney U test, P > 0.05) than the variation in points that were always clean (average similarity 17.1 ± 6.5%) or polluted (average similarity 18.9 ± 3.1%) (Fig. 6B).

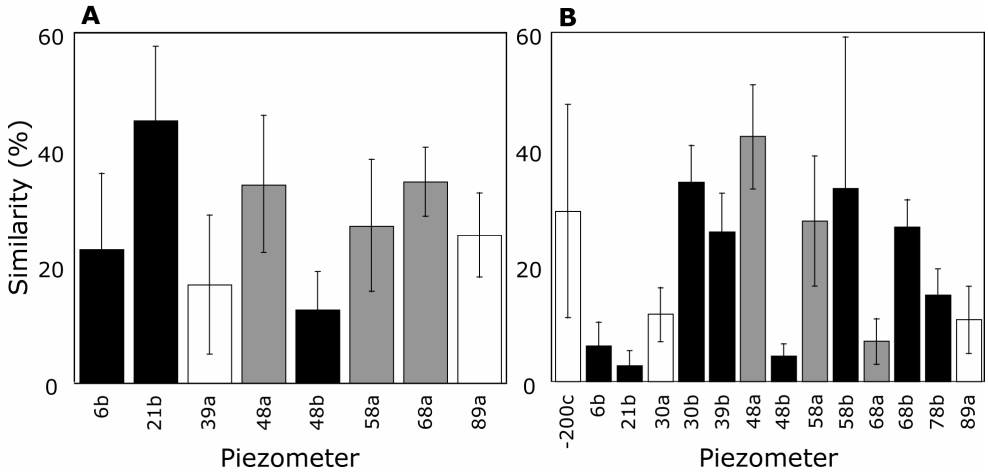

FIG. 6. Temporal variation per piezometer in bacteria (A) and eukaryotic (B) community in groundwater sampled from the aquifer at Banisveld in the period 1998-2004. Grey bars represent piezometers that became polluted over the period 1998-2004. Piezometers that remained over time either polluted or clean are represented by black and white bars, respectively.

Discussion

Changing abiotic conditions are important contributing factors to the spatiotemporal dynamics of microbial communities in the environment. Several reports confirmed that in marine settings (Fuhrmann et al., 2006), but also in surface polluted soils (Holtze et al., 2003) and sediments (Bissett et al., 2007), changes in the structure of microbial communities were directly related to variation of environmental factors. Also, for groundwater contaminated with monoaromatic hydrocarbons of the BTEX complex (Fahy et al., 2005) pollution decreased in time bacterial diversity, and long-term exposure of groundwater to benzene especially, led to temporal shifts in bacteria communities. These shifts were especially reflected in changes over time in the relative abundance of microbial groups, rather than in the presence or absence of specific groups of microorganisms. Likewise, temporarily rather stable microbial communities were encountered in another BTEX-contaminated aquifer, where the environmental characteristics remained relatively stable over time (Hendrickx et al., 2005). Slightly higher temporal changes in microbial community at one location in that BTEX-polluted aquifer were explained by changes in time of the groundwater temperature (Hendrickx et al., 2005).

In an aquifer polluted with organic materials the temporal variation in bacteria communities related to seasonal changes in the chemistry of groundwater (Franklin et al., 2000). The temporal changes in bacteria community structure were similar in magnitude and direction to changes in groundwater chemistry during the same period. Pollution, together with the local hydrological regime, was also responsible for spatial and temporal changes in the structure of microbial communities in an aquifer contaminated by fire-training activities (Haack et al., 2004). The recharge of this aquifer changed in time the structure of microbial communities. At aquifer recharge, the contaminants concentration can reduce as a result of dilution with uncontaminated groundwater. The recharge may also renew the electron acceptors in the aquifer, which could in absence of recharge, ultimately deplete (Chapelle et al., 1995).

In our study however, the temporal dynamics in the Banisveld aquifer microbial community structure were not clearly and directly linked to the overall hydrochemical changes and vice versa. While an upward migration of the pollution plume was noted over time in agreement with previous reported research (Chapter 2; Van Breukelen and Griffioen, 2004), the bacteria and eukaryotic communities did not seem to vary significantly more in locations that have become polluted over time. In contrast with the dynamic upper part of the plume, the amount of pollution did not seem to decrease notably inside the plume, as comparable levels of pollution were observed in 1998 and 2004. Despite the minor hydrochemical changes over time, the plume bacteria communities in 1998 appeared to relate more to each other than to those in later years.

The temporal variation in the Banisveld aquifer microbial communities was relatively high at all sampled locations. While higher temporal variation was noted in the plume bacteria communities compared to spatial variation, high variation in the eukaryotic communities was apparent over both time and space. In contrast with the study of Franklin and coworkers (2000), where higher temporal variation of the bacteria communities was found in anaerobic parts of the prospected aquifer in comparison to temporal variation in aerobic parts, the Banisveld bacteria communities appeared to vary in time more outside the pollution plume than within the plume. Franklin and coworkers (2000) had put the temporal variation in the structure of bacteria communities on the account of seasonal variation in the environmental characteristics (i.e. levels of pollution). For the aquifer at Banisveld however, these trends could not be observed as all three time points sampling occurred over the same season period each year (September - October).

In line with previous observations (Lin et al., 2005; Röling et al., 2000a; Röling et al., 2001), the bacteria community in polluted groundwater samples taken from the aquifer at Banisveld clustered separately from those in clean groundwater in 1998 and in 1999. Different bacteria communities in polluted samples as compared to communities in uncontaminated samples were observed also for other aquifers adjacent to landfills (Ludvigsen et al., 1999; Röling et al., 2000b). In a

hydrocarbon-polluted aquifer (Takahata et al., 2006) the pollution determined the spatial dynamics of bacteria communities. In another polluted aquifer (Fahy et al., 2005), bacteria communities were similar in uncontaminated groundwater, whereas the communities in benzene-polluted groundwater samples were different from each other and from those in the uncontaminated samples. Also, bacteria communities in the anaerobic zone of a shallow coastal aquifer polluted with organic materials (Franklin et al., 2000) were different from those in the aerobic zones.

In our study, the spatial and temporal variation in the eukaryotic communities was always higher than that of the bacteria. The similarity in bacteria communities decreased with distance between sampling points and along the flow path from the landfill. On the contrary, no such relationship was found for the eukaryotic communities. In groundwater polluted by the Banisveld landfill, but also in the associated sediments (Chapter 2), the eukaryotes seem more randomly and heterogeneously organized compared to bacteria. While heterogeneity is an apparent characteristic of the sediment-attached microbial communities in the aquifer at Banisveld (Chapter 2; Röling et al., 2001), groundwater, which contains the pollutants in solution was assumed to confer a more homogenous environment. Nevertheless, the large differences in bacteria and the eukaryotic community profiles obtained in the present study in different years from a certain groundwater well, suggest large temporal heterogeneity in the aquifer community structure. For the polluted aquifer at Banisveld in particular, the general hydrochemical changes over time appeared thus not to contribute substantially to microbial communities dynamics. Instead, spatial and especially the temporal heterogeneity in the structure of microbial communities seemed to govern their distribution and dynamics.

Acknowledgements

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Chapter 5

Nutrient recycling by protozoan predation can enhance overall bacterial degradation of organic matter*

Abstract

The combined influence of predation by the flagellate protozoan *Ochromonas* sp. DS and type of nutrient limitation on biodegradation of organic matter was studied mathematically and experimentally. Mathematical modeling, subsequently confirmed experimentally, indicated that predation can enhance carbon mineralization, and leads to higher overall activity (activity per unit sample), under nitrogen limiting conditions. Under carbon limitation, predation had a negative influence on organic matter degradation, in comparison with when predation was absent. The information obtained in this study can be of significance for the degradation of organic contaminants in oligotrophic ecosystems (e.g. aquifers), where nutrients like nitrogen or phosphorus generally become rapidly depleted. By their predation on bacteria, protozoa can recycle limiting nutrients and may stimulate indirectly pollutant biodegradation.

Keywords

predation, protozoa, nutrient recycling, biodegradation

*In preparation as Röling, W. F. M., T. Brad, and N. M. van Straalen

Introduction

In polluted subsurface environments, bacterial degradation of contaminants is the only mechanism that can really decrease the mass and toxicity of pollutants (Röling and van Verseveld, 2002). Therefore, microbiological studies on biodegradation have mainly addressed bacteria and their abilities to transform pollutants. A group of organisms in subsurface ecosystems, that has received much less attention than bacteria, are the protozoa. Protozoa abundance and diversity generally increase upon pollution (Kinner et al., 2002; Madsen et al., 1991; Novarino et al., 1997). Predation by protozoa on bacteria can enhance bioremediation by maintaining the hydraulic conductivity of polluted aquifers (Sinclair et al., 1993). By feeding on bacteria, protozoa play important roles in groundwater food webs, making bacterial productivity available to higher trophic levels (Novarino et al., 1997). Grazing pressure, especially exerted by the smallest protozoa, the flagellates, can control bacterial abundance (Hahn and Höfle, 1999; Jardillier et al., 2005; Kinner et al., 1997; Kinner et al., 1998; Kinner et al., 2002; Matz et al., 2002), and influence the species composition of bacterial communities (Hahn and Höfle, 2001; Jürgens et al., 1999; Matz and Jürgens, 2003). The bacterial roles in the mediation of element cycles and fluxes in the environment are thus likely influenced by protozoan predation.

In marine settings (Beardsley et al., 2003), but also in groundwater environments (Kota et al., 1999; Travis and Rosenberg, 1997), predation by protozoa can have negative effects on biodegradation rates by reducing the numbers of degraders. On the other hand, predation is also known to stimulate biodegradation rates under particular conditions. Increased bacterial activity can arise by greater accessibility to nutrients in larger pores (Wright et al., 1995), as a result of reduced soil aggregation (Kota et al., 1999) and decreased bacterial agglomeration in sediment pores (Mattison et al., 2002) due to protozoan predation. Furthermore, protozoa may stimulate biodegradation by altering community composition for example by selectively grazing those bacteria that normally outcompete pollutant degraders (Tso and Taghon, 2006). Predation by protozoa can stimulate

the per-bacterium activity and contribute indirectly to degradation of alkylbenzenes (Mattison and Harayama, 2001; Mattison and Harayama, 2005). Protozoa excrete nutrients, such as nitrogen and phosphorus, upon digesting their prey (Dolan, 1997; Ferrier-Pagès et al., 1998; Ratsak et al., 1996; Selph et al., 2003; Sherr and Sherr, 2002). Nitrogen and phosphorus generally become the growth-limiting nutrients in organic materials-contaminated environments (Chapelle, 2001), and thus nutrient recycling may lower nutrient limitations to bacteria.

In the present study, we examined mathematically and experimentally how predation by protozoa can enhance the overall bacterial degradation of organic matter and whether and how this stimulation relates to protozoan recycling of the growth-limiting nutrients under aerobic and anaerobic conditions. By using a mathematical modeling approach based on continuous culturing of bacteria under nutrient limiting conditions, we examined how microbial parameters, such as the bacterial maximum specific growth rate, influence the predator-induced stimulation of organic matter biodegradation. This type of information could be useful in bioremediation of contaminants in the environment.

Mathematical modeling

The degradation of organic matter by *Paracoccus denitrificans* under predation pressure of *Ochromonas* sp. DS and different nutrient limitations, was studied by the construction of a continuous culturing-based mathematical model. The model (Fig. 1, for parameters and their units see Table 1) includes microbial growth rate (μ), growth yield (Y) and affinity for substrate (K).

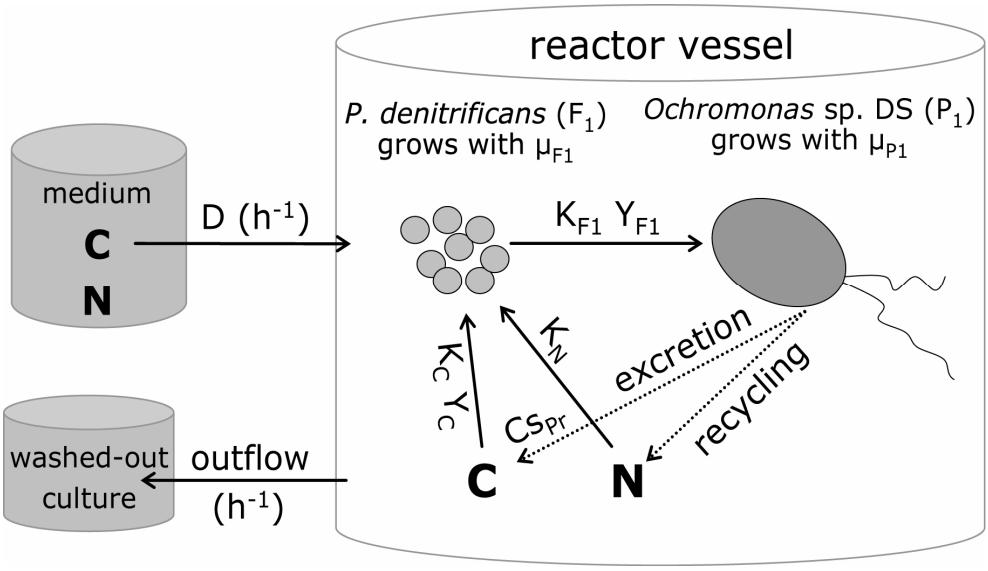

FIG. 1. Schematic view of the microbial components and parameters in mathematical models to describe the interactions between nutrients, prey and predator in a chemostat. The nutrients (C and N) are pumped from the medium reservoir into the reactor vessel at a particular dilution rate (D). The bacteria (F_1) consume nutrients, for which they have certain affinities (K_C and K_N), and grow according to their growth rates (μ_{F1}). Bacterial yield is biomass formed per substrate consumed. The bacteria are in turn being eaten by the flagellates (P_1) with an affinity constant (K_{F1}), and flagellate yield produced per amount of bacteria consumed. The flagellates excrete nutrients (N and C) that are recycled back to bacteria. The excess of culture in the reactor vessel is pumped out of the reactor at a rate equal to D.

