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Microbial Biofilms in Groundwater Ecosystems

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Because in the end,
you won't remember
the time you spend
working in the office,
or mowing your lawn.
Climb that goddamn mountain.

Jack Kerouac

Abstract

Microbial biofilms control biogeochemical processes and the fluxes of organic carbon in most aquatic and terrestrial ecosystems, thereby effecting ecosystem services and human health. In groundwater, a variety of attached microbial aggregates have been described to date. These descriptions have determined that these aggregates depend mainly on nutrient inputs and water flow. Nevertheless, a comprehensive understanding of groundwater biofilm community structure and functioning is still lacking. The 'Biofilm Initiative', funded by the HelmholtzZentrum München, was initiated as a multidisciplinary network studying microbial biofilms in biological systems relevant to environmental and human health. Within this network, this specific thesis project has addressed three central hypotheses concerning the importance of microbial biofilms in ground- and drinking water systems: (i) reactive mineral surfaces can alleviate nutrient limitations and select for specific communities of attached microbes in aquifers; (ii) cave systems with upwelling reduced waters offer a unique gradient habitat for the development of lithotrophic biofilms; (iii) attached microbes in drinking water wells provide specific ecological niches affecting the spread and survival of microbes in drinking water systems.

In this thesis, I aimed to characterize natural groundwater biofilm communities, to identify key bacterial constituents, and to examine their role in groundwater ecosystem functioning. Biofilm communities were investigated using microscopic and cultivation-based approaches, as well as by PCR amplification of diverse taxonomic or functional marker genes. T-RFLP fingerprinting, amplicon sequencing and quantitative polymerase chain reactions (qPCR) were applied. Hydrogeochemical characteristics of biofilm systems were recorded by elemental analysis, compound-specific isotope analysis (CSIA), gas chromatography and basic water chemistry (ion chromatography, dissolved organic carbon analysis).

Despite their importance, biofilms have hardly been studied in nutrient deprived, oligotrophic systems such as pristine aquifers. In the first experiment of my thesis, distinct and potentially reactive mineral coupons were exposed in a natural aquifer, after which the microbes attached to different mineral surfaces were tested for different metabolic capabilities and variations of community composition. A flow-through mesocosm was designed to expose the mineral coupons in natural groundwater, allowing for colonization of the substratum over the course of 15 months. Four different minerals (apatite, pyrite, magnetite and granite) were selected as representing potential sources of nutrients, electron donors and electron acceptors. T-RFLP fingerprinting, used in combination with pyrotag sequencing of the bacterial 16S rRNA genes, allowed for the comparison of composition and identification of key bacterial populations within

attached communities. Biofilms were also removed from the mineral surfaces and used to inoculate a carbon substrate utilization test array (Ecoplate Biolog). Imaging of colonized and weathered mineral surfaces via scanning electron microscopy (SEM) allowed for detailed visualizations of the attached microorganisms. The microbial communities attached to the mineral surfaces showed only minor distinctions, except for the sulfide mineral biofilms, where a high abundance of *Zoogloea* spp. was detected. Mineral weathering was observed for some substrata but no continuous coverage of the surfaces was discovered. Although phosphate limitation was apparent within the aquifer, the apatite minerals did not modify the communities compared to non-phosphate bearing minerals. Substrate usage patterns differed even between highly similar communities but biofilm activities were generally low. Surprisingly, the biofilm on pyrite displayed the lowest substrate uptake potential. Even though it had the most distinct community, highest cell abundance and greatest sign of weathering, it consumed the least carbon substrates. The results indicate that inorganic electron donors (sulfides) not specific nutrients (phosphate), have the potential to alleviate growth limitations and select for distinct attached microbiota in oligotrophic aquifers. Thus, strict electron donor limitation in pristine aquifers has the potential to override all other potential benefits of attached growth for microbes.

The second topic of this thesis focuses on massive microbial biofilms formed in a cave fed by an iodine-rich, former medicinal spring with high thermogenic methane loads stemming from deep formation water. The massive biomass produced by the microbes and exopolysaccharide (EPS) slime completely covers the walls and ceilings of the cave, the latter bearing bacterial snottites of up to 10 cm in length. However, the nature of these unique subsurface biofilms, as well as their ecophysiology, has not been investigated to date. Here, I provide primary evidence for the role of methane and potentially also methyl halides (iodomethane) as electron donors in this unique habitat. A surprisingly diverse microbial community was found within these biofilms, indicating a complex network of bacteria and archaea within the sticky matrix. Sequencing revealed distinct methanotrophic and methylotrophic populations within the *Alpha-*, *Beta-* and *Gammaproteobacteria*, which dominated the biofilms. *Methylophilaceae* and *Methylococcaceae* were abundant on the walls and especially in the cavern water but not in ceiling biofilms. Here, ribosomal and functional gene analysis hinted at methylotrophic and potentially also iodine-cycling populations, supporting a postulated production and oxidation of iodomethane in this system. A functional gene analysis of the methyltransferase (*cmuA*) also supported the presence of methyl halide-oxidizing bacteria. Geochemical gradients in the cave were reflected within biofilms and stoichiometric composition gave insights into biochemical fluxes from the spring to the cavern exit. In contrast to our initial expectation, biofilms seemed to largely draw on methane as a

source of energy and not as a source of carbon. In fact, the massive EPS formation suggested its use as an electron sink by the biofilms in face of severe nutrient limitations. In conclusion, primary insights into a dominantly chemolithoautotrophic biofilm system are revealed, largely independent from phototrophic carbon and energy inputs and just several meters under our feet.
The third hypothesis was addressed by sampling microbiota from an active drinking water production system close to the city of Munich. Microbial monitoring of drinking water production and distribution systems is essential to assure water quality and for risk prediction. Chemical and biological characteristics of the produced drinking water are checked regularly; however, microbiological data is still predominantly based on classical culturing tests. Here, intrinsic groundwater microbiota were investigated before, during and after the mechanical restoration of an active drinking water production well. High pressure jetting and hydrofracturing are procedures routinely used to alleviate well clogging by biofilms and fine sediments. Variations were observed in bacterial communities between different wells of the same production system before maintenance, despite the wells having practically identical water chemistries. This may have reflected the distinct usage practices of the different wells, and also local aquifer heterogeneity. In contrast, well jetting preferentially purged only a subset of the dominating taxa, including lineages related to *Diaphorobacter*, *Nitrospira*, *Sphingobium*, *Ralstonia*, *Alkanindiges*, and *Janthinobacterium* spp, suggesting their tendency for growth in well-associated biofilms. Lineages of potential hygienic drinking water concern (i.e. *Legionellaceae*, *Pseudomonadaceae*, and *Acinetobacter* spp.) reacted distinctly to hydraulic jetting. Bacterial diversity was markedly reduced in drinking water two weeks after the cleaning procedure. The results of this study provide a better understanding of drinking water wells as a microbial habitat, as well as their role of attached microbes in this ultra-oligotrophic habitat as a seed bank for drinking water networks.
In summary, this thesis comprises a multifaceted approach to studying biofilm structures and their ecology in groundwater systems. Their role in the turnover of organic and inorganic substrates, as well as a potential refuge for drinking water pathogens, is dissected in a comprehensive manner. These insights substantiate the largely neglected relevance of biofilms in groundwater ecosystems, which is a crucial advance in our current perspective of the functional diversity and biogeochemical fluxes in our societies' most important drinking water resource

Zusammenfassung

Viele biogeochemische Prozesse und Stoffflüsse in aquatischen und terrestrischen Ökosystemen sind durch mikrobielle Biofilme kontrolliert. Im Grundwasser wurden bereits einige dieser sessilen mikrobiellen Gemeinschaften untersucht, allerdings vorwiegend in Bezug auf ihre Abhängigkeit von Nährstoffeinträgen und hydrologischen Parametern. Ein umfassenderes ökologisches Verständnis von Biofilmen im Grundwasser, ihrer Zusammensetzung und ihrer Funktionsweise fehlt jedoch bislang. Trotz ihrer Bedeutung für die Wasserqualität wurden Biofilme in oligotrophen Systemen wie Grundwasser noch kaum charakterisiert. Zur Untersuchung mikrobieller Biofilme und deren Relevanz in Umwelt und Gesundheit wurde deshalb in 2009 am Helmholtz Zentrum München ein multidisziplinäres Forschungsprojekt initiiert, die sog."Biofilm Initiative".

Die vorliegende Doktorarbeit behandelt drei zentrale Hypothesen, die speziell für mikrobielle Biofilme in Grund- und Trinkwasser relevant erscheinen: (i) der Aufwuchs auf reaktiven mineralischen Oberflächen kann wesentliche Nährstofflimitierungen im Grundwasser abmindern, somit ist eine spezifische Selektion anhaftender mikrobieller Gemeinschaften zu vermuten; (ii) Biofilme in oberflächennahen Grundwasser- und Quellsystemen sind vor allem an geochemischen Gradienten zu vermuten, dominiert durch lithotrophe Mikroben; (iii) Biofilme in Trinkwassersystemen stellen eine ökologische Nische dar, die die Ausbreitung und das Überleben von Mikroorganismen im Trinkwasser beeinflusst.

Um diese Hypothesen zu hinterfragen, wurden in dieser Doktorarbeit natürliche Grund- und Trinkwasser-Biofilme in mehreren Systemen charakterisiert, sowie ihre biogeochemische Funktion in den Habitaten untersucht. Anhand mikrobiologischer Methoden wie der Kultivierung von Mikroben oder des Nachweises verschiedener taxonomischer und funktioneller Marker-Gene über PCR wurden die Zusammensetzung der Biofilme dargestellt. Die Struktur der Gemeinschaften wurde mit molekularen Methoden (genetische Fingerabdrücke, Sequenzierung mikrobieller Marker-Gene, sowie mittels quantitativer PCR) untersucht. Die hydrogeochemischen Eigenschaften der Biofilme wurden über die Analyse ihrer elementaren Zusammensetzung und Isotopensignatur, sowie durch Gas- und Ionenchromatographie charakterisiert.

Im ersten Experiment wurden in einem natürlichen Grundwasserleiter verschiedene reaktive mineralische Aufwuchsoberflächen exponiert. Über 15 Monaten erfolgte eine Besiedlung dieser Oberflächen in einem von Grundwasser durchströmten Mesokosmos. Vier verschiedene Mineralien (Apatit, Pyrit, Magnetit und Granit) wurden aufgrund ihres Potentials, entweder als

Nährstoffquelle, Elektronendonor oder Elektronenakzeptor zu fungieren ausgewählt. Danach wurden die aufgewachsenen Biofilme bezüglich ihrer Zusammensetzung sowie unterschiedlicher metabolischer Potentiale verglichen.

Eine Kombination von genetischen Fingerabdrücken und Gensequenzierung ermöglichte dabei eine vergleichende Identifizierung der wichtigsten bakteriellen Biofilm-Populationen.Der Umsatz verschiedener Kohlenstoffsubstrate durch die Biofilme wurde mit dem Ecoplate Assay (Biolog) untersucht. Besiedelte mineralische Oberflächen wurden mittels Elektronenmikroskopie visualisiert. Mit Ausnahme Biofilme auf sulfidischem Substrat (Pyrit), waren die aufgewachsenen mikrobiellen Gemeinschaften sehr ähnlich. Erstere waren vor allem durch *Zoogloea* spp. dominiert. Obwohl die Besiedlung oft nur spärlich war, zeigte sich an einigen Oberflächen eine starke Verwitterung der Mineralien. Trotz einer nachgewiesenen Phosphat-Limitierung im untersuchten Grundwasserleiter zeigte die Besiedlung der Apatit-Oberflächen keiner im Vergleich zu den nicht-phosphathaltigen Mineralien veränderte Gemeinschaft. Unterschiedliche Muster der Substratnutzung wurden trotz generell geringer Aktivität der Biofilme sogar zwischen strukturell ähnlichen Gemeinschaften identifiziert. Obwohl die stärkste Besiedlung und Oberflächenverwitterung auf Pyrit zu verzeichnen war, zeigten die Biofilme hier das geringste Spektrum an verwertbaren Substratquellen. Offensichtlich wirkte Pyrit als anorganischer Elektronendonor selektiv auf die aufwachsenden Mikroorganismen, während mineralische Nährstoffquellen (Apatit) keine Veränderung der mikrobiellen Gemeinschaft bedingten.

In einer zweiten Studie wurden Biofilme in einem von jodhaltigem Formationswasser gespeisten Quellsystem untersucht. Die Wände und die Decke dieses einzigartigen Habitats sind komplett durch Biofilme bedeckt. An der Decke bilden sich hängende, tropfenförmige Schleimfäden, sogenannte "Snottiten" von bis zu zehn Zentimeter Länge. Obwohl dieses Phänomen bereits seit Jahrzehnten bekannt ist, sind die Ursachen für das Wachstum dieser einzigartigen mikrobiellen Gemeinschaften und deren Ökophysiologie noch nicht verstanden. In der vorliegenden Doktorarbeit wurde erstmals der Nachweis erbracht, dass thermogenes Methans als primärer Elektronendonor von diesen Biofilmen genutzt wird. Ein komplexes mikrobielles Netzwerk aus Bakterien und Archaeen wird für die Snottiten erstmals beschrieben. Mehrere bekannte methanotrophe und methylotrophe Populationen innerhalb der *Alpha*-, *Beta*-, und *Gammaproteobakterien* wurden identifiziert. An den Wänden und besonders im Quellwasser waren *Methylophilaceae* und *Methylococcaceae* dominant, während sie an der Decke kaum nachzuweisen waren. Zudem wird ein möglicher Jod-Kreislauf in der Höhle postuliert, in dem mögliche Jodmethan-oxidierende Populationen eine Rolle spielen könnten. Geochemischen Gradienten innerhalb der Höhle waren in der stöchiometrischen Zusammensetzung der Biofilme reflektiert.

Damit ergeben sie Hinweise auf die biogeochemischen Stoffflüsse von der Quelle bis zum Höhlenabfluss. Diese Ergebnisse beschreiben erstmals ein von chemolithoautotrophen Biofilmen dominiertes oberflächennahes Grundwasser-Ökosystem, welches größtenteils unabhängig von rezenten Kohlenstoff- und Energieeinträgen aus der Biosphäre ist.
Im dritten Projekt wurden die mikrobiellen Gemeinschaften einer aktiven Trinkwasserversorgungsanlage im Münchner Umland untersucht. Von behördlicher Seite werden hier regelmäßig mikrobiologische Untersuchungen über klassische Kultivierungs-Ansätze durchgeführt. In diesem Projekt sollten nun aber die intrinsischen bakteriellen Gemeinschaften mittels Hochdurchsatz-Sequenzierung erstmals abgebildet werden. Zudem wurden Proben eines Trinkwasserbrunnens während einer hydraulischen Reinigung untersucht. Mittels solcher Hochdruck-Verfahren werden Brunnen regelmäßig von Biofilmen und Feinsedimenten befreit. Die untersuchten Brunnen wiesen trotz einheitlicher wasserchemischer Parameter sehr unterschiedliche bakterielle Gemeinschaften auf. Dies könnte möglicherweise unterschiedliche Nutzungsprofile der Brunnen sowie lokale Heterogenitäten im Grundwasserleiter wiederspiegeln. Ein gewisser Anteil der zuvor dominanten Taxa (z.B. *Diaphorobacter*, *Nitrospira*, *Sphingobium*, *Ralstonia*, *Alkanindiges* und *Janthinobacterium* spp.) wurde durch die Reinigung entfernt. Dies weist auf deren bevorzugte Ansiedlung im Brunnenbereich, z.B. in Biofilmen hin. Taxa mit potentiell pathogenen Organismen (z.B. *Legionellaceae*, *Pseudomonadaceae*, und *Acinetobacter* spp.) wurden durch die hydraulische Behandlung nur teilweise eliminiert, was auf ihren Ursprung aus dem umgebenden Grundwasserleiter hinweist. Die Diversität der Bakterien im Brunnen war zwei Wochen nach der Reinigung deutlich reduziert. Die Ergebnisse dieser Studie tragen zum besseren Verständnis von Trinkwasserbrunnen als mikrobielles Habitat bei. In diesen ultra-oligotrophen Systemen stellen Biofilme eine wichtige Überdauerungsstrategie der Mikroben dar.
Zusammenfassend wurden in dieser Doktorarbeit Biofilm-Strukturen und deren Ökologie in unterschiedlichen Grundwassersystemen mit vielfältigen methodischen Ansätzen charakterisiert. Ihre Rolle im Umsatz organischer und anorganischer Substrate, sowie ihre Funktion als Refugium möglicher pathogener Mikroben im Trinkwasser wurde untersucht. Die hier gewonnen Erkenntnisse belegen die große Bedeutung mikrobieller Biofilme für Stoffkreisläufe im Grundwasser. Somit wird ein wesentlicher Beitrag zum besseren Verständnis mikrobiologischer Funktionen und bestimmender Faktoren der Wasserqualität in oberflächennahen Grundwassersystemen geleistet.

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1 Introduction

This PhD thesis was conducted at the Institute for Groundwater Ecology (IGOE), as part of the "Biofilm Initiative", started by the Helmholtz Zentrum München, in 2009. Here, I present a groundwater perspective of microbial biofilms in the framework of systems ecology. The relevance of these microbial assemblages for ecosystem services and human health is discussed. First, a general description and definition of biofilms in natural and anthropogenic systems is given. Important properties of the biofilm lifestyle are further emphasized. The relevance and impact of microbial biofilms in groundwater ecosystems is presented and previous studies on this subject are discussed in detail. Furthermore, I give an overview on state-of-the-art in biofilm research linked to water quality. Research gaps related to biofilms in groundwater systems are described and consequently approached in three different experimental and field settings.

1.1 Defining Microbial Biofilms

Most microbial processes occurring in the environment are achieved through collective activities of microbial communities (Wolfaardt *et al.*, 1994, Moller *et al.*, 1998). Microbial consortia and communities attached to a surface in a spatially defined manner are termed biofilms (Figure 1- 1).

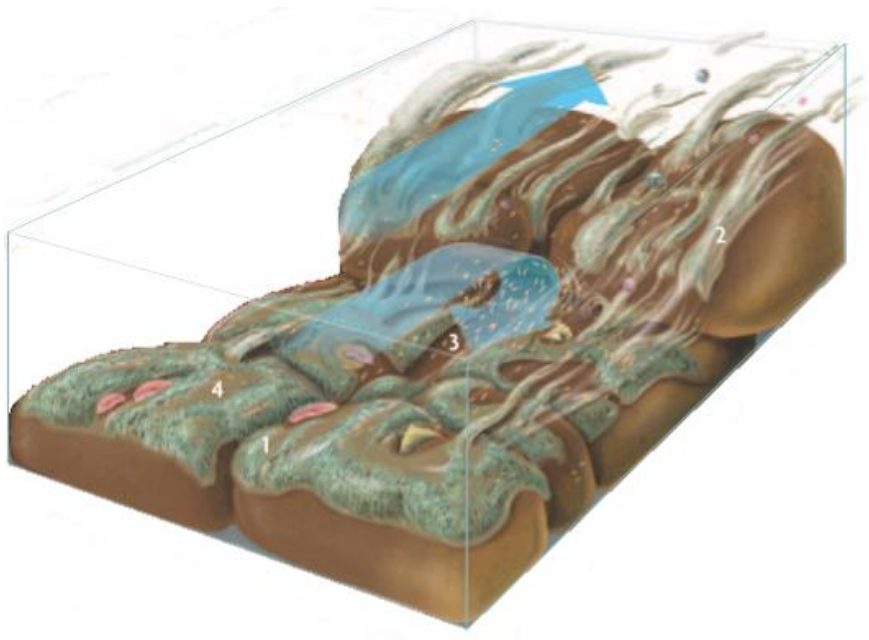


Figure 1- 1 Microbial biofilms display different structural characteristics induced by several abiotic and biotic factors such as slow flow (1), turbulent flow (2), as well as the dispersal and colonization (3) and grazing, creating a spatially heterogeneous landscape. Modified from Battin *et al.* (2007)

The IUPAC defines a biofilm as "an aggregate of microorganisms in which cells that are frequently embedded within a self-produced matrix of extracellular polymeric substance (EPS) adhere to each other and/or to a surface" (Vert *et al.*, 2012). In biofilms, microbial functions are dependent on a complex web of interactions (Hansen *et al.*, 2007). Surface-bound microbial populations and hence, the resulting spatial interdependence, facilitate interaction and mutualism. Individual microbial cells arrange in a way that facilitates interactions amongst themselves and can therefore be seen as an initiation of multi-cellularity (Wolfaardt *et al.*, 1994, Shapiro, 1998). Henrici (1933) was first to observe that aquatic bacteria grow mostly on submerged surfaces and not in the bulk water, describing the deposition of bacteria on exposed surfaces. The advantage of surface attachment for microbes was further recognized in the 1940s (Heukelekian & Heller, 1940, Zobell, 1943). In the seminal work of Claude Zobell (1943), several characteristics inherent to biofilms such as the accumulation and deposition of organic material and nutrients along surfaces and the increase in the local concentration of nutrients which enhance bacterial growth were described (Figure 1- 2).

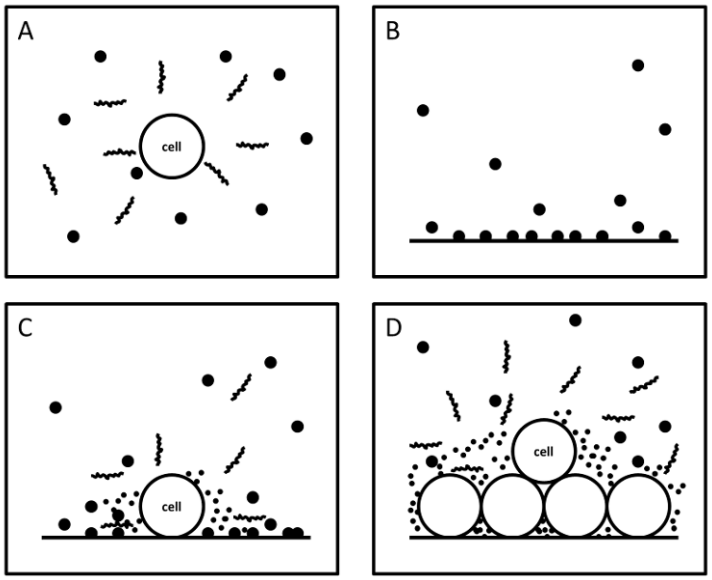


Figure 1- 2 A free-floating bacterial cell surrounded by particulate matter which must be hydrolyzed by exoenzymes (helicoidal line) before the resulting hydrolyzates (dots) can be ingestited and assimilated. **B** Particles of nutrients concentrated in a monomolecular layer in a solid surface. **C** Nutrient particles are more available to the cell on solid surfaces where the interstices at the tangent of the bacterial cell and the solid surface retard the diffusion of exoenzymes and hydrolyzates away from the cell. **D** Multiple cells form additional interstitial spaces. Taken from Zobell (1943).

The term "Biofilm" was first used in a publication in 1975 describing a diverse microbial community attached to a wastewater trickling filter (Mack *et al.*, 1975). A few years later,

Costerton *et al.* (1978) described to a greater audience the scientific shift of attention in applied and environmental microbiology towards the attached microbes and their extracellular glycocalyx. This extracellular matrix, termed EPS, gives a biofilm viscoelastic properties. Matrix polymers not only glue the biofilm to the surface but also enable spatial organization to be imposed on the community (Costerton *et al.*, 1987, McBain *et al.*, 2000). The revelation that attached bacteria differ in their physiological behavior and adaptability towards planktonic cells led to a rapid increase of publications (Figure 1- 3) addressing biofilms in ecology, biotechnology, health and industry.

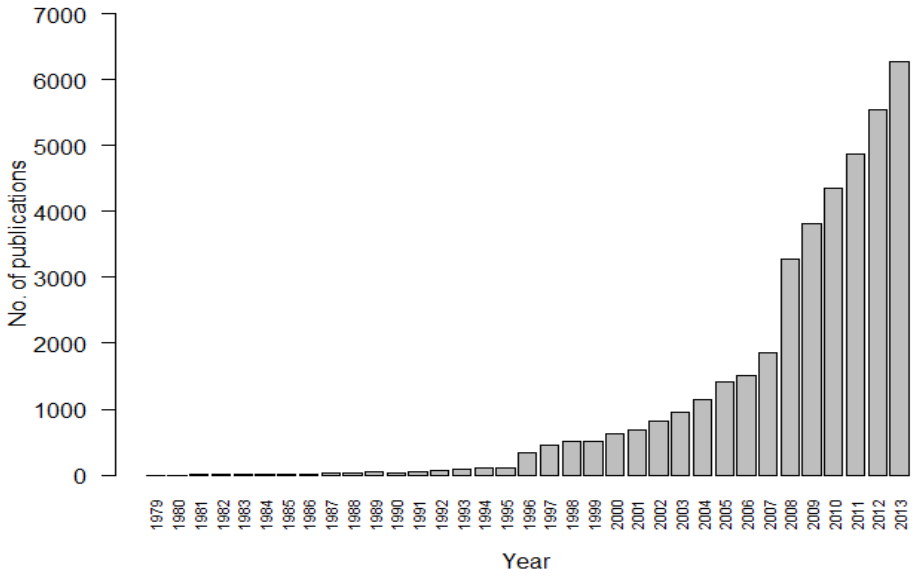


Figure 1- 3 Annual numbers of publications associated with the keyword biofilm, from 1979 – 2013 (Source ISI Web of Knowledge)

Important advances in the understanding of biofilms came from medical sciences studying dental plaques (Rickard *et al.*, 2003, Filoche *et al.*, 2010), and infections of catheters and implants where biofilms can form resistant sheaths (Hall-Stoodley *et al.*, 2004). The intensive study of the human microbiome deals with microbial assemblages in our digestive tracts (Koenig *et al.*, 2011), lungs, and skin (Costello *et al.*, 2009) improving medical treatment and diagnosis of diseases, many of which are related to biofilms. Wastewater treatment processes benefit from micro-gradients created by microbial assemblages (Hidalgo *et al.*, 2009), thereby reducing nutrient loads. Biogeochemical cycles are very much controlled by metabolic reactions induced by microbes that are attached to each other. In soils, bacterial cells will attach or even intrude into plant roots, affecting plant growth and viability. Mineral weathering is facilitated by bacteria, which increases the fertility of soils. It is presumed that the rhizosphere can be stimulated to prevent plant

pathogens and increase crop production (Morris & Monier, 2003). Stream biofilms covering riverbeds are now understood as bioreactors contributing to the turnover of transported carbon loads (Battin *et al.*, 2003a, Battin *et al.*, 2008). Marine snow is likely to be the most extensive biofilm assemblage on Earth, greatly impacting the oceanic carbon cycle (Azam & Malfatti, 2007).

There is even fossil evidence for the formation of biofilms dating back to 3.5 billion years ago, making them likely to be among the first life forms on Earth (Rasmussen, 2000, Altermann & Kazmierczak, 2003, Martin *et al.*, 2008). Biofilms in hydrothermal environments such as hot springs and deep-sea vents are extreme habitats, often harbouring "living fossils" of the most ancient lineages (Reigstad *et al.*, 2010, Williams *et al.*, 2013). In the context of evolution, biofilms provide homeostasis under fluctuating and harsh external conditions facilitating the development of complex interactions between individual cells (Hall-Stoodley *et al.*, 2004).

A plethora of biofilm properties and activities have been studied, providing in-depth knowledge on biofilm formation, the nature of the EPS, interactions and communication, pathogenicity, biofilms in industrial and medical applications, and many others (Hall-Stoodley *et al.*, 2004, Xavier & Foster, 2007, Karatan & Watnick, 2009, Flemming & Wingender, 2010) but research has been mainly based on simplified model systems mimicking nutrient rich environments. The examples of biofilm research presented here discuss ecological and environmental aspects of this microbial mode of life.

In the introduction of this PhD thesis, I provide at first an overview of the state-of-the-art of multispecies microbial biofilm ecology. Special attention is then given to biofilms in groundwater and water quality related issues. Three selected systems that allow addressing fundamental questions of biofilms in groundwater ecology to be addressed are introduced. This thesis aims to elucidate the role of microbial biofilms in groundwater ecosystems. Microbial biofilms and their ecological role are investigated and further discussed in light of their contribution to groundwater quality.

1.2 Biofilm Formation and Structure

Biofilm development includes colonization, maturation, maintenance, and dissolution (O'Toole *et al.*, 2000, Stoodley *et al.*, 2002). Structural development (Figure 1- 4) is therefore the net result of attachment, growth and detachment of microbial biomass, hydrodynamics, and substrate availability, as well as predation e.g. grazing, viral lysis (Battin *et al.*, 2003a). The different dispersal capabilities and microscale landscape patterns of biofilms affect dispersal-assembled communities (Battin *et al.*, 2007). Biofilm community dynamics involve a fine balance between

the forces of attachment and those associated with detachment and colonization resistance of the community (McBain *et al.*, 2000).

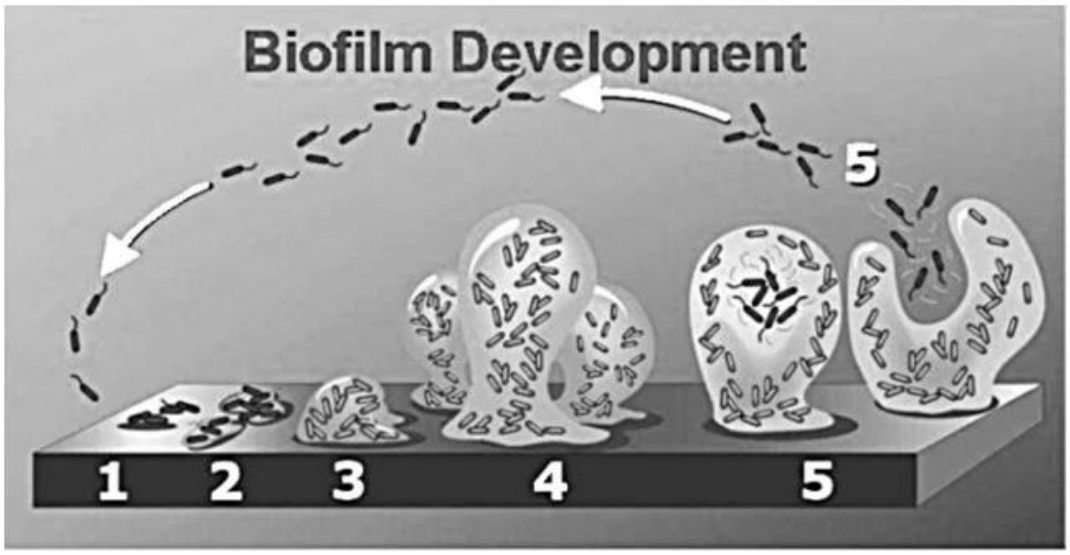


Figure 1- 4 Stages of biofilm development: The initial attachment (1) of microbial cells and subsequent production of EPS (2) resulting in "irreversible" attachment. The development of biofilm architecture (3) leads to maturation of the biofilm (4).Mature biofilms represent a seed bank proliferating cells via dispersion. Taken from Stoodley *et al.* (2002)

Although biofilm microbes are not strictly sessile organisms, they are primarily dependent on dispersal, which is a primary process regulating population dynamics. The seed-dispersion pattern not only determines the potential area of colonization, but also controls subsequent processes, such as predation, competition and concurrence (Nathan & Muller-Landau, 2000). The important role of physical transportation in regulating the supply of recruits to an area has been emphasized in aquatic ecology (McNair *et al.*, 1997, Leff *et al.*, 1998). The continous flux of individuals to and from regional dispersal pools and their residence times profoundly impact local assemblage dynamics (Palmer *et al.*, 1996). The selective advantage of bacterial adhesion favors the localization of surface- bound bacterial populations in nutritionally favorable, non-hostile environments and at the same time provides some level of protection (Dunne, 2002). In general, biofilm surface colonization can occur through at least three different mechanisms: One is by the redistribution of attached cells by surface motility, second is from the binary division of attached cells, and third is aggregation by the recruitment of planktonic cells from the bulk fluid to the developing biofilm (Stoodley *et al.*, 2002).

When microorganisms migrate to a surface, attachment is determined by physical and chemical interactions, which may be attractive or repulsive, depending upon the complex interplay of the

chemistries of the bacterial and substratum surfaces, and the aqueous phase (Figure 1- 5) (An & Friedman, 1998, Bos *et al.*, 1999, Katsikogianni & Missirlis, 2004).