For the flagellate predator *Ochromonas* sp. DS (P_1, biomass concentration in mM N-biomass), these parameters were obtained from literature (Mattison and Harayama, 2001), while the parameters for *P. denitrificans* (F_1, biomass concentration in mM N-biomass) were estimated based on previous experiments in our laboratory (Table 1).

TABLE 1.

Physiological and kinetic parameters for the bacterium *Paracoccus denitrificans* (F_1), growing on succinate, and for the growth of *Ochromonas* sp. DS (P_1) on bacteria*, as used in mathematical models to describe prey-predator-substrate interactions under aerobic and anaerobic conditions.

PARAMETER	SYMBOL AND UNIT		aerobic	anaerobic
Bacteria maximum growth rate	$\mu_{max}F_1$	(h^{-1})	0.5	0.5
Protozoa maximum growth rate	$\mu_{max}P_1$	(h^{-1})	0.1	0.1
Affinity constant of bacteria for N	K_N	(mM N)	0.042	0.042
Affinity constant of bacteria for C	K_C	(mM C)	0.04	0.04
Affinity for C-source recycled upon predation	K_{CSPr}	(mM C)	0.05	0.05
Affinity of protozoa for bacteria	K_{F1}	(mmol N-bacterial biomass l^{-1})	0.1	0.1
Efficiency (recycled N available to bacteria)	efficiency		1	1
Bacterial yield on N	Y_{F1}	(mmol N-biomass mmol N^{-1})	1	1
Bacterial yield on C	Y_C	(mmol C-biomass mmol C^{-1})	0.5	0.1
Protozoan yield per bacteria consumed	Y_{P1}	(mmol N-biomass · mmol N-bacterial biomass^{-1})	0.5	0.1
N concentration in medium reservoir	$N_{reservoir}$	(mM N)	0.2	0.2
C concentration in medium reservoir	$C_{reservoir}$	(mM C)	4	20

*Growth parameters of *Ochromonas* sp. on bacteria were taken from Mattison and Harayama, 2001

The model was run for both aerobic and anaerobic conditions. The bacterial growth rate (μ_{F1}) followed a simple Monod equation (Monod, 1958) when carbon (C, in mM) was the growth-limiting nutrient supplied:

$$\mu_{F1} = \mu_{maxF1} \frac{C}{K_C\left(1 + \frac{C}{K_C}\right)} \quad (1a)$$

For non-C limiting conditions, a double Monod equation was used with one term reflecting the carbon source and the other a non-competitive nutrient (N, in mM), which can be nitrogen, phosphate or an electron acceptor:

$$\mu_{F1} = \mu_{maxF1} \frac{N \times C}{K_N\left(1 + \frac{N}{K_N}\right) \times K_C\left(1 + \frac{C}{K_C}\right)} \quad (1b)$$

A modified Monod equation was used to reflect the utilization by the bacteria of a second carbon source (C_{Spr}, in mM) competitive with that supplied via the medium reservoir (C, in mM), and which is released by protozoa upon predation:

$$\mu_{F1} = \mu_{maxF1} \frac{N}{K_N + N} \times \frac{\frac{C}{K_C} + \frac{C_{Spr}}{K_{CSpr}}}{1 + \frac{C}{K_C} + \frac{C_{Spr}}{K_{CSpr}}} \quad (1c)$$

The growth rate of the flagellate protozoa was described by a Holling equation, which resembles the Monod equation. The growth-limiting substrate for the predator flagellate *Ochromonas* sp. DS was the bacterium (*P. denitrificans*):

$$\mu_{P1} = \mu_{maxP1} \frac{F_1}{K_{F1}\left(1 + \frac{F_1}{K_{F1}}\right)} \qquad (1d)$$

The changes in biomass of prey and predators are described by equations 2a and 2b, respectively, where D (in h^{-1}) is the chemostat dilution rate:

$$\frac{dF_1}{dt} = \mu_{F1} \times F_1 - \frac{\mu_{P1} \times P_1}{Y_{P1}} - D \times F_1 \qquad (2a)$$

$$\frac{dP_1}{dt} = \mu_{P1} \times P_1 - D \times P_1 \qquad (2b)$$

When the predators are absent under nitrogen limitation, or N compounds excreted by the predator are not utilizable by *P. denitrificans*, the overall rate of nitrogen (N) consumption can be described by:

$$\frac{dN}{dt} = - \frac{\mu_{F1} \times F_1}{Y_{F1}} + D(N_{reservoir} - N) \qquad (3a)$$

Here, $N_{reservoir}$ is the concentration of the nitrogen source (in mM) supplied via the medium reservoir and N is the concentration of nitrogen source in the reactor vessel. When N originates from both medium reservoir and protozoan excretion, the rate of N consumption is modeled as:

$$\frac{dN}{dt} = - \frac{\mu_{F1} \times F_1}{Y_{F1}} + efficiency \times (1 - Y_{P1}) \times \frac{\mu_{F1} \times P_1}{Y_{P1}} + D(N_{reservoir} - N) \qquad (3b)$$

where 'efficiency' is the fraction of N excreted by protozoa that is available for bacterial growth, it ranges from 0 to 1. This recycled N is assumed to be the same compound as the growth-limiting nutrient supplied via the medium reservoir. The rate of carbon consumption was calculated for a C:N ratio of 5:1 in biomass as:

$$\frac{dC}{dt} = D(C_{reservoir} - C) - 5\frac{\mu_{F1} \times F_1}{Y_C \times Y_{F1}} \qquad (3c)$$

The consumption rate of the second carbon source, competitive with that supplied via the medium reservoir, was modeled as:

$$\frac{dC_{Spr}}{dt} = 5\left[(1 - Y_{P1}) \times \frac{\mu_{P1} \times P_1}{Y_{P1}}\right] - \frac{5}{Y_C} \times \frac{\mu_{F1} \times F_1}{Y_{F1}} - D \times C_{Spr} \qquad (3d)$$

where μ_{F1} is that calculated in equation 1c.

The model was written in Maple 7.0 (Waterloo Maple Inc.).

Material and methods

Organisms and media

The organisms used for experiments in this study were strains of *Paracoccus denitrificans*, *Escherichia coli*, *Klebsiella edwardii*, *Bacillus cereus*, *Pseudomonas* sp. PS+, *Pseudomonas putida*, as prey organisms, and the facultative mixotrophic flagellate *Ochromonas* sp. DS (*Chrysophyceae*, *Ochromonadales*) as predator. *Ochromonas* sp. DS was isolated from Lake Constance (Southern Germany) (Hahn and Höfle, 1998), and preliminary analysis of its 18S rRNA gene revealed that the flagellate relates most closely to *Poteriochromonas malhamensis* (M. W. Hahn, personal communication). Prior to full characterization of this flagellate, including the exact taxonomic affiliation, it has been suggested that the name of *Ochromonas* sp. DS should be used (Hahn, M. W., personal communication). Mixotrophy is facultative in *Ochromonas* sp. DS. The flagellate (4-6 µm in diameter) can grow entirely heterotrophically (bacterivorously) in the dark, while in the light, it is unable to maintain growth based on photosynthesis alone (Hahn and Höfle, 1998). *Ochromonas* sp. DS has an interception feeding strategy and forms pseudopods to take in food (Boenigk and Arndt, 2002).

Prior to the experiments, an axenic culture of *Ochromonas* sp. DS, which was maintained for a longer period at 16°C under conditions of light, was transferred into the dark at 20°C and fed twice during one week with heat-killed cells of *P. denitrificans*. The medium used for maintaining the axenic cultures of *Ochromonas* sp. DS was inorganic basal medium (IBM). The IBM medium contained (mg l^{-1}) $MgSO_4 \cdot 7H_2O$ (75.0), $Ca(NO_3)_2 \cdot 4H_2O$ (43.0), $NaHCO_3$ (16.0), KCl (5.0), $K_2HPO_4 \cdot 3H_2O$ (3.7), Na_2EDTA (4.4), $FeCl_3 \cdot 6H_2O$ (3.2), H_3BO_3 (1.0), $MnCl_2 \cdot 4H_2O$ (0.2), $NiCl_2 \cdot 6H_2O$ (0.1), $ZnSO_4 \cdot 7H_2O$ (0.02), $CuSO_4 \cdot 6H_2O$ (0.02), $CoCl_2 \cdot 6H_2O$ (0.01), and $Na_2MoO_4 \cdot 2H_2O$ (0.006).

The medium used for experiments throughout the present study contained (mM) $Na_2MoO_4 \cdot 2H_2O$ (30), $MgSO_4 \cdot 7H_2O$ (0.8), Titriplex I (0.25), and $CaCl_2 \cdot 2H_2O$ (0.3). 65 ml of 1 M potassium-phosphate buffer (pH 7.0) and 1 ml of Lawford trace elements solution (Lawford et al.,

1976) were autoclaved separately, then added to 1 l of sterile medium. The carbon source consisted of 2.5 mM of either sodium-succinate ($Na_2C_4H_4O_4 \cdot 6H_2O$), sodium-acetate ($CH_3CO_2Na$), or glucose ($C_6H_{12}O_6$). Nitrogen limitation was obtained by using 0.2 mM of NH_4Cl, while medium with nitrogen in excess, and carbon as limiting compound, contained 2 mM of NH_4Cl.

Growth conditions and experimental design

Culturing experiments were performed for examining the degradation of organic matter by various bacterial strains in the presence and absence of the predator *Ochromonas* sp. DS, and under either carbon or nitrogen limitation. Bacterial precultures were grown overnight at 30ºC in an orbital shaker at 150 rpm, and 0.5 ml aliquots were used to inoculate bottles containing 50 ml medium (either N- or C-limited). *P. denitrificans* was used to study the degradation of succinate, while the degradation of acetate and glucose was performed with *E. coli*, *K. edwardii*, *B. cereus*, *Pseudomonas* sp., and *P. putida*. In the case of predation, 2 ml of the axenic culture of *Ochromonas* sp. DS was added. All treatments were performed in triplicate. The bottles were incubated in the dark at room temperature (20°C), and shaken at 50 rpm.

Measurements

The cultures were inspected for the presence and activity of flagellates by using an inverted microscope with a magnification of 600×. 2-ml aliquots were removed for biomass determinations and chemical analysis. Optical density (OD) was measured spectrophotometrically at 660 nm wavelength. Only bacterial cells were assumed to be responsible for the measured OD_{660} values, as bacteria-free cultures of *Ochromonas* sp. DS had similar OD_{660} values compared to sterile medium as blank. For total organic carbon (TOC) determinations, a 1-ml sample was removed from each culture and centrifuged for 10 min at 10.000 rpm. The supernatant was then transferred to a clean tube, and TOC was measured using a Dohrmann DC-190 TOC analyzer (Rosemount Analytical, Santa Clara, CA) after removing of the dissolved inorganic carbon (DIC) by adding 50 µl of 37% HCl to 1 ml of sample. Determinations of OD_{660} and residual TOC in each culture were performed after 1, 2, 3, 4, 7 and 48 days of incubation.

Results and discussion

Modeling results

The model revealed that under carbon-limiting conditions, in both aerobic and anaerobic circumstances, predation always (irrespective of dilution rate) had a negative influence on the amount of carbon source degraded. The C source was consumed almost entirely in the absence of predators, but not in their presence (Fig. 2B and 2D). In contrast, under N-limiting conditions, a relative increase in carbon degradation was noted when nitrogen was recycled through predation. The carbon source was best consumed under N-limiting conditions and in the presence of predators, in comparison with situations when nitrogen was not recycled by the predator, or predation was absent (Fig. 2A and 2C, Fig. 3A and Fig. 3B).

At similar dilution rates (Fig. 3, D = 0.02 for aerobic and D = 0.01 for anaerobic conditions), the rate of C degradation, calculated as $D\times(C_{reservoir} - C_{reactor\ vessel})$, was highest when predation was present under both aerobic and anaerobic N-limiting conditions (Fig. 3A and 3B, respectively). The effect of predation on C source degradation under N limitation was not always positive, but dependent on the maximum growth rate of the bacteria, relative to protozoa. At a low maximum specific growth rate (0.1 h^{-1}), predation had a negative effect on the rate C degradation under both aerobic (Fig. 4A) and anaerobic (Fig. 4C) conditions, even in the presence of N-recycling. As already demonstrated in Figs. 2 and 3, positive effects were instead registered in case of faster growing bacteria (maximum growth rate 0.5 h^{-1}) predated by protozoa under either aerobic (Fig. 4B) or anaerobic (Fig. 4D) N-limiting conditions.

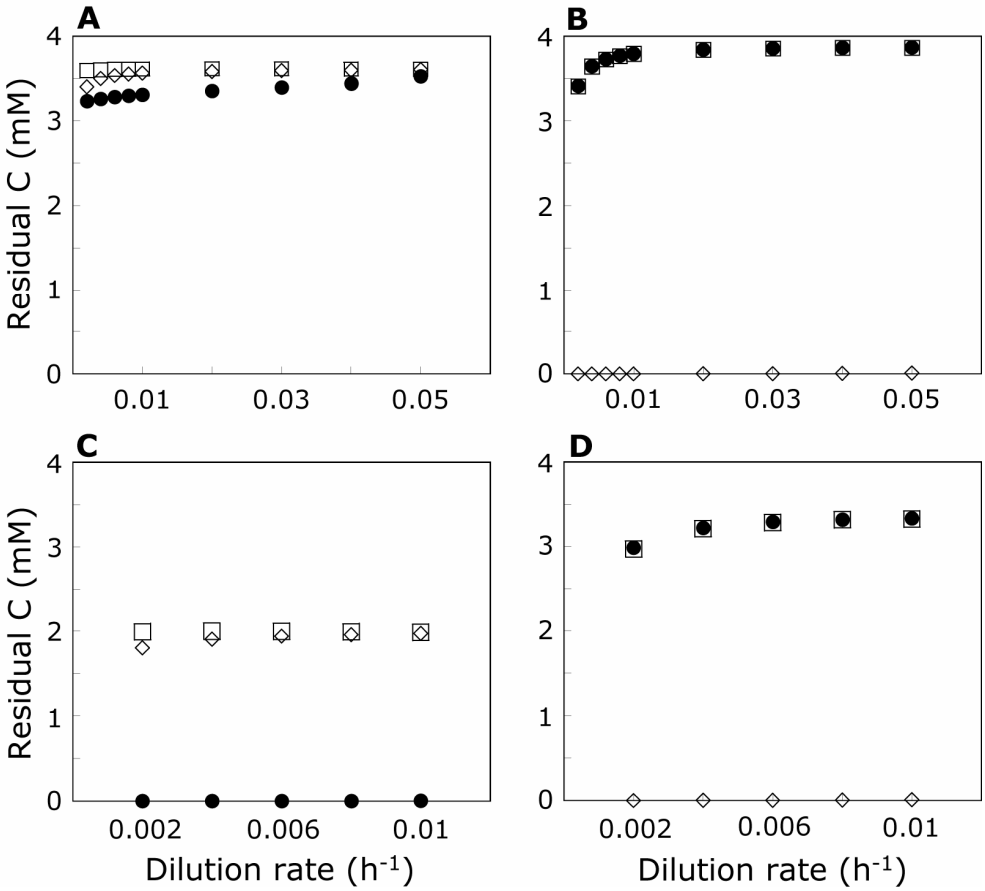

FIG. 2. Modeled residual carbon concentrations at various dilution rates under aerobic N-limiting (A) and C-limiting (B) conditions, and under anaerobic situations in N-limited (C) or C-limited (D) medium. ◇ represents the situation when predation was absent; □ predator was present but nutrients were not recycled; and ● predator was present and nutrients were recycled upon predation.

The relative stimulation of C degradation by predation under N limitation was higher under anaerobic conditions compared to that under aerobic conditions (Fig. 3, 4). A lower bacterial yield was used to model predation under anaerobic conditions compared to that under aerobic conditions: more C source will be needed to produce the same amount of energy and biomass (per N assimilated) under anaerobic conditions, compared to aerobic conditions. This is because under anaerobic conditions the gain in ATP per mole carbon source dissimilated is less than under aerobic conditions.

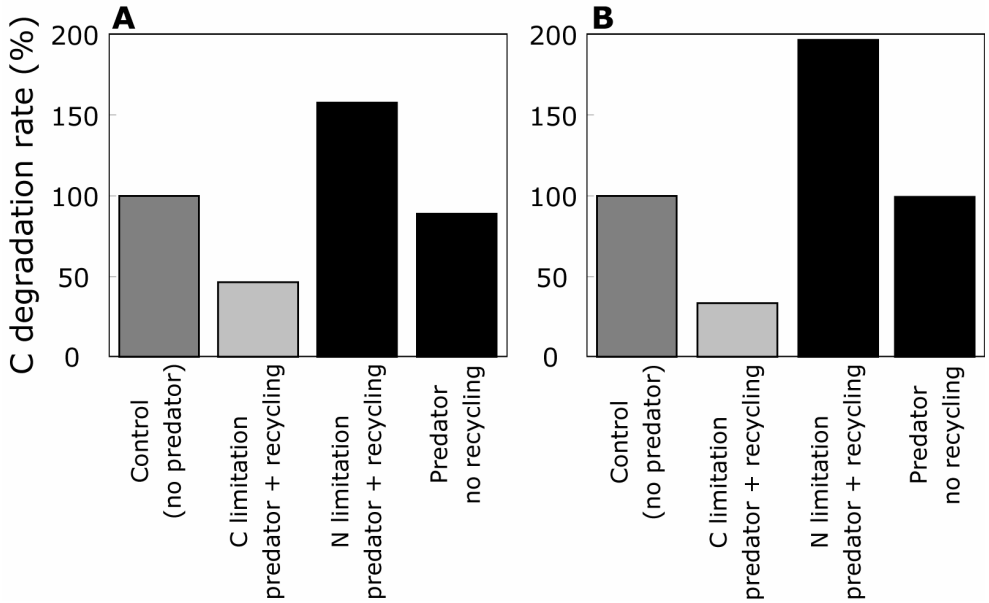

FIG. 3. Rate of C degradation modeled as $D \times (C_{reservoir} - C_{reactor\ vessel})$ under A. aerobic ($D = 0.02\ h^{-1}$) and B. anaerobic ($D = 0.01\ h^{-1}$) conditions. The influence of predation, under C limitation, and under N limitation, with and without N-recycling, on carbon degradation, is reported relative to the situation where predation is absent (set at 100%).

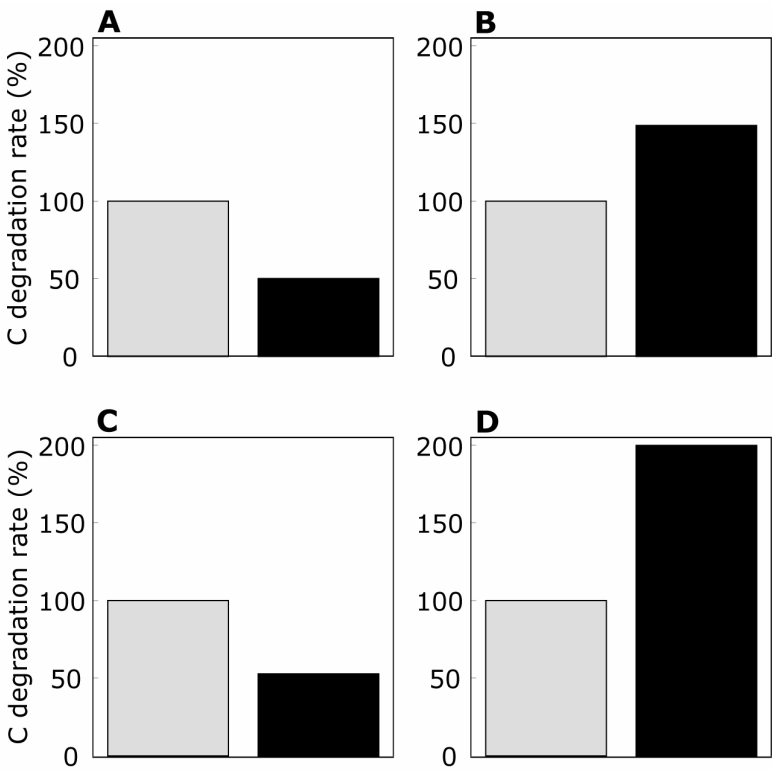

FIG. 4. Influence of maximum growth rate on rate of carbon degradation under N-limiting conditions. Maximum growth rates of bacteria of 0.1 h^{-1} (A and C) and 0.5 h^{-1} (B and D) were used in mathematical models. The aerobic situations are shown in A and B, anaerobic situations in C and D. Grey bars indicate the rate of carbon degradation in the absence of predation, set at 100%, while the relative degradation rates in the presence of predators is represented by black bars.

At comparable dilution rates, predation pressure reduced bacterial biomass (Fig. 5) and enhanced bacterial growth rates (Fig. 6) under both N and C limiting conditions, and under either aerobic or anaerobic situations. Thus, higher activity was noted per unit biomass. Higher activity per unit biomass in the presence of predators was also noted in the experiments of Mattison and coworkers (Mattison and Harayama, 2001; Mattison and Harayama, 2005). Bacterial biomass is reduced by the predation pressure, leaving more substrate left over that allow for a higher growth rate.

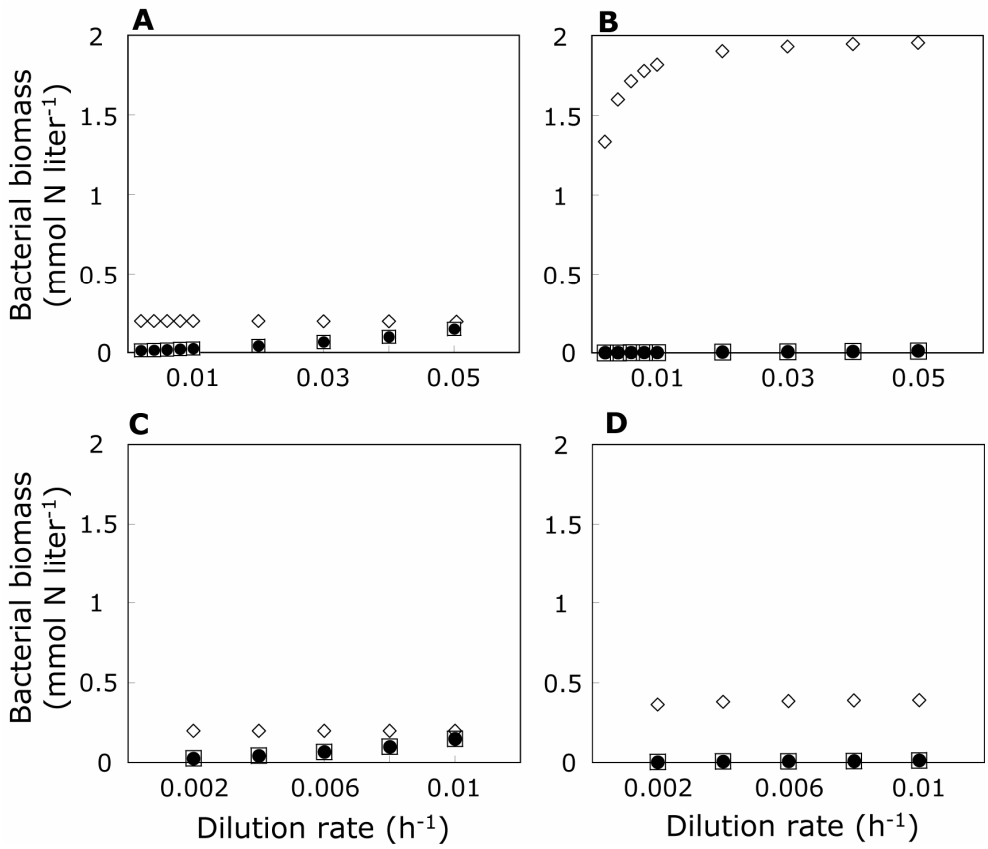

FIG. 5. Modeled bacterial biomass formed in the reactor vessel at different dilution rates under aerobic N-limiting (A) and C-limiting (B) conditions, and under anaerobic nitrogen- (C) and carbon-limiting (D) conditions. ◇ represent the situation when predation was absent, □ predator was present but nutrients were not recycled, ● predator was present and nutrients were recycled upon predation.