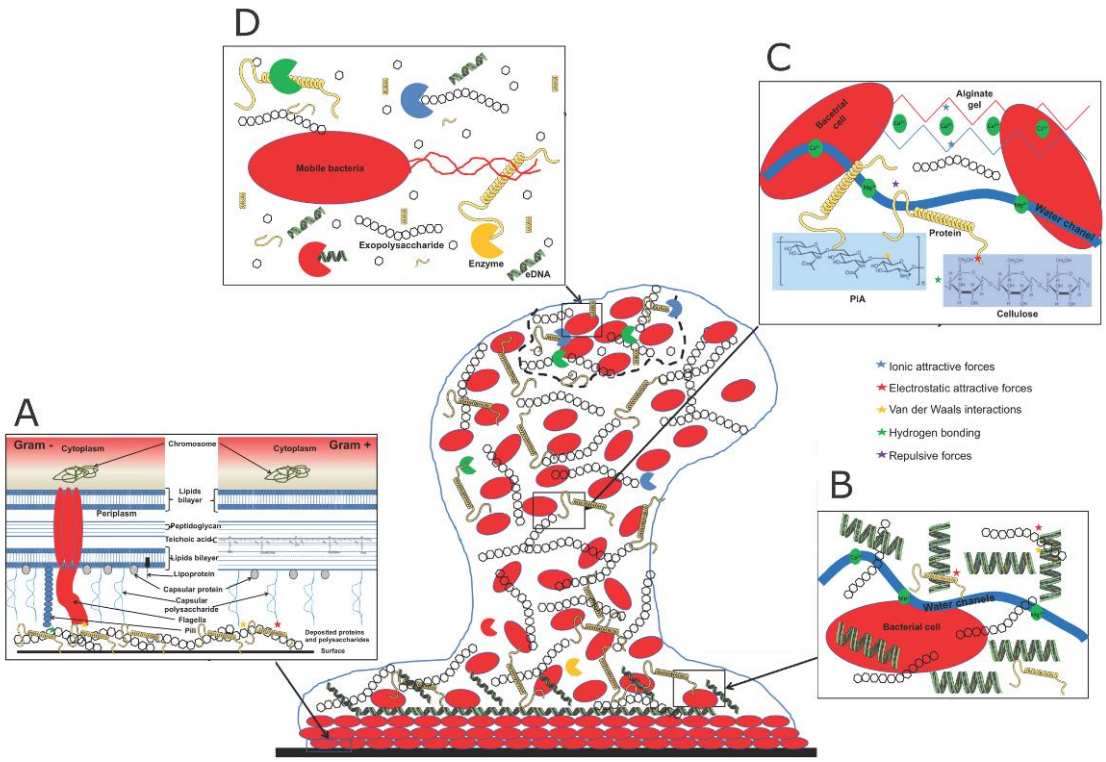


Figure 1- 5 A mature biofilm at the solid – liquid interface: attached bacteria embedded in EPS. **A** At contact, the microbial cells can interact with the surface via several protein and polysaccharide appendages such as pili, flagella, capsular polysaccharides. **B** Extracellular DNA (eDNA), protein, and polysaccharides are important in early biofilm formation. **C** Water channels and void spaces allow the distribution of ions and nutrients across the biofilm matrix. **D** Exoenzymes solubilize the exopolymeric matrix and release planktonic cells. Modified from Lembre *et al.* (2012)

In the case of primary colonisation, biofilm formation is initiated with the adsorption of a conditioning film comprised of polysaccharides, proteins, lipids, humic acids, nucleic acids and amino acids to which the colonizing bacteria subsequently adhere (Loeb & Neihof, 1975, Bakker *et al.*, 2003, Siboni *et al.*, 2007, Tang *et al.*, 2013). The division of the initial microbial adhesion process in two phases continues to be the dominant perspective (An & Friedman, 1998, Hermansson, 1999, Garrett *et al.*, 2008). Cells are initially attracted towards the surface due to van der Waals attraction forces, Brownian motion, gravitational forces, electrostatic charges and/or hydrophobic interactions (Busscher *et al.*, 1991, Bos *et al.*, 1999). The relative contribution of specific and non-specific mechanisms, that play an important role in the ability

of cells to attach to surfaces, likely depends on surface properties as well as the associated flow conditions (Katsikogianni & Missirlis, 2004).

In the second phase of adhesion, molecular and cellular interactions between bacterial surface structures and substratum surfaces govern the attachment which benefits from microbial surface polymeric structures and appendages such as capsules, fimbriae or pili and EPS (Bullitt & Makowski, 1995, Pratt & Kolter, 1998, Mayer *et al.*, 1999), leading to irreversible adehsion. This adhesion sequence is then followed by population growth. Production of bio-polymers 'glue' the cell and its daughter cells onto the surface until detachment takes place (Hermansson, 1999, Mack, 1999, O'Gara & Humphreys, 2001).

Highly organized patterns with relatively regular cell spacing have been observed in single species biofilms (Stoodley *et al.*, 2002). An organized spatial structure is certainly necessary to allow the evolution of cooperation in biofilms (Kreft, 2004). The spatial patterns formed in microbial communities are important in order to understand species interactions and dispersal, and to develop ecological networks and theory (Battin *et al.*, 2007, Hanski, 2007, Rani *et al.*, 2007, Xavier *et al.*, 2009).

At maturity, biofilms are challenged by invading planktonic cells from the bulk liquid (Kadouri & O'Toole, 2005, Kim *et al.*, 2013). These might constitute individual cells that have grown in suspension or ones that have been derived from biofilms upstream of the community. Immigrant organisms depend upon their ability to displace, compete or co-operate effectively with the resident biofilm (McBain *et al.*, 2000). The encounter between invading cells and a surface can have several outcomes:

- The surface may be hostile to the potential colonizer due to lack of available/unoccupied binding sites and the immigrant will therefore fail to bind.
- The invading cells may physically displace one of the early colonizers by virtue of a higher binding affinity for a common binding site. This is most likely to occur during the initial attachment phase of film formation and before the deposition of polymer cements. The duration of this phase will therefore be indirectly related to the metabolic potential at each colonized site.
- Both the invading species and the primary colonizer are retained at the surface, either at separate sites or attached to each other or to matrix polymers. Where a surface is co-colonized, then the degree of interaction between the colonizers will be minimal in the first instance but will increase as the community grows and adjacent micro-colonies come into closer proximity. Such interactions might be mediated through the production of

cell-cell signaling compounds, specific and nonspecific inhibitors, or competition for available nutrients (McBain *et al.*, 2000).

Indeed, these interactions appear to be essential for the attachment, growth and survival of species at a site (Rickard *et al.*, 2002). In addition to possible patterns during adhesion, movement of cells within the biofilm matrix was described (Stoodley *et al.*, 2002, Gloag *et al.*, 2013). Motility of bacterial cells over surfaces by gliding, twitching and swarming has been reported (Fenchel, 2002, Harshey, 2003, Kaiser, 2007). It is evident that many different environmental factors influence the settling and adherence of particles. Thus the spatial patterns of organisms primarily result from abiotic factors, and organisms physically alter their environment, thereby creating spatial heterogeneity (Tolker-Nielsen & Molin, 2000, Turner, 2005, Hanski, 2007). Selection pressures are exerted disproportionately throughout the biofilm, leading to the establishment of mosaics of sub-communities within the global biofilm architecture (McBain *et al.*, 2000, Stewart & Franklin, 2008, McDougald *et al.*, 2012). Detachment and active dispersal of cells from biofilms can be triggered by a multitude of biotic and abiotic drivers such as temperature fluctuations, nutrient and oxygen limitations, quorum sensing and other cell-cell signals (Karatan & Watnick, 2009, Kaplan, 2010, McDougald *et al.*, 2012). The process of detachment is of outmost importance regarding the dispersal abilities and structural integrity of biofilms.

The structure and architecture of biofilms in aquatic and subsurface environments has been the subject of many recent studies (Battin *et al.*, 2003b, Wilmes *et al.*, 2008, Besemer *et al.*, 2009, Ziegler *et al.*, 2009). The spatial arrangement of microorganisms is generated by intrinsic (e.g. vegetative division of immotile cells, invasion and detachment processes) as well as extrinsic (e.g. flow of liquids, temperature, pH, salinity, oxygen, accessibility of nutrients) factors. The spatial configuration of biofilms has a direct influence in regulating community structure-function relationships through processes such as dispersal, cell-cell signalling and horizontal gene transfer (Tolker-Nielsen & Molin, 2000, O'Donnell *et al.*, 2007). The coupling of structural properties and their functions have been emphasized in biofilm ecology studies (Stoodley *et al.*, 1999).

Biofilms are highly hydrated open structures often containing a large fraction of EPS and large void spaces between micro-colonies (Lawrence *et al.*, 1991, Tolker-Nielsen & Molin, 2000). Secondary structures such as mushroom-like caps, filamentous streamers and migratory ripples separated by channels and voids can be seen as adaptations of biofilm structure for survival in varying environments of high nutritional input (Hall-Stoodley *et al.*, 2004) while oligotrophic habitats are likely to contain only patchy single-layer micro-aggregates (Karatan & Watnick, 2009).

Biofilms can serve as environmental refugia by enclosing colloidal particles such as bacteria and virus-sized particles from the surrounding bulk fluid (Drury *et al.*, 1993, Flood, 2000, Searcy *et al.*, 2006). This is especially of interest regarding the fate of pathogens. A positive relationship between particle deposition and biofilm surface roughness (Battin *et al.*, 2003b), as well as biofilm thickness (Drury *et al.*, 1993), has been shown. The internal biofilm channel system and the highly hydrated matrix constitute important transient storage zones (Battin *et al.*, 2003b) facilitating the deposition of nutrients and storage.
Biofilm formation influences the interplay of microbial constituents with their environment. Surfaces that are colonized can be living tissue, other microbial cells or abiotic structures. In groundwater habitats, inorganic surfaces such as minerals are likely to outweigh other possible sites for attachment. Certainly those surfaces cannot be seen as inert, and while in surface waters the surrounding liquids transport most of the nutrients and carbon responsible for biofilm growth, microbial weathering is likely to play a governing factor affecting community composition and metabolic properties in nutrient deprived environments such as the subsurface. This implies that a holisitc approach including hydrodynamics, geochemistry and molecular sciences is needed to understand biofilm formation in groundwater. Mechanisms describing biofilm adhesion at the solid-liquid interface appear universal and can be adapted for groundwater biofilms. Biofilm formation has been also observed in artifical sediment columns using novel imaging techniques (Martiny *et al.*, 2003, Keller & Auset, 2007, Rockhold *et al.*, 2007, Davit *et al.*, 2011). Nonetheless, there is still a lack of knowledge regarding the existence and distribution of biofilms in oligo- and ultraoligotrophic systems. The reported cell numbers in these nutrient deprived environments infer that substantial surface areas are not covered by microbes (Baveye *et al.*, 1992, Griebler *et al.*, 2002, Goldscheider *et al.*, 2006).

1.2.1 The Social Life in Biofilms

Biofilms have been referred to as "cities of microbes" (Watnick & Kolter, 2000) illustrating the manifold interactions within these multifaceted communities and the importance of spatial organization. Microorganisms communicate and cooperate in biofilms, forming a complex system of inter-species interactions, in order to perform a wide range of multicellular behaviours (West *et al.*, 2006, Freilich *et al.*, 2010). Ecological interactions in microbial communities range from symbiotic interactions such as parasitism, commensalism and mutualism, to antagonistic interactions via competition or predation (Little *et al.*, 2008). Cell-cell signalling induced by small diffusible molecules secreted and detected by resident microorganisms modulates several interactions. This communication, known as quorum sensing or diffusion sensing, is thought to

be used as a proxy for population density and monitoring the extent of diffusion in their microenvironment, thereby tuning the behaviour of other cells (Redfield, 2002, Hense *et al.*, 2007, Nadell *et al.*, 2009). Understanding the extent of coordination and cooperation in biofilms is a prerequisite to acquire insights into the functions of these entangled communities. In groundwater, it is conceivable that many obligate mutualistic interactions (cell-cell signalling, syntrophy) are only possible in biofilms, due to the patchiness of aquifers as microbial habitats. Comprehensive evidence, however, remains undeveloped. Hence, studying such interactions will provide valuable insights regarding ecological and evolutionary roles in structuring communities.

1.2.2 Rheology – Forming Biofilms

The development of multispecies biofilms at solid-liquid interfaces occurs both in quiescent water and under conditions of high shear forces (Rickard *et al.*, 2004). Turbulent flow controls transport processes through exchange with the water-column. Turbulence involves complex, three dimensional fluid motions in which the paths of individual fluid packets are unpredictable (McNair *et al.*, 1997). On the contrary, laminar flow is characterized by low Reynolds numbers and unidirectional flow. In theory, the flow velocity immediately adjacent to the substratum/liquid interface, termed the hydrodynamic boundary layer, is negligible (Donlan, 2002). Nevertheless, shear rates over surfaces have been reported (Rickard *et al.*, 2004) to directly influence bacterial composition and govern the abilities of individual species to immigrate to biofilms and colonize surfaces, thus controlling the diversity of multispecies biofilms. Ultimately, hydrodynamic conditions will influence biofilm structure (Purevdorj *et al.*, 2002) and mechanisms that facilitate bacterial adhesion (e.g. co-aggregation) might be flow dependent (Rickard *et al.*, 2004). The biofilm shape varies with the growth cycle of resident microbes and with variations in fluid shear (Stoodley *et al.*, 1999, O'Toole *et al.*, 2000). Changes in biofilm shape will affect its porosity and density and therefore the transfer of solutes into and through the biofilm (Stoodley *et al.*, 1999). Shear-mediated migration of biofilms represents one possible mechanism of dissemination in flowing systems (Purevdorj et al. 2002).

In groundwater systems, diverse flow regimes can establish themselves. However, most groundwater bodies are characterized by slow flow and little shear rates, while turbulent flow occurs in zones of high hydraulic-conductivity and engineered environments e.g. drinking water pipes. Thus, biofilms must contend with the varying flow conditions that shape the community structure as well as biofilm arrangement. The shape and structure of biofilms differs substantially in different habitats (Figure 1- 6) as they are formed by a feedback of external forces and the

microorganisms that actually build the biofilms (Besemer *et al.*, 2009) through attachment and exudation of their sticky matrix.

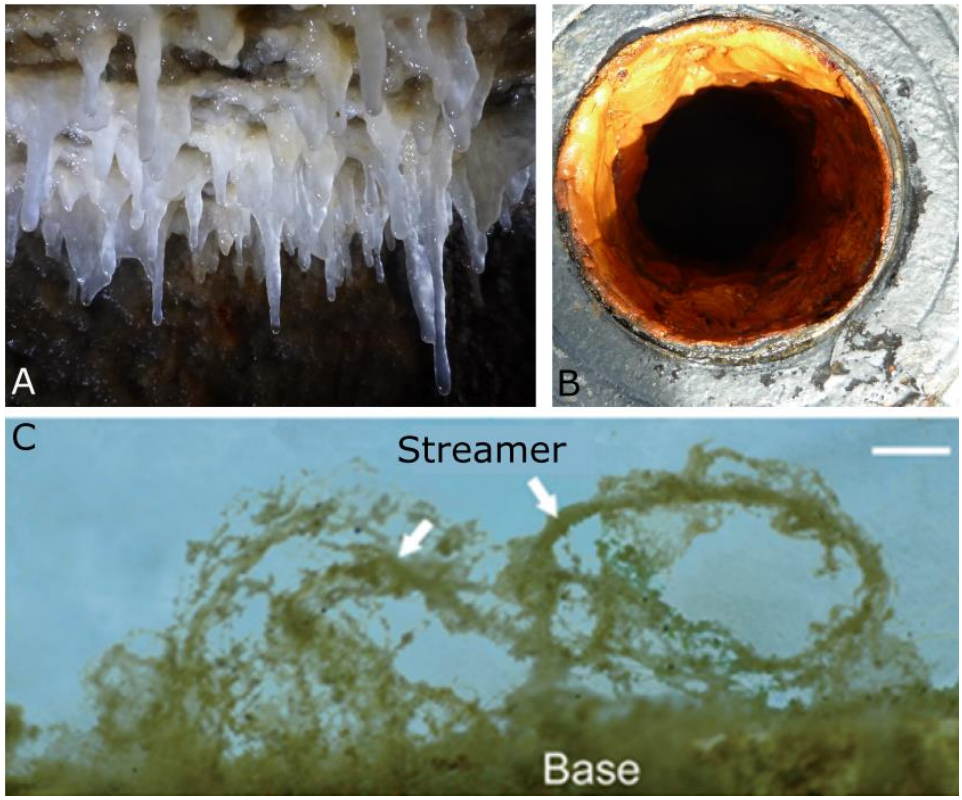


Figure 1- 6 Diverse biofilm structures found in different aquatic habitats. **A** Subaerial "snottites" encountered in cave ecosystems. (Photo: Karwautz) **B** Incrustations and biofilm formation within a drinking water distribution pipe. (Photo: Karwautz) **C** Oscillating benthic stream biofilms. Taken from Besemer *et al.* (2009)

1.2.3 Extracellular Polymeric Substances - EPS

Once attached to a surface, microbial cells change their specific gene expression patterns (Prigent-Combaret *et al.*, 1999, Beloin & Ghigo, 2005) and embed themselves in adhesive exopolymeric substances, which provide structural support. It appears that most microorganisms are able to form extracellular polymeric substances through multiple pathways (Linton, 1990, Branda *et al.*, 2005). Potential functions of the EPS are related to attachment and biofilm formation, genetic transfer, nutrient retention and accumulation. Furthermore, juxtaposition within the matrix allows diverse interactions (e.g. quorum sensing, syntrophy). Exopolymers provide a physical and chemical barrier, which creates microenvironments shaped by the exudation of metabolic products as well as the influx of new substances (Figure 1- 7) (Wolfaardt

et al., 1999, Donlan, 2002, Stewart & Franklin, 2008). Exudates include a wide range of organic polymers that may be arranged into loose associations or into discrete structures (Wotton, 2011). The composition of the EPS varies with the organisms that produce them and hence differ in their chemical and physical properties (Sutherland, 2001). Moreover, biopolymer configuration can vary depending on environmental conditions such as nutrient composition and availability (Sheng *et al.*, 2010) as well as biofilm age (Leriche *et al.*, 2000).

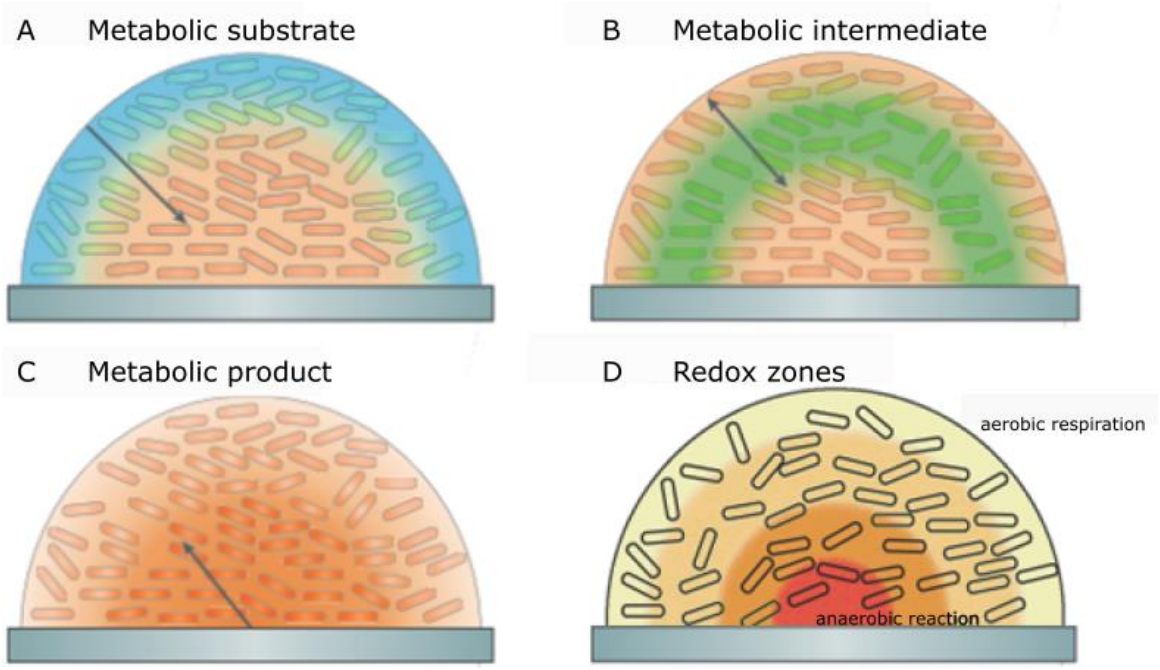


Figure 1- 7 Chemical heterogeneity in biofilms. Distinct patterns arise from reaction–diffusion interactions of a metabolic substrate creating a gradient from the source outside the biofilm, which decreases with depth (blue; **A**), a metabolic intermediate which is produced and consumed within the biofilm, thereby creating local maxima (green; **B**), a metabolic product concentrated inside the biofilm diffuses outwards (orange; **C**) and redox zones describing the reduction potential due to the depletion of electron acceptors towards the biofilm base (red; **D**). Modified from Stewart & Franklin (2008).

Polysaccharides were initially thought to be the essential components of the EPS, but proteins, nucleic acids, lipids and humic substances have also been found in substantial amounts (Wingender *et al.*, 1999, Flemming & Wingender, 2010). Exo-biopolymers form and maintain the three-dimensional, viscoelastic, locally charged biofilm matrix. One key feature of EPS is its ability to bind water after secretion (Sutherland 2001), becoming highly hydrated and likely to contain colloidal and dissolved compounds. Once the mucus is excreted, inorganic particles and biogenic material are entrapped, potentially involved in the cross-linking of EPS and thus in polymer network formation (Denkhaus *et al.*, 2006).

The role of extracellular DNA (eDNA) within the matrix is especially fascinating since it is not only important during cell adhesion (Figure 1- 5), but also appears to be an essential exudate of live cells, maintaining the structural integrity of the matrix (Flemming & Wingender, 2010). Several features of eDNA are likely to enhance cooperation and interaction within biofilms. Extracellular DNA can be used in gene transfers, creating extremely dynamic genomes (Ochman *et al.*, 2000). Furthermore, it is a source of nutrients and even facilitates motility, since it is crucial in the assembly and coordination of the collective behaviour of cells (Gloag *et al.*, 2013).

The EPS provides a microbial public good, an excreted secondary metabolite, coming at a cost for the producing individuals (Xavier & Foster, 2007, Hibbing *et al.*, 2010). The ATP requirement may even exceed cell production at the same growth rate (Linton 1990). In addition to establishing a protective environment, the viscous matrix also changes a multitude of parameters for the microbes. Substrates and gases can only diffuse through the EPS, creating steep gradients (Figure 1- 7) (De Beer et al. 1994, Stewart and Franklin 2008). On the other hand, excreted metabolites e.g. signaling molecules and extracellular enzymes can accumulate, facilitating complex interactions and concerted actions.

The production of metabolites and EPS in biofilms is often triggered by high carbon to nitrogen ratios or nitrogen and/or oxygen limitations (Wrangstadh *et al.*, 1986, Babel, 1992). Exopolymer production and biofilm formation of methylotrophic bacteria and organisms that oxidize and assimilate C_1-compounds have been especially studied in great detail (Linton *et al.*, 1986, Linton, 1990, Hilger *et al.*, 2000). Methylotrophs produce sugars as intermediates, providing a direct route of formaldehyde removal, balancing the utilization and production of this toxic compound that can be readily build into EPS (Southgate & Goodwin, 1989, Linton, 1990). Bacteria have also been shown to produce polyhydroxybutyrate (PHB) granules under high carbon to nitrogen ratios and limited O_2, providing an energy source that can be made available at a later stage (Babel, 1992, Müller *et al.*, 1999, Shi *et al.*, 2007). In this way, EPS offers an electron sink to microbes that are provided with excess supply of electrons they cannot use for growth and division due to the limitation of other essential nutrients.

Extracellular polymeric substances have been identified as a defining property of biofilms. They substantially influence the proximate environment of cells living within the matrix. In groundwater systems, extensive EPS production is rare as high-energy demand is associated with its formation. Nevertheless, biofilms with significant amounts of EPS have been found in drinking water pipes (Pedersen, 1990, Percival & Walker, 1999) and groundwater caves (Engel *et al.*, 2004b, Macalady *et al.*, 2007). Several factors are likely to influence the absence of EPS. Classical drivers of biofilm formation such as the availability of energy and nutrients, as well as

the necessity to firmly adhere to the surface due to high shear forces are absent in most aquifers. Other functions, such as the buffering of fluctuating environmental conditions and the prevention of desiccation, are also not crucial for starved subsurface organisms. However, EPS production can be expected to commence as soon as shear increases or energy sources become available to microbial communities.

1.3 Biofilms in Groundwater Ecosystems

Pristine aquifers are often ultra-oligotrophic systems exhibiting low temperatures and characterized by several specific conditions such as the lack of light and therefore the absence of photosynthesis and a requirement for allochthonous energy inputs and chemosynthesis. The presence of electron donors and acceptors, carbon sources, and nutrients are essential for microbial growth. Fast turnover rates of dissolved free amino acids and carbohydrates indicate that these compounds are generally important carbon, nitrogen, and energy sources for heterotrophic microbes in groundwater (Poindexter, 1981, Hazen *et al.*, 1991, Egli, 2010). More than 90 percent of the microbial biomass in aquifers is attached to matrix surfaces, thereby altering their environments considerably. The number of attached microbes in groundwater systems can be in the range of 10^4 and 10^8 cells cm^{-3} of sediment (Griebler & Lueders, 2009). Biofilms are usually taken to be composed of highly active and growing bacteria, while in natural, especially nutrient deprived habitats, a substantial amount of the standing biomass will generally be composed of dormant, dead or slow growing cells under starving conditions (Morita, 1990, Sherr *et al.*, 1999, Wilhartitz *et al.*, 2009). In sum, high variability in bacterial growth efficiency is reported at low growth rates when substrates are limiting (Del Giorgio & Cole, 1998). Microorganisms attached to minerals produce very reactive microenvironments, accelerating the dissolution of minerals and releasing potential nutrients (Röling & van Verseveld, 2002, Rogers & Bennett, 2004, Gadd, 2010). Thus, access to growth limiting substrates and nutrients is enhanced via surface attachment. Bioleaching of minerals is dependent on the reactivity of the substrate, the environmental conditions, and the colonizing microbial community (Viles, 1995). The biogeochemical microenvironment and resource availability generated within attached microorganisms is significantly different from compartments where sediments are not colonized (Tolker-Nielsen & Molin, 2000, Jackson, 2003, Gadd, 2010). Microbial leaching and solubilization of essential nutrients (Bennett *et al.*, 2001, Boswell *et al.*, 2001), is facilitated by attachment and excretion of exopolymers. Microorganisms can accumulate and utilize metals either as electron acceptors (Boon *et al.*, 1998, Johnson, 1998, Tebo & Obraztsova, 1998, Rohwerder *et al.*, 2003) or electron donors (Ehrlich, 1997, Emerson *et al.*, 2010) for energy

metabolism. (Figure 1- 8). This suggests a competitive advantage for surface bound communities over their planktonic counterparts (Kjelleberg *et al.*, 1982, Hazen *et al.*, 1991), if specific resources are limiting. It is assumed that biofilm communities also control many important processes in the subsurface (Griebler *et al.*, 2002), although comprehensive knowledge is still lacking.

In aquifers, available nutrient pools have to be utilized as efficiently as possible. Bacteria in low nutrient environments may use several organic substrates simultaneously (Del Giorgio & Cole, 1998, Egli, 2010), expressing a wide range of uptake and catabolic systems, even for compounds that cannot serve as a sole energy source. In carbon-limited continuous systems, mixed substrate growth is the rule rather than the exception during slow growth. Several stress response systems are expressed at higher levels during slow growth in biofilms (Kjelleberg & Hermansson, 1984, Schimel *et al.*, 2007, Hengge, 2009, Karatan & Watnick, 2009). The advantage of exploiting several carbon compounds simultaneously improved kinetic performance and metabolic/physiological flexibility resulting in a competitive advantage (Gottschal *et al.*, 1979).

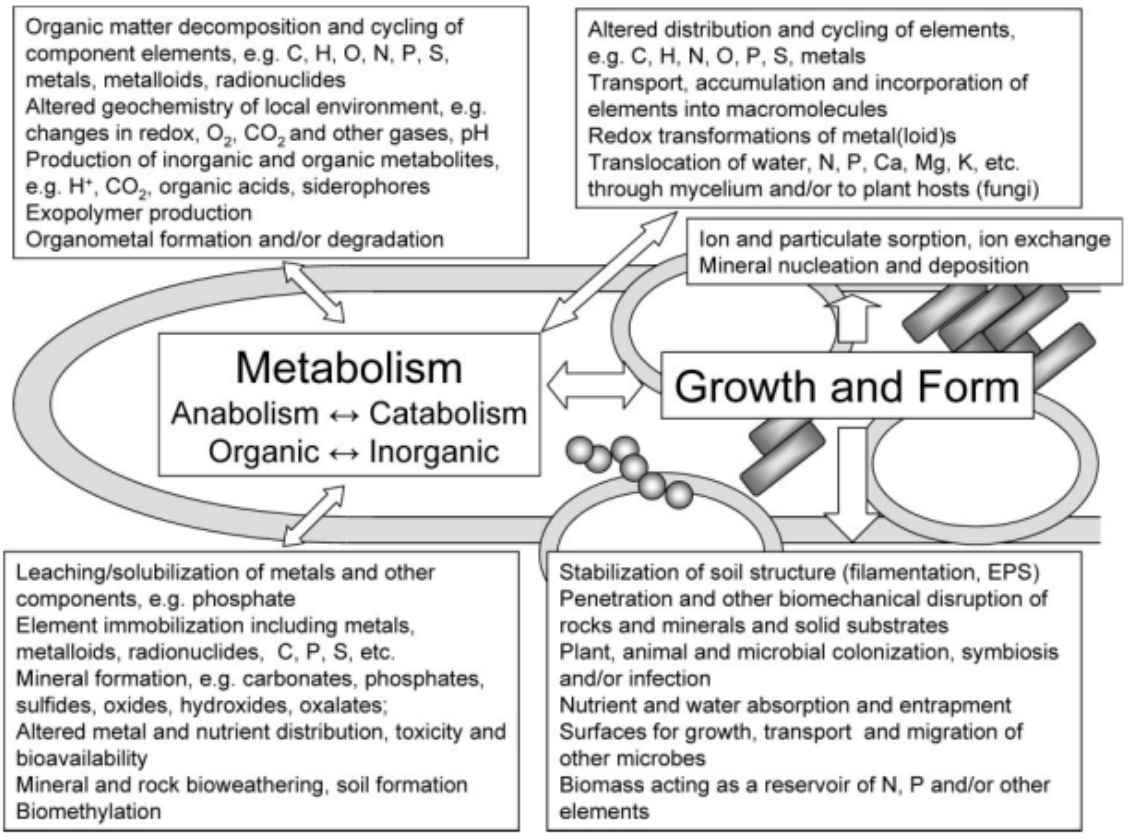


Figure 1- 8 Biogeochemical processes modified by microbial actions lead to altered distributions of elements. Present microbial populations and physico-chemical factors affect the activity and processes controlling the turnover of organic carbon, the solubilizations and availability of nutrients as well as influence the speciation of metals. Taken from Gadd (2010)

Bacteria can consume large amounts of energy directed towards other processes than growth; for example, secondary metabolite production, especially when growth is constrained (Linton, 1990, Russell & Cook, 1995). Though there is evidence that microbes also attach to surfaces under starving conditions (Morita & ZoBell, 1955, Pedersen, 1997, Griebler *et al.*, 2002), the formation of multilayer assemblages is nevertheless unlikely and the exudation of secondary metabolites too costly (Brockhurst *et al.*, 2008). In contrast, it seems possible that small patches of greater microbial activity exist along preferential flow paths in groundwater. There, microbial interactions also facilitate the degradation of recalcitrant compounds, which could provide important advantages in contrast to starving, planktonic cells.

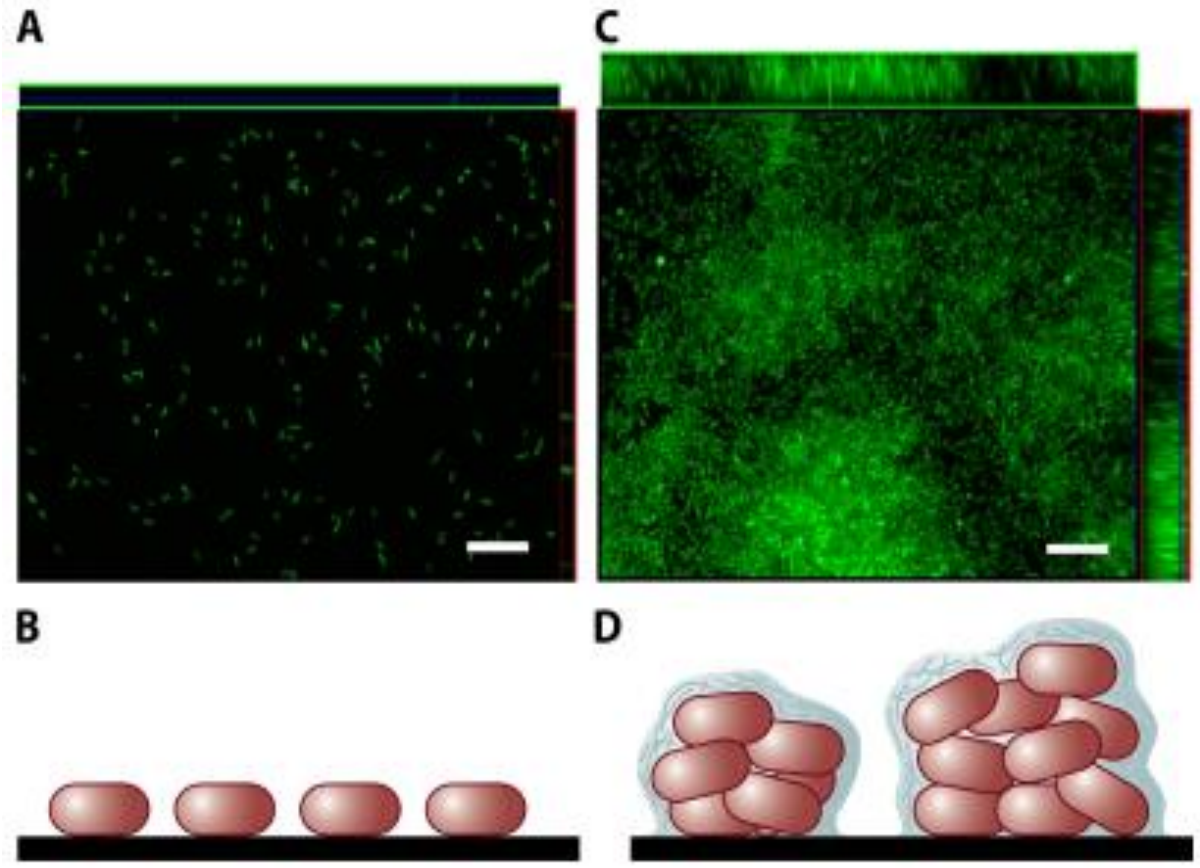


Figure 1- 9 Microscopic cross-sections through monolayer (A) and multilayer (C) biofilms and illustrated side views of the monolayer (B) and multilayer (D) biofilms encased in an EPS matrix. Taken from Karatan and Watnick (2009)

Biofilms may be an important prerequisite for the survival and growth of specific microbes in the subsurface, because they facilitate the most efficient use of available resources and provide an especially protective environment (Tolker-Nielsen & Molin, 2000). Also, the importance of the "biofilm-mode-of-life" in structuring the diversity and spatial distribution of microbial

communities in the subsurface by facilitating access to limiting nutrients and resources remains to be verified and is one of the research questions that shall be addressed in this thesis.
Much of the biofilm research mentioned in this introduction has dealt with non-nutrient limited, multilayer, often mono-species biofilms (Figure 1- 9). In contrast, multi-species monolayer biofilms, may be the most prevalent form in oligotrophic groundwater environments (Karatan & Watnick, 2009).

1.3.1 Heterotrophic vs. Autotrophic Metabolism

Heterotrophic biofilms establish themselves in groundwater systems based on the turnover of allochthonous organic carbon. Despite nutrient limitations, many heterotrophic biofilms display great microbial diversity (Pedersen, 1997, Newman & Banfield, 2002, Castelle *et al.*, 2013) and metabolic versatility (Hazen *et al.*, 1991, Castelle *et al.*, 2013) in subsurface sediments. In the absence of oxygen, or nitrate, in subsurface sediments. In the absence of oxygen or nitrate, iron oxides and sulfate are reduced during anaerobic metabolism in contaminated or deep subsurface aquifers (Lovley & Chapelle, 1995, Meckenstock *et al.*, 2004, Winderl *et al.*, 2008, Pilloni *et al.*, 2011) and even within biofilms where oxygen may become depleted (Bishop & Yu, 1999). In groundwater systems, the importance of microbe-mineral interactions is recognized as an important component of biogeochemical cycles , but knowledge of the microbial key players mediating these processes is mainly obtained for very particular, often contaminated groundwater habitats (Reardon *et al.*, 2004, Hug *et al.*, 2013).
Autotrophic biofilms, which derive their energy from oxidizing ammonia, ferric iron, hydrogen sulphide or methane, present an alternative mode of life. Highly specialized microbial communities have been observed in cave systems (Bond *et al.*, 2000, Hose *et al.*, 2000, Northup *et al.*, 2003, Macalady *et al.*, 2007, Ziegler *et al.*, 2009). The present analysis of biofilm communities has indicated that chemolithotrophic microorganisms constitute the primary producers in many of these systems (Peck, 1986). Sometimes extreme biofilm production has been observed in these caves not only at the solid-liquid interface but also in subaerial biofilms covering walls and ceilings (Bond *et al.*, 2000). Organic carbon concentrations in caves are usually low, so that carbon assimilation is based mostly on chemolithoautotrophic processes. Oxidation of sulfur and iron compounds as well as oxidation of gases (e.g. methane) must therefore provide sufficient energy for extensive biofilm accumulation (Holmes *et al.*, 1995, Angert *et al.*, 1998, Macalady *et al.*, 2008, Ziegler *et al.*, 2009). The impact of these biofilms on cave formation (speleogenesis) and mineral dissolution, as well as biomineralization processes, is the subject of several studies (Peck, 1986, Engel *et al.*, 2004a, Macalady *et al.*, 2006, Barton & Northup, 2007). Microbial life in caves

can be influenced by the mixing of communities populating habitats below as well as communities seeping through the overlaying soil layers (Goldscheider *et al.*, 2006). Parallels regarding the metabolism of microbes in deep subsurface communities and autotrophic groundwater communities can be drawn.

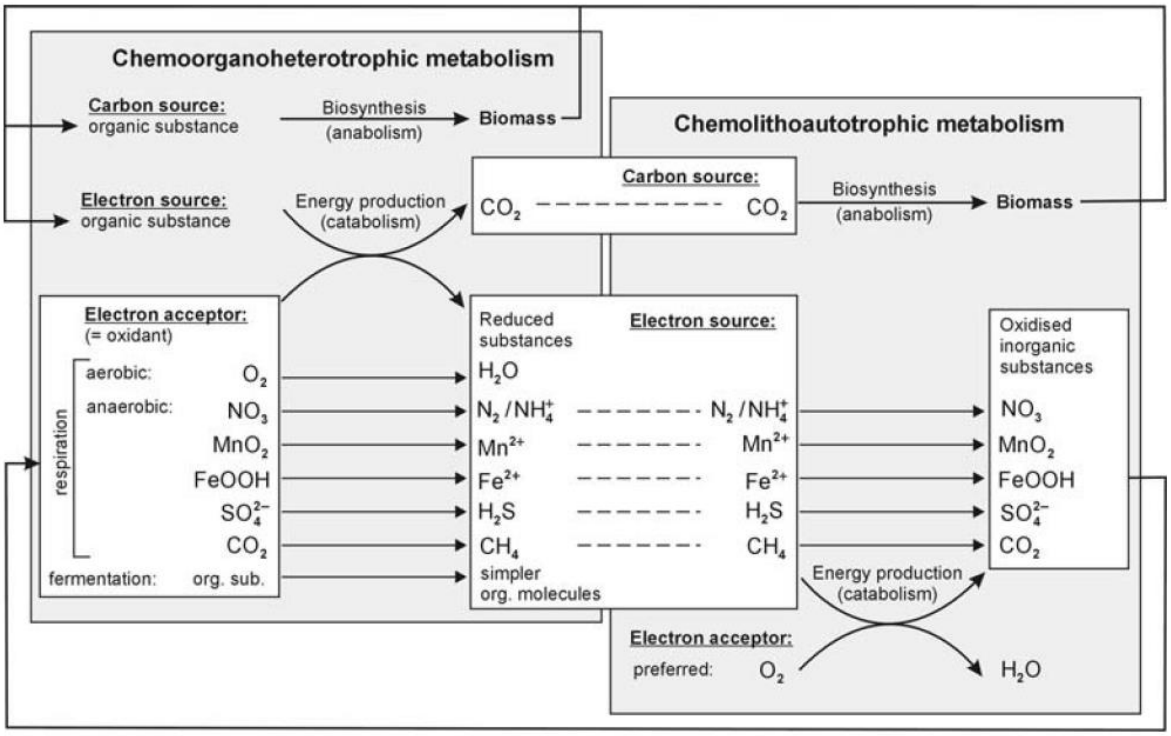


Figure 1- 10 Autotrophic and heterotrophic metabolism:. Autotrophs fix the energy from inorganic sources such as atmospheric CO_2, while heterotrophs depend on energy and carbon fixed by other organisms. Taken from Goldscheider *et al.* (2006)

Based on the central role of carbon metabolism in groundwater ecosystems, a differentiation into autotrophic and heterotrophic biofilms appears instinctive (Figure 1- 10). Previous findings from biofilm studies in oligotrophic environments can be used to establish a framework of biofilm functionality and community structure in subsurface sediments. It is the purpose of this thesis to address the complexity of microbial biofilms in groundwater regarding their microbial diversity and functionality.

1.3.2 Role of Biofilms in Water Quality

Although substantial data on microbes in drinking water systems is available, little is known on the microbiology of groundwater extraction wells and the ecology of the native, attached microbes therein. In the few studies available, it appears that apart from a core groundwater

community dominated by oligotrophic taxa predominantly related to *Proteobacteria* but also *Bacteroidetes*, specialized organisms depending on the availability of specific nutrients (e.g. iron, nitrogen) shape the inherent communities (Hong *et al.*, 2010, Pinto *et al.*, 2012, Navarro-Noya *et al.*, 2013). Additionally, potential pathogens commonly appear and disperse in proximity of drinking water production systems (Kwon *et al.*, 2011). Microbial monitoring of drinking water from the well to the tap is essential to assure water quality and to predict possible risks (Bockelmann *et al.*, 2009, Miles *et al.*, 2009).

Besides their beneficial ability to stimulate bioremediation, other studies have demonstrated the resistance of natural, indigenous biofilms against pathogens in drinking water systems and flow cell chambers (Habimana *et al.*, 2009, Gião *et al.*, 2011). On the other hand biofilms can contribute to the persistence of pathogens protected within its matrix, being highly resistant to antimicrobial and cleaning treatments (Davies, 2003, Gião *et al.*, 2008). Thus, biofilms impact public health (Costerton *et al.*, 1999, Hall-Stoodley *et al.*, 2004). The role of potential pathogens and their interactions within the biofilm community needs further examination. Triggers for outbreaks, transmission routes and the influence of biofilms as reservoirs are still enigmatic. Waterborne disease outbreaks have been frequently associated with microbial pathogens entering drinking water via natural groundwater (Hoffman *et al.*, 2009).

Processes in drinking water biofilms are often dominated by heterotrophic bacteria but are usually associated with autotrophic organisms which are beneficial because they remove nitrate (Kindaichi *et al.*, 2004). In a long-term study (Martiny *et al.*, 2003), different successional stages of biofilm formation were observed over the course of three years and temporal variation was shown to impact community composition. In the study, the authors suggest that dominating microbes are reduced in older biofilms allowing the emergence of organisms that might benefit from the exudates generated within the biofilm (Martiny *et al.*, 2003). There is evidence for ecological succession of biofilms in groundwater and even seasonal variation in shallow aquifers (Feris *et al.*, 2004). Assessment of these patterns might give clues on ecological strategies of the studied taxa.

Temporal variations in microbial communities could also be of interest to drinking water providers that aim to consistently provide water of continuously high quality. Drinking water production systems have to be understood as highly engineered environments, which provide different conditions to groundwater biofilms than those in aquifers. While the milieu in aquifers seems to be mainly governed by sediment heterogeneity and microbe-mineral interactions as well as nutrient availability, distribution systems in drinking water production environments are mainly governed by extreme hydrodynamics and pipe material characteristics (Yu *et al.*, 2010). To

date, drinking water biofilms have mainly been studied within the framework of a distribution system (Block *et al.*, 1993, Williams *et al.*, 2004, Henne *et al.*, 2012, Lautenschlager *et al.*, 2013), but the link between aquifers and technical systems is not very well understood, at least from a microbiological perspective.

Representative microbiota sampled from clogged wells have mainly been methanogenic bacteria, algae (Bubela, 1985), iron oxidizers (e.g *Gallionella ferruginea*) (Søgaard *et al.*, 2001) inducing precipitation of ferrihydrate, as well as iron-, nitrate- and sulfate-reducing bacteria (Beek & Kooij, 1982, Ralph & Stevenson, 1995, Baveye *et al.*, 1998) that can reduce hydraulic conductivity in the proximity of drinking water wells through the production of exopolymeric slime and precipitates.

In this thesis, attached microbes in drinking water wells and their impact on drinking water proliferation will be addressed. Special focus was put on biofilm composition and diversity as well as the presence of putative pathogenic bacteria.

1.3.3 Examples of Biofilm Systems in Groundwater Investigated in this Thesis

In subsurface sediments, microbes are directly linked to water quality issues. The composition of biofilms can have far-reaching impacts affecting ecological processes in the subsurface such as the turnover of organic matter, the transformation of xenobiotic compounds and even the dispersal capabilities of species. Sediment characteristics and surface-microbe interactions are major factors shaping community structure and thus biogeochemical processes. Biofilms determine activities at microbe-mineral interfaces, such as the biomineralization and dissolution of metals and minerals (Lünsdorf *et al.*, 2000, Rogers & Bennett, 2004). Thus, microbes are responsible for the build-up and dissolution of geological formations, thereby forging subterranean karst landscapes and caves (Northup & Lavoie, 2001, Newman & Banfield, 2002).

In this thesis, three major aspects of microbial biofilms in subsurface systems were investigated: microbe-mineral interactions, autotrophic cave biofilms, and attached microbes in drinking water wells.

1. First, microbial colonization of minerals in a natural aquifer was investigated within an experimental mesocosm system. Since mineral surfaces can be attachment surfaces and potential substrates at the same time, the interplay of these two factors requires detailed understanding. Access to growth limiting substrates and nutrients is enhanced via surface attachment (Rogers & Bennett, 2004, Wu *et al.*, 2008). Microbial leaching allows the cells

in biofilms to gain nutrients and transfer electrons to the mineral interface, which can provide a competitive advantage over free-living microbes, if the resource is limited (Fredrickson & Zachara, 2008). So far, studies on natural, porous aquifer biofilms lack substantial data regarding the influence of prevalent mineral surfaces and their impact on the microbiology of biofilms. The chosen experimental approach provides identical environmental conditions and colonizing taxa to a series of mineral surfaces. Selected mineral surfaces were: 1) Granite, as the most common mineral in the Earth's crust but representing a rather inert surface, 2) Apatite, a phosphate-bearing mineral which could provide this often limiting nutrient to attached microorganisms, 3) Magnetite, which may provide iron oxides as electron acceptors, and 4) Pyrite (FeS_2), as well as other metal sulphides (ZnS) where attachment, leaching and oxidation of Fe^{2+} and S^{2-} as electron donors should be expected (Edwards *et al.*, 1999, Rodríguez *et al.*, 2003). According to the tenet of Baas-Becking (1934); "Everything is everywhere, but the environment selects". Thus, the minerals should exert selective forces on the attaching communities. Deterministic selection rather than stochastic processes should mainly determine community assembly. Correlation of subsurface microbial diversity and carbon-utilization patterns provides insights into the interactions of community diversity, geology and function. The long-term character of the study, running for 15 months, allows the integration of possible short-term fluctuations in the natural seeding community.

2. Second, massive biofilms that have been discovered in a spring cave were investigated regarding extrinsic energy sources and biofilm community assembly. Here, a fundamental ecological question was posed as to how environmental heterogeneity is reflected in the composition of biofilm species. Physicochemical gradients that establish within cave, by outgassing methane and their influence on biofilm community composition were investigated. The main focus was on methane oxidizing bacteria and identifying potential key players in biofilm formation. Questions regarding the spatial organization within these communities were addressed by means of microscopic examination and labelling of key taxonomic groups with fluorescence in situ hybridization (FISH). Special interest was placed on spatial distinctions within cave biofilms regarding possible energy sources and metabolic functionality. The characterization of the cave regarding the input of allochthonous carbon sources, the chemistry of the mineral spring water and its inherent microbial community provide a primary microbiological description of this unique habitat. The co-occurrence of *Bacteria*, *Eukarya* and *Archaea* was investigated, identifying possible interaction partners for further research.

3. In the third part of this thesis, active drinking water wells were selected to investigate biofilms in an engineered environment. Inter-well comparability, aquifer heterogeneity and observation of the attached indigenous microbiota within the wells were in focus. The biofilms at the intersection of an aquifer and a drinking water distribution system provide a reservoir for microorganisms and even for downstream dispersal and colonization of potential pathogens. This study aimed to characterize the microbial community in order to find dominant populations as well as species of potential pathogenicity. A purging event of the microbes during hydraulic well restoration, conducted in the form of high pressure jetting, was monitored for the first time. Time resolved analysis of the community composition during cleaning allowed sampling of the inherent attached well community. Comparison of the well water before and after the restoration provided details on the transported aquifer microbes. The influence of the attached well bacteria on the microbial drinking water community was elucidated.

In summary, the three main hypotheses that were tackled in these separate projects were:

1. Reactive mineral surfaces can alleviate nutrient limitations and select for specific communities of attached microbes in aquifers.
2. Cave systems with upwelling, reduced waters offer a unique gradient habitat for the development of lithotrophic biofilms.
3. Attached microbes in drinking water wells provide specific ecological niches, affecting the spread and survival of microbes in drinking water systems.

In essence, this thesis aims to elucidate the role of microbial biofilm communities in groundwater ecosystems. I present an extensive synopsis on attached microorganisms in the subsurface and their potential role in biogeochemical processes, and provide a framework for future investigations.

1.3.3.1 Study 1: Mineral Surfaces Exposed to Colonization of Microbial Communities from a Porous Aquifer

Biofilm growth on mineral surfaces can alleviate important energy and nutrient limitations for microbes in aquifers (Bennett *et al.*, 1996, Bennett *et al.*, 2001, Wu *et al.*, 2008). Coevolution of mineral substrates and biofilms leads to disintegration and dissolution via several processes such as acidolysis, alkaline hydrolysis, enzymolysis, ligand degradation, and extracellular polysaccharide redox effects (Beveridge *et al.*, 1997, Rohwerder *et al.*, 2003, Sand & Gehrke, 2006, Uroz *et al.*, 2009). Microbial assemblages in groundwater are likely to build isolated islands (Rittmann, 1993,

Wanger *et al.*, 2006, Mueller *et al.*, 2010), creating micro-niches of high reactivity involved in weathering, biomineralization, oxidation or reduction reactions of metals, carbon, nitrogen and sulfur (Edwards *et al.*, 1999, Sand & Gehrke, 2006, Fredrickson & Zachara, 2008, Gadd, 2010). The microbes responsible for the weathering of mineral surfaces display different abilities of dispersal, colonization, competition and dissolution of recalcitrant compounds.
In this study, a mesocosm filled with several differing mineral coupons was continuously fed with natural groundwater from a porous aquifer. The surfaces represent rather common minerals in the subsurface constituting potential electron donors and acceptors, as well as nutrient sources. They included phosphate bearing apatite, pyrite (FeS_2; the most common sulfide mineral and a potential electron donor), magnetite ($Fe^{2+}Fe_2^{3+}O_4$; an important iron oxide), and granite (a mixture of silicates including feldspar and quartz, the two most abundant minerals in the Earth's crust, both quite inert). The chosen experimental approach allowed for the comparison of biofilm communities exposed to the same abiotic and biotic conditions and potentially seeding planktonic microbes. The abundance of taxa for each surface biofilm and the structure and diversity of each community was investigated to show the selectiveness exerted by the minerals. The 15 months duration of the study allowed for the integration of possible fluctuations in the natural seeding community and permitted sampling of mature groundwater biofilms. Sub-hypothesis for this study were that (i) minerals rich in nutrients (apatite) or electron donors (pyrite/sphalerite) increase biofilm biomass. (ii) Phylogenetic diversity and metabolic flexibility are interrelated. Minerals providing essential nutrients (apatite) may sustain a more diverse community with higher metabolic flexibility; while a more specialized community would be found on highly specific surfaces (pyrite).

1.3.3.2 Study 2: Karst and Cave Biofilms

Caves are known to host a rich diversity of microorganisms (Angert *et al.*, 1998, Northup *et al.*, 2003, Engel *et al.*, 2004a, Barton & Jurado, 2007, Macalady *et al.*, 2008, Engel, 2010, Shabarova & Pernthaler, 2010), many of which are organized as microbial biofilms (Hose *et al.*, 2000, Macalady *et al.*, 2006, Macalady *et al.*, 2007, Engel, 2010). Such ecosystems can be mainly sustained by chemolithoautotrophic microbial activities within biofilms covering the walls, as well as microbial mats floating on cave water (Engel *et al.*, 2004b, Kumaresan *et al.* 2014). Extremophiles, including acidophilic, thermophilic and sulfidophilic microbes have been found to produce massive biofilms of various shapes and even snottites in caves (Angert *et al.*, 1998, Hutchens *et al.*, 2003, Northup *et al.*, 2003, Macalady *et al.*, 2008, Ziegler *et al.*, 2009). Deep subsurface energy inputs such as upwelling formation water, rich in hydrocarbons and outgassing thermogenic gas can provide substrates to cave biofilms (Hill, 1995, Hutchens *et al.*, 2003, Engel *et al.*, 2004b,

Shabarova & Pernthaler, 2010). Consequently, the microbial communities found in cave habitats reflect the inputs of different inocula, such as soil leakage carrying microbes especially during storm events (Rusterholtz & Mallory, 1994, Goldscheider *et al.*, 2006), and upwelling groundwater microbes originating from deeper strata (Farnleitner *et al.*, 2005).

The Sulzbrunn spring cavern (Bavaria, Germany) presents a unique model system for biofilm research. Massive biofilm growth has been observed on the walls and ceiling along the man-made gallery up to the natural cavern where mineral spring water with high iodine loads emerge (Ditterich, 1863) into a pool filling the cave half way up the wall (Figure 1- 11).

Situated in the fault region of the northern alps, gas seeps releasing thermogenic methane are fairly common (Etiope, 2009, Etiope *et al.*, 2010). The occurrence of iodine in groundwater is an indicator for formation waters which have been in contact with fossil oil or gas reservoirs (Moran *et al.*, 1995).

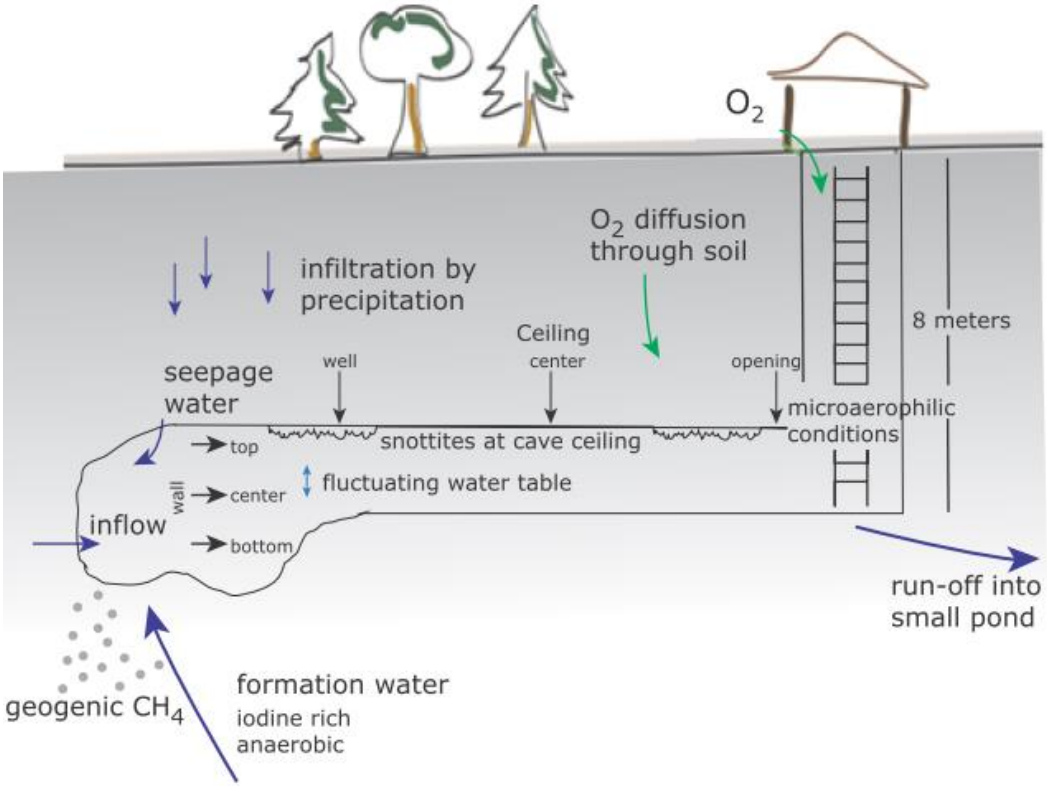


Figure 1- 11 The Sulzbrunn spring cave system: sampling locations and assumed fluxes. Two transects, (1) along the ceiling from the well towards the cave opening, and (2) a vertical profile of wall biofilms, covering potential physicochemical gradients in the cavern were sampled. Water mainly enters the cavern in the form of spring water but a substantial amount of seepage water also trickles down from the ceiling adding to the cavern water.

The role of iodine and associated microbial activities are still poorly understood but the accumulation and oxidation of iodide as well as the reduction of iodate in microbes has been shown mainly in marine systems (Councell *et al.*, 1997, Amachi *et al.*, 2007b, Amachi, 2008, Arakawa *et al.*, 2012). Specifically, the production of methyl iodide via volatilization of iodine (Hughes *et al.*, 2008, Fujimori *et al.*, 2012) and subsequent oxidation (McDonald *et al.*, 2002, Cox *et al.*, 2012) of these carcinogenic compounds could play a role in this cave.

Some characteristic, abundant taxa of uncontaminated alpine karst waters are *Beta-* and *Deltaproteobacteria*, *Acidobacteria*, *Nitrospira*, and *Firmicutes* (Engel, 2010). Besides these common groundwater bacteria, some extremophilic taxa have been found within biofilms and corrosion residues, affiliated with e.g. *Hyphomicrobium* sp., *Pedomicrobium* sp. and *Leptospirillum* sp., but rather directly related to acidic and sulfidic conditions (Hose *et al.*, 2000, Northrup *et al.*, 2003).

Snottites, subaerial pendulous biofilm structures, have been found in acid mine drainage karst environments at low pH (Bond *et al.*, 2000, Hose *et al.*, 2000, Ziegler *et al.*, 2009), but to date have not been encountered in less extreme environments. In a cave in Romania, active methanotrophs were found next to the dominating sulfur-oxidizing bacteria, representing the first report of methane oxidizing bacteria in caves, but no biofilm structures were reported (Hutchens *et al.*, 2003, Chen *et al.*, 2009, Kumaresan *et al.*, 2014). Thus, the Sulzbrunn cavern system has the potential to host the first methane driven snottite formation in a cave system described at circum-neutral pH.

Methylotrophic organisms are phylogenetically diverse and several "modules" of these pathways utilizing single-carbon compounds have been discovered (Anthony, 1982, Chistoserdova, 2011). These include the group of aerobic methanotrophs, which are able to thrive on methane as a sole carbon source. They are found within the *Alpha-* and *Gammaproteobacteria* and *Verrucomicrobia* (Hanson & Hanson, 1996, Op den Camp *et al.*, 2009, Dedysh & Dunfield, 2011). In addition, anaerobes oxidizing methane with sulfate (Boetius *et al.*, 2000, Thauer, 2011) and iron (Sivan *et al.*, 2011), and a special form of anaerobic methane oxidation driven by nitrite (Ettwig *et al.*, 2010) in oxygenic bacteria have also been described. Biofilm formation of methanotrophic microbes has been investigated in terms of overflow metabolism and EPS production (Linton *et al.*, 1986, Babel, 1992), if excess electrons are provided in the form of methane. After the initial oxidation of methane, toxic formaldehyde could accumulate but be shunted off via sugars that are built in the ribulose monophosphate pathway (Linton, 1990). These could provide building blocks for the exopolymeric substances, thereby providing an electron sink and nutrient storage (Branda *et al.*, 2005, Shi *et al.*, 2007).

The following research questions are addressed in this subproject: (i) What drives massive biofilm growth and who are the key players in the Sulzbrunn biofilm communities? If methane is present, methanotrophs should be abundant as well as a long tail of methylotrophs that consume methylated compounds. (ii) Do microbial biofilm communities in the cave reflect intrinsic physicochemical gradients?

1.3.3.3 Study 3: Technical and Drinking Water Systems

The microbiome of drinking water produced from groundwater is primarily influenced by the influx of microbes from the surrounding aquifer, as well as by biofilms established in the drinking water wells and the distribution network itself. Bacterial communities in drinking water distribution networks and also the impacts of disinfection and filtration have been intensively studied (Schmeisser *et al.*, 2003, Williams *et al.*, 2004, Poitelon *et al.*, 2009, Revetta *et al.*, 2010, Henne *et al.*, 2012, Pinto *et al.*, 2012, Zhang *et al.*, 2012, Lautenschlager *et al.*, 2013). Here, although classical cultivation-based approaches are a powerful tool to detect specific indicator taxa in potable water, they are known to allow for only a very limited grasp of total microbial communities (Szewzyk *et al.*, 2000).

Proteobacteria are the most dominant bacterial phylum in drinking water habitats, comprising sometimes up to 90 percent of total communities, represented mostly by *Alpha-, Beta-*, and *Gammaproteobacteria* (Schmeisser *et al.*, 2003, Pinto *et al.*, 2012, Zhang *et al.*, 2012). There are even some typical genera such as *Aquabacterium*, *Sphingomonas* and *Polaromonas* (Ultee *et al.*, 2004, Williams *et al.*, 2004, Loy *et al.*, 2005, Pinto *et al.*, 2012) that have been associated with extremely oligotrophic conditions. Other characteristic lineages frequently reported for drinking water communities are within the phyla *Bacteroidetes*, *Actinobacteria*, and *Nitrospira* (Henne *et al.*, 2012, Pinto *et al.*, 2012, Liu *et al.*, 2013). Some genera harbouring potential pathogens can also be frequently observed, such as *Aeromonas*, *Mycobacterium*, and *Legionella* (Szewzyk *et al.*, 2000). *Legionella* spp. seem to be especially ubiquitous in ground- and drinking water systems, even at low temperatures, but not necessarily connected to a pathogenic risk (Costa *et al.*, 2005, Wullings *et al.*, 2011).

Hydraulic well restoration by high pressure jetting is a routine maintenance method that can be used in drinking water production to maintain well productivity, by dislodging inorganic and organic deposits in the well casing (DVGW, 2007). Such a purging event offered a unique possibility to access the microbes established in the well vicinity, and to discriminate them against the base influx of microbes from the surrounding aquifer. To the best of my knowledge, such high-pressure jetting has never been followed from a microbial community perspective. It was expected that (i) before treatment, microbial communities from closely related wells in the

same aquifer should be rather similar, dominated by lineages typical for low nutrient groundwater. (ii) The taxa specifically dislodged during the maintenance procedure were distinct well microbiota established potentially as biofilms, in the well matrix. Moreover, it was tested whether (iii) high pressure jetting had a beneficial (= negative) effect on the detectability of bacterial lineages of potential pathogen affiliation after well restoration.

These questions were addressed in an operative drinking water production system in southern Germany during a routine hydraulic well purging event by the application of 454 pyrotag sequencing of bacterial 16S rRNA gene amplicons (Pilloni *et al.*, 2012) to suspended microbes, resulting in an extensive level of microbial identity data on the microbiota in this oligotrophic habitat.

2 Materials and Methods

The methods described here aim to characterize and comprehend the community structure and possible environmental controls of biofilms in groundwater systems. The author of this thesis together with Dr. Tillmann Lueders selected the sampling sites and developed the sampling design and experiments. All sampling and sample analysis was conducted by the author of this thesis if not stated otherwise.