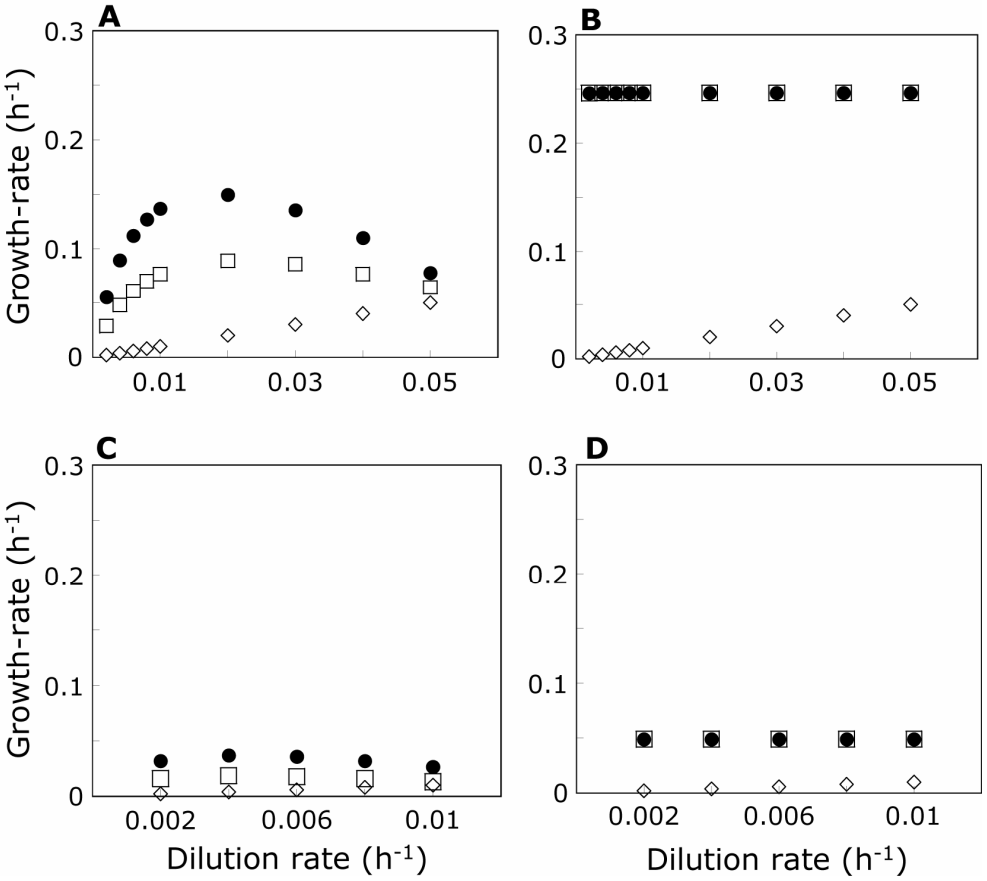

FIG. 6. Modeled growth rates of bacteria at different dilution rates under aerobic N-limiting (A) and C-limiting (B) conditions, and in anaerobic conditions under nitrogen (C) and carbon (D) limitations. ◇ represent the situation when predation was absent, □ when predator was present but nutrients were not recycled, and ● symbolizes the situation when the predator was present and nutrients were recycled upon predation.

Experimental results

Laboratory experiments on the degradation of organic matter by different bacterial strains under nutrient limitations and predation under pressure by the flagellate protozoan *Ochromonas* sp. DS confirmed the trends predicted by the mathematical modeling approach. Like for the mathematical modeling, the experiments involved the presence of one bacterium and one protozoan species, and the degree of degradation of organic materials depended particularly on the type of nutrient limitation present. Under carbon-limiting conditions, predation did not have a notable influence on the overall degradation of organic matter under carbon-limiting conditions. Almost complete degradation of succinate by *Paracoccus denitrificans* occurred under carbon limitation regardless of predation by *Ochromonas* sp. DS (Fig. 7A). The remaining unmineralized carbon relates most probably to the presence in the medium of the C-containing chelator agent EDTA. In agreement with results obtained by mathematical modeling, the organic matter (succinate) degradation by *P. denitrificans* was however enhanced by the presence of predation under nitrogen-limiting conditions, in comparison to non-predation situations (Fig. 7A). The C-source decreased notably already after two days of incubation. The presence of predation influenced positively the extent and rate of degradation of organic matter only under N limiting conditions, while under C limitation carbon source was slower consumed under predation pressure compared to non-predation situations.

A slight decline of *P. denitrificans* biomass was registered after the second day of incubation when predation was absent under carbon-limiting conditions. When nitrogen was limiting, and predation was absent, bacterial biomass increased until the 7^{th} day of the experiment (Fig. 7B). Predation by protozoa generally decreases bacteria densities (Kota et al., 1999; Mattison and Harayama, 2001). Also in this study a dramatic decline of the *P. denitrificans* population was noted after the 4^{th} day of incubation under both C and N limiting conditions with predation present (Fig. 7B).

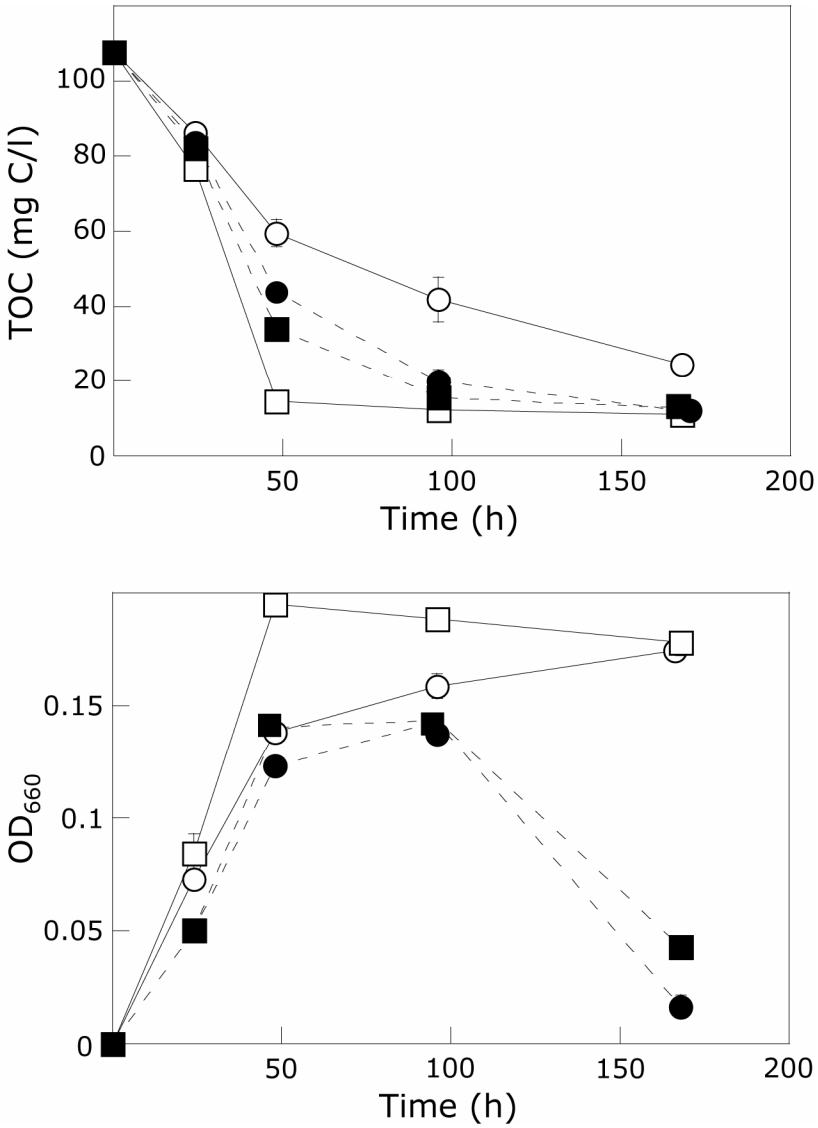

FIG. 7. Changes in TOC concentrations (A) and optical density (B) of *P. denitrificans* over time, in the absence or presence of a predator. Open symbols represent the situation when predation was absent, while filled symbols stand for the presence of the predator *Ochromonas* sp. DS. Circles represent the nitrogen limiting conditions, while the carbon limiting situations are represented by squares.

The trends observed for the effect of predation on organic matter (succinate) biodegradation by *P. denitrificans* were also found for other bacterial strains exploited by the same protozoa under nitrogen limiting conditions. The degradation of acetate and glucose by *E. coli*, *K. edwardii*, *B. cereus*, *Pseudomonas* sp. PS+ and *P. putida* was enhanced by the presence of the predator *Ochromonas* sp. DS. A considerable drop of the residual carbon was registered after 3 days of incubation. The carbon sources were almost completely used by bacteria after 48 days of incubation, especially in situations where predation was present (Fig. 8). Here, the smallest stimulating effect of predation was noted for the two *Pseudomonas* strains.

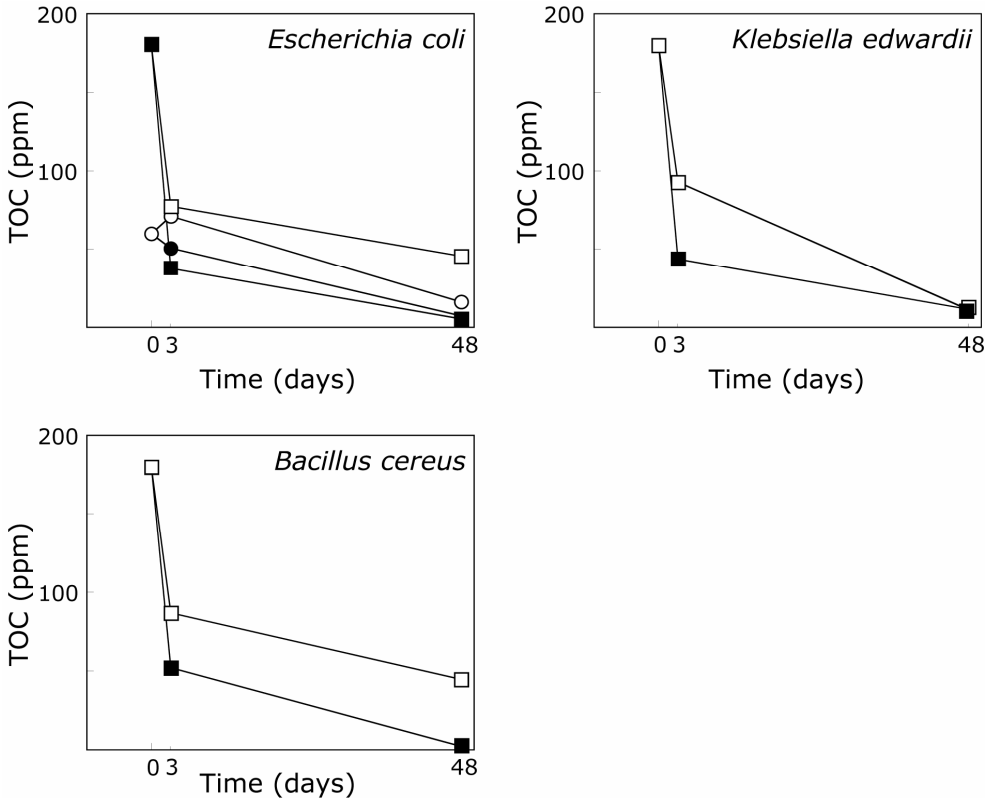

FIG. 8. TOC consumption over time by different bacterial strains (species name is shown in each graph) predated by *Ochromonas* sp. DS, under N limiting conditions. Open symbols stand for the absence of predation, while full symbols for the presence of predation. The carbon source consisted of either Na-acetate (circles) or glucose (squares).

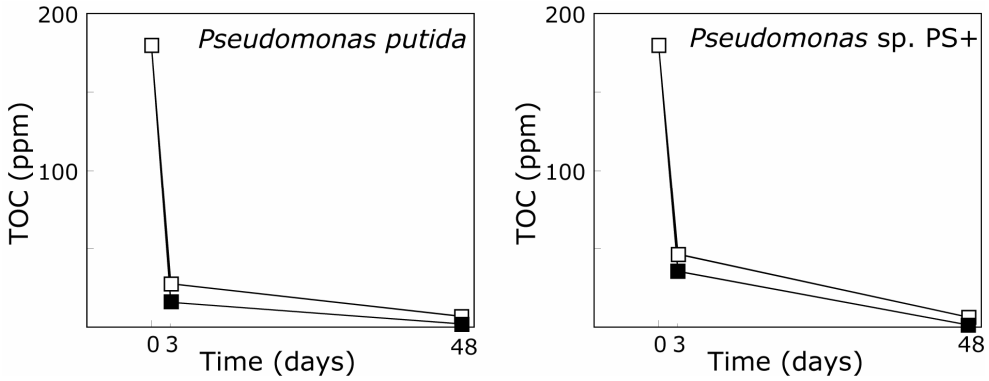

FIG. 8. Continued

The mathematical modeling approach employed in this research was based on continuous culturing of bacteria in a nitrogen-limited environment in the presence of protozoan predation, and used simple equations to describe microbial growth. The batch-culture experiments, which were performed in liquid medium with either carbon or nitrogen limitations, were designed to test the model predictions, and have involved the presence and activity of the above-modeled organisms. The two different approaches (i.e. mathematical modeling and experiments) revealed the same trends of increased carbon mineralization in conditions of recycling of the limiting nitrogen upon protozoan predation.

As flagellate protists are important predators of bacteria (Hahn and Höfle, 2001; Jürgens and Matz, 2002), they influence bacterial abundances and activity (Sherr and Sherr, 2002; Weisse, 2002). Flagellate protozoa contribute to carbon (Caron et al., 1985), phosphorus and nitrogen (Goldman et al., 1985; Eccleston-Parry and Leadbeater, 1995) cycling, and to the transfer of carbon to higher trophic levels (Novarino et al., 1997). For polluted environments in particular, this type of information can have important implications, as by their predation on bacteria and recycling of limiting nutrients, flagellate protozoa could be used in bioremediation of organic matter contaminated sites, where important nutrients, such as nitrogen or phosphorus are often the limiting nutrients. This research was limited to the interaction of one organic matter-degrading bacterium species with one predator protozoan species, and cannot mimic the complexity of

microbial communities in natural environments. Nevertheless, the simple food chain studied here, provides proof of principle for enhanced predator-stimulated bacterial degradation of organic matter in nitrogen-limited environments.

Acknowledgements

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Chapter 6

Thesis synthesis and general discussion

Pollution of groundwater is an apparent phenomenon wherever human presence and activity exist. Although groundwater is the world main drinking water reservoir (Shiklomanov, 1993), pollution of groundwater by anthropogenic activities suggests a lack of awareness manifested by the society of today. For example, 75% of the European Community citizens depend on groundwater as clean drinking water supply (Gibert et al., 1994), but paradoxically, in many places, industrial, agricultural, or residential area practices still do not operate according to regulations meant to protect and use groundwater sources in a sustainable manner. Unfortunately, the soil and subsurface are still major deposits for various wastes originating from different sources, such as industrial activities and storage, spreading of fertilizers and pesticides through agricultural practices, leaking sewers in residential areas and urban run-offs, or landfills. Soil pollution stemming from such sources is a major source of groundwater deterioration.

Landfills constitute particular threats to the environment by polluting the neighboring aquifers (Christensen et al., 2001; Kjeldsen, 1993). Until very recently, the construction of landfills did not foresee lining of the sites, and even today old unlined landfills still operate, and threaten the environment. Percolating rain water mobilizes the pollutants in landfills, and carries with the groundwater flow sometimes impressive plumes of pollution towards nature reserves, residential areas or other places of public interest, contaminating the drinking water supplies. The composition of landfill-leachate plumes depends primarily on the types of deposited wastes, which can be of municipal, commercial or industrial origin (Christensen et al., 1994). The organic charge of landfill-leachate plumes is in general high, and hazardous contaminants such as monoaromatic hydrocarbons of the BTEX complex (benzene, toluene, ethylbenzene and xylenes), naphthalene, halogenated hydrocarbons and other chlorinated compounds, phenols or heavy metals, are also present (Christensen et al., 2001; Kjeldsen et al., 2002).

Once percolated into the underlying aquifers, landfill leachate is subjected to dilution due to mixing with the groundwater. Sequentially, natural attenuation-related biological and physicochemical processes, such as biodegradation, dissolution, precipitation, complexation, ion exchange or sorption (Christensen et al., 2001), can reduce the spreading of leachate and lead to a decrease in pollutants concentration away from the source (Rügner et al., 2006; Van Breukelen, 2003). Of the natural attenuation processes, microbial biodegradation is the only means of really decreasing the concentration and toxicity of pollutants (Röling and Van Verseveld, 2002). Microbial activities are also responsible for the development of redox zones downgradient of the landfill. For that, the available electron acceptors will be used preferentially in sequence, from the free oxygen and nitrate in groundwater, to oxidized iron and manganese compounds and sulfate, and finally methanogenesis may occur by fermentation and carbon dioxide reduction (Christensen et al., 2000).

One of the approximately 4000 old landfills in The Netherlands is the Banisveld landfill, which is located in the province of Noord-Brabant, 5 km southwest of the town of Boxtel. This landfill operated for over 12 years and contains mostly household refuse, but organic micropollutants (i. e. benzene, ethylbenzene, xylene and naphthalene) and heavy metals have also been detected (Van Breukelen et al., 2003).

Thesis main findings

Natural attenuation of organic pollutants was noted in the Banisveld aquifer under anaerobic iron-reducing conditions, and the process was attributed mainly to microbial biodegradation (Botton, 2007; Röling et al., 2001; Van Breukelen et al., 2003). While for the aquifer at Banisveld, but also for other polluted aquifers, the presence and activities of prokaryotes in relation to biodegradation of contaminants had intensively been addressed, the occurrence and functions of eukaryotic organisms in this type of environments seemed to have been largely disregarded. The present thesis placed emphasis on the presence and roles of eukaryotes in the aquifer downgradient of the Banisveld landfill. The microeukaryotic community structure and composition were regarded in relation with bacteria community and environmental factors, especially with the pollution with landfill leachate. The roles and contribution of eukaryotes (flagellate protists) to biodegradation of organic matter were studied in systems where nutrients like carbon or nitrogen were limiting.

The sediment-associated eukaryotic and bacteria communities in the polluted Banisveld aquifer, were complex and diverse, and varied greatly over horizontal and vertical sections in the aquifer. The spatial heterogeneity of microbial communities in the aquifer was apparent and seemed to relate to the large heterogeneity in environmental characteristics. Due to the lack of spatial autocorrelation in the structure of eukaryotic and bacteria communities, and the large differences in their community profiles obtained in neighboring sediment samples, it is proposed to sample at scales smaller than 1 m for fully characterizing the microbial communities in polluted heterogeneous environments like the Banisveld aquifer (Chapter 2). Larger groundwater fauna (stygobiontic invertebrates) was not detected in this aquifer due to the fine sediments, lack of oxygen and pollution degree. Instead, a diverse microeukaryotic community composed of flagellate protists, unicellular fungi and algae was present, and related to the occurrence of pollution with landfill leachate. The heterotrophic nanoflagellate *Heteromita globosa* dominated the sediments in the most polluted part of the aquifer downgradient of the landfill (Chapter 3). A general upward

migration of the pollution plume was noted over time, especially in places at larger distances from the landfill. The mass of polluting substances did not seem to decrease in the contaminant plume during a six-year investigation period. The pollution with landfill leachate and the general hydrochemical changes over time did not seem to contribute considerably to spatiotemporal dynamics in bacteria and eukaryotic communities. But it was rather the spatial and especially the temporal heterogeneity in the structure of communities and environmental characteristics that governs the distribution and dynamics of microbial communities in the Banisveld aquifer (Chapter 4). The presence and activity of flagellate protozoa in the contaminated aquifer at Banisveld can be of importance in the biodegradation of organic materials at this site. By their predation on bacteria, the flagellates can recycle limiting nutrients back to their prey. In this way, flagellates can increase the overall activity of bacteria and the rates of organic matter biodegradation under nitrogen limiting conditions (Chapter 5).

Relation of eukaryotic community structure and diversity with bacteria community and the Banisveld polluted environment

The polluted aquifer downgradient of the Banisveld landfill harbors complex and diverse eukaryotic and bacteria communities. In general, the structure of eukaryotic communities in groundwater and sediments did not provide an accurate reflection of the distribution of environmental factors (i.e. pollution with landfill leachate) in the aquifer. On the other hand, pollution appeared to have a strong influence on the aquifer bacteria communities; apparently selecting for certain types of these microorganisms in both sediments and associated groundwater. In leachate-affected sediments, the bacteria and eukaryotic communities were distinct from those in clean sediments (Chapter 2). In groundwater, bacteria communities were more similar to each other in polluted locations than in clean areas (Chapter 4; Lin et al., 2005; Röling et al., 2000a; Röling et al., 2001). Eukaryotic communities appeared to be more mobile and more heterogeneously distributed in groundwater, and they did not seem to be influenced particularly by the presence of pollution (Chapter 4).

Spatial heterogeneity was assumed to be a characteristic primarily of the sediment-attached eukaryotic communities, while groundwater would confer a more homogenous environment. The heterogeneity in eukaryotic community structure was associated to the large heterogeneity in environmental sediment characteristics in the aquifer (Chapter 2). However, large spatial variation and heterogeneity was also noted in the eukaryotic communities in polluted and pristine groundwater from Banisveld (Chapter 4). Here (Chapter 4), the eukaryotic and bacteria communities in groundwater proved to be quite heterogeneous also over time. A large variation in their community profiles was noted in different years from the same location, and appeared not to relate rigorously to the overall hydrochemical changes in the aquifer.

The eukaryotic and bacteria communities related to each other in polluted sediments from Banisveld (Chapter 2), but not in groundwater (Chapter 4). As the sediment-associated bacteria are generally more abundant (Albrechtsen and Winding, 1992; Holm et al., 1992) and different from those drifting freely in groundwater (Röling et al., 2001), possibly the bacteria-grazing protozoa agglomerate more around sediment particles for feeding than in flowing groundwater, where their food (the bacteria) may be also less accessible compared to that in sediments.

The food web observed in the polluted Banisveld aquifer was simple, and consisted of bacteria and fungi as potential decomposers of organic matter, and protozoa as grazers and top predators. Larger groundwater invertebrates were absent from this groundwater ecosystem due to the fine sediments, lack of oxygen, and presumably the presence of pollution. When samples taken at 3 m were compared, the diversity of microeukaryotic community was highest in the most polluted part of the Banisveld aquifer close to the landfill. This aspect was especially revealed in Chapter 3 of this thesis, where at depths of 3 m in the aquifer and distances from the landfill of 21 to 30 m, sequences of flagellate protozoa, yeast-like fungi and algae where identified. In contrast, in sediments at larger distances from landfill, as well as in clean reference sediments at comparable depths, only fungal sequences were detected. It has been shown that, also for other contaminated

environments, the abundance and diversity of eukaryotes increase generally upon pollution (Chapter 3; Madsen et al., 1991; Novarino et al., 1997). A higher charge of organic pollution, like that in the proximity of the Banisveld landfill, means higher diversity and larger amounts of carbon sources, which support greater diversity and growth of bacteria and fungi compared to less polluted or clean locations in the aquifer. It was previously shown that for the aquifer at Banisveld the functional diversity was higher within the pollution plume in comparison to that outside the plume (Röling et al., 2000a). On the contrary, research on other aquifers polluted with BTEX revealed richer bacteria communities in uncontaminated locations than in polluted ones (Fahy et al., 2005; Hendrickx et al., 2005). However, higher abundance and diversity of bacteria in the polluted Banisveld aquifer close to the landfill, indicates more food for bacterivorous protists, and in consequence increased protozoan productivity at these locations. Here, closer to the landfill, the bacterivorous nanoflagellate *Heteromita globosa* dominated the microeukaryotic community at larger depths (i.e. 5 m deep). Moreover, this flagellate seemed to prefer more the polluted locations, where more food (the bacteria) is available as a result of higher organic charge, compared to places less contaminated by the leachate (Chapter 3).

Potential for natural attenuation and resilience of the polluted Banisveld aquifer. Possible roles of eukaryotes

Research at Banisveld began in 1998 and focused on understanding the functional mechanisms of natural attenuation at this site in particular and for landfill-leachate polluted aquifers in general. The term *natural attenuation* refers here to the observed reduction of contaminants concentration and migration, as contaminants move away from their source in aquifers or other environments. For relying on natural attenuation, as the cheapest means of bioremediation of polluted environments like that from Banisveld (Röling and Van Verseveld, 2002), one has to obtain knowledge on the occurrence of this process *in situ* and determine the involved mechanisms. Furthermore, the process has to be properly monitored over time in order to formulate predictions on whether and how the concentration of a particular contaminant

decreases, and on whether and how long it takes before its concentrations drop to an acceptable level. The chemical composition of the leachate originating from the Banisveld landfill was determined, and the biogeochemical and redox processes associated to the attenuation of contaminants were identified (Van Breukelen et al., 2003; Van Breukelen and Griffioen, 2004; Van Breukelen et al., 2004). Although not very effective (Van Breukelen et al., 2003), it has been revealed that natural attenuation of organic pollutants occurs in the polluted Banisveld aquifer, especially at locations in the nearby proximity of the landfill. Here, an abrupt decrease in the concentration of organic micropollutants (benzene, ethylbenzene, xylene and naphthalene) was observed in comparison with their concentrations at locations further downgradient of the landfill (Van Breukelen et al., 2003). This decrease was put on the account of microbial biodegradation, the only natural attenuation way of decreasing the mass and toxicity of pollutants (Röling and Van Verseveld, 2002), as the high concentration of chlorides (used as conservative tracers) remained relatively constant along the flow path from the landfill. Dilution of contaminants along the flow path could therefore be excluded (Röling et al., 2001; Van Breukelen et al., 2003). Further microbiological and biogeochemical research confirmed the importance of microbial processes in pollutant attenuation. It has been determined that biodegradation of the total organic carbon (TOC) in general (Van Breukelen et al., 2003) and of benzene and ethylbenzene in particular (Botton, 2007) occurs in the polluted Banisveld aquifer *in situ*.

While the presence and activities of prokaryotic microorganisms has thus extensively been addressed especially in relation to contaminant biodegradation in the Banisveld aquifer, the occurrence of eukaryotes in this aquifer had only been assumed by the detection of an archaean endosymbiont of an anaerobic protozoan (Röling et al., 2001). In the present thesis, a complex and diverse community of eukaryotes was revealed in the polluted aquifer and described in detail.