2.1 Sampling, Sites and Experimental Setup

2.1.1 Colonization of Mineral Surfaces

The "mineral surfaces" experiment was conducted at the Helmholtz Centre Munich. Groundwater was pumped from a filtered well, situated in the Munich gravel plain at a depth of 16 meters and fed into a thermally isolated barrel (volume 300 liter) with a flow rate of 136 mL s^{-1} to provide a reservoir. The aquifer is characterized by sandy sediments and fractions of gravel and silt (Figure 2- 1 **A**). Effective porosity, defined as the mobile fraction of the water, was calculated to be 22 % (Karolak, 2010).

An experimental box was designed to allow for the exposure of mineral coupons under controlled groundwater inflow. Constant flow to the experimental box was attained by peristaltic pumps (IPC8 30 W, Ismatec, Switzerland), which transported water from the reservoir to 4 inlets of the box. The box was constructed from acryl with a size of 200 x 340 mm (Figure 2- 1 B & C) and was installed in the well pit at a depth of three meters to assure constant temperature at ~12 °C. The box was fixed vertically so the inlets were situated at the bottom and outlets at the top. The first 3 cm within the box were filled with glass beads with a diameter of 7 mm packed to a porosity of n=0.42 to ensure homogenous spreading of the water. Beforehand, glass beads were soaked with 1 M HCl and rinsed with double-distilled (bidest) water, then baked at 600 °C for 4 hours to remove residual carbon. Water flow was verified using a visual tracer (uranine solution). Mean water velocity was adjusted to ~40 centimeters per day. Within the box, sterile coupons (in total 11 coupons per mineral species) were arranged, one mineral type per row facing in alternating directions, to prevent transfer from adjacent communities.

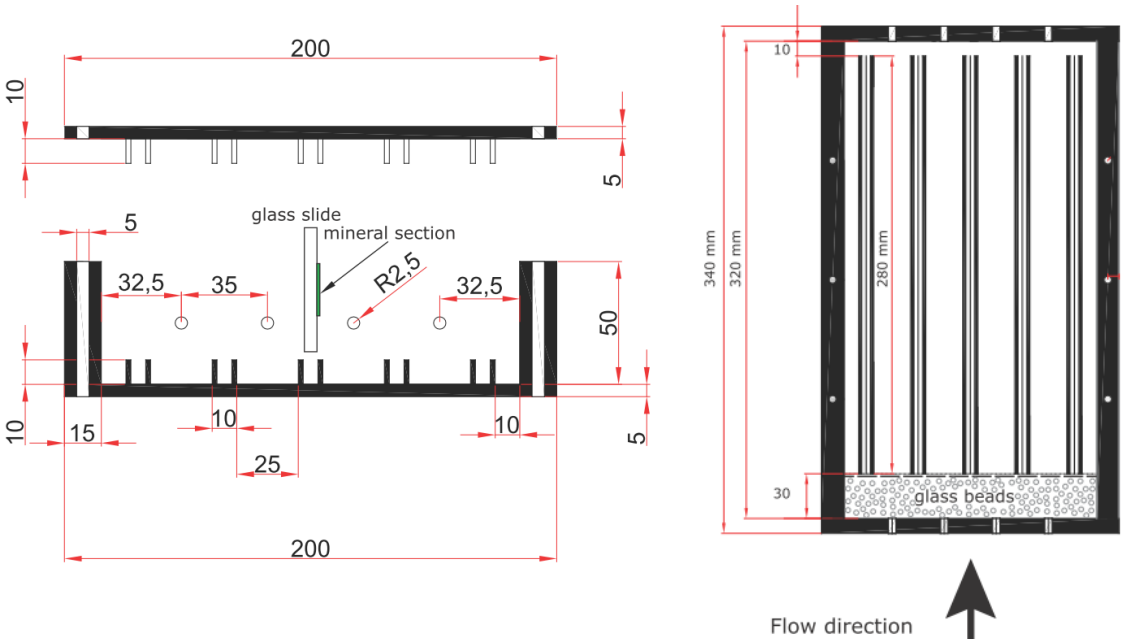


Figure 2- 1 Cross-section and top view of the mesocosm system. Glass beads at the inlet ensured homogenous flow. Glass slides with mounted mineral sections were fixed in an upright position.

Granite, apatite, pyrite and magnetite were attained from Wards Natural Science (USA) (Table 2-1). Mineral sections, which were cut and fixed on glass slides covering roughly 2 cm^2 (prepared by Franziska Häuser, Department of Earth and Environmental Sciences, LMU Munich) were fixed in an upright position into the flow through box. After 15 months, sample slides were removed with sterile tweezers and stored in 5 mL plastic tubes (Becton-Dickinson Biosciences). The slides were selected by stratified sampling dividing the mesocosm length into a front (slides 2 - 4), center (slides 5 - 7) and back section (slides 8 - 10), taking minerals in random order at least three for each analysis.

Table 2- 1 Minerals used in the colonization experiment and selected characteristics

Mineral Name	Typical Configuration	Potential	Category
Apatite	$Ca_5(PO_4)_3F_3$	Phosphate donor	Phosphate mineral
Magnetite	$Fe^{2+}Fe_2^{3+}O_4$	Electron acceptor	Oxide mineral
Pyrite / Sphalerite	FeS_2 / ZnS	Electron donor	Sulfide mineral
Granite	$KAlSi_3O_8$;$KAl(AlSi_4O_{10})OH$	"inert"	Igneous rock

2.1.2 Biofilms in a Spring Cavern

Cavern biofilms were sampled close to Sulzbrunn (N 47°40', E 10°21'), in the vicinity of the Allgäu Alps of Bavaria. Situated at 875 m altitude, the location is influenced by relatively high precipitation (1274 mm a^{-1}) and a mean annual temperature of 8 °C. A small mixed forest and a building surround the cavern entry. The cave entrance is closed by a metal cover plate (Figure 2- 2 **A**). An eight meter descent accesses a manmade gallery into the base of the cavern (Figure 1- 11). The corridor is 150 cm in height and 100 cm wide, it extends 8 meters into the hillside from where it drops about 150 cm into the natural cavern. The cavern is approximately 300 cm in height and 400 cm wide. On the cavern floor, a small pool is formed, filled with a layer of fine sediment. A stainless steel sampling flume has been installed from a fissure at the foot of the pool, but mineral water also seeps from below directly into the cavern bottom. Gas bubbles out at several spots within the cavern pool (Figure 2- 2 **B**). In addition to the mineral water, there is a constant inflow of hillslope seepage from the ceiling.

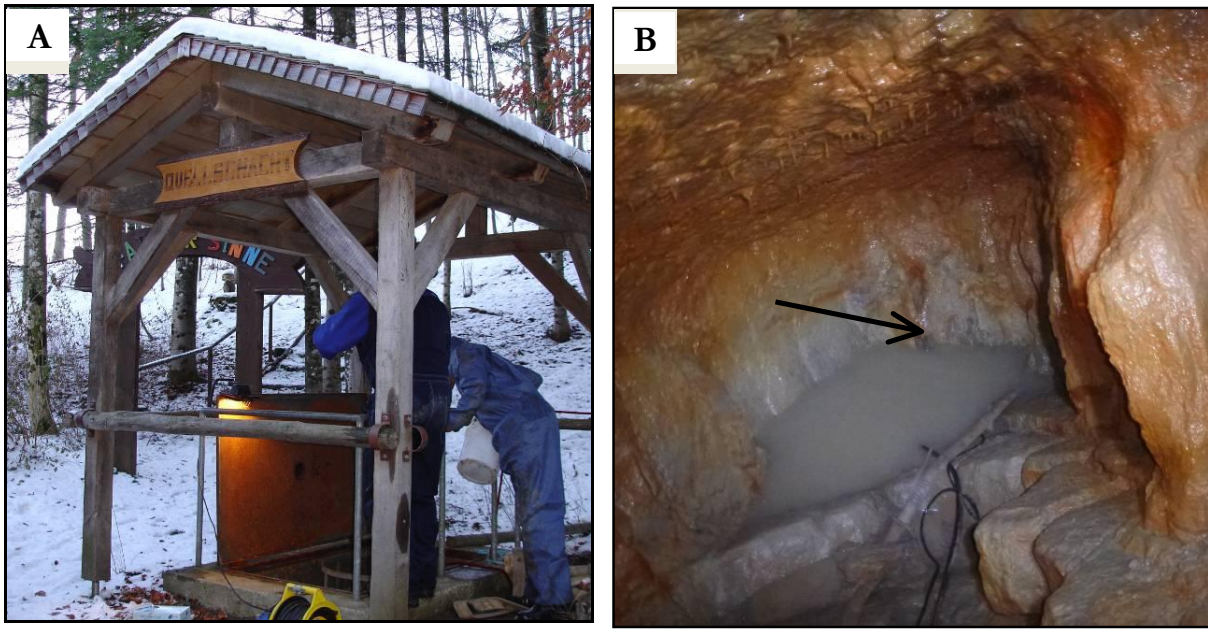


Figure 2- 2 A The entrance to the cavern is roofed and closed with a metal cover; photo from the sampling campaign in December 2013. **B** The collected mineral water enters the cavern through a sampling flume (arrow) in the cave wall. Outgassing methane and sediment was collected from the cavern pool. (Photo: Karwautz)

The ceiling is covered throughout with pendulous biofilms of up to 15 cm in length and 1 cm diameter at the base, termed "snottites". At the same time, the walls are covered with thick

biofilms from the water table up to the entrance, displaying different volume and hydration status. The water level within the cavern fluctuates depending on precipitation but an overflow at the entrance prohibits the water level from rising above 100 cm in the gallery.
Biofilm samples were collected after pumping water out for 24 hours and opening the metal cover to allow fresh air to fall into the cavern. Samples were directly transferred into 50 mL Falcon tubes (BD Biosciences, USA) and cooled until storage at -20 °C. The selected sampling points were located on the cavern ceiling with increasing distance from the mineral spring and a vertical profile of the biofilms covering the wall. Water samples were taken in sterile, one-liter glass bottles by collecting either spring water from the sampling flume or seepage water which trickles from the ceiling. The mixed cavern water was collected during separate sampling campaigns using a Ruttner sampler. Gas samples of the undisturbed cavern atmosphere were taken by installing a hose connected to a laboratory pump, filling sealed 120 mL serum bottles (Glasgerätebau Ochs, Germany).
Water samples in the immediate vicinity were taken from a small stream, which passes within 20 meters of the cave, and a second sample was taken from a close-by spring (~ 900 m distance) providing reference groundwater.

2.1.3 Drinking Water Wells

Drinking water wells were investigated at an operational drinking water production system in Baldham, close to Munich, Bavaria. A commercial service provider performed the well restoration of two wells in the course of two days. Samples were taken in cooperation with the local water supplier before, during and after hydraulic well restoration at an operational drinking water production unit east of Munich, Germany. Three groundwater extraction wells (Figure 2-3) located in close proximity to each other (~ 50 m) were sampled in the summer of 2010. The wells extend ~37 m below the ground into the Munich gravel plain. The groundwater table is ~18 m below surface and the aquifer extends 9 to 14 m down to an underlying impermeable tertiary clay layer. Well 2 is distinct from the others in that it extends through a ~10 m layer of more sandy gravel. While well 3 is permanently used for drinking water supply (pumping rate ~16 L s^{-1}), well 1 and 2 are backup facilities and are only operational for ~2 h per month (pumping rate ~100 L s^{-1}). Well 2 had developed signs of reduced hydraulic conductivity over several years.
Four days before the actual purging event, fresh drinking water was collected from each well via dedicated monitoring faucets, into previously sterilized 5 L glass bottles and immediately transferred to the lab. Samples taken during high pressure jetting were collected directly from the

operative suction hose at the beginning of the purging process, after 15 min, and after 60 min. The high pressure pump (up to 420 bar), operated by the service provider was combined with a submersible rotating jet forcing water out of several nozzles at a speed of up to 180 m s^{-1}. A last sample was taken two weeks after the procedure. Suspended solids purged during jetting (at the start, after 15 and 45 min of jetting) were collected in sterile 1 L glass bottles, transported to the lab and centrifuged at 5000 rpm for 15 min to collect suspended solids and attached biomass.

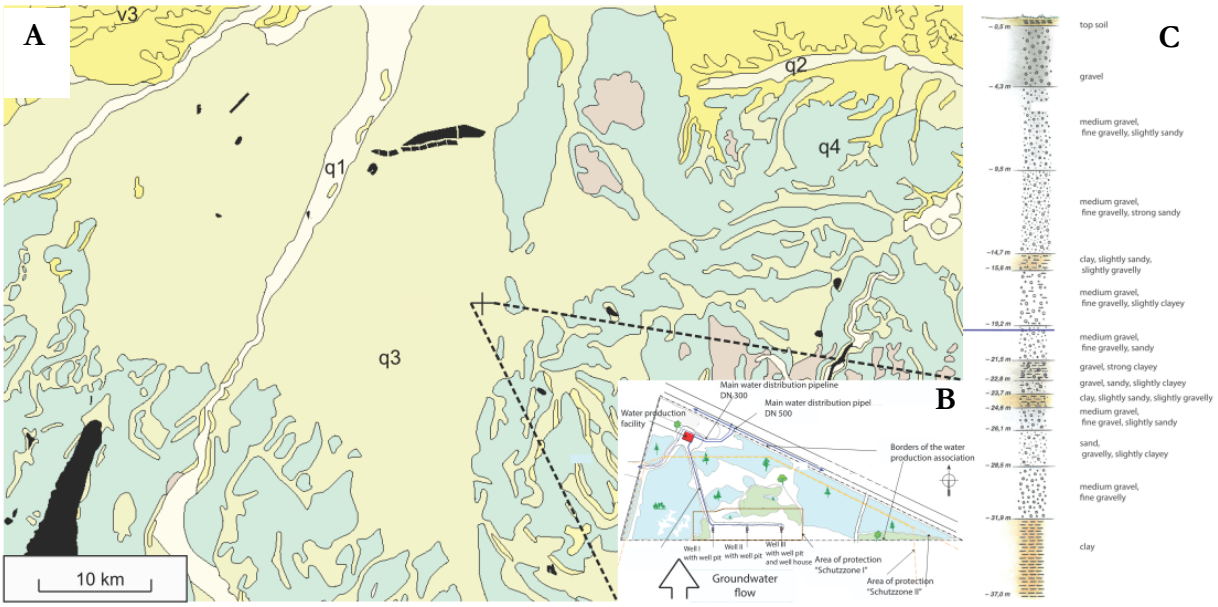


Figure 2- 3 A Hydrogeological map of parts of the catchment area. Taken from Landesamt für Digitalisierung, Breitband und Vermessung - http://geoportal.bayern.de/bayernatlas/.

Legend: q3 fluvial-glacial sediments, q4 morainic sediments, q1 quaternary river gravel, q2 quaternary river gravel and sands, v3 gravel and gravelly sands of the Bavarian upper freshwater molasse

Inlay B Aerial view of the water production area facility including production wells and indicated groundwater flow. Modified from the homepage of Wasserverband Baldham: http://www.wasserverband-baldham.de/sektionen/technik/grundstueck.gif.

C Groundwater well profile of well 2 taken from Ortner (1999).

2.2 Biogeochemical Analyses

2.2.1 Water Properties and Chemistry

In each of the three studies, common physicochemical water properties were evaluated. Water parameters such as pH, dissolved oxygen (DO), and specific conductivity were measured with calibrated field sensors (Hach Company, Germany). Water for dissolved organic carbon (DOC) analysis and ion chromatography was filtered through 0.45 μm syringe driven filter units (Merck Millipore, Germany) and stored at 4 °C in sterile, pretreated (soaked overnight in bidest water) glassware containing no residual carbon respectively collected in polystyrene vials for analysis by ion chromatography (IC).

DOC samples that were not analyzed immediately were acidified using 1 M HCl to a final concentration of 100 mM. DOC in carbon deprived, environmental samples was analyzed using high temperature combustion with infrared detection of CO_2 on a TOC-V (Shimadzu, Japan) with an ASI-V autosampler having a detection limit of 0.1 mg L^{-1}. If higher concentrations were anticipated, a TOC-5000 (Shimadzu, Japan) with a detection limit of 1 mg L^{-1} was used based on the same detection method (Dickson *et al.*, 2007). Major cations (calcium, magnesium, potassium, ammonium, sodium) and anions (nitrite, nitrate, chloride, bromide, sulfate) were measured on a DX-100 (Dionex, USA) ion chromatograph equipped with a CS12 and AS14A Ion Pac columns (Faye *et al.*, 2005) equipped with an AS40 auto-sampler.

2.2.2 Gas Samples

Gas samples were taken either directly from leaking bubbles within the Sulzbrunn cavern, collected into inverted bottles subsequently sealed with vinyl stoppers, or were sucked from outside the cavern using a laboratory pump (N 86, KNF Neuberger GmbH, Deutschland) with a flow rate of 25 mL s^{-1} and directly transferred into gas traps and sealed with stopcocks (Rotaflo, England). For this reason, plastic tubes were installed within the cave and placed at different locations. Tubing volumes were exchanged 5 times before sample collection. Samples for quantification were analyzed within two days, while those for isotope analysis were kept dark at 4 °C until measurement.

2.2.2.1 GC-TCD/HID

For methane concentration analysis, a 100 µL gas sample was manually injected into the gas chromatograph (GC) (SRI Instruments, USA) at constant helium flow. Gas samples were separated on a 6 meter 1/8" S.S. HayeSep D column (SRI Instruments, USA) at 50 °C. Measuring time was 4 min, allowing for the recording of nitrogen, methane and carbon dioxide. For quantification, a 5-point standard curve (10000 – 1000 ppm) was generated ($R^2 = 0.98$) using mixtures of N_2, CH_4, and CO_2 (all gases Linde, Germany). Measurements were taken in triplicates. Signals were recorded simultaneously on a helium ionization detector and a thermal conductivity detector set to high.

2.2.2.2 Isotope Analysis of Methane Gas Samples by GC-IRMS

Measuring carbon and hydrogen isotope values of sampled methane was accomplished by the candidate with the advice of the staff of the "Environmental Isotope Chemistry" group at the Institute for Groundwater Ecology, Helmholtz Zentrum München. Compound specific isotope ratios were measured using a TRACE GC Ultra gas chromatograph (GC) (Thermo Fisher Scientific; Italy), coupled to a FinniganTM MAT 253 IRMS (Thermo Fisher Scientific, Germany) connected by a FinniganTM GC Combustion III Interface as previously described (Bergmann *et al.*, 2011). The combustion oven temperature was 980 °C for carbon isotope analysis, while for hydrogen isotopes a pyrolytic interface was used (1390 °C). The GC was equipped with a programmable temperature vaporizer (PTV) injector (Optic3, ATAS GL International B.V.; Netherlands) heated to 100 °C. For isotope analysis of methane, the GC was equipped with a RT –QPLOT capillary column (30m x 0.32, Restek, USA) connected to fused-silica pre- and postcolumns (FS-Methyl-Sil, 2 m x 0.32 mm and 1 m x 0.32 mm, respectively; CS Chromatographie Service GmbH, Germany). For carbon analysis, carrier gas flow was set to 1.4 mL min^{-1} and a 100 µL sample was injected for each measurement making three measurements per run (800 s). Gas samples were injected by hand into the heated 100 °C injection port. Oxidation of carbon was achieved at 980 °C.
For hydrogen isotope analysis, the same settings and columns were used but carrier gas flow was set to 1.2 mL min^{-1} and 500 µL was injected for each measurement. The GC-oven was programmed to permanently hold at 40 °C.

2.2.3 Biofilm Characterization

2.2.3.1 Isotope Analysis of Biofilm Samples Using an Elemental Analyzer

Analysis via published methods (Bernstein *et al.*, 2010) was executed by Harald Lowag of the "Environmental Isotope Chemistry" group at the Institute for Groundwater Ecology, Helmholtz Zentrum München. Isotope analysis of biofilm samples was carried out on an elemental analyzer (EA; Euro Vector SPA, Italy) coupled with a combustion unit (Hekatech, Germany) connected to a Finnegan Mat 253 isotope-ratio mass spectrometer (IRMS; Thermo Fisher Scientific, Germany) to assess the carbon and nitrogen isotopic signature. Samples were lyophilized by means of freeze-drying and then ground to powder which was weighed into tin capsules for analysis. The temperature of the oxidation tube in the EA was 1000 °C and the temperature of the reduction tube was 600 °C. The temperature of the combustion tube was 1480 °C. For calibration, three different standards were analyzed in triplicate before and after the analysis of the samples. Standards used for $\delta^{13}C$ and $\delta^{15}N$ calibration were caffeine (IAEA-600) and l-glutamic acid (USGS-40 and USGS-41). In addition, a standard was analyzed after every seventh sample to calibrate shifts in the measurements. The caffeine standard was used for this purpose.

Equations and calculations

The isotope values generated by the IRMS for a given compound are relative to the isotopic composition of an international standard. This isotope ratio, δ, is measured in per mil units (‰).

$$\delta x = \frac{R_x - R_{std}}{R_{std}} \times 1000$$

where δx is the isotope ratio of the investigated compound, R_x and R_{std} are the ratios between the heavy and light isotopes in the investigated compound and in the international standard.

2.2.3.2 Trace Elemental Analysis

Peter Grill and Prof. Dr. Bernhard Michalke of the "Research Unit Analytical BioGeoChemistry" at the Helmholtz Zentrum München executed this published method. In brief, freeze-dried sample material was extracted by means of ashing under pressure at 170 °C in a Seif device with HNO_3 respectively with tetramethyl ammonium hydroxide (TMAH) for the determination of iodine. Elements in the extracts were determined using ICP-OES (inductively coupled plasma optical emission spectrometry) (Schramel, 1994)

2.3 Microbiological and Molecular Analyses

2.3.1 Coliform Screening on Commercial Agar Plates

Screening for coliform bacteria in drinking water, sampled directly at drinking water wells, was done by filtering 100 mL of the liquid sample onto Endo KS plates (Sartorius, Germany) which were incubated at 36 °C for 24 hours (EC Council Directive 1998).

2.3.2 Estimating Active Microbial Biomass via Adenosine Triphosphat (ATP) Measurement

Viable microbial biomass was estimated from ATP concentration as determined in water samples and cell suspensions attained from the mineral colonization experiment. Cellular ATP was measured using the BacTiter-Glo Microbial Viability Assay reagent (Promega Corporation, USA) and a luminometer (Glomax, Turner Biosystems, USA) following a modified protocol of Hammes *et al.* (2010). The ATP bioluminescence assay uses the reaction of the substrate luciferin catalyzed by the luciferase enzyme requiring ATP, oxygen and magnesium ions resulting in the emission of photons. Emitted light is measured quantitatively and correlated with ATP extracted from the microbial community. All reagents and samples are warmed to 38 °C prior to the measurement. A standard curve from 16.5 nM to 16.5 fM of ATP (Promega, USA) was used for calibration.

To measure total ATP, 1 mL of water sample and 50 µL of BacTiter-Glo reagent containing ATP releasing agents and the luciferase were mixed in sterile 2 mL reaction tubes (Eppendorf, Germany). After one minute of incubation at 38 °C, luminescence was measured. Extracellular ATP was measured by centrifuging aliquots of the same water sample at 7500 g for 20 min. The incubation and measurement procedure was repeated for the supernatant, which represented the cell-free ATP concentration in the water sample. Data were collected as relative light units (RLU) and further converted to ATP [M] by means of a calibration curve with known ATP standard concentrations (Roche, Germany) The conversion factors taken to calculate active biomass from ATP concentration were: 1 ng mL^{-1} ATP equals 250 ng mL^{-1} cell carbon and 2.95 x 10^{-9} nmol ATP relates to 1 μm^3 biovolume of a microbial cell (Hammes *et al.*, 2010). Finally, the cell carbon of a microbial cell from groundwater was set to 20 fg C for calculations (Karl, 1980).

2.3.3 Fluorescence In Situ Hybridization (FISH) with Labelled Oligonucleotide Probes

Samples from the Sulzbrunn cavern were immediately fixed with 4 % formaldehyde, pH 7 (2 g Paraformaldehyde in 50 mL PBS, 1N NaOH to adjust pH) for at least 12 hours and subsequently stored at 4 °C. After incubation, liquid samples were spun down (10 min 4000 g), the supernatant was discarded and fixed cells were resuspended in 500 µL 1X PBS. This was repeated two more times to ensure the removal of formaldehyde. Finally, samples were resuspended in a 1:1 mix of EtOH and 1x PBS. Glass slides were rinsed with H_2O bidest, dried and dipped into a prewarmed (70 °C) gelatin solution (0.1 % w/v gelatin and $KCr(SO_4)_2x12H_2O$) and then air-dried, enhancing the adherence of samples. Biofilm samples were fixed in a formaldehyde solution (4 %, pH 7) which was removed after 14 hours with a pipette and samples were washed with PBS (1X, pH 7.6) prior to embedding (see 2.3.4).
All subsequent steps were performed swiftly to avoid temperature fluctuations and unnecessary exposure of the probes to light.

Hybridization

Formamide concentration in the hybridization buffer (Table 2- 2) was adjusted to the salt concentration of the washing buffer (Table 2- 3). 10 µL of the liquid environmental sample was put on the prepared glass slide and dried at 37 °C. Frozen biofilm sections were also dried at 37 °C.
In order to further dry out the cells, sample preparation required soaking the samples in an ethanol series with increasing concentrations (50, 80, absolute) for 3 minutes each. Samples were dried and 9–18 µL (depending on sample dimensions) of pre-warmed (37 °C) hybridization buffer were spread on the specimen followed by incubation at 46 °C for 15 min in the hybridization oven (HB-1000 Hybridizer, UVP, USA). For this, glass slides were put into 50 mL tubes (Falcon, Becton-Dickinson, USA) and filled with clean, moistened paper towels. After 15 min, 1–2 µL of the specific probe were quickly added keeping the glass slide warmed on a heated metal block. Samples were then hybridized for 2 hours at 46 °C.

Table 2- 2 Hybridization buffer taken from Manz *et al.* (1992)

Reagent	Volume	Final concentration in hybridization buffer
Formamide concentration [%]		Depending on probe
5 M NaCl	360 µL	900 mM
1 M Tris HCl	40 µL	20 mM
20 % SDS, (w/v)	1 µL	0.01 %
H_2O bidest	Add to 2 mL	

Washing Procedure

Plastic tubes were filled with washing buffer (Table 2- 3) and warmed to 48 °C in a water bath. Specimens were removed from the oven, immediately rinsed with washing buffer, then immersed in pre-warmed 48 °C washing buffer for 15 minutes. Finally, they were rinsed with deionized water and dried. If needed, samples were counterstained with 10 µL DAPI solution (1 µg mL^{-1}) and incubated for 10 min at room temperature. The staining solution was washed off again with deionized water. To prevent rapid photo bleaching, dried samples were embedded in Citifluor (Citifluor Ltd, U.K) and fixed with a cover glass. Samples were either directly used for microscopy or stored at -20 °C in the dark.

Table 2- 3 Washing buffer for a hybridization reaction using 35 % formamide

Formamide concentration [%]	35
5M NaCl [µL]	700
TRIS / HCl buffer [µL]	1000
EDTA 0.5 M pH 8	500
20 % SDS, (w/v) [µL]	25
H_2O bidest [mL]	add 50

Table 2- 4 Names and position, nucleotide sequences, targeted organisms and references of FISH probes used

Probe name	Position *	Formamide Conc. [%]	Sequence 5' - 3'	Specificity	Reference
EUB338/I	338	0 - 50	GGC TGC CTC CCGTAGGAGT	Most *Bacteria*	Amann *et al.* (1990)
EUB338/II	338	0 - 50	GCA GCC ACC CGT AGG TGT	*Planctomycetales*	Daims *et al.* (1999)
EUB338/III	338	0 - 50	GCT GCC ACC CGT AGG TGT	*Verrucomicrobiales*	Daims *et al.* (1999)

ARCH345	345	0 - 30	TTC GCG CCT GST GCR CCC CG	*Archaea*	Rudolph (2004)
ARCH915	915	0 – 30	GTG CTC CCC CGC CAA TTC CT	*Archaea*	Stahl (1991)
ARCH1044	1044	0 – 30	GGC CAT GCA CCW CCT CTC	*Archaea*	Rudolph (2004)
ALF968	968	20	GGT AAG GTT CTG CGC GTT	*Alphaproteobacteria* except of *Rickettsiales*	Neef (1997)
BET42a	1027	35	GCC TTC CCA CTT CGT TT	*Betaproteobacteria*	Manz *et al.* (1992)
GAM42a	1027	35	GCC TTC CCA CAT CGT TT	*Gammaproteobacteria*	Manz *et al.* (1992)
Mg705	705	20	CTG GTG TTC CTT CAG ATC	Type I methanotrophs	Eller *et al.* (2001)
Mg84	84	20	CCA CTC GTC AGC GCC CGA	Type I methanotrophs	Eller *et al.* (2001)

* Positions refer to the 16S rRNA gene numbering of E.coli (Brosius *et al.*, 1978)
Further details on oligonucleotide probes are available at probeBase (Loy *et al.*, 2003)

Probes EUB338 I, EUB338 II, and EUB338 III (Table 2- 4) were used in equimolar concentrations as "EUBMix". Probes ARCH345, ARCH915, and ARCH1060 were used in equimolar concentrations as "ARCMix". Probes Mg705 and Mg84 were used in equimolar concentrations in "MgMix". The specified probes were labelled with the fluorescent dyes RhodaminGreen, DyLight 488, Cy 3 or Cy 5, as synthesized from Eurofins (Germany) as well as FITC and Cy3 (Biomers, Germany)

2.3.4 Cryosection - Biofilm Sections at the Micrometer Scale

For spatial analysis, biofilms were embedded in an O.C.T. compound (Sakura Finetek Europe B.V., Netherlands) and stored frozen at –20 °C until cryosectioning and further analysis. Cryosections were performed on a Cryostat (Leica CM 1860 UV, Leica Biosystems, Germany) by cutting slices of 10–25 μm thickness at a -20 °C working temperature. Those were then transferred onto poly-l-lysine coated microscope slides, air-dried and again stored at -20 °C until further analysis.

2.3.5 Image Acquisition using Epifluorescence Microscopy and Electron Microscopy

2.3.5.1 Epifluorescence microscopy of biofilms

Images of fluorescently labelled samples were obtained using an epifluorescence microscope with Apotome (Zeiss Imager AxioImager.Z1) with an 80 x Neoplanar objective. For excitation

of the fluorophores (Table 2- 5), a mecury lamp (Xcite 120, Olympus, Germany) was used. Pictures were recorded with a 5 mega pixel digital camera (Zeiss AxioCam MRc5) and optical sections were acquired using a confocal laser scanning microscope (Zeiss LSM 510 META, Zeiss, Germany) equipped with 488 and 633 nm laser lines.

Table 2- 5 Fluorophores and filters used for epifluorescence Microscopy

Fluorophore	Ex / Em	Filter	Excitation	Beam Splitter	Emission
DAPI	359 / 457	49	G 365	FT 395	BP 445/50
Dy488 /FITC	493 / 518	25	TBP 400 + 495 + 570	TFT 410 + 505 + 585	TBP 460 + 530 + 625
Cy3	549 / 562	43 HE	BP 550 / 25 (HE)	FT 570 (HE)	BP 605/70 (HE)
Cy5	646 / 664	50	BP 640 / 30	FT 660	BP 690 / 50

2.3.5.2 Scanning Electron Microscopy (SEM) of Mineral Coupons

Handling and image acquisition was carried out by Dr. Marianne Hanzlik from the Institute of Chemistry at the TUM in Garching. Mineral surfaces were scanned for indications of bioleaching and microbial colonization. Samples were rinsed with 0.2 µm filtered water, then frozen at -20 °C and subsequently air dried before investigation. Samples were sputtered with gold (Bal-Tec SCD005 Sputter Coater, Liechtenstein) for 70 seconds. The ~3 nm resolution microscope (JEOL JSM-5900LV, Japan) was equipped with an EDX detector which allowed for an elemental analysis of the samples.

2.3.6 Counting Microbial Cells Applying Flow Cytometry

Measurements were conducted by the author with the help of Nina Weber, a technician at the IGOE. Environmental samples were fixed with a 25 % glutardialdehyde solution to a final concentration of 2.5 % and stored at 4 °C. Samples were further processed as described in Hammes *et al.* (2008). In brief, counting beads (TrueCount Tubes, Becton-Dickinson, Germany) were dissolved with 1 mL sterile filtered 1x PBS to a final concentration of 49800 beads mL^{-1}, vortexed and added for standardization. A 250 µL aliquot of the bead suspension and 3 µL of SybrGreen (conc. 1000x) was added and mixed to each environmental sample (1 mL) and a control, containing only PBS buffer. Samples were then incubated for 15 minutes in the dark. Before measurement, abiotic particles had to be removed via a filter top system (CellTrics Partec, Germany). Total cell counts were quantified in a flow cytometer (Beckmann Coulter FC 500,

USA) equipped with a 488 nm and 633 nm laser. Each sample was always measured in duplicates and counted until 200 beads were counted in each sample.