Of high significance is the dominance of the bacterivorous flagellate protozoan *Heteromita globosa*, in polluted sediments close to the landfill (Chapter 3), where microbial contaminant biodegradation seemed to occur at relatively higher rates. The occurrence of this flagellate

appeared to be related to the presence of pollution. The finding and dominance of *H. globosa* in polluted sediments can be of importance in biodegradation processes at the Banisveld site. This bacterivorous flagellate, which was retrieved in aerobic and anaerobic enrichments inoculated with polluted sediments from the aquifer at Banisveld (Chapter 3), was shown to influence positively the biodegradation of alkylbenzenes (Mattison and Harayama, 2001; Mattison and Harayama, 2005) by enhancing the activity of bacteria. Also, experiments and mathematical modeling (Chapter 5) showed that bacterivorous flagellates (such as, for example, *H. globosa*) can enhance the overall activity of degrading microorganisms by recycling of limiting nutrients, and in turn the overall rates of organic matter biodegradation in systems where nutrients like nitrogen are present in limited amounts. Therefore, further studies involving this particular organism (*Heteromita globosa*) and degrading bacteria present in the Banisveld plume, will be of importance in understanding of how biodegradation of organic contaminants works in this system. Microcosms and continuous culture experiments can provide information and allow for predictions on the rates of contaminant biodegradation, and the actual contribution of flagellate protists to stimulating biodegradation processes. The study of larger groundwater fauna in polluted aquifers in relation with the degrading bacteria community, will add valuable information to how natural attenuation functions in groundwater ecosystems. As the diet of these stygobiontic invertebrates consists primarily of microorganisms, they most likely contribute to contaminant microbial biodegradation.

Of significance for biodegradation of organic contaminants in the Banisveld aquifer is also the presence of fungi. It has been shown that basidiomycote fungi like those encountered (i.e. *Rhodotorula* sp., *Cryptococcus* sp. and *Leucosporidium* sp.) are capable of degrading monoaromatic hydrocarbons under aerobic conditions (Middelhoven, 1993; Middelhoven et al., 1992a), while ascomycote fungi (e.g. *Candida* sp.) can degrade aerobically phenols and hydroxybenzoates (Middelhoven et al., 1992a). As these types of fungi can also grow anaerobically (Ekendahl et al., 2003; Visser et al., 1990) and have been identified in anaerobic environmental settings (Chapter 3; Lou et al., 2005), further research on their role in anaerobic contaminant biodegradation will be of importance in bioremediation of polluted

environments like the Banisveld aquifer. Experiments involving yeast-like fungi (such as those encountered at Banisveld) degrading organic compounds analogous to those present in landfill leachate, can emphasize their contribution to contaminant biodegradation.

Another trait favorable to natural attenuation in the polluted Banisveld aquifer can be the large spatial heterogeneity observed in the microbial community composition (Chapter 2 and 4). It is assumed that the contaminants that flow with the groundwater will pass over more types of microorganisms if the environment is more heterogeneous, and have thus greater chances to become degraded in comparison with the situation when the environment is more homogenous. The large heterogeneity in rRNA genes in sediments (Chapter 2) and groundwater (Chapter 4) sampled from the aquifer at Banisveld does not reflect however the diversity of and heterogeneity in functional genes involved in biochemical pathways of contaminant biodegradation. Identification of this type of genes in polluted environments, such as the benzylsuccinate synthase-encoding gene (*bssA*) detected in BTX-degrading enrichments inoculated with aquifer material from Banisveld (Botton, 2007), will provide information on the capabilities of microorganisms with respect to contaminant biodegradation.

The groundwater ecosystem from Banisveld seems to be resilient to landfill leachate contamination. *Resilience* is seen here as the ability of ecosystems to cope with pollution threatening, and recover to a functional state similar to that prior to disturbance. The groundwater ecosystem from Banisveld reposts to pollution threats with a wide range of responses and has the premises for naturally attenuate the contaminants, of which microbial biodegradation is the most important. Predation by protozoa and subsequent recycling of limiting nutrients are factors potentially stimulating the contaminant biodegradation in the aquifer. Whether it is resilient to contamination with landfill leachate, the groundwater ecosystem from Banisveld must be shielded by a buffer zone where pollution attenuation takes place. If microbial communities involved in contaminant biodegradation can indeed reduce the mass and toxicity of pollutants, they cannot prevent the landfill leachate to form and migrate. Therefore, further research is needed to investigate whether this buffer zone is of acceptable size, contaminants attenuation

really occurs therein, and this shield does not increase in time or change its position in unacceptable ways.

However, several issues make this particular ecosystem (the groundwater ecosystem) quite vulnerable to pollution. According to the general ecological theory, an ecosystem with short food chains, simple food webs and high nutrient inputs, like is the Banisveld aquifer ecosystem, is considered to be relatively little resilient (Cottingham and Carpenter, 1994), as high nutrient load generally leads to disturbed ecosystems with unstable communities (Chapter 4; Borgmann et al., 1988; Hessen and Nilssen, 1986; Luckinbill, 1974; Luckinbill, 1979). In most cases, groundwater ecosystems are oligotrophic, and will remain stable and resilient as long as the nutrient inputs are kept limited.

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Subsurface landfill leachate - home to complex and dynamic eukaryotic communities

Summary

Research on natural attenuation in groundwater ecosystems has mainly addressed the presence and abilities of prokaryotic microorganisms to reduce the mass and toxicity of pollutants. The occurrence and possible roles of eukaryotes in contaminant biodegradation has so far largely been overlooked. The present thesis places an emphasis on the presence of eukaryotes in a polluted aquifer downgradient of a landfill (Banisveld, The Netherlands) and discusses their potential contribution to natural attenuation of contaminants. The structure of microeukaryotic communities in sediments and groundwater from this aquifer was examined in relation to bacteria community structure and environmental factors, particularly with the presence of landfill leachate pollution.

Chapter 1 provides an introduction to this thesis where a background on the importance of groundwater is presented, along with the threatening sources of groundwater pollution, of which landfills receive primary attention, and the possible ways of natural attenuation of contaminants in the subsurface. This chapter contains also a brief description of the research location and employed methodology.

The structure of sediment-associated eukaryotic communities was examined using culture-independent methods in **Chapter 2** in relation to bacteria community and the heterogeneity in environmental factors. Eukaryotes were detected all over the aquifer; their diversity was high and community structure complex. The eukaryotic community structure did not seem to relate to depth in the aquifer or distance from the landfill. The diversity in eukaryotic 18S rRNA genes decreased with sampling depth, and was significantly lower in polluted samples than in clean samples. The eukaryotic, and also bacteria, community profiles associated more to each other in polluted sediment samples than in clean samples. The bacteria community profiles obtained in surface

sediment samples clustered together and separately from those in subsurface sediments. The belowground bacteria profiles clustered location-specific. No significant autocorrelation of bacteria or eukaryotic communities in sediments was observed over 1-m depth intervals per sampling location. This may suggest that sampling should be performed at smaller scales than 1 m, if one aims at fully characterizing the microbial communities in heterogeneous aquifers like that from Banisveld. Spatial heterogeneity in sediment-associated microbial communities was apparent and seemed much larger than that in groundwater. Spatial heterogeneity can complicate the assessment of microbial community structure and functioning, but in turn it provides better opportunities for natural attenuation. While transported with the groundwater flow, the landfill leachate contaminants will pass over more types of microorganisms in a heterogeneous environment, and thus have greater chances of being degraded, in comparison with more homogeneous settings.

As the general fingerprinting method (18S rRNA gene based Denaturing Gradient Gel Electrophoresis) used in Chapter 2 cannot indicate which types of organisms are present, in **Chapter 3**, culture-independent phylogenetic analysis of 18S rRNA gene fragments and culturing were employed on six sediment samples belonging to the same set as used in Chapter 2. A diverse microeukaryotic community was revealed in five sediment samples obtained from 3 to 5 m depth along a transect (21-68 m) downgradient of the landfill, and from one reference location unaffected by the leachate. The microeukaryotic community consisted of heterotrophic nanoflagellates, yeast-like fungi and green algae. Fungal sequences, especially belonging to yeasts of the *Basidiomycota*, dominated most clone libraries. Sequences most closely related to *Ascomycota*, *Chytridiomycota* and *Zygomycota* had also been encountered. Green algae (*Chlorophyta*) were detected in polluted locations close (<30 m) to the landfill, and were believed to have been introduced in this aquifer by the percolating rain water or other surface recharges, as this type of green algae were not described to be capable of growth in absence of light. The bacterivorous nanoflagellate *Heteromita globosa* (*Cercozoa*) was retrieved in aerobic and anaerobic enrichments, and its sequences dominated the clone library derived from the polluted aquifer at 5 m depth, and 21 m downgradient of the

landfill. The presence and activity of these bacterivorous protists can be of high importance in bioremediation of polluted environments by predating on bacteria and subsequent recycling of limiting nutrients. The number of culturable eukaryotes ranged from 10^2 to 10^3 cells/g sediment, whereas culture-independent quantification revealed slightly higher numbers. Groundwater mesofauna was not detected in the aquifer; the absence of larger stygobiontic invertebrates was due to the fine aquifer sediments, lack of oxygen and presence of landfill leachate pollution. The food web in the polluted Banisveld aquifer is simple; the food chains are short consisting of prokaryotes and fungi as decomposers of organic matter and protozoa as primary consumers of prokaryotes, and top predators in this particular groundwater ecosystem.

While in Chapter 2 large spatial heterogeneity was apparent in sediment characteristics and related microbial community, in **Chapter 4** the spatial and temporal heterogeneities and dynamics of microbial communities were studied in groundwater samples, with the aim of identifying whether the variation of bacteria and eukaryotic communities in time and space related to the overall spatial and temporal hydrochemical changes. The core plume of pollution was hydrochemically rather stable in time; similar pollution levels were detected in the plume in the interval 1998-2004. The plume upper fringe appeared to have moved up to the surface, especially at distances greater than 48 m from the landfill. Complex and heterogeneous bacterial and eukaryotic communities were resolved using DGGE of 16S and 18S rRNA gene fragments. Over the period 1998 to 2004, large fluctuations were noted in the eukaryotic communities associated with polluted and clean groundwater. The bacterial profiles of polluted samples were more similar to each other than to those in clean groundwater in 1998 and 1999, but no longer in 2004. Unlike the eukaryotic profiles, the 1998 bacteria profiles in polluted groundwater samples were more related to each other than to those recovered from polluted samples obtained in 1999 and 2004. The temporal variation in bacteria and eukaryotic communities was greater than the spatial variation at all sampled locations in the aquifer. Pollution with landfill leachate seemed to have a smaller contribution to the distribution of microbial communities in the

aquifer, in comparison with temporal fluctuations and heterogeneity in their community structure and environmental settings.

The presence or protists (i.e. *Heteromita globosa*) was evident in contaminated part of the aquifer at Banisveld (Chapter 3), a role in bioremediation was suggested. **Chapter 5** of this thesis presents the combined influence of predation by another bacterivorous flagellate (i.e. *Ochromonas* sp. DS) and type of nutrient limitation on biodegradation of organic matter. Mathematical modeling, subsequently confirmed experimentally, indicated that predation by this flagellate can enhance carbon mineralization, and leads to higher overall activity (activity per unit sample), under nitrogen limiting conditions. Under carbon limitation, predation had a negative influence on organic matter degradation, in comparison with when predation was absent. The information obtained in this chapter can be of significance for the degradation of organic contaminants in oligotrophic ecosystems (e.g. aquifers), where nutrients like nitrogen or phosphorus become in general rapidly depleted. By their predation on bacteria, protozoa can recycle limiting nutrients and may stimulate indirectly pollutant biodegradation.

Chapter 6 of this thesis makes a synthesis and discusses the obtained results. The groundwater environment from Banisveld experienced pollution threatening by landfill leachate for over four decades. This ecosystem appears to be resilient to landfill leachate pollution; ecosystem resilience is described here as the ability of ecosystems to cope with pollution threatening, and recover to a functional state similar to that prior to disturbance. The polluted aquifer at Banisveld possesses the potential for biodegradation of pollutants. The large heterogeneity in sediment characteristics and microbial community structure can be favorable to natural attenuation. Degrading microorganisms (bacteria and fungi) are present and capable of degrading organic materials. Protozoan predation on bacteria and subsequent recycling of limiting nutrients, are factors that may enhance the biodegradation of organic pollution in the aquifer. Several ecological aspects may make however this groundwater ecosystem quite vulnerable to pollution. The groundwater ecosystem is usually oligotrophic, with communities little or not adapted to large fluctuations in environmental conditions in their

habitat. As the groundwater food webs are in general simple with short food chains, large organic charge like that in the Banisveld aquifer may lead to a disturbed ecosystem with unstable communities.