2.3.7 Biolog - Community-Level Physiological Profile (CLPP)

This method provides a community-level approach for assessing patterns of carbon source utilization in mixed microbial communities. An inoculum was prepared by cleaning three surface coupons into a plastic tube with a sterile electronic toothbrush for 30 seconds and then rinsing with 0.2 μm filtered groundwater before collecting a 10 mL cell slurry. The toothbrush was washed again with 80 % ethanol and subsequently thoroughly rinsed with water (bidest). Bacterial biomass was estimated employing ATP concentration measurements. The inoculum for the carbon utilization assay was normalized by diluting the samples to equal starting concentrations based on measured ATP concentration.
Sample slurries taken from the mineral surfaces were inoculated to microtiter plates (Biolog™ Ecoplate, Biolog Inc., USA) composed of 96 wells containing 31 different sole carbon sources and nutrients plus controls without a carbon source in triplicates. Through direct inoculation of the environmental samples into the microtiter plates, a color formation from the reduction of a tetrazolium dye was generated. Oxidation of the carbon source is indicated by the reduction of 2,3,5-triphenyl tetrazolium chloride (TTC) to the colored, insoluble formazan. To assess utilization of the separate sole carbon sources, plates were inoculated with 150 μL of the normalized sample and incubated at 16 °C. The development of color was measured spectrophotometrically at 595 nm on a VICTOR3 plate reader (Perkin Elmer, USA) periodically, for 5 days (132 hours). The plate reader started by shaking the plate and measured each well in duplicates. Well color development was calculated by subtracting the absorbance of the control wells filled with no additional carbon substrate from the respective absorbance value. Median values were taken for the two measurements of the three well replicates (n=6). The well color development (Garland & Mills, 1991) was calculated for each time point measurement taking median values of the corrected absorbance values of each plate. Calculations were similarly performed for carbon sources which were grouped into chemical guilds as suggested (Zak *et al.*, 1994, Preston-Mafham *et al.*, 2002) and diversity indices were calculated (see data 2.4.2).

2.3.8 DNA Extraction

A modified extraction protocol based on Lueders *et al.* (2004) was used for water and sediment samples. Cave biofilms were pretreated specifically (Cury & Koo, 2007) to interrupt the EPS

matrix. 1–2 g samples (wet weight) were mixed with 7 mL sterile filtered 1x PBS and the supernatant was carefully removed after 5 min. The samples were then re-suspended with 7 mL PBS, vortexed for 30 s, sonicated for 30 s (35 kHz, Sonorex RK102; Bandelin Electronic GmbH & Co, Germany) and subsequently the sample was centrifuged 10 min at 5500 g at 4 °C. The supernatant was then removed and the previous three steps (vortex, sonicate, centrifuge) were repeated two more times. Finally, 500 µL of the cell slurry was transferred to a bead beating cup and processed as described for all other samples.

Solid samples were weighed and 0.2–0.4 g of a sample (wet weight), or cut water filter respectively (Corning, USA) with filtered sample material was added to bead beating cups filled with 0.2 mL of a 1:1 (v/v) mixture of 0.1 mm zirconia/silica beads and 0.7mm zirconia beads (Biospec Products Inc., Bartlesville, USA).

750 µL PTN buffer (pH 8) containing 120 mM $NaPO_4$, 125 mM Tris-HCl and 25 mM NaCl, were vortexed for 10 s and subsequently 40 µL lysozyme with a concentration of 50 mg mL^{-1} in 1x TE (10 mM Tris-HCl and 1 mM EDTA) as well as 10 µL Proteinase K (10 mg mL^{-1} in 1x TE) were added. Samples were then mixed manually and incubated at 37 °C for 15 min in a Thermomixer (Eppendorf, Germany). An additional 100 µL of 20 % (w/v) sodium dodecyl sulfate (SDS) was added and again incubated this time at 65 °C by shaking at 500 rpm for another 15 min. The cells were lysed using a combination of the phenol chloroform extraction and bead beating. In all of the subsequent steps, the samples were kept on ice during handling. All chemicals used were molecular grade from Sigma-Aldrich (St. Louis, USA), if not stated otherwise. A 100 µL phenol-chloroform-isoamylalcohol (25:24:1, pH 8) (PCI) solution was added followed by a first round of bead beating. Bead beating was performed on a cell disruptor (FastPrep24, MP Biomedicals, USA) for 45 s at a setting of 6 m s^{-1}. After centrifugation (5 min, 4 °C, 6000 g), 600 µL of the supernatant was stored in a separate vial and the remaining sample was mixed with up to 300 µL PTN and again extracted via bead beating for 20 s at 6.5 m s^{-1}. The suspension was again centrifuged and 300 µL of the resulting supernatant was pooled with the previous. Further extraction was accomplished by vigorous shaking with an equal volume of PCI (25:24:1, pH 8) and the phases were separated again by centrifugation (4 min, 4 °C, 12000 g). To remove the phenol, the liquid phase was transferred to a Phase Lock Gel Heavy 2 mL vial (5 Prime, Germany) and mixed with an equal volume of chloroform-isoamylalcohol (24:1) followed by centrifugation (4 min, 4 °C, 12000 g). Afterwards, the DNA from the extracted liquid phase was precipitated by mixing with two volumes of 30 % (w/v) PEG solution (polyethylene glycol 6000, 1.6 mM NaCl). Samples were then stored for a minimum of 6 hours at 4 °C and subsequently centrifuged for 2 hours at 4 °C and 12000 g. The DNA pellet was then washed

with ice cold 70 % (v/v) ethanol, air dried and dissolved in 25-40 µL elution buffer (Qiagen GmbH, Germany), depending on the expected yield.

2.3.9 Polymerase Chain Reaction (PCR)

Preparation and mixing of the reagents and sample DNA were performed under sterile conditions (UV Box, UVP, USA), reagents were kept on ice.
The PCR was scaled to 50 µL comprising of 5 µL of 10 x PCR buffer, 3 µL of 1.5 mM $MgCl_2$, 0.5 µL of 0.1 mM dNTPs, 0.25 µL (5 units μL^{-1}) of recombinant Taq polymerase (all from Thermo Fisher, USA), 0.5 µL of 0.2 $\mu g\ \mu L^{-1}$ bovine serum albumin (BSA) (Roche, Germany), 0.3 µL of each primer (50 µM) (Biomers, Germany) and 1 µL of template DNA. For primers see Table 2- 6 and for temperature profiles Table 2- 7.

2.3.10 Gel Electrophoresis and Amplicon Purifaction

DNA extracts quality and integrity was inspected by gel electrophoresis. 5 µL of raw DNA extract was mixed with 1 µL of loading dye (Thermo Fisher, USA) and applied to a 1.5 % agarose gel. Gel electrophoresis was done with 1x Tris-acetate-EDTA (TAE) buffer at 90 V and 150 W for 45 minutes. Afterwards, the gel was stained for 10 minutes with 3 % GelRed (Biotium, USA) and DNA bands were visualized under UV light. The same procedure was followed to visualize amplified DNA. Clearly visible bands of the expected size and lack of contamination by humic acid were used as criteria for further usage of the amplicon.
Amplicons were purified of primers, nucleotides, enzymes, salts and residual impurities with the commercial PCRExtract kit (5PRIME, Germany). This kit contains a silica membrane binding DNA in a high-salt buffer and elution with a low-salt buffer or water.

Table 2- 6 Primer names and amplification direction, nucleotide sequences, targeted organisms or functional genes and original reference of primers used in PCR and qPCR

Name direction	Sequence 5' - 3'	Specificity	Reference
27f forward*	AGA GTT TGA TCM TGG CTC AG	16S rRNA most Bacteria	(Suzuki & Giovannoni,
907r reverse	CCG TCA ATT CCT TTG AGT TT	16S rRNA most Bacteria	Amann *et al.* (1992)
519r reverse	TAT TAC CGC GGC KGC TG	16S rRNA most Bacteria	Lane (1991)
Ar109f forward	ACK GCT CAG TAA CAC GT	16S rRNA most Archaea	Whitehead & Cotta (1999)

Ar912rt reverse*	CTC CCC CGC CAA TTC CTT TA	16S rRNA most Archaea	Lueders & Friedrich (2000)
Euk20f*	TGC CAG TAG TCA TAT GCT TGT	18S rRNA Most Eukarya	Kowalchuk *et al.* (1997)
Euk516r	ACC AGA CTT GYC CTC CAA T	18S rRNA Most Eukarya	Amann *et al.* (1990)
A189f forward*	GGN GAC TGG GAC TTC TGG	*pmoA*	Holmes *et al.* (1995)
mb661 reverse	CCG GMG CAA CGT CYT TAC C	*pmoA*	(Costello & Lidstrom, 1999)
A682 reverse	GAA SGC NGA GAA GAA SGC	*pmoA*, *amoA*	Holmes *et al.* (1995)
mxaf1003 forward	GCG GCA CCA ACT GGG GCT GGT	methanol dehydrogenase	
mxar1561 reverse	GGG CAG CAT GAA GGG CTC CC	methanol dehydrogenase	McDonald *et al.* (1996)
V170f	GGA TWG ATT GGA AAG ATM G	*pmoA Methylacidiphilum*	Sharp *et al.* (2012)
V613b	GCA AAR CTY CTC ATY GTW CC	*pmoA Methylacidiphilum*	Sharp *et al.* (2012)
cmuA929f forward	AAC TAG CTG CTG AGG TTG GCT AYA AYG GNG G	Methyltransferase / corrinoid-binding	McAnulla *et al.* (2001a)
cmuA1669r reverse	CAA CGT ATA CGG TGG AGG AGT TNG TCA TNA C	Methyltransferase / corrinoid-binding	McAnulla *et al.* (2001a)

* indicates FAM labelled primers used in T-RFLP

Temperature Profiles

Amplification reactions were performed on preheated thermal cyclers using either a Mastercycler EP Gradient (Eppendorf, Germany) or a SureCycler 8800 (Agilent, USA).

Table 2- 7 Temperature profiles and the number of cycles used in Polymerase Chain Reactions

Amplicon	**16S rRNA**	**18S rRNA**	***mxaF***	***pmoA***	***cmuA***
Initial denaturation	94 °C 5 min	94 °C 5 min	94 °C 5 min	92 °C 5 min	94 °C 5 min
Number of cycles	28 *Bact.* 30 *Arch.*	28	28	25	30
Denaturation	94 °C 30 s	94 °C 30 s	94 °C 60 s	94 °C 60 s	94 °C 60 s
Annealing	52 °C 30 s	55 °C 30 s	55 °C 60 s	55 °C 60 s	55 °C 60 s
Elongation	72 °C 60 s	70 °C 60 s	72 °C 60 s	72 °C 60 s	72 °C 60 s
Final elongation	70 °C 5 min	70 °C 5 min	72 °C 5 min	72 °C 5 min	72 °C 10 min
Reference			Dumont & Murrell (2005).	Costello & Lidstrom (1999)	Miller *et al.* (2004)

2.3.11 Terminal Restriction Fragment Length Polymorphism (T-RFLP) Fingerprinting

The bacterial, archaeal, and eukaryotic community were analyzed by 16S and 18S rDNA gene-targeted terminal restriction fragment length polymorphism (T-RFLP) fingerprinting (Liu *et al.*, 1997) with primers specified (Table 2- 6). Forward primers were labelled at the 5' end with 6-FAM (6- carboxyfluorescein), which has a maximum absorbance at 492 nm and emission maxima at 517 nm, binding to the amplified DNA. PCR was conducted as described above (2.3.9).

Restriction

In total, a volume of 8.7 µL amplified DNA (9.2 ng μL^{-1}) was cut by adding 0.3 µL restriction enzymes (10 units μL^{-1}) and 1 µL buffer (all Thermo Fisher, USA) and incubating the mixture for 2 h at 37 °C (bacteria, eukarya) or 65 °C for archaea. The restriction enzymes used were *MspI* and *AluI* for bacteria (Liu *et al.*, 1997), and *Bsh1236I* for eukarya (Euringer & Lueders, 2008). Restriction of the amplified and labelled *pmoA* genes was performed using *MspI* and *HhaI* (Costello & Lidstrom, 1999).

Fragments were subsequently desalted with DyeEx 2.0 Spin Kit columns (QIAGEN, Germany). Aliquots of 1 and 3 µL were combined with a mixture of high definition formamide and 6-carboxy-X-rhodamine-labeled MapMarker 1000 ladder (BioVentures, USA) in a 1:400 dilution. Fragments were denatured at 95 °C for 5 min and stored in the fridge (4 °C) until fragment analysis.

Automated fragment electrophoresis was performed at the genome analysis center, where fragments were separated by capillary electrophoresis on an ABI 3730 DNA analyser (Applied Biosystems, Germany). Electrophoresis was executed with POP-7 polymer in a 50 cm capillary array under the following conditions: 10 s injection time, 2 kV injection voltage, 7 kV run voltage, 66 °C run temperature and 63 min analysis time.

The electropherograms were then evaluated with the Gene Mapper 5.1 software (Applied Biosystems, Germany) and T-RFLP data was analyzed with the online T-RF analysis software T-REX (Culman *et al.*, 2008). Background noise filtering (Abdo *et al.*, 2006) selected all peak heights above ±1 of the standard deviation and the clustering threshold for aligning peaks across the samples was set to 2, binning all peaks within two base pairs. Relative T-RF abundance was inferred from peak heights. For reduction of data complexity, T-RFs that occurred in less than 5 % of the samples were excluded from further analysis.

2.3.12 Quantitative PCR (qPCR)

To estimate bacterial gene abundance of samples from the Sulzbrunn cave, qPCR was prepared for 16S rRNA genes with a Stratagene MX3000P qPCR cycler (Agilent, USA). Gene copy numbers per DNA extract were measured for three dilutions in triplicate. Dilution factors were $1x10^{0}$, $1x10^{-1}$ and $1x10^{-2}$. Quantitative PCR was performed using the PCR settings described above (1.1.1) while also adding the fluorescent dyes SybrGreen (0.25 µL 1/500) for DNA detection and ROX (0.75 µL 1/500) (both Life Technologies, USA) for equilibration and adding 2 µL sample instead of 1 µL. Quantitative PCR was performed at an initial denaturation temperature of 94 °C (3 min) followed by 40 cycles of denaturation (94 °C, 30 s), annealing (52 °C, 30 s) and elongation (70 °C 30 s), and subsequent denaturation (95 °C, 1 min), reassociation (55 °C, 30 s) and a dissociation ramp (55 °C to 95 °C, 30 min). The specificity of the PCR products was verified by melting curve analysis. A full length 16S rRNA of *Azoarcus* sp. strain T, with a known concentration, was used in a dilution series between $1x10^{7}$ and $1x10^{1}$ molecules per µL as a standard curve to convert measured threshold cycles to rRNA gene copy numbers (Kunapuli *et al.*, 2007).

2.3.13 Amplicon Pyrosequencing

2.3.13.1 Amplicon Pyrotag PCR

Barcoded amplicons were generated for forward and reverse reads with the same PCR conditions as specified (Table 2- 7). Primers were fused with A or B adapters (for forward and reverse discrimination) and multiplex identifiers (MID), a small barcode sequence (Roche, 2013b). PCR products were purified with the NucleoSpin clean-up kit (Macherey - Nagel, Germany) and Agencourt AMPure magnetic beads (Beckman Coulter, Germany) as specified by the manufacturers. Amplicons were extracted twice with 20 µL 1x TE buffer. After the first purification a second purification step was necessary to guarantee the removal of all short fragments. Furthermore, each amplicon was checked for primer dimer contamination and correct fragment size using the Bioanalyzer2100 (Agilent, USA) by loading High Sensitivity DNA assay chips (Agilent, USA), as described by the manufacturer.

The PicoGreen® dsDNA quantification assay (Life Technologies, USA) was used to exactly measure amplicon quantity based on standard curve concentrations (2.5, 2, 1.5, 1, 0.5, 0.1, 0.05 and 0 µg/mL). Samples were measured in two dilutions and each in duplicates with the Stratagene MX3000P qPCR cycler (Agilent, USA).

All amplicons were diluted to 1x 10^{9} molecules μL^{-1} by applying the following equation:

$$sd_{pooled} = \sqrt{\frac{var_{boot} + var_{permut}}{2}} \text{ molecules } \mu L^{-1} = \frac{\text{sample conc. [ng/}\mu\text{L]} \quad 6.022 \quad 10^{23}}{656.6 \quad 10^{9} \quad \text{amplicon length [bp]}}$$

where 6.022 x 10^{23} is Avogadro's number in molecules mol^{-1}, and 656.6 is the average molecular weight of nucleotide pairs, in g mol^{-1} (Roche, 2013b).

Samples with differing MIDs (20–26 samples) were then pooled for subsequent emulsion PCR (emPCR). Pooled amplicon libraries were then diluted to 1x 10^{7} molecules μL^{-1}. emPCR and sequencing was done by Katrin Hörmann (Molecular Ecology, IGOE, HMGU) as recommended by the manufacturer in the emPCR Method Manual (Roche, 2013a). To find the best bead enrichment percentage (8 %) for plate loading, emulsion titration was done before each run in three different concentrations. For emulsion PCR, capture beads were loaded with amplicons, amplification mix and emulsion oil was added. To ensure encapsulated PCR amplification for every bead, the emulsion was established by shaking with a tissue lyser before amplification. Beads were washed and recovered and amplicon bearing beads were bound to magnetic enrichment beads that retained the beads with the amplified DNA on a magnet. In this step, beads without amplified material are removed with the surrounding buffer. Finally, sequencing primers for forward and reverse reads were annealed to the amplicons.

2.3.13.2 Pyrosequencing

Sequencing itself was done by the EGEN research unit environmental genomics at the HMGU, with a 454 GS FLX pyrosequencer and Titanium chemicals (Roche). Amplicons fixed to DNA beads were mixed with packing beads and filled into the picoTiterPlate (PTP) in between two layers of enzyme beads. A layer with PPiase beads preventing interferences between wells was placed on top. Next, sequencing took place with 100s automated rounds of alternating additions of either dATP, dTTP, dGTP or dCTP and washing. Each successful incorporation of a nucleoside resulted in the release of PPi which was then coupled to adenosine 5´ phosphosulfate by ATP sulfurylase. ATP, in turn, was hydrolysed again by luciferase and a fluorescent signal was emitted indicating base by base the nucleotide sequence of the amplicon (Margulies *et al.*, 2005).

2.4 Data Processing

2.4.1 Processing Pyro-tag Sequence Reads

Raw data was transformed to conventional sff files using a linux script written by Marion Engel (EGEN, HMGU). Data analysis was performed either using a protocol developed in-house (Pilloni *et al.*, 2012) or via the mothur pipeline (Schloss *et al.*, 2009).

2.4.1.1 In-house Pipeline (Pilloni *et al.*, 2012)

Sequences from pooled amplicon libraries were de-multiplexed according to their sample and primer barcode, generating fasta and qual files which were further used to trim data with respect to its quality scores using the TRIM function of the GreenGenes web application (DeSantis et al. 2006). Default settings (good quality threshold: 20, window size: 40, percentage: 90) were applied. Sequences shorter than 250 base pairs (bp) were eliminated with BioEdit (Hall 1999). These sequences were then classified by the web application RDP classifier (Wang et al. 2007).

Contig Assembly and TRF Prediction

Sequences were split into forward and reverse reads in BioEdit using primer nucleotide information. "Contigs" were assembled from overlapping reads of closely related taxa in each single sample with SEQMAN II (DNAStar, USA) using an assembly threshold of 98 % sequence similarity in a window size of 50 bp. Contigs containing at least one forward and reverse read were used for further analysis. The generated sequences were again submitted to the RDP classifier (Wang et al. 2007) after setting the taxonomic classification threshold to 80 %. T-RFs were computed with TRiFLe (Junier et al. 2008) based on contig sequences, specifying primer sequences and restriction enzymes used for original T-RFs.

2.4.1.2 Mothur Pipeline

Processing of sff files was conducted with the software "mothur" (Schloss *et al.*, 2009). The standard script (Schloss *et al.*, 2011) was modified. In brief, flowgrams were denoised using the implemented PyroNoise algorithm (Quince *et al.*, 2011), by setting the flow length to 360 and 720 flows, and then allowing two primer differences and one difference in barcode sequences. Next, sequences were trimmed selecting only sequences longer than 250 bp, with less than 8 homopolymers, allowing only one primer mismatch. Sequences were aligned to the SILVA-compatible alignment database. Sequence artifacts were identified with the implemented Chimera.uchime (Edgar *et al.*, 2011) and removed. Remaining sequences were binned into operational taxonomic units (OTUs) at a 97 % sequence similarity cut-off, using the average

neighbor-clustering algorithm. Figure 3- 12 shows the pyrosequencing results obtained from the Sulzbrunn cavern system prepared with Krona, an interactive visualization program (Ondov *et al.*, 2011).

2.4.2 Data Handling of Substrate Utilisation Assay

Absorbance values at 595 nm were used to evaluate the well colour development over time (Garland & Mills, 1991). Measurements for each plate at the beginning of the experiment (t0) were used as blank values. The median value of replicate well measurements (n=3) and repeated measurement after vigorous shaking (in total 6 values) was computed for each well. For each median substrate value, the median blank value of water was subtracted. Negative values were set to zero. Well colour development was calculated by taking the sum of all absorbance values (n=31) for each time point. Error bars for the well colour development plots (Figure 3- 2) were comprised of the standard deviations of all measurements. Diversity was expressed either as substrate use richness, by counting all positive well values or as Shannon diversity (H') index $H' = -\Sigma\, p_i \ln p_i$ (Shannon, 1948), where p_i is the proportion of a measured value belonging to the i^{th} value in the dataset.

2.4.3 Multivariate Analysis

All calculations were performed using the open-source platform R (version 3.1.0) and several of the available packages. The vegan package (Oksanen *et al.*, 2013) was especially used to calculate diversity indices and perform ordination techniques as described below.

2.4.3.1 Principal Coordinate Analysis (PCA)

PCA was used for the ordination of T-RFLP data of *pmoA* fragments as well as the pyrosequencing results acquired in the well restoration field sampling. A PCA transforms variables into new uncorrelated variables called principal components or factors. Multivariate datasets can be reduced to a few components to find patterns or structures. The scores for each object on each component are called z-scores (Quinn & Keough, 2002). Within the PCA plot, the eigenvalues of parameters (e.g. abundance of OTUs) are depicted as vectors. The directions and length of a vector represent the correlation with the principal component (Ramette, 2007).

2.4.3.2 The Additive Main Effects and Multiplicative Interaction Model (AMMI)

This double-centered PCA, implemented in the T-REX software (Culman *et al.*, 2008), using used to find differences in the T-RFLP dataset of the microbial communities sampled from the

mineral surfaces. First, an ANOVA is calculated to estimate the variation of captured or minimal assumptions about the data. AMMI creates an interaction matrix by removing sources of variation (Gauch Jr, 1992).

2.4.4 Network Construction and Network Analysis

A network was constructed using the open-source platform Cytoscape (version 3.1.1.) and the CONET plugin (Faust *et al.*, 2012). The cave biofilm community dataset generated with pyro-tag sequencing was used to find possible interactions partners at the family level. The network was constructed following the description of Faust *et al.* (2012). In brief, the data matrix containing relative abundance at the family level was filtered to remove all taxa appearing in less than 5 samples and was then normalized for each sample. Four measures of correlation or dissimilarity (Pearson, Spearman, Kullback-Leibler, Bray Curtis) were calculated to compute pair-wise scores. The top and bottom 100 edges were kept for each measure. The network was recomputed with 1000 permutations. Next, 1000 bootstrap matrices were computed by sampling columns of the original matrix with replacement, thereby constructing confidence intervals around the edge scores. Edges with scores below the 95 % confidence interval were discarded. A measure- and edge-specific p-value was obtained from a Gauss curve defined by the mean and standard deviation of the bootstrap distribution. Variances of the bootstrap and permutation distribution were pooled by taking the standard deviation of the null distribution into account.

$$sd_{pooled} = \sqrt{\frac{var_{boot} + var_{permut}}{2}})$$

Moreover the p-value was computed as:

$$p\text{-val} = pnorm(mean_{permut}, mean=mean_{boot}, sd= sd_{pooled})$$

High p-values correspond to negative relationships (low similarities and high distances) and were converted into low p-values by computing 1 - p-value for all p-values above 0.5.
Each edge is supported by a set of measure-specific p-values, which are dependent, since the measures are correlated. P-values were merged with the Sime's method (Dai *et al.*, 2012), which kept the minimum p-value as the merged p-value of the edge. Merged p-values were then corrected for multiple hypothesis testing using the false discovery rate control (Benjamini & Hochberg, 1995). Edges with p-values above a confidence interval of 0.05 were discarded.

3 Results

The experiments and field studies described here provide insights into the multifaceted appearance and role of biofilms in groundwater ecosystems. This thesis focuses on the analysis of microbial communities based on established fingerprint methods such as T-RFLP and state-of-the-art community sequencing. These molecular tools were used to target marker genes, such as the 16S rRNA of bacteria, 18S rRNA of eukaryotes and functional genes like *pmoA* for methane oxidizing bacteria. Additional parameters e.g. water chemistry, elemental composition, gas concentrations and isotope signatures were analyzed to understand the ecological niches within these biofilm communities. Datasets were mined using multivariate statistics and results are presented in integrative plots and tables. Images from epifluorescence microscopy and electron microscopy (EM) are provided for an additional discussion of biofilm features.

3.1 Mineral Surfaces Attachment Experiment

The experiment was set up in a mesocosm system equipped with mineral coupons to grow near natural biofilms. Groundwater was pumped from a depth of 16 meters from an aquifer in the Munich gravel plain. The water was filled from a thermally isolated reservoir (Figure 3- 1) at the surface to ensure constant water flow. Subsequently, the groundwater was fed into the mesocosm using a peristaltic pump (Ismatec, Switzerland), applying low in situ flow velocities. A characterization of the physicochemical parameters of the groundwater is presented in Table 3-1, followed by a microbiological analysis of the seeding community and the biofilm communities growing on the mineral coupons after 15 months.

3.1.1 Water Analyses

Tertiary groundwater pumped at high rates (~490 L h^{-1}) into the first container displayed constant physicochemical parameters throughout the year (Table 3- 1). The water was oxygenated and had a circum-neutral pH and constant temperature. Dissolved organic carbon in the groundwater was low (0.5 mg L^{-1}) and was only marginally higher in the outflow of the mesocosm (1.1 mg L^{-1}). Groundwater was pumped through the mesocosm with a constant flow rate of 1.6 L h^{-1}, which corresponds to a mean residence time of 1.7 hours.

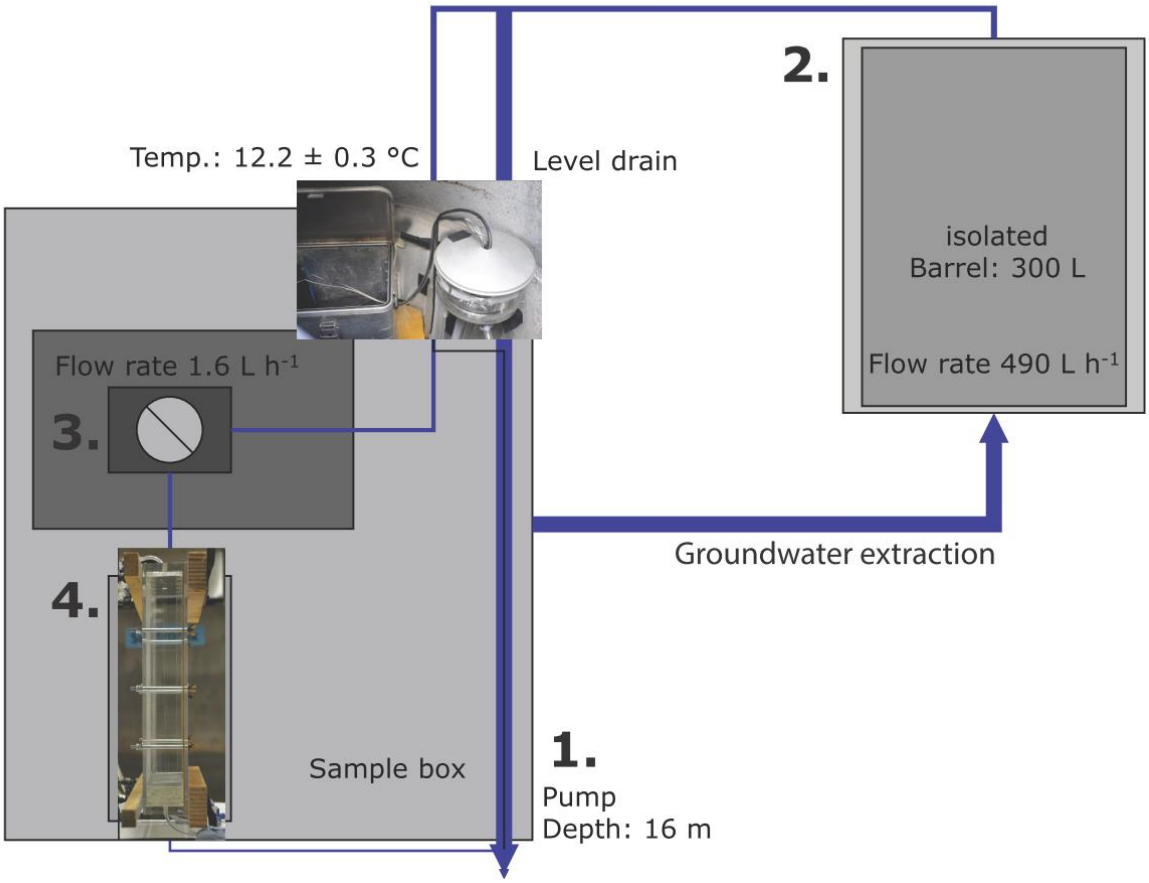


Figure 3- 1 A schematic representation of the experimental setup including: (1.) the extraction pump, (2.) the water reservoir on the surface, (3.) a 4-channel peristaltic pump in the well pit and (4.) the experimental mesocosm box.

Table 3- 1 Physiochemical water characteristics of the groundwater collected over 2 years (mean ± sd, n= 29)

Sample	pH	Oxygen [mg L^{-1}]	EC[µS cm^{-1}]	Temperature [°C]
Groundwater	7.7 ± 0.2	6.3 ± 0.4	542 ± 1	12.2 ± 0.3

A comparison of major ion concentrations in the groundwater and water sampled from the mesocosm showed only little differences (Table 3- 2). Nitrate and sulfate concentrations were relatively high compared to that of other essential nutrient sources measured as nitrite, ammonium and phosphate, which were negligible in both systems.

Table 3- 2 Concentrations of major ions in groundwater and mesocosm water samples (mean ± sd, n=8)

Ion species	Groundwater	Mesocosm
Na^+ [mg L^{-1}]	6.2±2.8	6.3±2.5
NH_4^+ [mg L^{-1}]	<0.01*	0.1±0.1
K^+ [mg L^{-1}]	1.0±0.03	1.0±0.04
Mg^{2+} [mg L^{-1}]	28±4.4	26.6±4
Ca^{2+} [mg L^{-1}]	68.8±3.9	61.8±9.7
Cl^- [mg L^{-1}]	13±1.8	14.8±3.6
NO_2^- [mg L^{-1}]	<0.01*	0.01
NO_3^- [mg L^{-1}]	17.2±2.1	16.6±1.4
HPO_4^- [mg L^{-1}]	<0.1	<0.1
SO_4^- [mg L^{-1}]	19.9±1.8	21.9±3.6

* indicates values below the detection limit

3.1.2 Microbial Community and Carbon Utilization Assay

At the end of the colonization experiment, the active biomass in the groundwater and mesocosm water was evaluated by ATP quantification (n=3) yielding 1.7 x 10^3 ± 6.5 x 10^2 cells mL^{-1} and 3.3 x 10^4 cells mL^{-1} respectively. Directly after removing the cells from the mineral surface, it was determined that the attached microbial biomass was similar for most minerals (magnetite 9.9 x 10^5 ± 5.1 x 10^3, apatite 1.54 x 10^6 ± 1.9 x 10^5, granite 6.4 x 10^5 ± 9.6 x 10^4 cells cm^{-2}) but the biomass was considerably higher on pyrite (5.9 x 10^6 ± 1.5 x 10^5 cells cm^{-2}). Detached cells were diluted to a starting concentration of 6.4 x 10^5 cells mL^{-1} for the incubation of the carbon substrate utilization assay.