Vuilstort perkolaat in de ondergrond - plek van complexe en dynamische eukaryotische gemeenschappen

Samenvatting

Onderzoek naar de natuurlijke afzwakking van vervuiling in grondwater heeft zich voornamelijk bezig gehouden met de aanwezigheid en de vermogens van prokaryotische microorganismen om de hoeveelheid en giftigheid van de verontreinigingen te reduceren. Het voorkomen en de mogelijke rol van eukaryoten in de afbraak van vervuiling heeft tot nu toe weinig aandacht gekregen. Dit proefschrift legt de nadruk op de aanwezigheid van eukaryoten in een vervuilde, waterhoudende grondlaag stroomafwaarts van een vuilnisstortplaats (Banisveld, Nederland) en bespreekt hun mogelijke bijdrage aan de natuurlijke afzwakking van verontreiniging. De structuur van microeukaryotische gemeenschappen in sedimenten en grondwater van deze waterhoudende grondlaag werd bestudeerd in relatie tot de structuur van bacteriële gemeenschappen en omgevingsfactoren, in het bijzonder de aanwezigheid van vuilstort perkolaat.

Hoofdstuk 1 geeft een introductie van dit proefschrift. De achtergrond van het belang van grondwater wordt gepresenteerd, samen met de dreigingen van grondwatervervuiling, waarbij vuilnisstortplaatsen met name aandacht krijgen, en de mogelijke natuurlijke afzwakking van verontreinigingen in de ondergrond. Dit hoofdstuk bevat ook een korte beschrijving van de onderzoekslocatie en de gebruikte methodes.

De structuur van eukaryotische gemeenschappen die aan het sediment verbonden zijn, werd bestudeerd met behulp van cultuuronafhankelijke methodes in **Hoofdstuk 2**, en in relatie gebracht met de bacteriële gemeenschap en de heterogeniteit in milieufactoren. Eukaryoten werden in de gehele water-verzadigde grondlaag aangetroffen; hun verscheidenheid was hoog en de structuur van de gemeenschap complex. De structuur van de gemeenschap van eukaryoten leek geen relatie te hebben met de diepte in de waterige grondlaag of de afstand

tot de vuilnisstortplaats. De verscheidenheid in eukaryotisch 18S rRNA genen nam af met de diepte waarop monsters werden genomen, en was significant lager in vervuilde monsters dan in schone monsters. De eukaryotische en bacteriële gemeenschaps profielen lijken meer op elkaar in monsters van vervuilde sedimenten dan in schone monsters. De bacteriële gemeenschaps profielen, verkregen in oppervlakte monsters clusteren samen, en zijn duidelijk verschillend van die uit sediment-monsters genomen onder het oppervlak. De profielen van bacteriën onder het grondoppervlak clusteren locatie afhankelijk. Er werd geen significante autocorrelatie gevonden van bacteriële of eukaryotische gemeenschappen in sedimenten over 1 meter diepte intervals per monsterlocatie. Dit suggereert dat monstername mogelijk zou moeten worden uitgevoerd op een kleinere schaal dan 1 meter, wanneer het doel is de microbiële gemeenschappen in heterogene ondergrond zoals die bij de Banisveld stort volledig te karakteriseren. Ruimtelijke heterogeniteit in microbiële gemeenschappen in sediment werd waargenomen en leek veel groter dan die voorheen waargenomen in grondwater. Ruimtelijke heterogeniteit kan het interpreteren van de structuur en het functioneren van microbiële gemeenschappen bemoeilijken, maar levert daarentegen ook betere mogelijkheden op tot natuurlijke afzwakking. Tijdens hun transport met de grondwaterstroom zullen de vervuiling uit vuilnisstortplaatsen meer typen microorganismen in een heterogene omgeving tegenkomen, en zo een grotere kans hebben afgebroken te worden vergeleken met meer homogene plaatsen.

Omdat de algemene methode van fingerprinting (op 18S rRNA gen gebaseerde Denaturing Gradient Gel Electroforesis) zoals gebruikt in Hoofdstuk 2 geen informatie verschaft welke typen organismen aanwezig zijn, werden in **Hoofdstuk 3** cultuur onafhankelijke fylogenetische analyses van 18S rRNA fragmenten en cultures toegepast op zes sedimentmonsters, die behoorden tot dezelfde set die gebruikt was in Hoofdstuk 2. Een diverse microeukaryotische gemeenschap werd waargenomen voor vijf sedimentmonsters die verkregen waren op een diepte van 3 tot 5 meter over een transect (21 tot 68 meter) in een neerwaartse gradiënt vanaf de vuilnisstortplaats, en van een referentielokatie die niet vervuild was. De microeukaryotische gemeenschap bestond uit heterotrofe nanoflagellaten, gistachtige schimmels en groene algen. Schimmelsequenties, vooral behorende tot

de gisten van de *Basidiomycota*, overheersten de meeste kloonbanken. Sequenties die het meest leken op die behorende tot *Ascomycota*, *Chytridiomycota* en *Zygomycota* werden ook aangetroffen. Groene algen (*Chlorophyta*) werden aangetroffen in vervuilde locaties dicht bij (<30 meter) de vuilnisstortplaats, en aangenomen werd dat zij in de waterige grondlaag waren geïntroduceerd door infiltratie van regenwater of ander oppervlakte water, daar beschreven is dat dit type van groene algen niet in staat zou zijn te groeien in de afwezigheid van licht. De bacterie-etende nanoflagellaat *Heteromita globosa* (*Cercozoa*) werd aangetroffen in aërobe en anaërobe verrijkingscultures, en zijn sequenties domineerden de kloonbank die was verkregen van de vervuilde ondergrond op een diepte van 5 meter en op 21 meter afstand van de vuilnisstortplaats. De aanwezigheid en activiteit van deze bacterie-etende protisten kunnen van groot belang zijn bij het biologisch herstel van vervuilde milieus door de predatie op bacteriën en de daarmee samenhangende recycling van beperkende voedingsstoffen. Het aantal kweekbare eukaryoten varieerde van 10^2 tot 10^3 cellen/gram sediment, terwijl cultuur onafhankelijke kwantificering wat hogere aantallen opleverde. Er werd geen grondwater mesofauna aangetroffen; de afwezigheid van grotere stygobiontische invertebraten was zeer waarschijnlijk een gevolg van de fijne korrel-structuur van de sedimenten, gebrek aan zuurstof en de aanwezigheid van vervuiling. Het voedselweb in de vervuilde waterige grondlaag van Banisveld is eenvoudig; de voedselketens zijn kort en bestaan uit prokaryoten en schimmels als afbrekers van organisch meteriaal en protozoa als eerste consumenten van prokaryoten en top predators in dit grondwater ecosysteem.

Terwijl in Hoofdstuk 2 grote ruimtelijke heterogeniteit aanwezig scheen in de karakteristieken van sedimenten en aanverwante microbiele gemeenschappen, werden in **Hoofdstuk 4** de ruimtelijke en tijdelijke heterogeniteit en dynamiek in microbiële gemeenschappen bestudeerd in grondwatermonsters, met als doel er achter te komen of de variatie van bacteriële en eukaryotische gemeenschappen in tijd en ruimte afhing van hydrochemische veranderingen over tijd en ruimte. Het centrum van de vervuilingspluim was hydrochemisch vrij stabiel in de tijd; vergelijkbare niveaus van vervuiling werden aangetroffen in het interval 1998-2004. De bovenrand van de pluim van vervuiling bleek

naar het oppervlak te zijn opgeschoven, in het bijzonder op afstanden groter dan 48 meter van de vuilnisstortplaats. Complexe en heterogene bacteriële en eukaryotische gemeenschappen werden waargenomen, op basis van DGGE van 16S en 18S rRNA genfragmenten. Over de periode 1998 tot 2004 werden grote fluctuaties in de eukaryotische gemeenschappen in zowel vervuild als schoon grondwater. De profielen van bacteriën van vervuilde monsters leken meer op elkaar dan die in schoon grondwater in 1998 en 1999, maar in 2004 niet meer. In tegenstelling tot de profielen van de eukaryoten leken de profielen van de bacteriën in vervuild grondwater in 1998 meer op elkaar dan die verkregen waren van vervuilde monsters uit 1999 en 2004. De variatie in de tijd van bacteriële en eukaryotische gemeenschappen was groter dan de ruimtelijke variatie in alle bemonsterde locaties in de ondergrond. Vervuiling door de vuilnisstortplaats leek een relatief kleinere bijdrage te hebben op de verspreiding van microbiële gemeenschappen in de waterige grondlaag, in vergelijking met fluctuaties in de tijd en de heterogeniteit in hun gemeenschapsstructuur en milieu-condities.

De aanwezigheid van protisten (bv *Heteromita globosa*) was duidelijk in de vervuilde ondergrond bij Banisveld (Hoofdstuk 3), en zou protozoa zouden mogelijk een rol kunnen hebben in biologische herstel. **Hoofdstuk 5** van dit proefschrift laat de gecombineerde invloed van predatie door een andere bacterie-etende flagellaat (Ochromonas sp. DS), en het type nutriënt limitatie, zien. Wiskundige modeling, vervolgens experimenteel bevestigd, toonde aan dat predatie door deze flagellaat koolstof mineralisatie kan vergroten en tot hogere algehele activiteit (activiteit per eenheid monster) kan leiden onder stikstof-limiterende omstandigheden. Onder koolstof-limiterende omstandigheden heeft predatie een negatieve invloed op de afbraak van organisch materiaal vergeleken met de situatie waarin er geen predatie was. De in dit hoofdstuk verkregen informatie kan belangrijk zijn voor de afbraak van organische vervuiling in oligotrofe ecosystemen (b.v. de waterverzadigde ondergrond), waar voedingstoffen zoals stikstof of fosfor in het algemeen snel uitgeput raken. Door hun predatie op bacteriën kunnen protozoa beperkende voedingsstoffen recyclen en indirect biologische afbraak van vervuilende stoffen stimuleren.

In **Hoofdstuk 6** van dit proefschrift is een synthese van de resultaten in de onderzoekshoofdstukken 2 tot en met 5 gemaakt. De grondwateromgeving van Banisveld heeft de dreiging met vervuiling uit de vuilnisstortplaat meer dan 40 jaar ervaren. Dit ecosysteem lijkt veerkrachtig te zijn in relatie tot deze vervuiling. De veerkracht van het ecosysteem wordt hier beschouwd als het vermogen van ecosystemen om om te gaan met de dreiging van vervuiling en terug te keren naar een functionele staat die gelijk is aan die van voor de verstoring. De vervuilde ondergrond te Banisveld bezit het vermogen om vervuilingen biologisch af te breken. De grote heterogeniteit in de karakteristieken van sediment en microbiële gemeenschapsstructuren kan gunstig zijn voor natuurlijke afzwakking. Afbrekende micro-organismen (bacteriën en schimmels) zijn aanwezig en in staat organische materialen af te breken. Predatie van bacteriën door protozoa en daarop volgende recycling van beperkende voedingstoffen zijn factoren die de biologische afbraak van vervuilingen in de waterige grondlaag mogelijk vergroten. Echter, verscheidene ecologische aspecten maken dit grondwater ecosysteem kwetsbaar voor vervuiling. Grondwater ecosystemen zijn meestal oligotroof met gemeenschappen die weinig of helemaal niet zijn aangepast aan grote fluctuaties in milieuomstandigheden in hun habitat. Omdat de voedselnetwerken in grondwater in het algemeen eenvoudig zijn met korte voedselketens, kan een grote organische belasting zoals die bij de Banisveld vuilstort leiden tot een verstoord ecosysteem met instabiele gemeenschappen.

Scurgerile în subteran dintr-o groapă de gunoi - mediu pentru comunităţi complexe şi dinamice de eucariote

Rezumat

Cercetările asupra atenuării naturale din apele subterane se axează în principal pe evidenţierea prezenţei şi abilităţii organismelor procariote de a reduce masa şi toxicitatea compuşilor poluanţi. Rolul organismelor eucariote în atenuarea naturală a poluanţilor din apele subterane a fost până în prezent ignorat. În teza de faţă, accentul se pune pe prezenţa microrganismelor eucariote într-un acvifer poluat situat în aval de o groapă de gunoi (Banisveld, Olanda) şi se discută posibilele roluri ale acestor microrganisme în atenuarea naturală a poluării. Structura comunităţii microeucariote din sedimentele şi apa subterană ale acestui acvifer a fost examinată in relaţie cu comunitatea bacteriană prezentă şi factorii de mediu, în mod particular cu prezenţa în acvifer a poluării cu scurgeri de la gropa de gunoi amintită.

Capitolul 1 face introducerea tezei şi prezintă importanţa vitală a apelor subterane, a surselor de poluare care le ameninţă, atenţie specială fiind acordată gropilor de gunoi, cât şi posibilele căi de atenuare naturală a poluării în subteran. Acest capitol conţine de asemenea o descriere a zonei de cercetare şi metodologia folosită.