The time-resolved monitoring of carbon-utilization showed the highest versatility and activity of the microbial community when associated with the magnetite mineral surface, steadily increasing until the end of the Biolog plate incubations after 132 hours (Figure 3- 2 **B**). A similar pattern was observed for the microbial community attached to granite, while the activities of the apatite grown microorganisms reached a plateau after ~100 hours of incubation. High variability was observed in the absorbance of different replicate wells filled with microbes of the magnetite and granite associated communities, especially at later time points. Carbon usage activity measured for pyrite-attached microorganisms was negligible.

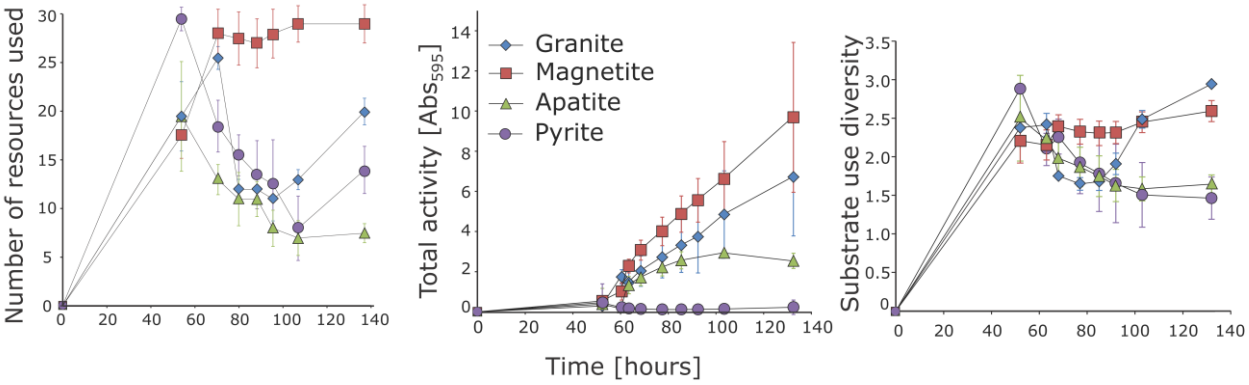


Figure 3- 2 Utilization profiles of 31 distinct substrates (see Table 3- 3) for the attached microbial communities removed from mineral coupons. Substrate usage was analyzed on the Biolog plate (n=3). The median value was taken to depict **A** the number of utilized substrates for each community exceeding values measured in wells without carbon substrate (blank) at given time points, **B** the activity of the community measured taking the sum of all absorbance values (at 595 nm) indicating total activity and **C** the diversity of substrate usage calculated as $\mathbf{H'} = - \Sigma\, p_i \ln p_i$., where p_i is one absorbance value at a time point. Error bar represent the standard deviation (n=3). Error bars are not shown if the symbol size is greater than the standard deviation.

Magnetite associated microbes were actually able to utilize all of the presented substrates (30) but one (2-hydroxy benzoic acid) showed no measurable absorbance at 595 nm by the end of the experiment. Microbes from granite mineral surfaces converted more than half (20) of the available substrates, whereas pyrite grown cells actively used 14 different compounds (Figure 3- 2 **A**). The inoculum from the apatite mineral surfaces converted eight different substrates, most notably converting malic acids by the end of the experiment. Substrate utilization patterns were most diverse in the assay inoculated with microbial communities taken from magnetite and granite surfaces (Figure 3- 2 **C**), followed by apatite and pyrite. Granite increased utilization diversity recovering from a small drop after two days of incubation. A more detailed look at the utilized substrate guilds (carbohydrates, carboxylic and acetic acids, polymers, amines and amino acids) helped to differentiate between community capabilities.

In the assay containing the granite associated microbial community, carboxylic and acetic acids were preferentially utilized (Figure 3- 3) while the least used compounds where amino acids. The magnetite profiles showed the highest affinity for amines, where activity was twice as high as for carboxylic and acetic acids, which topped amino acids and polymer utilization development. Carbohydrates were the least converted substrates in those communities. A similar pattern but with less activity was found in the apatite associated substrate assay which differed in that amines and carbohydrates were not utilized at all.

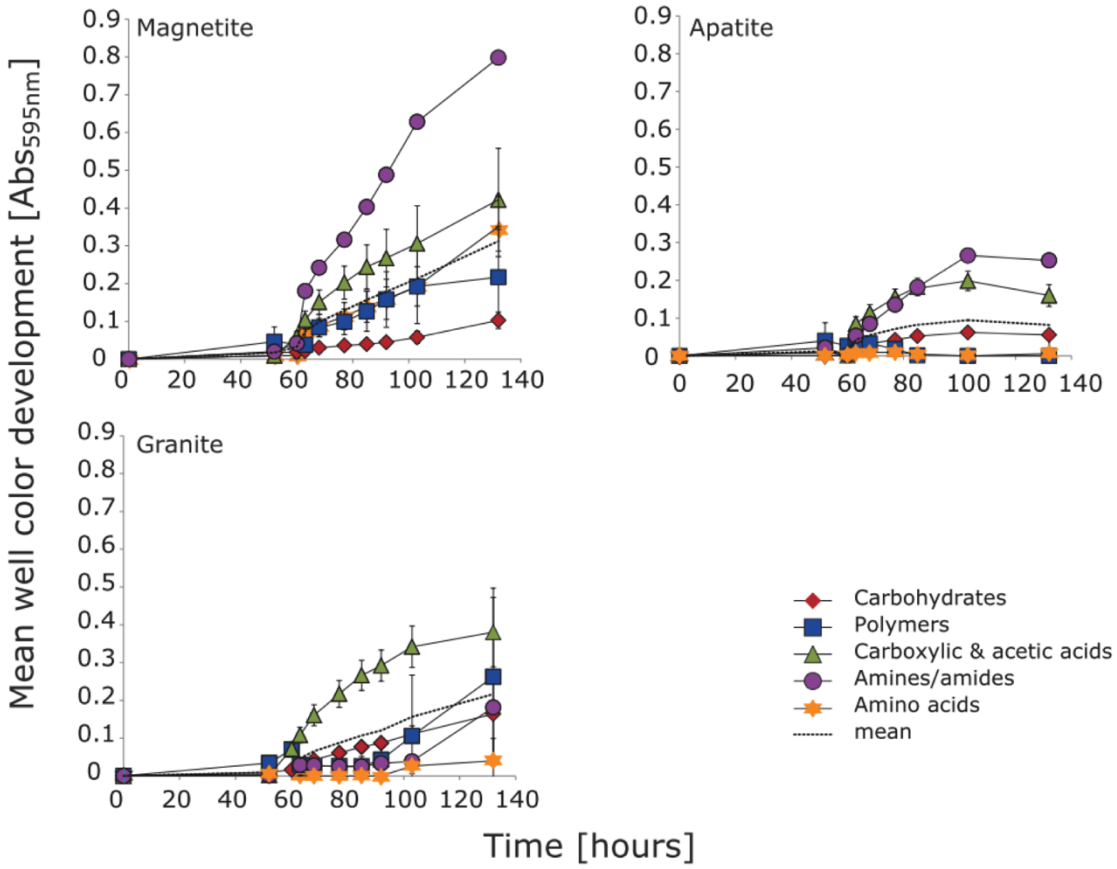


Figure 3- 3 Substrates condensed to substrate guilds, which were utilized by the microbial communities attached to mineral surfaces. Sums of activities were normalized, dividing the sum of absorbance values for each chemical guild by the number of substrates per guild. Error bar represent the standard deviation (n=3). Error bars are not shown if the symbol size is greater than the standard deviation.

Table 3- 3 Substrates presented to the detached biofilm communities on the Ecoplate of the Biolog assay grouped into chemical guilds

Amines & amides	Amino acids	Carbohydrates	Carboxylic & acetic acids	Polymers
Phenylethylamine	L-Arginine	Pyruvic acid methyl ester	D-Glucosaminic acid	Tween 40
Putrescine	L-Asparagine	D-Cellobiose	D-Galactonic acid-γ-lactone	Tween 80
	L-Phenylalanine	α-D-Lactose	D-Galacturonic acid	α-Cyclodextrin
	L-Serine	β -Methyl-D-glucoside	2-Hydroxy benzoic acid	Glycogen
	L-Threonine	D-Xylose	4-Hydroxy benzoic acid	
	Glycyl-L-glutamic acid	i-Erythritol	γ-Hydroxybutyric acid	
		D-Mannitol	Itaconic acid	
		N-Acetyl-D-glucosamine	α-Ketobutyric acid	
		Glucose-1-phosphate	D-Malic acid	
		D,L- α –Glycerol phosphate		

Preferential consumption of malic acid was observed in granite, magnetite, and apatite samples. Furthermore, granite communities converted methyl pyruvate, D-galactonic acid- γ -lactone, and 4-hydroxy benzoic acid in substantial amounts. Microbial communities taken from magnetite surfaces utilized efficiently L-asparagine and putrescine, as well as 4-hydroxy benzoic acid.
The substrate utilization patterns of the planktonic community was not directly compared to that of the attached microbial communities since cell density of the inocula was much lower (1.7 x $10^3 \pm 6.5 \times 10^2$ cells mL^{-1} for the groundwater and 3.3 x 10^4 cells mL^{-1} for the mesocosm water). Taking the low cell numbers into account, the number of utilized substrates was high (Figure 3- 4 **A**) and did not show the pronounced break down of activity as observed for detached biofilms. The microbial community taken from the mesocosm bulk water utilized up to 28 substrates and the groundwater community was capable of using 26 different carbon substrates (Figure 3- 4 **A**). The differences in inoculum density were also reflected in the low substrate use activity assessed by summarized well color development.

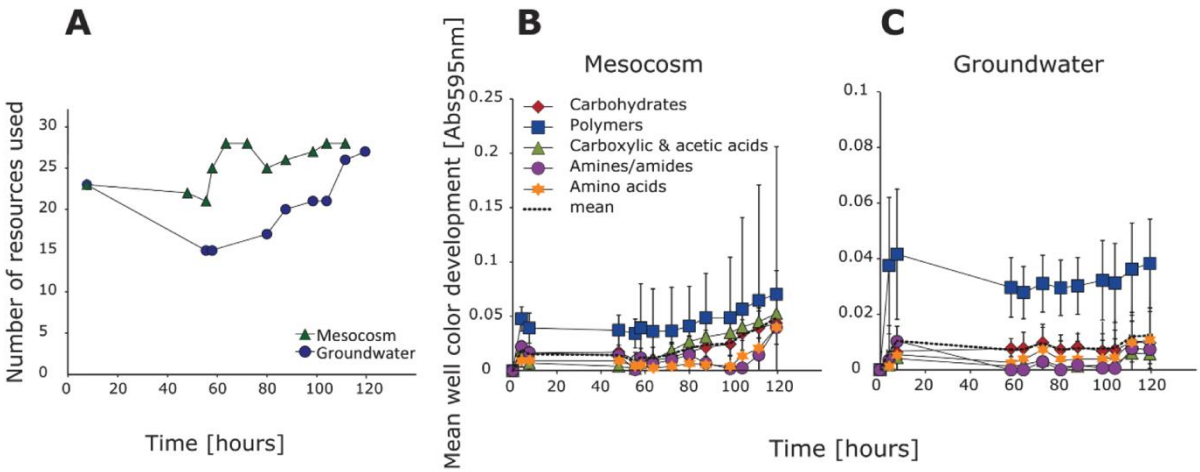


Figure 3- 4 Substrate utilization patterns from planktonic samples of groundwater and mesocosm bulk water sampled at the end of the colonization experiment. **A** shows the number of utilized substrates in the course of the incubation. **B** and **C** depict the normalized sum of activity for the defined substrate guilds. Error bar represent the standard deviation (n=3). Error bars are not shown if the symbol size is greater than the standard deviation.

Microbial activity for each substrate guild (Figure 3- 4 **B** and **C**) displayed low absorbance values, indicating that only small amounts of substrates were oxidized. Again, amines and amides were the least used substrates, while carbohydrates and polymers were converted fastest and considerably more efficiently than all of the other substrates. The mesocosm community displayed high variability by the end of the Biolog assay. Nonetheless, there was a trend of

increasing activity in the assays (Figure 3- 4 **B**). While the number of resources used increased, the activity in the groundwater-inoculated assays did not change overtime (Figure 3- 4 **C**).

3.1.3 Biofilm and Planktonic Community Composition

Detached communities from mineral coupons were subjected to T-RFLP fingerprinting. For selected samples, pyro-tag libraries were constructed in order to identify dominant OTUs. Several key taxonomic groups were identified as *Proteobacteria*, which was the most abundant phylum. Additionally numerous other bacterial phyla *Bacteroidetes*, *Actinobacteria*, *Nitrospira* and *Planctomyceta* were also characteristic of the attached communities (Figure 3- 5). Most of the Proteobacteria were affiliated to the subgroup *Betaproteobacteria*. As shown in the ordination, differences amongst fingerprints of apatite, granite and magnetite associated bacterial communities were marginal (Figure 3- 6). The main taxa associated with these surfaces were *Rhodocyclaceae* apparent *Sulfuritalea* spp., but also *Comamonadaceae* and several *Gammaproteobacteria*. The *Comamonadaceae,* most likely *Acidovorax* spp. had the same T-RF length of 488 bp as *Thiotrichales* which were present and abundant on the pyrite surfaces. Furthermore the bacteria attached to pyrite differed substantially and were dominated by *Rhodocyclaceae* (related to *Zoogloea* spp. but at low sequence identity of ~40 %). Interestingly, *Flavobacteriaceae* (*Bacteroidetes*) were found throughout all samples except for the apatite mineral surfaces. The composition of the planktonic community in the mesocosm was more similar to that of the pyrite biofilms, but taxa were more evenly distributed and a higher number of low abundance taxa were found in the water samples. The groundwater community appeared mostly distinct when compared to all other communities.

Table 3- 4 Diversity estimates of attached and planktonic bacterial communities based on 16S rRNA gene fingerprinting ($n_{Apatite,Granite}=2$, $n_{Magnetite,\ Pyrite}=3$, $n_{Mesocosm}=3$, $n_{Groundwater}=5$)

Sample	Apatite	Granite	Magnetite	Pyrite	Mescosm	Groundwater
Richness	59	48	48±16	55±6	64±15	45±18
Shannon H'	3	2.8	2.9±0.9	2.2±0.2	3.2±0.1	2.9±0.1
Simpson D	0.89	0.88	0.9±0.03	0.77±0.0	0.92±0.01	0.9±0.01
effective no. of OTUs	19.4	17	18.9±4	9.2±1.4	25.6±3.5	17.6±3.2

Most notably, *Planctomycetes* were more abundant here than in any other sample. Community diversity based on the T-RFs was highest for the planktonic communities (Table 3- 4). The similarity of the attached communities of the three minerals apatite, granite and magnetite was

reflected in the diversity indices. The few dominating taxa attached to the pyrite surfaces resulted in lower diversity measures, although the number of total OTUs was the highest of all mineral associated bacterial communities.

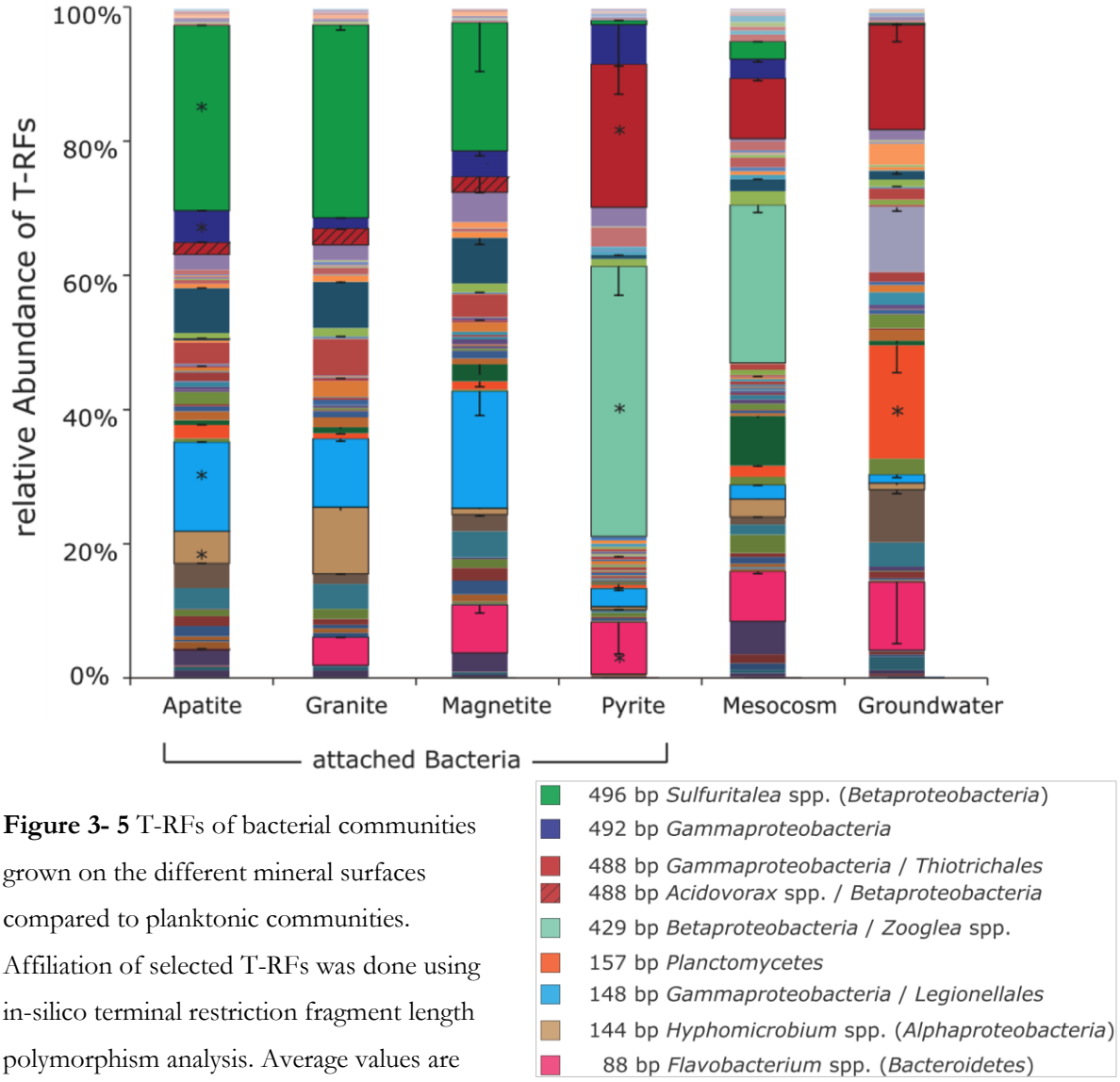


Figure 3- 5 T-RFs of bacterial communities grown on the different mineral surfaces compared to planktonic communities. Affiliation of selected T-RFs was done using in-silico terminal restriction fragment length polymorphism analysis. Average values are shown. Error bars represent the standard deviation of two biological replicates and two technical replicates, each representing the microbes detached from three mineral coupons. The first taxa given in the legend have more than 98 % identity with the OTU, while the second taxa given further specify possible affiliations.

* indicates T-RFs identified via pyrotag libraries

A double-centered PCA (Figure 3- 6) was chosen to display distinctions in community composition between the different mineral surfaces. A great amount of variance was represented on the first axis (69.3 %). Almost no differences were observed between the granite, apatite and

magnetite communities. In contrast, the pyrite associated bacterial community was most different to those biofilms. The planktonic communities are discriminated on the second axis, positioning the groundwater community further away from the surface biota than the mesocosm water community.

T-RFs with the highest loadings (scaled eigenvectors) towards the three similar minerals were *Gammaproteobacteria* (closely related to *Legionellales*), as well as the *Betaproteobacteria* (*Sulfuritalea* spp.) and alphaproteobacterial *Hyphomicrobium* spp.. The 429 bp T-RF (*Rhodocyclaceae*) was clearly indicative for the pyrite surfaces.

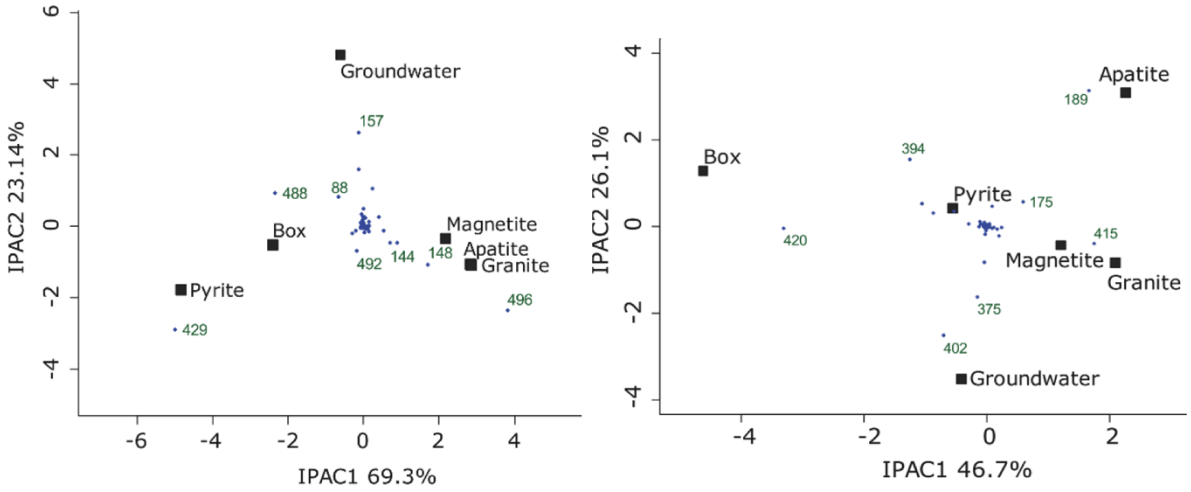


Figure 3- 6 A double centered PCA depicting bacterial (left) and eukaryotic (right) community similarity, identifying discriminative T-RFs being characteristic for the specific minerals Number of samples for *Bacteria*: $n_{Apatite, Granite}=2$, $n_{Magnetite, Pyrite}=3$, $n_{Mesocosm}=3$, $n_{Groundwater}=5$ and *Microeukaryotes*: $n_{Apatite}=6$, $n_{Granite}=3$, $n_{Magnetite, Pyrite}=5$, $n_{Mesocosm, Groundwater}=3$

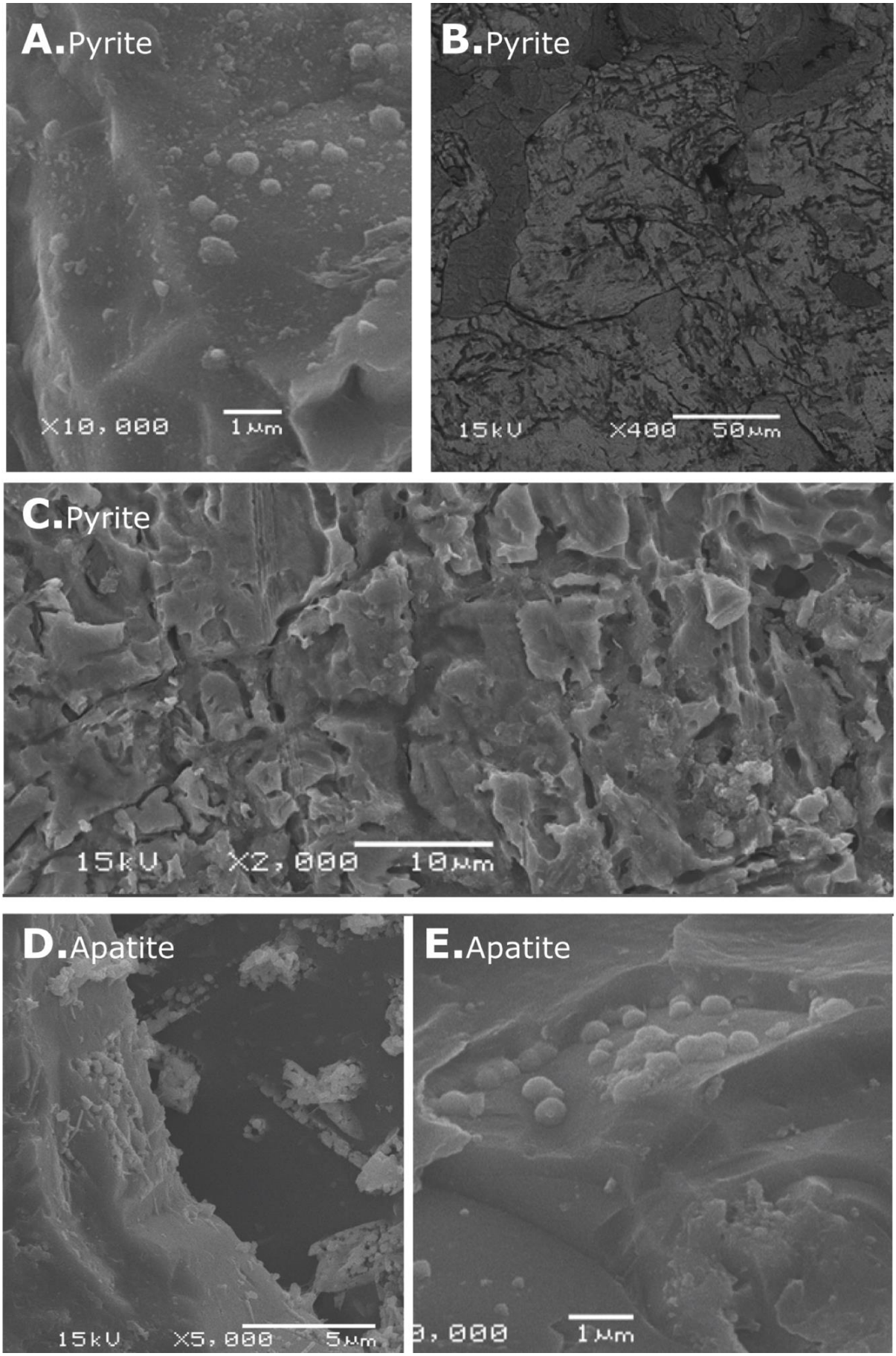
In addition to the bacterial fingerprints, micro-eukaryotic communities were also analyzed for diversity of samples (Table 3- 5) and in the ordination plot (Figure 3- 6). The highest diversity of microeukaryotic T-RFs was found associated to the magnetite surfaces, while the lowest diversity was encountered on the granite and apatite samples. In general, diversity indices indicated similar diversity patterns for eukaryotic and bacterial communities with the exception of pyrite, where the microeukaryotes were by far more diverse.

Table 3- 5 Diversity estimates of the eukaryotic communities based on T-RFs from the 18S rRNA gene fingerprint ($n_{Apatite}$ =6, $n_{Granite}$=3, $n_{Magnetite, Pyrite}$ =5, $n_{Mesocosm, Groundwater}$ =3)

Sample	Apatite	Granite	Magnetite	Pyrite	Mescosm	Groundwater
Richness	26±11	22±7	40±7	33±5	31±10	32±7
Shannon H'	2.1±0.4	2.2±0.1	2.6±0.4	2.6±0.1	2.4±0.3	2.2±0.1
Simpson D	0.81±0.1	0.81±0.01	0.85±0.1	0.9±0.01	0.84±0.1	0.85±0.02
effective no. of OTUs	8.8±2.9	8.8±0.9	13.7±5.0	13.2±1.3	11.4±0.13.4	9.3±1.3

3.1.4 Mineral Weathering

After incubation, coupons of each mineral species were frozen and later dried to allow for an imaging of weathering processes, which was done by Dr. Marianne Hanzlik at the Institute of Electron Microscopy, TUM Garching. The examination of mineral surfaces using scanning electron microscopy showed interesting features of attached microbes and mineral weathering. In general, colonization was low which could be due to the treatment of mineral surfaces where coupons were rinsed with water trying to separate loose and planktonic cells directly after recovering the mineral coupons from the mesocosm, although no electron microscopic images of surfaces before exposure were made. The pyrite mineral surface appeared considerably altered after exposure, showing grooves and channels (Figure 3- 7). Elemental analysis of the surface probed with EDX revealed the prevalence of zinc over iron inclusions in the sulfide mineral. Surprisingly, no evidence for biofilm formation was found. In contrast, apatite surfaces were clearly colonized and several microbial cell clusters were found preferentially in cavities of the mineral. Only sparse colonization of the granite surface was found, but again cells appeared in clusters. The sample handling noticeably collapsed cells and differentiation of morphologies was no longer feasible. Nonetheless, the cell walls displayed a coarse surface, which was especially true for granite and magnetite attached microbial cells. The magnetite surface appeared the most densely colonized mineral. Several colonies of approximately 50–80 cells were distributed in distances of about 150 μm to each other. SEM allowed the examination of mineral surfaces at a resolution relevant for microbial growth. Even though no clear evidence for differences in the colonization behavior of the selected minerals was observed, the SEM allowed the examination of mineral surfaces at a resolution relevant for microbial ranges.


A.Pyrite
X10,000
1µm
B.Pyrite
15kV
X400
50µm
C.Pyrite
15kV
X2,000
10µm
D.Apatite
15kV
X5,000
5µm
E.Apatite
1µm

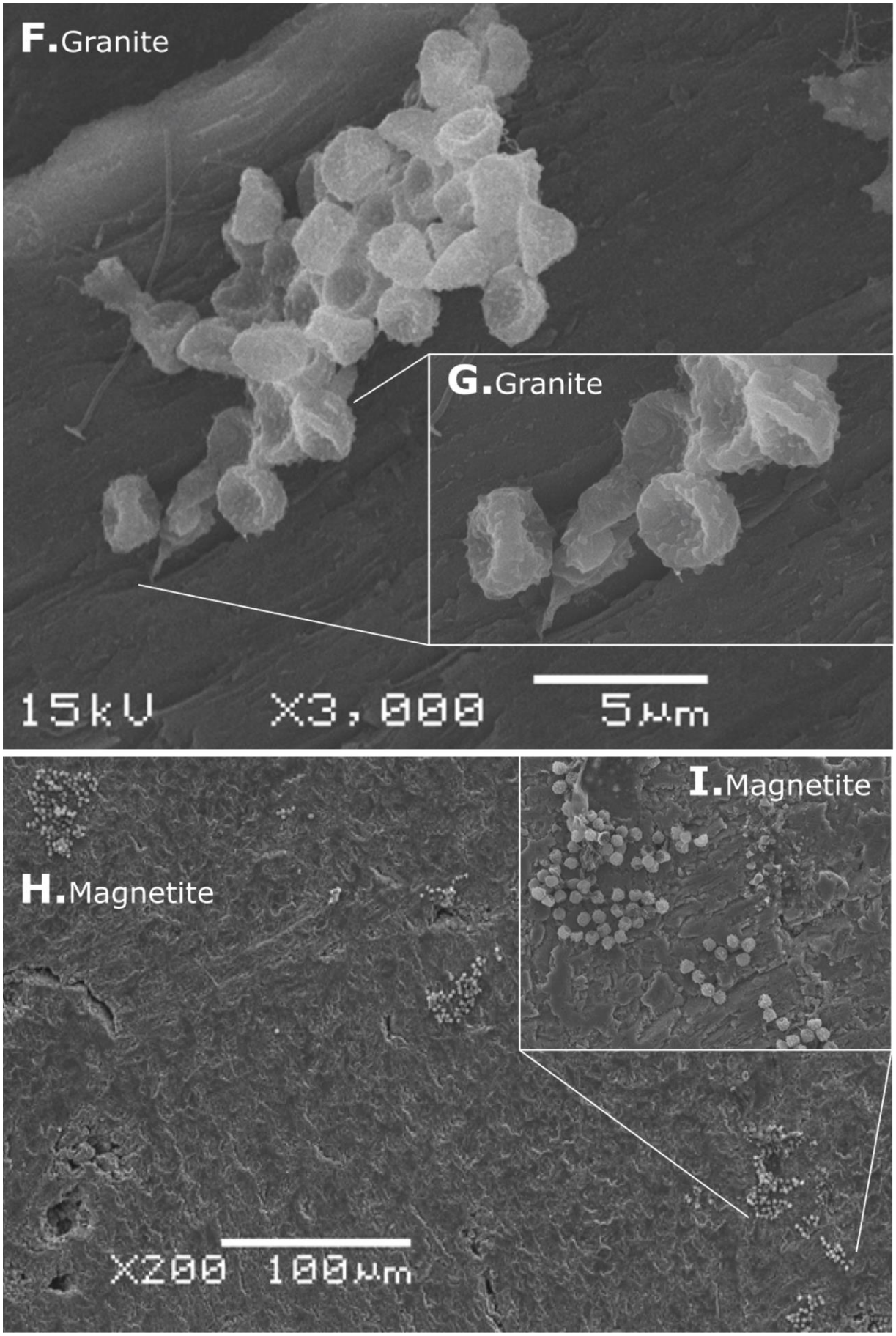

F.Granite
G.Granite
15kV X3,000 5µm
H.Magnetite
I.Magnetite
X200 100µm

Figure 3- 7 A - I Electron microscopy images show different levels of mineral weathering and colonization of surface coupons exposed in groundwater mesocosms. **A** Single cell-like structures were observed on the pyrite mineral coupons. **B** Examination of the pyrite surface shows a heterogeneous mixture of different mineral species observed as lighter and darker areas. **B, C** These coupons were clearly weathered showing increased surface roughness after exposure. **D, E** An organic film covered most of the apatite minerals after exposure. **E** Microbial cells cluster within a depression of the apatite. **F** Micro-colonies were observed on the quartz material surfaces. **G** The inlay presents a magnification of this colony indicating cell surface structures. **H** Several micro-colonies were also detected on the magnetite coupons. **I** The close distances between attached cells would enable cell-cell interactions within the cell clusters. Sample preparation and SEM imaging was done by Dr. Marianne Hanzlik of the Institute of Chemistry of the TUM in Garching.