Structura comunităţii eucariote din sedimentele acviferului Banisveld a fost examinată în **Capitolul 2** în relaţie cu comunitatea bacteriană şi factorii de mediu. S-au folosit metode independente de cultivarea microorganismelor. S-a identificat o diversitate mare a organismelor eucariote şi o structură complexă a comunităţii lor. Diversitatea în gene eucariote ARNr 18S a scăzut cu adâncimea de la care au fost prelevate eşantioanele şi a fost semnificativ mai mică în probele poluate decât în cele nepoluate. Profilele comunităţii eucariote şi ale celei bacteriene din sedimentele poluate s-au grupat separat de cele obţinute din probele de sediment nepoluat. Profilele comunităţii bacteriene din sedimentele de la suprafaţă s-au grupat împreună şi separat faţă de cele din subteran,

care, la rândul lor, s-au grupat pe baza locaţiei de unde au fost obţinute. Nu a fost identificată o autocorelaţie semnificativă a comunităţilor eucariote şi bacteriene obţinute din fiecare staţie la intervale de adâncime de 1 m. Acest fapt poate sugera că eşantionarea ar trebui realizată la intervale de adâncime mai mici de 1 m, dacă se urmăreşte caracterizarea completă a comunităţilor microbiene din acvifere heterogene, ca acviferul Banisveld. Heterogenitatea spatială a comunităţilor microbiene din sedimentele acviferului Banisveld a fost evidentă şi mai pronunţată decât cea din apa subterană la aceleaşi staţii de eşantionare. Heterogenitatea spatială poate complica evaluarea structurii şi funcţiilor comunităţilor microbiene, dar în acelaşi timp poate oferi bune oportunităţi pentru atenuarea naturală din subteran. În timpul migrării datorate curgerii apelor subterane, poluanţii din scurgerile de la gropile de gunoi vor întâlni mai multe tipuri de microorganisme într-un mediu heterogen şi astfel, vor avea o şansă mai mare de a fi degradate, în comparaţie cu situaţia dintr-un mediu mai omogen.

Metodele generale de profilare a comunităţilor microbiene, ca şi cea folosită în Capitolul 2 (DGGE - Denaturing Gradient Gel Electrophoresis - asupra genelor eucariote de tip ARNr 18S), nu indică tipul de organisme prezente. De aceea, în **Capitolul 3** s-a folosit analiza filogenetică a genelor eucariote de tip ARNr 18S, cât şi cultivarea organismelor eucariote prezente în şase probe de sediment din acelaşi set de probe folosit în Capitolul 2. În cinci probe de sediment obţinute de la adâncimi cuprinse între 3 şi 5 m de-a lungul unui transect (21-68 m) în aval de groapa de gunoi şi într-una din probele control prelevate dintr-o staţie neafectată de scurgerile de la groapa de gunoi, s-a identificat o comunitate eucariotă diversă, compusă din protozoare flagelate heterotrofe, ciuperci unicelulare de tipul drojdiilor şi alge verzi. Secvenţele fungilor, în special a celor aparţinând drojdiilor bazidiomicete, au dominat majoritatea bibliotecilor de clone realizate. Pe lângă bazidiomicete, au mai fost identificate sevenţe ale ascomicetelor, chitridiomicetelor şi zigomicetelor. Algele verzi (*Chlorophyta*) au fost identificate în staţiile poluate situate la mai puţin de 30 m aval de groapa de gunoi. Prezenţa algelor verzi în acvifer se explică prin introducerea acestora în subteran prin intermediul apelor de percolaţie. Algele mixotrofe (alge verzi care, în absenţa luminii, se hrănesc cu

celule bacteriene) nu au fost identificate în acvifer. Flagelatul bacterivor *Heteromita globosa* (Cercozoa) a fost izolat din probele de sediment poluat şi crescut în culturi aerobe şi anaerobe. Secvenţele acestui flagelat au dominat biblioteca de clone generată din acviferul poluat de la o adâncime de 5 m şi o distanţă faţă de groapa de gunoi de 21 m. Prezenţa şi activitatea acestui protozoar bacterivor poate avea o importanţă notabilă în procesul de bioremediere a mediilor naturale poluate prin prădarea asupra bacteriilor şi recircularea nutrienţilor cu caracter limitativ. Numărul eucariotelor cultivabile a variat între 10^2 şi 10^3 indivizi/g sediment, în timp ce cuantificarea eucariotelor, independentă de cultivarea lor, a oferit valori uşor mai ridicate. Nevertebrate stigobionte (organisme care se întâlnesc numai în apele subterane) nu au fost găsite în acviferul Banisveld, absenţa lor fiind cauzată de sedimentele fine şi puternic compactate, lipsa oxigenului şi incărcăturii organice mari provenite de la groapa de gunoi. Reţeaua trofică din acviferul poluat Banisveld este una simplă; lanţurile trofice scurte sunt compuse din microorganisme procariote şi ciuperci ca descompunători ai materiei organice şi protozoare cu rolul de consumatori primari şi prădatori de vârf pentru acest ecosistem acvatic subteran particular.

Dacă în Capitolul 2 heterogenitatea spaţială a fost evidentă în caracteristicile sedimentelor şi în structura comunităţilor microbiene asociate sedimentelor subterane, în **Capitolul 4** s-a examinat heterogenitatea spaţială şi temporală a comunităţilor microbiene în eşantioane de apă subterană. Scopul acestui studiu a fost de a evidenţia variaţiile în spaţiu şi timp ale comunităţilor eucariote şi bacteriene, asociate cu schimbările hidrochimice generale spaţiale şi temporale din acvifer. Miezul norului de poluare din subteran a fost din punct de vedere hidrochimic relativ stabil în timp. Nivelele de poluare din acest nor au fost asemănătoare în intervalul 1998-2004. Partea superioară a norului de poluare a suferit în timp o deplasare către suprafaţă, în mod special la distanţe mai mari de 48 m faţă de groapa de gunoi. Comunităţile eucariote şi bacteriene au fost evidenţiate folosind aceeaşi metodă de profilare ca în Capitolul 2 (DGGE). Comunităţile eucariote şi bacteriene profilate pentru intervalul 1998-2004 au fost complexe şi heterogene. Schimbări notabile au fost identificate atât în structura comunităţii eucariote din apa subterană poluată, cât şi a celei din apa

subterană nepoluată. Profilele DGGE ale comunităţii bacteriene din probele poluate s-au grupat separat faţă de cele din probele nepoluate în 1998 şi 1999. Acest aspect nu a mai fost evident în 2004. Spre deosebire de profilele comunităţii eucariote, profilele bacteriene din probele de apă subterană poluată obţinute în 1998 s-au grupat separat de cele din probele de apă subterană poluată eşantionată în 1999 şi 2004. Fluctuaţiile temporale ale comunităţilor eucariote şi bacteriene au fost mai pronunţate decât cele spaţiale la toate staţiile de eşantionare din acvifer. Poluarea cu scurgeri de la groapa de gunoi Banisveld a avut o contribuţie mai mică la distribuţia comunităţilor microbiene în acvifer, în comparaţie cu fluctuaţiile temporale şi heterogenitatea din structura acestora şi cea a factorilor de mediu.

Prezenţa protozoarelor (i.e. *Heteromita globosa*) a fost evidentă în partea contaminată a acviferului Banisveld (Capitolul 3), iar rolul acestora în procesul de atenuare naturală a poluanţilor a fost sugerat. **Capitolul 5** prezintă influenţa prădării unui alt protozoar flagelat bacterivor (i.e. *Ochromonas* sp. DS) împreună cu influenţa tipului de limitare în nutrienţi asupra procesului de biodegradare a materiei organice. Modelarea matematică, susţinută apoi experimental, a indicat faptul că prădarea poate spori mineralizarea carbonului organic şi conduce la o activitate bacteriană mai intensă în condiţiile limitării în azot. Când carbonul a fost nutrientul cu caracter limitativ, prădarea a avut o influenţă negativă asupra degradării materiei organice în comparaţie cu situaţia în care factorul prădare a lipsit. Informaţiile obţinute în acest capitol pot avea o semnificaţie importantă pentru degradarea poluanţilor organici în ecosistemele oligotrofe (e. g. acvifere), unde nutrienţi ca azotul şi fosforul se epuizează, în general, rapid. Prin prădarea asupra bacteriilor, protozoarele pot recicla aceşti nutrienţi cu caracter limitativ, stimulând astfel indirect procesul de biodegradare.

Capitolul 6 face o sinteză şi discută rezultatele obţinute. Mediul acvatic subteran de la Banisveld a fost supus poluării cu scurgeri de la groapa de gunoi de peste 40 de ani. Acest ecosistem acvatic subteran pare a fi însă rezilient, rezilienţa fiind descrisă aici ca abilitatea ecosistemului de a suporta ameninţarea poluării şi de a se recupera la un nivel funcţional similar cu cel anterior poluării. Acviferul poluat Banisveld are potenţial

pentru biodegradarea poluanţilor. Heterogenitatea importantă a caracteristicilor sedimentelor şi structurii comunităţilor microbiene poate fi favorabilă atenuării naturale. Microorganismele descompunătoare (bacteriile şi ciupercile) sunt prezente şi capabile de degradarea materiei organice. Prădarea asupra bacteriilor de către protozoare şi reciclarea nutrienţilor cu caracter limitativ sunt factori care pot spori biodegradarea poluării organice din acvifer. Câteva aspecte ecologice pot face, însă, acest ecosistem acvatic subteran vulnerabil la poluare. Ecosistemul acvatic subteran este de obicei oligotrof, cu comunităţi puţin sau deloc adaptate la fluctuaţii mari ale condiţiilor de mediu în habitatul pe care îl ocupă. Reţelele trofice din apele subterane sunt în general simple cu lanţuri trofice scurte; astfel, o încărcătură organică mare, ca şi cea din acviferul Banisveld, poate duce la deranjarea ecosistemului şi la dezvoltarea comunităţilor instabile.

Life history.
Instead of curriculum vitae, few words about me

I was born on May 10, 1977 in Alba Iulia, Romania.

I followed the primary, secondary and high (Colegiul Naţional *Horea, Cloşca şi Crişan*) school in Alba Iulia. My first connection with natural sciences was made in high school where I had followed the section of Chemistry-Biology.

After baccalaureate, at the age of 18, I asked myself:
"Now what? What am I now going to do? What do I know to do? Should I go to university, or should I stay in my home town and follow a preacademic school of dentistry technicians?"

And I answered my question after one month of working in a Coca-Cola deposit as crate manipulator:
"No; I will firstly try passing the admission exam at the University of Cluj-Napoca, and to what other Faculty should I go if not Biology?"

Hence, in the autumn of 1995 I started my studies in Ecology and Protection of Environment at the Faculty of Biology and Geology, Babeş-Bolyai University, Cluj-Napoca. Soon after I have been enlightened and decided to do this type of work, in natural sciences, biology, for the rest of my life. I must confess I did not shine during my studies; my grades were sometimes not too high. And that was probably because some disciplines were either not very exciting, or, maybe more likely, they were not taught in a too exciting manner, or probably I cared less about grades.

I graduated in the summer of 1999 and I made my diploma work on groundwater fauna from a cave (Huda lui Papară, Apuseni Mountains). This was practically my beginnings in the study of subsurface life. And I owe this to my dear colleague Dr. Oana Moldovan who firstly gave me a net and jars for collecting groundwater fauna.

In the autumn of 1999 I started my master courses in System Ecology and Biodiversity Conservation, same Faculty, and finished them in February 2001 with a more limnological dissertation on the zooplankton community of a lake. In the same time, December 1st, 2000, I was appointed as research assistant at the Institute of Speleology "Emil Racoviţă" in Cluj. This was another decisive moment of my life. Here, at the Institute of Speleology, I continued my studies on groundwater fauna and I looked especially to stygobiontic amphipods. I collected fauna from caves, but also from other groundwater habitats, such as the rivers hyporheic or phreatic (streams and wells). Then I could not do much with this fauna but sorting it out of samples and identifying the amphipods. Because…

...because in spring 2001 I got an email from my dear friend Jos Notenboom saying that Henk and Nico want somebody to look for and at the groundwater fauna in a polluted aquifer in Southern Netherlands. An interview at the Vrije Universiteit Amsterdam followed, where I remained simply impressed of such research industry, how I called it at that time, and where I was about to work for the next four years. Then probably the most intensive and nice four years of my life past, when besides others I followed two of my father's advises. He once said "son, follow the school, as you are not good for working the land" and "son, go away and see the world".

Following those four beautiful years, in December 2005, I came back to The Institute of Speleology in Cluj, where after one year and a half I finished writing this thesis.

List of publications

Publications related to my PhD research

1. **Brad**, T., B. M. van Breukelen, M. Braster, N. M. van Straalen, and W. F. M. Röling. *Spatial heterogeneity in sediment-associated bacterial and eukaryotic communities in a landfill leachate-contaminated aquifer*. Submitted
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3. **Brad**, T., M. Braster, B. M. van Breukelen, N. M. van Straalen, and W. F. M. Röling. 2006. *Modern tools for assessing, monitoring and predicting natural attenuation of contaminants in karst*. 14th International Karstological School "Classical Karst" - Sustainable Management of Natural and Environmental Resources on Karst, June 27 - July 2, 2006, Postojna, Slovenia
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5. **Brad**, T., M. Braster, B. M. van Breukelen, N. M. van Straalen, and W. F. M. Röling. 2005. *Relationships and contribution of eukaryotes to subsurface contaminant biodegradation*. Netherlands Scientific Symposium "Soil and Water", June 1-2, 2005, Zeist, The Netherlands
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