3.2 Iodine Spring Cavern Biofilms

Here, a unique, semi-artificial cave augmented by mineral water was studied for the first time, focusing on microbial biofilm communities and the physiology of inherent biofilms. An in-depth analysis of the biofilm communities, not only by molecular but also by chemical methods, provided primary insights into this fascinating habitat.

3.2.1 Biogeochemical Analyses

Water Samples

Michael Stöckl (IGOE, HMGU) and the author collected water samples from spring and seepage water in November 2012 and December 2013. Additional samples of nearby water bodies (river, groundwater well) and the mixed cavern water were also taken. Water chemistry of the mineral spring water was analyzed by Michael Stöckl, and compared to data recorded over the previous 2 years and in the 1950s (Table 3- 6).

Table 3- 6 Ion composition and dissolved organic carbon content of the spring water recorded by several independent studies. Mean values ± sd are given

Water parameters	Souci & 1950	MUVA Kempten 2011	LfU ‡ 2010-2012 (n=4)	This study 2012-2013 (n=2)
Na^+ [mg L^{-1}]	453	1380	1210±105	1140
K^+ [mg L^{-1}]	6	10.2	9.5±0.4	8.6
Mg^{2+} [mg L^{-1}]	24	56.5	48.5±4.4	55.3
Ca^{2+} [mg L^{-1}]	82.1	12.5	113.5±11.4	61.8
Fe [mg L^{-1}]	0.6	0.6	0.9±0.2	*NA*
Cl^- [mg L^{-1}]	685	2360	2075±96	2224
Br^- [mg L^{-1}]	*	*NA*	20*	19.2
I^- [mg L^{-1}]	7.2	20	21±1.4	20
NH_4^+ [mg L^{-1}]	*NA*	*NA*	*NA*	<0.01*
NO_2^- [mg L^{-1}]	*NA*	0.02	*NA*	<0.01*
NO_3^- [mg L^{-1}]	*NA*	0.4	< 3*	0.19
SO_4^- [mg L^{-1}]	5.1	2.5	< 3*	0.85
DOC [mg L^{-1}]	*NA*	*NA*	7.4±11.7	1.45

* Indicates at least one measurement below detection limit; *NA* not assessed
† Accredited water analytical laboratory, data made available by Franz Hösle, Jodbad Sulzbrunn
‡ Bavarian Environment agency, data made available by Günter Kus, LfU Bayern

Little difference was observed, though variability in the concentration of bromide was found, which is likely due to analysis thresholds. Elevated salinity was mainly owing to chloride, sodium and potassium, which were also reflected by the measured conductivity (Table 3- 7). Nutrients or electron acceptors such as nitrate, phosphate and sulfate were negligible in the well water. Dissolved organic carbon measured in the spring water showed some variability and is likely to be influenced by mixing of formation water and meteoric seepage water. Iodine concentrations remained stable at 20 mg L^{-1} in the spring water, and 925 µg L^{-1} in the mixed cavern water.

Table 3- 7 Chemistry of water samples (n=1) taken at different locations and dates in the vicinity of the Spring. Given standard deviations are of technically replicated measurements (n=6).

Parameter	Spring water 17.12.2012	Seepage 17.12.2012	Cavern water 18.11.2013	Surface water 18.11.2013	Nearby groundwater 18.11.2013
Na^+ [mg L^{-1}]	1131.01	18.71	328.55	1.38	4.25
K^+ [mg L^{-1}]	8.64	1.39	3.01	0.67	0.75
Mg^{2+} [mg L^{-1}]	55.32	22.18	28.87	23.45	24.05
Ca^{2+} [mg L^{-1}]	111.74	76.81	93.27	83.60	85.38
Cl^- [mg L^{-1}]	2248.22	4.51	606.48	0.39	2.09
NO_2^- [mg L^{-1}]	<0.01	0.01	<0.01	<0.01	<0.01
Br^- [mg L^{-1}]	19.20	0.03	4.69	<0.01	<0.01
NO_3^- [mg L^{-1}]	0.19	3.95	1.77	5.32	5.12
HPO_4^-[mg L^{-1}]	0.01	<0.01	<0.01	<0.01	<0.01
SO_4^- [mg L^{-1}]	1.70	3.42	1.14	2.38	1.97
pH	7.9	8.3	8	*NA*	7.5
EC [µS cm^{-1}]	6250	526	2040	*NA*	537
O_2 [mg L^{-1}]	2.85	8.5	5	*NA*	10.5
Temp. [°C]	7.2	*NA*	8.6	*NA*	7.5
$\delta^{18}O$ [‰]	-7. 8 ± 0.1	-11 ± 0.1	-10.2 ± 0.1	*NA*	*NA*
δ^2H [‰]	-66.9 ± 0.3	-75.4 ± 0.2	-73.2 ± 0.3	*NA*	*NA*

Water stable isotope analysis was done by Petra Seibel, Institute of Groundwater Ecology.

The mixing ratio of the two waters (seepage water and mineral well water) calculated from $\delta^{18}O$ (‰) and δ^2H (‰) values, measured by Petra Seibel (IGOE, HMGU), indicated a high input of seepage water. The analysis of $\delta^{18}O$ and δ^2H provided estimates for the amount of seepage water in the cavern being 75.3 % and 73.3 % of the total volume, respectively. This mixing ratio was

also supported by chloride data (Table 3- 7), giving a volume of 73 % originating from seepage inflow.

Gas Samples

Tillmann Lueders, Michael Stöckl, Franz Hösle (responsible person at the Jodbad Sulzbrunn) and the author obtained gas samples in the course of two sampling campaigns (November 2012, December 2013), by collecting gas bubbles with an inverted glass bottle directly from the cavern pool. In addition, gas samples from the undisturbed cave atmosphere were drawn at three more occasions using a vacuum pump connected to a tube entering the main cavern. High methane concentrations of up to 50 % were measured from the emerging gas bubbles directly in the well pool. The average methane concentration sampled from the cave atmosphere was 3000 ppm. The measured CO_2 concentrations of 8000 ppm were 20-fold higher than the natural average concentration of the Earth's atmosphere. Compound specific stable isotope analysis (CSIA) revealed relatively heavy $\delta^{13}C$ values of -43.6 ± 0.2 ‰ (n =6) for the outgassing methane, and of -33.2 ± 0.1 ‰ (n=6) for the $\delta^{13}C$ of CO_2. The hydrogen isotopic composition of methane collected directly from the bubbles was -164.9 ± 2.2 ‰ (n=10).

Biofilm Samples

Cave biofilm samples were collected in November 2012 by Tillmann Lueders, covering three ceiling samples from the well towards the cavern opening at distances of ~5 meters as well as three samples representing a wall gradient from the bottom towards the top (~1.80 m height) of the wall. In addition, a sample was drawn from the sediment layer directly at the spring water inflow. An extra biofilm sample was taken inside a pipe collecting the outflow of the cavern.
A stable isotope analysis of freeze dried biofilms, carried out by Harald Lowag (IGOE, HMGU), allowed the comparison to known carbon and nitrogen sources as well as processes potentially influencing the values (Whiticar, 1999). The only sample having a lighter carbon isotope ratio ($t=5.87$, $p<0.05$) than the measured gas (-43.6 ‰) was the biofilm at the bottom of the wall gradient (-44.4 ‰ ± 0.12), which is normally submerged. The sample taken at the wall center, above the water table, had a $\delta^{13}C$ value of -37.7 ‰ and the sample at the top of the wall (-30.8 ‰) was indistinguishable from $\delta^{13}C$ values of the ceiling biofilms (-31.08 ‰ ± 1.2). There was no obvious trend observed in the carbon signature of ceiling biofilms (Figure 3- 8). The mean $\delta^{13}C$ value of the sediment sample was -11.6 ‰ but showed substantial variation (Figure 3- 8). A similar pattern, but less pronounced, was given by the nitrogen isotope values (Figure 3- 8). Wall biofilms displayed a gradient of increasing (heavier) nitrogen values towards

the top spanning from -11.3 ‰ to -0.4 ‰. The average value for the ceiling biofilms and sediment was 0.15 ‰ respectively 0.2 ‰.

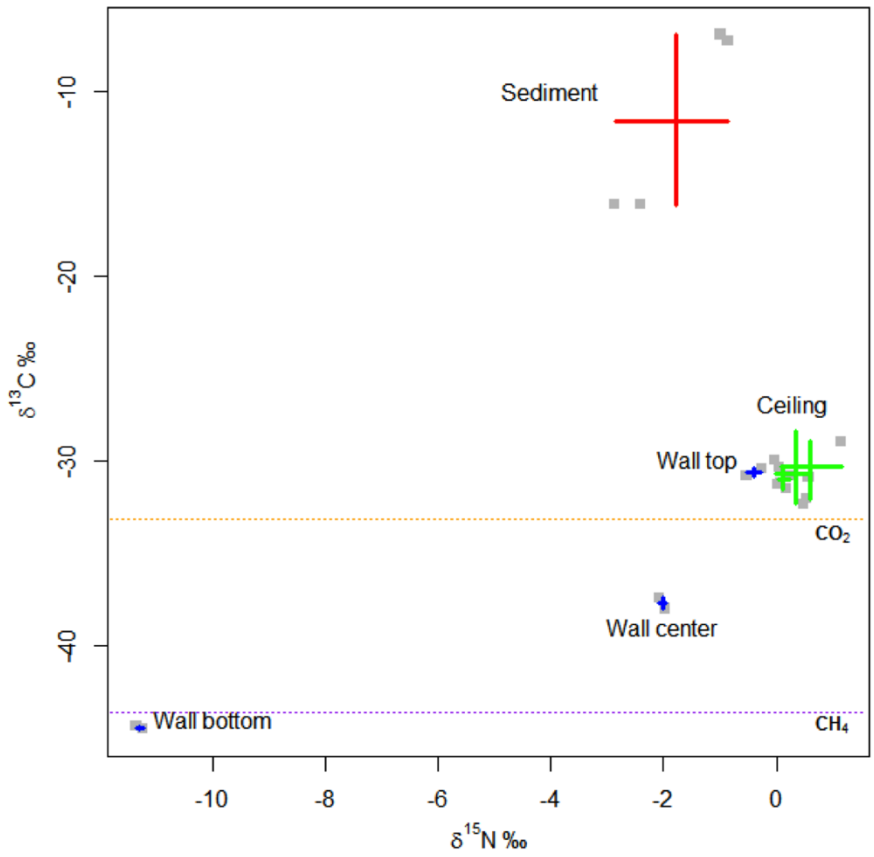


Figure 3- 8 Carbon and nitrogen stable isotope ratios as measured in biofilm samples. The length of the crosshair depicts the standard deviation of averaged measurements (n=3). Stable isotope analysis was done by Harald Lowag, Institute of Groundwater Ecology.

An elemental analysis of the biofilms, conducted by Peter Grill and Bernhard of the Research unit Analytical BioGeoChemistry, provided information on the composition of biofilms (total carbon, nitrogen, phosphorous, and sulfur), as well as on iron and iodine accumulation. High carbon ratios were found in the ceiling biofilms (Figure 3- 9), reaching from 273 to 426 mg g^{-1} dry weight. Nitrogen and phosphate concentrations were higher in wall biofilms, which was also the case for iron and sulfur (Table 3- 8). The high values of iron at the bottom and center of wall biofilms were especially noticeable. Iodine values did not display a clear gradient in the biofilm biomass, and were 4290 ppm on average.

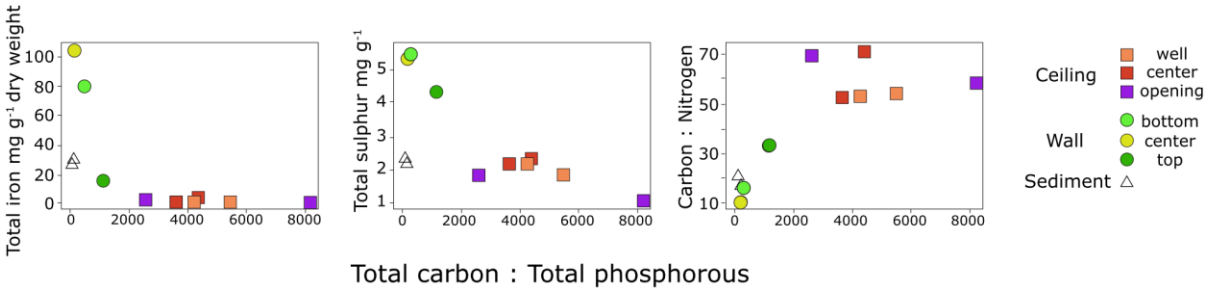


Figure 3- 9 Correlation of the concentration of specific elements to the carbon to phosphorous ratio in lyophilized cave biofilms along sampling gradients ($n_{Ceiling,Sediment}$=2; n_{Wall}=1). The elemental composition was determined by Bernhard Michalke, Research Unit Analytical BioGeoChemistry.

High carbon ratios were found in the ceiling biofilms (Figure 3- 9), reaching from 273 to 426 mg g^{-1} dry weight. Nitrogen and phosphate concentrations were higher in wall biofilms, which was also the case for iron and sulfur (Table 3- 8). The high values of iron at the bottom and center of wall biofilms were especially noticeable. Iodine values did not display a clear gradient in the biofilm biomass, and were 4290 ppm on average.

Table 3- 8 An elemental analysis of lyophilized biofilm samples. The carbon and nitrogen values were derived from isotope analysis (by Harald Lowag, IGOE) on the Elemental analyzer (n=3), all others by ICP-MS (Bernhard Michalke, Research Unit Analytical BioGeoChemistry) (n_{Wall}=1, $n_{Ceiling,Sed}$=2)

	Location	C [mg g^{-1}]	N [mg g^{-1}]	P [mg g^{-1}]	Fe [mg g^{-1}]	S [mg g^{-1}]	I [mg g^{-1}]
Wall	Bottom	183	11.9	0.5	80	5.6	0.3
	Center	138.4	13.4	0.6	106	5.6	0.7
	Top	283.8	8.7	0.2	18.1	4.4	0.2
Ceiling	Well	273.5±15.3	5.2±0.2	0.1	1.1	2.2	0.4
	Center	416.8±43.3	6.3±1.3	0.1	1.9	2.4	0.4
	Opening	426.6±21.9	6.8±0.5	0.1	2.3	1.5	0.6
Sediment		43.1±7.5	2.4±1.1	0.5	31.2	2.2	0.2

3.2.2 Molecular Analyses of Water and Biofilm Communities

Cells numbers in the upwelling water determined by flow cytometry (n=3) were 1.3 x 10^5 cells mL^{-1}. This value is consistent with rRNA gene quantification with qPCR (n=6) (Figure 3- 10) and reveals high gene abundance, especially in the lower wall biofilms, as well as in the biofilm of the cavern outflow. Clear gradients in the abundance of 16S genes were found. Bacteria

decreased in numbers as the height of the wall transect increased and slightly increased along the ceiling transect towards the cell opening.

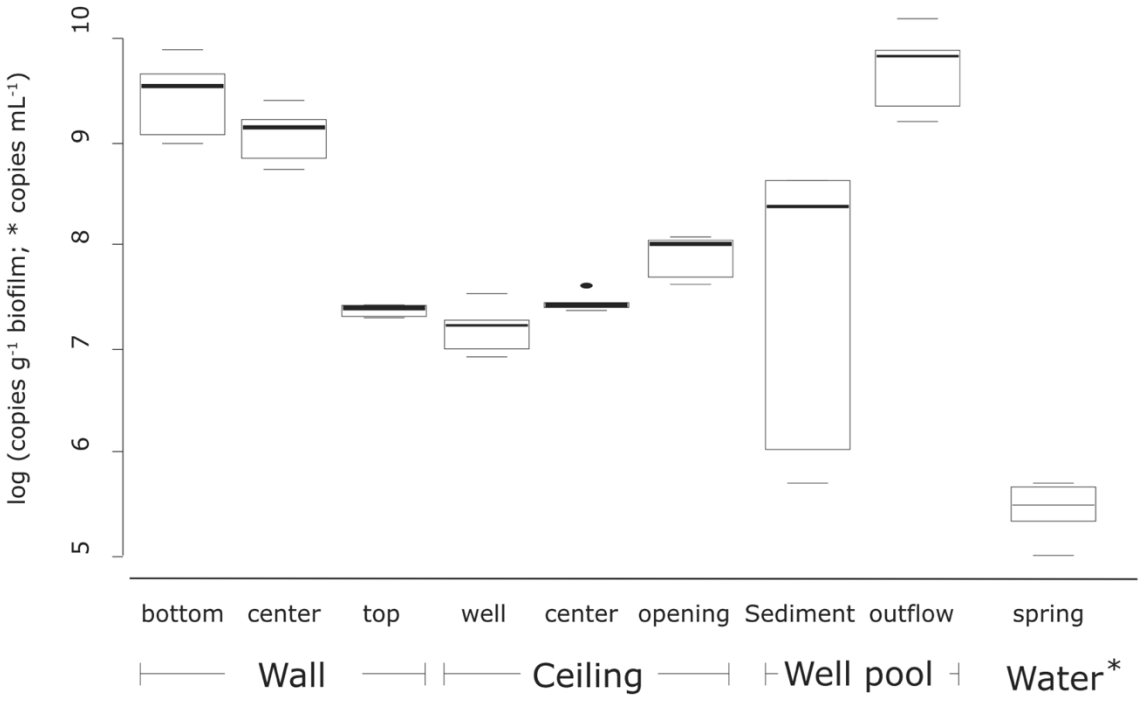


Figure 3- 10 Boxplot of qPCR results from biofilms, sediments and outflow, as well as spring water. The maximum and minimum values are represented by whiskers, the boxes encompass quartiles and the line indicates the median value for gene copy numbers (n=6)

PCR screening for functional marker genes indicative of methylotrophic and methanotrophic physiology provided tentative evidence for the presence of microbes carrying such functional potentials. All tested samples gave positive results for genes enconding methanol dehydrogenase (MDH) and the membrane-associated particulate methane monooxygenase (pMMO). The methane oxidation gene cluster A (*mxaF*) encodes an enzyme containing a pyrroloquinoline quinone (PQQ) cofactor that oxidizes methanol to formaldehyde in the second step of the methane oxidation pathway, while *pmoA* encoding the pMMO is the marker gene for all obligate methanotrophs (Hanson & Hanson, 1996, Anthony, 2000). The *cmuA* gene encodes a corrinoid-binding / methyltransferase - responsible for the initial step of methyl halide oxidation (Hanson & Hanson, 1996, Anthony, 2000, Schäfer *et al.*, 2005). The *cmuA* gene encodes a C-terminal corrinoid-binding domain and an N-terminal methyltransferase responsible for the initial step of methyl halide oxidation (McAnulla *et al.*, 2001b, Schäfer *et al.*, 2005). Detection of the *cmuA* gene was positive only for the lower wall biofilms and for sediment samples (Table 3- 9).

Table 3- 9 Qualitative PCR results of *mxaF*, *pmoA*, and *cmuA* marker genes amplified from DNA extracted from different biofilm ($n_{Ceiling,Wall}$=6), water (n=4) and sediment (n=4) samples. A check mark indicates a positive amplification of an expected amplicon, while the cross expresses lack of successful amplification.

Protein-encoding gene	Ceiling	Wall	Well water	Sediment
mxaF	✓	✓	✓	✓
pmoA	✓	✓	✓	✓
cmuA	X	✓	X	✓

Molecular fingerprinting (T-RFLP analysis) of the *pmoA* gene pool revealed low diversity among the wall biofilms, and clustering of biofilm towards the ceiling. The sediment community was most different from the other samples (Figure 3- 11). Potential candidate lineages for T-RF affiliation via fragment lengths given in the literature are shown in (Table 3- 10)

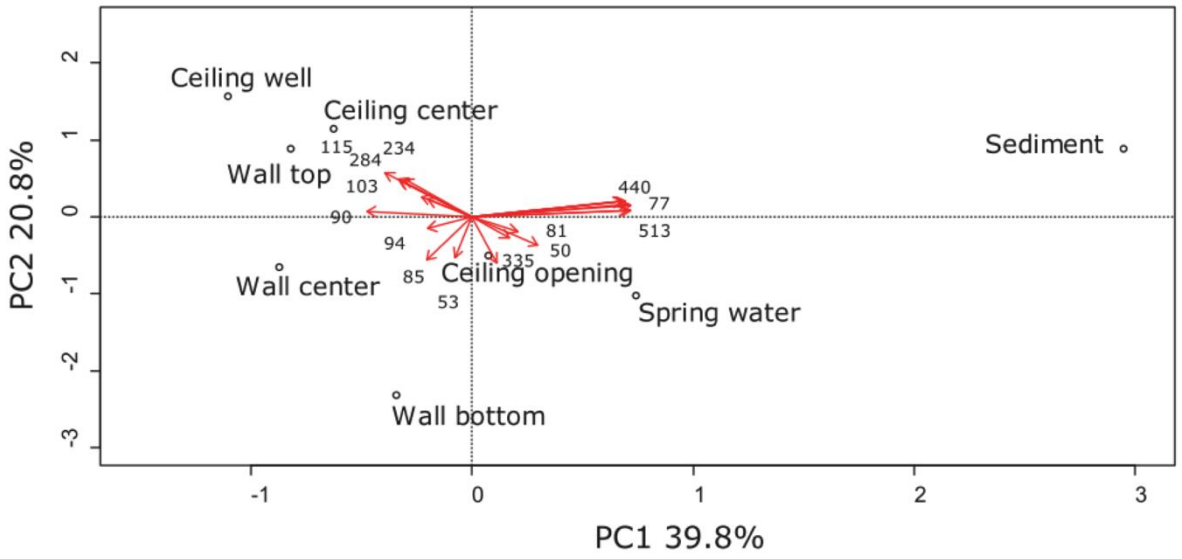


Figure 3- 11 A Principal Component Analysis: Ordination of aerobic methane oxidizers in biofilms based on *pmoA* fingerprinting using *Msp*I as the restriction enzyme.

Table 3- 10 Methanotrophic taxa and corresponding lengths of associated *pmoA* gene terminal restriction fragments (T-RFs) with *Msp*I digestion Taken from Reim *et al.* (2012).

Methanotroph	Fragment length [bp]
Methylomonas	437
Methylobacter	508
Type I b	79
Type I a	349
Type I a	241

3.2.2.1 Pyrosequencing Analysis of Water and Biofilm Communities

Sequence reads were obtained from the same biofilm samples used for biogeochemical analysis. Due to resource limitations and feasibility, only samples from ceiling biofilms and spring water were sequenced in replicate. Bioinformatic processing of the pyrotag libraries resulted in an average of 3272 ± 914 reads per sample affiliated to bacteria. *Proteobacteria* represent the most abundant phylum in all samples, while other phyla often linked to groundwater and soils e.g. *Planctomycetes*, *Bacteroidetes* and *Verrucmicrobia* also substantially contributed to the community. A total of 11 phyla were present at >1 % abundance in any sample. Unclassifiable reads accounted for up to 6 % in sediment and well water samples.

Table 3- 11 Diversity indices of sequencing libraries of bacterial 16S rRNA gene amplicons of different cavern and biofilm DNA extracts (n=1).

	Wall			**Ceiling**			**Spring**	**Sediment**	**Outflow**
	Bottom	Center	Top	Well	Center	Opening	**water**		
Richness	73	105	59	68	66	71	132	199	53
Shannon H´	2.9	3.5	3.1	2.9	3.2	3.3	2.5	3.9	2.7
Simpson D	0.91	0.96	0.93	0.9	0.92	0.95	0.85	0.96	0.9
effective no. of OTUs	18.2	34	23.3	18.4	24.3	28.4	12.5	49.9	15.2

Diversity given in Hill's numbers (Table 3- 11), also known as the effective number (Jost, 2007) and used here for bacteria at the family level, was highest in the sediment (49.9). Regarding the biofilm samples, the center of wall biofilms was most diverse (34) while the lowest wall biofilm was the least diverse (18.2). In general, the spring water sample (12.5) was less diverse than biofilm samples. The sediment community had by far the highest number of taxa (199).

Within the *Proteobacteria*, *Alphaproteobacteria* were almost equally frequent in all wall and ceiling biofilms (29 ± 4 %), but almost absent (<1 %) in the mineral spring water. *Gammaproteobacteria* were highly abundant in almost all samples, foremost in the ceiling biofilms (37 ± 9 %) and in the outflow sample (51 %) (Figure 3- 12). *Betaproteobacteria* were highly abundant (62 %) in spring water and biofilms forming on top of the cavern pool. Notably, the lower wall communities were also rich in *Betaproteobacteria* (27 %), while their abundance was low (4 ± 1 %) in all other cave biofilms. *Planctomycetes* were generally more abundant in upper biofilms (ceiling, wall center and wall top) with the exception of the ceiling biofilm close to the source.

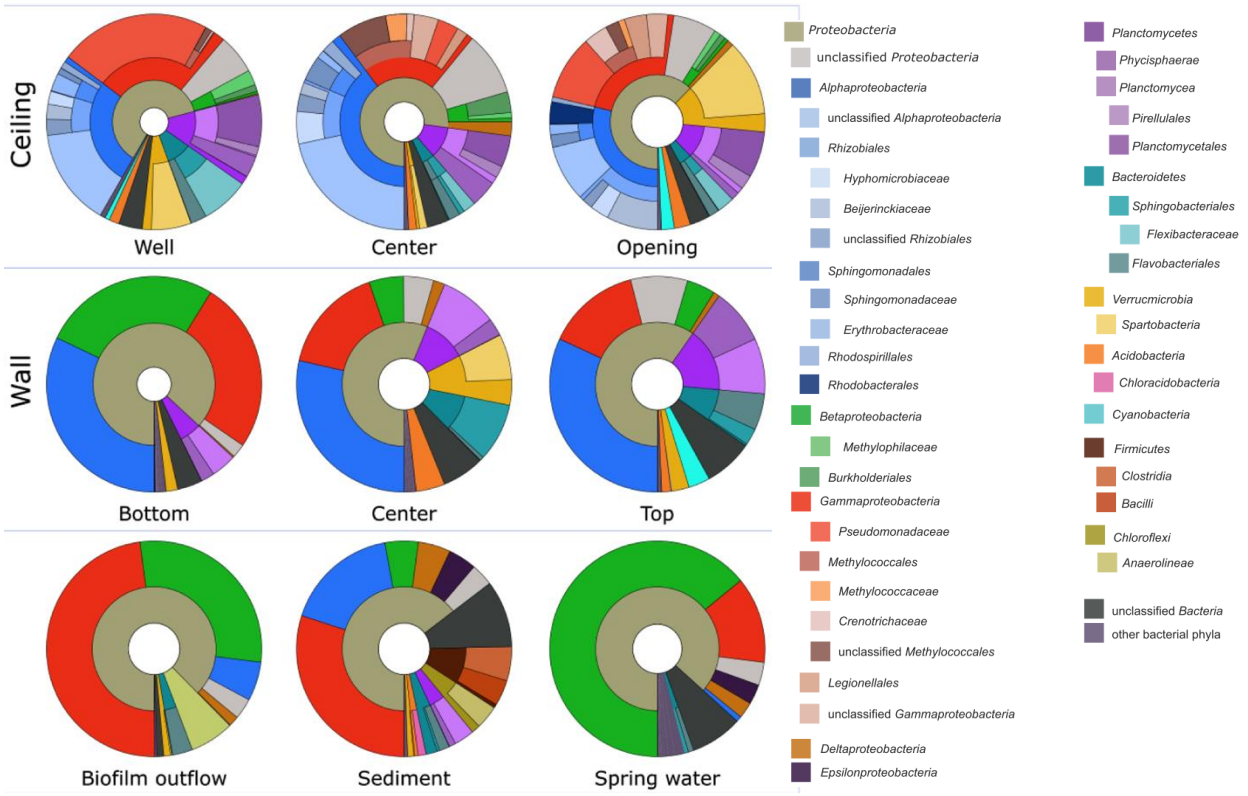


Figure 3- 12 Bacterial community composition of biofilm and cavern samples (n=1) at the phylum level with selected lineages zoomed down to family level, as displayed via Krona diagramms (Ondov *et al.*, 2011).

An in-depth taxonomic analysis revealed the main putative methylotrophic, and especially methanotrophic populations, within the biofilms (Figure 3- 12). Potential methylotrophic *Alphaproteobacteria* were apparent as *Beijerinckiaceae*, *Hyphomicrobiaceae*, *Rhodobacteraceae*, and *Erythrobacteraceae*, but there was also a substantial amount of unclassified *Alphaproteobacteria* in all biofilm samples. *Beijerinckiaceae* (3 ± 1 %) and *Hyphomicrobiaceae* (4 ± 3 %) were present in ceiling biofilms but almost irrelevant (<1 %) in all other samples. Wall biofilms were rich in

Rhodobacteraceae (bottom: 6 %/ center: 3 %/top: 2 %) and *Erythrobacteriaceae* (15 %/3 %/3 %), whose relative abundance decreased with height (Figure 3- 13). An inverse trend was found for unclassified *Alphaproteobacteria* (8 %/11 %/19 %). *Erythrobacteriaceae* (3 %) and unclassified *Alphaproteobacteria* (6 %) were also present in the sediment sample. The contribution of all these taxa to the community composition in any water sample was negligible.

Within the *Betaproteobacteria*, *Methylophilaceae* were abundant (10 %) in the lower wall biofilms, in the spring water (4 %) and the outflow water sample (6 %). Dominant taxa within this group were affiliated to *Methylotenera* sp.. Most notable was the dominance of *Gallionellaceae* (25 %) within the spring water and, at the same time, a similarly high fraction of unclassified *Betaproteobacteria* (25 %), both absent in all other samples. *Rhodocyclaceae* contributed to the well water with 7 % but were also abundant in the lower wall biofilms (8.5 %) and the outflow (8 %). The most abundant alphaproteobacterial family in the outflow was *Comamonadaceae* (16 %).

The high abundance of *Gammaproteobacteria* in ceiling biofilms was mainly due to *Pseudomonadaceae* contributing up to 44 % of the total relative abundance. This contribution was of minor importance in wall biofilms (max 3 %) and almost absent in water and sediment samples. Interestingly, *Legionellaceae* increased in higher wall biofilms (3 %/5 %/6 %) but were less abundant in ceiling biofilms (~1 %). Unclassified *Gammaproteobacteria* were found in all samples in relatively high abundance. There was an increase of this group towards the top in the wall biofilms (2 %/4 %/8.5 %), and their fraction accounted for 6.5 % within ceiling biofilms. Water and sediment samples contained ~3 % of unclassified *Gammaproteobacteria*.

Methylococcaceae were the most abundant putative methanotrophic group found in the biofilm samples. Highly abundant reads within the water samples were affiliated to *Methylobacter* sp. and *Methylosarcina* sp.. These type I methanotrophs were common especially within the lower wall biofilms (17 %) but were drastically reduced (<1 %) towards the ceiling (Figure 3- 13). In the well water (10 %) and outflow water (17 %), those taxa were also highly represented.

The *Planctomycetes* were mostly affiliated to *Planctomyces* spp. and unclassified *Phycisphaeraceae* and to a minor extend to *Pirellulaceae*. The amount of *Planctomyces* spp. in ceiling biofilms increased towards the cave entrance (1 %/6 %/12.5 %) and wall biofilms in the center (4 %) and top part of the cave (4.5 %).

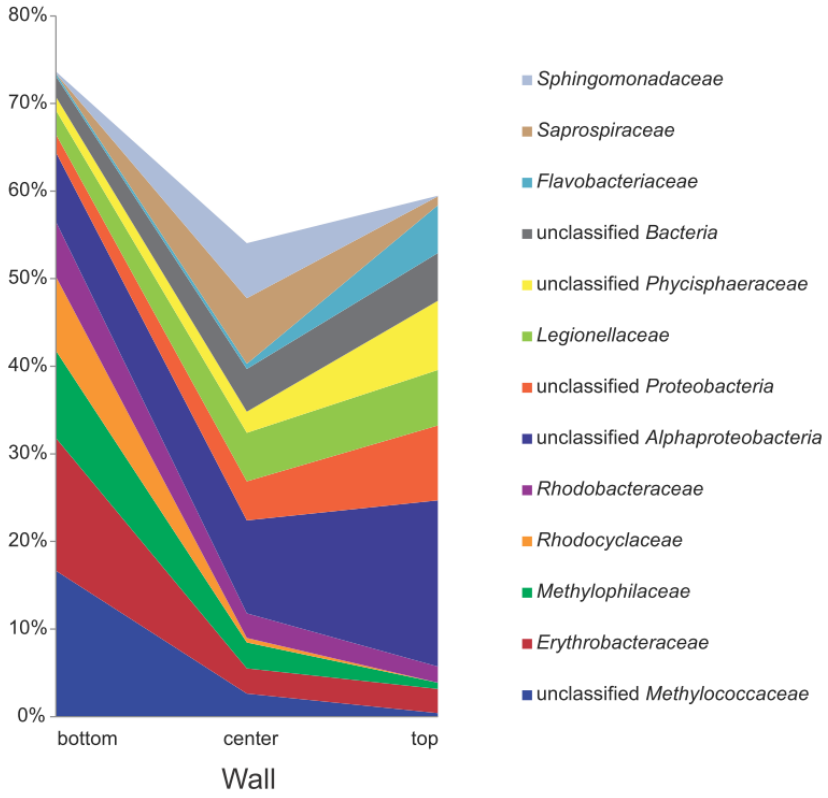


Figure 3- 13 Selected taxa (abundance >4 %) displayed a more or less pronounced gradient along the wall transect (n=1).

Verrucomicobia were represented primarily by *Spartobacteriaceae* and were only found in higher numbers (>5 %) in ceiling biofilms. The recently discovered candidate phylum NC10 and its described anaerobic, methanotrophic taxa *Methylomirabilis* sp. (Ettwig *et al.*, 2010), was found but only in negligible amounts (<1 %). The appearance of *Epsilonproteobacteria* in sediment and spring water samples is especially interesting since those taxa are absent in most freshwater habitats but have been found in other cave (Engel *et al.*, 2004b) and groundwater systems (Moissl *et al.*, 2002), mainly linked to the cycling of sulfur. Archaeal populations within biofilms were also sequenced, but so far only very preliminary data is available and not included here.

3.2.2.2 Fluorescence In Situ Hybridization (FISH)

Specific fluorescently labeled probes were selected based on the results of sequencing. Staining of specific taxonomic groups via FISH and microscopic examination, done by the author, was greatly impaired by strong autofluorescence due to mineral inclusions in the matrix and probe delivery through the thick EPS. Possible particles could be iron precipitates in the wall biofilms. Probes Gam42a and ArcMix for *Gammaproteobacteria* and *Archaea* gave the best results.

Counterstaining with DAPI revealed interdispersed gammaproteobacterial cells throughout the biofilm, while archaeal taxa seemed to be confined to the inner parts of the biofilm (Figure 3- 14)

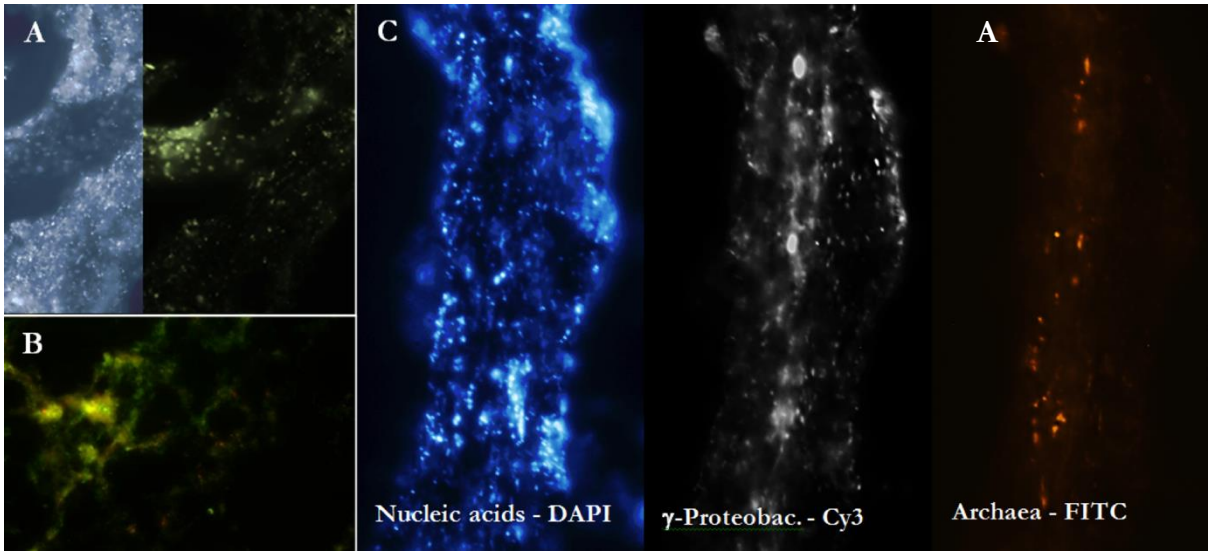


Figure 3- 14 A Biofilm thinsection stained with nucleic acid stain DAPI and a *Gammaproteobacteria* specific probe **B** Composite picture of a cave biofilm stained with specific fluorescence probe for *Gammaproteobacteria* (Cy 3 – green) and *Archaea* (FITC – red). **B** Wall biofilm series: all nucleic acids stained with DAPI (blue), *Gammaproteobacteria* (grey) and *Archaea* (red).

3.2.2.3 Network Analysis of Bacterial Co-occurrence in Water and Biofilms

A co-occurrence network was constructed based on the co-existence or mutual exclusion of taxa in all samples. The analysis could reveal possible cooperation between taxa or indicate competition through exclusion (Barberan *et al.*, 2012, Faust *et al.*, 2012). The bacterial network (Figure 3- 15) revealed potential cooperation of specific taxa. In total, 54 taxa made up the nodes of this network. Several basic network parameters are presented (Table 3- 11).

The co-occurrence network did not indicate central involvement of methanotrophs in cooperation with other taxa, in contrast to several putative methylotrophs. This could also have been due to the low sample size and relatively strict settings for network construction. In contrast, several of the potential methylotrophic taxa, especially within the *Betaproteobacteria*, were negatively correlated to *Alphaproteobacteria,* which themselves were co-occurring.

Table 3- 12 Parameters describing the network of bacterial co-occurrence patterns in the biofilm samples

Parameter	Description	Value
Clustering coefficient	$C_n = \frac{2e_n}{k_n(k_n - 1)}$	0.247
Network diameter	largest distance between two nodes (n,m)	12
Average number of neighbors	connectivity of a node in the network	3.11
Network centralization	Centralized = 1, decentralized = 0	0.096
Characteristic path length	expected distance between two connected nodes	5.271
Network heterogeneity	Tendency to have hub nodes	0.668
Shortest paths distribution	L(n,m) = k	2454 (85 %)

k_n is the number of neighbors of n
e_n is the number of connected pairs between all neighbors of n

Pseudomonadaceae, which were highly abundant in ceiling biofilms, co-occurred with *Beijerinckiaceae* and *Flexibacteraceae*, both of which have been associated to methane rich, often marine environments (Kobayashi *et al.*, 2008, Chistoserdova, 2011). Additionally, *Hyphomicrobiaceae* appeared centrally in the biofilms, co-occurring with several other methylotrophic taxa mainly belonging to the *Alphaproteobacteria* but also the *Planctomycetaceae.* The latter should also be highlighted, as they might also be involved in the turnover of methane (Bauer *et al.*, 2004, Chistoserdova *et al.*, 2004) but are also regularly encountered in bacterial communities of cave ecosystems(Pasic *et al.*, 2010, Northup *et al.*, 2011).

Thus, primary insights into a dominantly chemolithoautotrophic biofilm system largely independent from phototrophic carbon and energy inputs were revealed.

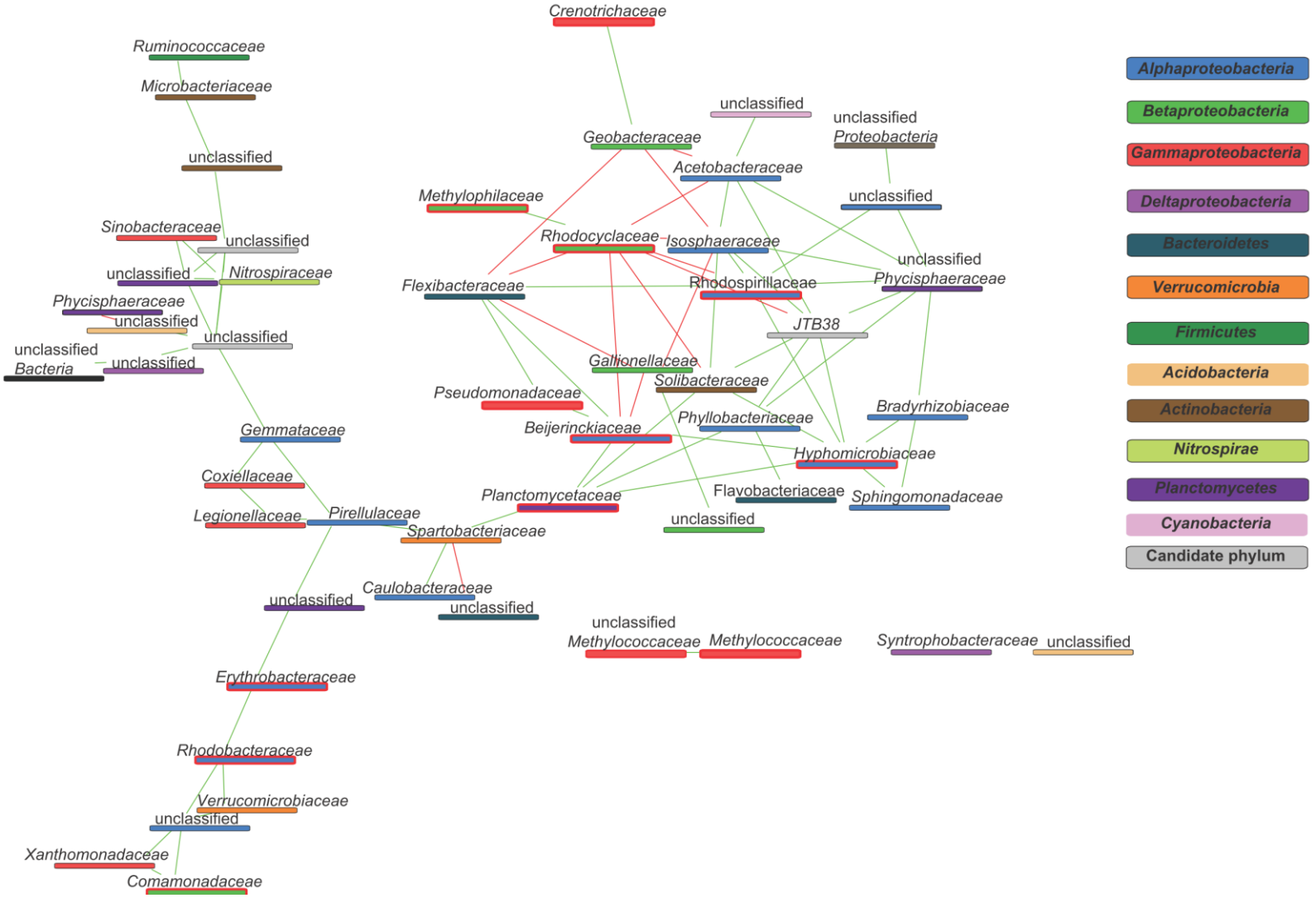


Figure 3- 15 The network diagram indicates community interactions such as co-occurrence patterns of bacterial families in all samples from the Sulzbrunn cave. Green nodes indicate co-occurrence, while red nodes show exclusion. Phylum affiliation is color-coded, possible methylotrophic taxa are circled red

3.3 The Microbiology of Drinking Water Wells

The molecular monitoring of drinking water wells provides a valuable tool to assess water quality and enable risk analysis. Here, three fully functional and active drinking water wells were investigated before the hydraulic removal of fine sediments and well filter biofilms. A series of samples taken during and after the restoration provided new insights into the distribution of well microbiota and the impact of such a procedure. Special focus was placed on the appearance of potential pathogenic taxa.

3.3.1 Water Analyses

The drinking water produced at the site was of moderate mineralization, which is characteristic of the region (Ca^{2+} 76.1 mg L^{-1}, Mg^{2+} 21.9 mg L^{-1}, HCO_3^- 324.8 mg L^{-1}). Between-well variability of hydrochemical parameters was minimal (Table 3- 13). The aquifer can be described as a well-oxygenated, oligotrophic system (~0.5 mg L^{-1} DOC). $S0_4^{2-}$ (8.6 mg L^{-1}) and NO_3^- (13.9 mg L^{-1}) were present as potential alternative electron acceptors for microbial respiration, whilst ammonium, nitrite and phosphate were below detection limits. Standard screening for coliform indicator bacteria in the drinking water via cultivation in this study also gave no positive results, consistent with results of regular inspection by certified labs.

3.3.2 Variability of Bacteria in Drinking Water Wells

Before purging, bacterial communities in the well systems were analyzed via bidirectional amplicon pyrotag sequencing. Although the reproducibility and semi-quantitative rigor of pyrosequencing libraries is still a matter of debate, a strong reproducibility of taxon abundances across biologically replicated DNA extracts for the pyrotag workflow was recently reported (Pilloni *et al.*, 2012) and shown that relative abundances can indeed be semi-quantitatively meaningful for taxa with a relative abundance between 0.2 % and 20 %. Therefore, but mainly for reasons of resources available for the project, analyses of replicated water samples per well or time point were not performed in this study.

Table 3- 13 Hydrochemical parameters [mg L^{-1}] of drinking water produced from the three wells 4 days before the well restoration (n=3). Standard deviations are not shown.

Sample	Ca^{2+}	Cl^{-}	Mg^{2+}	Na^{+}	NO_3^{-}	SO_4^{2-}	DOC
Well 1	76.4	7.4	21.9	2.6	13.9	8.6	0.53
Well 2	76.2	7.5	21.9	2.7	14	8.6	0.56
Well 3	75.8	7.4	21.8	2.6	13.8	8.5	0.41

First, it was the aim to assess the inter-well variability of drinking water bacteria between parallel wells. Since the hydrochemical parameters were highly similar (Table 3- 13), one would expect the same for the recovered microbial communities. After processing and quality filtering of all reads, sequencing provided 5109 ± 933 reads per library and sample (Table 3- 14), of which 99.9 ± 0.04 % were assigned to bacteria. Overall, in the three wells, 12 out of the 47 identified bacterial phyla contributed to the community with more than 1 % relative abundance to at least one library. The phylum-level read abundances between wells already indicated some variability between well communities (Figure 3- 16). The inverse Simpson diversity measure indicated the highest diversity within well 1 ($1/\lambda$ = 62.7, PIE = 41), but lower values for well 2 ($1/\lambda$ = 42.3, PIE = 38.8) and well 3 ($1/\lambda$ = 29, PIE = 30.7). At the same time, weighted unifrac suggested differences between overall community structures just above significance thresholds (W = 0.97, p-Value = 0.06).

Proteobacteria predominated the communities contributing half (57.2 ± 5.6 %) of all reads within the wells before maintenance (Figure 3- 16). Within the *Proteobacteria*, the *Alphaproteobacteria* (20.3 ± 6.5 %), *Gammaproteobacteria* (17.3 ± 3.6 %) and *Betaproteobacteria* (13.3 ± 5.9 %) were most abundant. A sub-phylum level perspective revealed the prevalence of members of the *Sphingomonadaceae* (5 ± 1 %), *Comamonadaceae* (4.2 ± 2.9 %), *Legionellaceae* (4.4 ± 3.9 %), and *Pseudomonadaceae* (3.4 ± 4.6 %) in all wells, the latter two being of interest as ubiquitous lineages harboring potential drinking water pathogens. In contrast, and as suggested already by cultivation-based coliform screening, members of the *Enterobacteriaceae* and coliform bacteria were of extremely low abundance (<0.05 %), or not detected at all, respectively. *Actinobacteria* contributed substantially only in well 1 (10.5 %) and well 2 (15.5 %). Well 3 harbored the lowest ratio of *Betaproteobacteria* (5.9 %) and the highest ratio of *Alphaproteobacteria* (29.5 %), dominated by *Rhodospirillaceae* (10.5 %). Surprisingly, well 3 also hosted a notable frequency of reads within the *Cyanobacteria* (4.1 %). Sequences obtained from candidate phyla adding up to more than one

percent in one of the communities were affiliated to the phyla *Thermi* ,TM7, SPAM, TG3, and WS3 (Figure 3- 16).

Table 3- 14 The number of trimmed and processed 454 sequencing reads of bacterial 16S rDNA gene pyrotag libraries from the drinking water wells (n=1). Diversity and richness indicators were inferred as stated.

Sequencing reads, Diversity Indices	**4 days before purging**			**During restoration of well 2**			
	Well 1	**Well 2**	**Well 3**	**Start**	**15 min**	**45 min**	**After**
Trimmed reads (f- & r-; >250 bp)	5406	6366	4718	5714	3695	7086	6687
Denoised reads (f- & r-; >250 bp)	4703	5746	4447	5269	3536	6085	5980
Inverse Simpson index (1/λ)	49.9	41	27.1	58.5	17.2	14.4	15.9
Rarefied species index (S_n)	265.8	245	155.1	241.4	118.5	251.8	106.7
Total species richness	392	389	232	372	165	447	189

3.3.3 Bacterial Dynamics During the Restoration of Well 2

One week after this initial assessment of well microbiota, the bacterial community dynamics during the hydraulic purging treatment were monitored in one of the wells (Well 2). The influence of high pressure jetting was clearly evident in community composition at the phylum level (Figure 3- 16). Before and upon the start of jetting, only minor changes in community composition compared to the initial status were observed, as shown also by principal component analysis of pyrotag data (Figure 3- 17). But then, within 15 minutes of jetting, the abundance of *Betaproteobacteria* increased from ~22 % to ~32 %, and especially members of the genus *Diaphorobacter* appeared preferentially purged.

Although almost absent before and at the beginning of purging, reads of *Ralstonia* and *Chryseobacterium* spp. emerged in the sample 15 min after jetting. All three taxa did not appear in higher numbers in later samples. Moreover, reads of *Acinetobacter* spp., *Nitrospira* spp. *and Sphingobium* spp. were most abundant in the purged samples on the first two time points. After 45 min of the maintenance procedure, reads of *Alkanindiges sp.* (22.9 %) and also unclassified cyanobacterial sequences (7.7 %) affiliated to *Bacillariophyta,* as well as *Janithobacterium* spp. (7.1 %) were suddenly observed (Figure 3- 16 & Figure 3- 17*).*

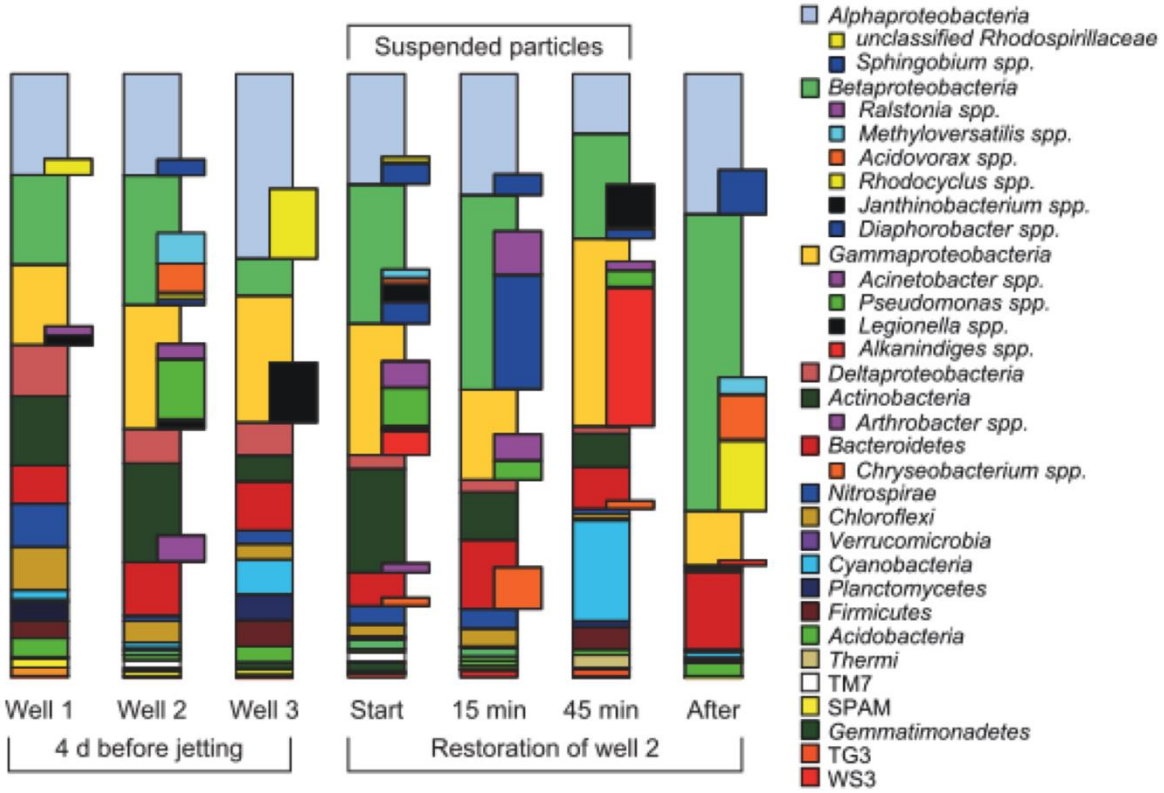


Figure 3- 16 Relative read abundance of major taxa in bacterial sequencing libraries (n=1) of drinking water wells. Communities were analyzed between the three wells (planktonic bacteria), as well as in course of the hydraulic restoration of well 2 (suspended particle-associated bacteria) and after the event (planktonic bacteria). All phyla or classes contributing more than 1 % abundance are depicted. Selected sub-phylum taxa mentioned in the text are highlighted.

Intriguingly, reads affiliated to *Pseudomonas* spp. constantly decreased in abundance during the maintenance and were hardly detectable after 2 weeks. The lowest bacterial diversity ($1/\lambda = 18.3$, PIE = 23.6) was observed in the water sample taken two weeks after high pressure jetting. Still, this community was more similar to well 2 before cleaning than samples towards the end of purging, according to the PCA (Figure 3- 17),. *Betaproteobacteria* (48.8 %) dominated the well community, while *Actinobacteria* were almost absent (0.7 %). The most abundant genus-level representatives were *Acidovorax* spp. (7.7 %), *Sphingobium* spp. (7.4 %), and *Rhodocyclus* spp. (11.7 %) as well as unclassified sphingobacterial sequences (11.1 %). *Chryseobacterium* spp. (*Bacteroidetes*) was the only taxon of hygienic concern, which increased in abundance (6.9 %) after the cleaning procedure.

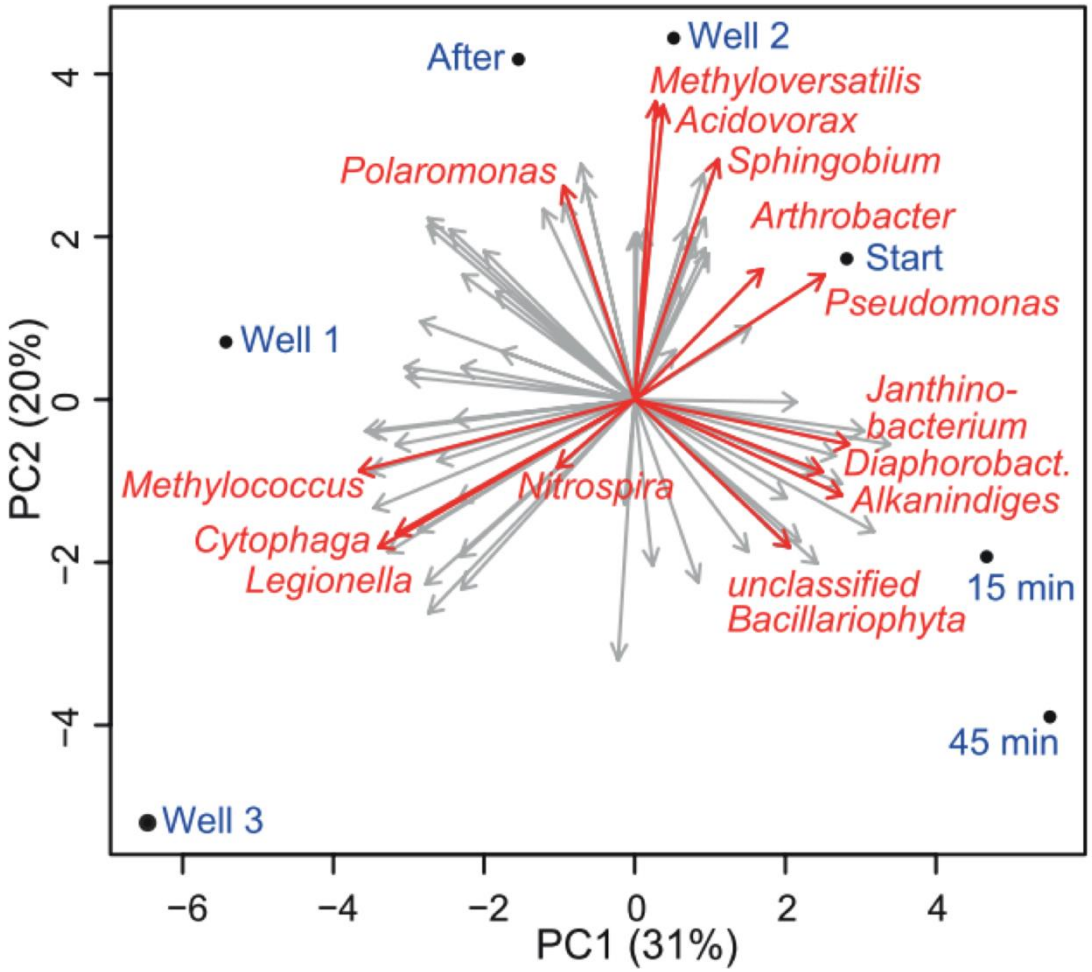


Figure 3- 17 Principal component biplots of community variability between wells and during the maintenance of well 2. Sample codes are the same as in Figure 3- 16. Selected taxa with high impacts on sample ordination are highlighted (arrows). Variance explanation ratios for each principal component (PC) are given.

This study demonstrated for the first time that drinking water wells act as a distinct microbial habitat and emphasize the role of attached microbes in this ultra-oligotrophic habitat as a seed bank for drinking water networks.

4 Discussion

4.1 Microbial Colonization of Mineral Surfaces

The weathering of minerals by microbial actions is ubiquitous in nature (Viles, 1995, Rogers & Bennett, 2004, Uroz *et al.*, 2009, Wang *et al.*, 2014). Porous aquifers present an immense surface area and potential habitat for microbial colonization. Still, the lack of easily available carbon sources and nutrients in pristine aquifers usually does not allow for high cell densities. The groundwater used in this study reflected these conditions displaying low dissolved organic carbon concentrations and the depletion of major nutrients (Table 3- 2). Up to 99 % of total microbial biomass has been found attached to geological structures in previous studies (Lehman *et al.*, 2001, Griebler *et al.*, 2002). However, a continuous coverage or even a multilayer biofilm growth on mineral surfaces is unlikely (Baveye *et al.*, 1992) in pristine aquifers. In the experiment conducted here, electron microscopy confirmed this scenario for all exposed surfaces. Nonetheless, patches of microbial colonization or colony development were found on most minerals (Figure 3- 7). Microbes attached to mineral surfaces establish local microenvironments through the exudation of secondary metabolites (e.g. EPS, exoenzymes) and interactions between cells (Norberg & Rydin, 1984, Johnson, 1998, Rohwerder *et al.*, 2003). Microbial aggregates can attack the surface of abiotic mineral particles by means of disintegration and dissolution by acidolysis, alkaline hydrolysis, enzymolysis, ligand degradation, and extracellular polysaccharide redox effects (Beveridge *et al.*, 1997, Sand *et al.*, 2001, Rohwerder *et al.*, 2003, Wu *et al.*, 2008) processes which are often summarized as bioleaching. Heavily weathered surfaces were observed in this experiment, most notably on the sulfide mineral. This is to be discussed in the following section (see "The colonization of minerals – Pyrite").

Community diversity and environmental factors such as pH, temperature and oxygen supply are essential in determining weathering processes (Johnson, 1998, Bennett *et al.*, 2001, Guidry & Mackenzie, 2003). In this experiment, groundwater fed to the mesocosm displayed circum-neutral pH, low temperatures and high oxygen concentrations (Table 3- 1). Knowledge of psychrophilic bacteria involved in mineral dissolution is rare. The availability of electron donors and electron acceptors in the form of natural minerals can have an especially fundamental impact on microbial communities. An investigation of silicate weathering (Bennett *et al.*, 2001) showed the colonization of silicates and the leaching of potential nutrients. Minerals also used in this study (apatite and magnetite) were shown to be colonized and weathered in *in situ* microcosms from an oil-contaminated aquifer (Table 4- 1).

Table 4- 1 Microbial colonization and weathering of mineral surfaces within an oil-contaminated aquifer: Increasing magnitude of colonization, EPS production and weathering are symbolized by + to +++, - if no such process was observed. Taken from Bennett *et al.* (2001)

Mineral	Colonized	Weathered
Apatite ($Ca_5(PO_4)^3$ (F,Cl,OH)	+	++
Magnetite (Fe_3O_4)	+++	+
Quartz (SiO_2)	++	+
Anorthoclase ((Na,K) $AlSi_3O_8$)	+++	+++
Plagioclase ($NaAlSi_3O_8$ – $CaAl_2Si_2O_8$)	-	-

The experiment conducted here gives a new perspective of the influence of mineral surfaces on community composition as well on biofilm functioning. Nutrient limitations, specifically low phosphate concentrations, were thought to increase weathering and colonization of the phosphate-bearing mineral. However, this was not observed. All of the different minerals do not select for distinct communities, rather only the one substratum providing the most limiting resource. In this case, the pyrite surface acts as an electron donor. This provides essential evidence that electron donor limitation dominates all other drivers of community selection. Other surfaces, such as the sulfide mineral, could provide an electron acceptor to colonizing microbes. Attachment has also been considered to be more influenced by physical surface condition (e.g. surface roughness, interaction forces) than by nutritional selection of bacteria (Katsikogianni & Missirlis, 2004). The bacterial cell wall influences mineral-microbe interactions (Beveridge *et al.*, 1997). Active cell wall turnover increases the surface area-to-volume ratio of microbial aggregates. Therefore, cell growth and division rates impact overall charge capacity. Gram-positive cell walls display especially high structural complexity and coinciding chemical complexity. Gram-positive cell walls are also known to sequester dilute metal ions and are often covered by charged mineral particles (Beveridge & Murray, 1976). At circum-neutral pH, carboxylates and phosphates are first to be converted as they are used in the turnover of cell walls. Gram-negative cell walls are also capable of interacting with metals (Li & Logan, 2004). Essentially, lipopolysaccharides surrounding the cells control the physicochemical behavior. Other bacterial surfaces, such as capsules, sheaths and biofilms can react with mineral surfaces (Beveridge & Graham, 1991, Beveridge *et al.*, 1997). All the dominant taxa identified in the experiment (Figure 3- 5) possess gram-negative cell walls.

Many of these attached cells have increased metabolic activity, which is reflected in their internal ATP.

4.1.1 The Colonization of Minerals

Apatite

The possibility of leaching limiting nutrients from mineral surfaces presents a considerable opportunity for microbes to alleviate nutrient deprivation. Soluble phosphate (PO_4^{3-}) is especially scarce and tightly cycled within the microbial community in many groundwater systems (Madsen & Ghiorse, 1993). Microbial phosphate solubilization has been extensively studied, emphasizing its essential role in soil fertility (Rodríguez & Fraga, 1999). The apatite surfaces could potentially provide phosphate to attached microorganisms (Goldstein, 1986, Bennett *et al.*, 2001, Welch *et al.*, 2002). In this study, neither the community composition nor specific taxa indicated exploitation of the apatite mineral surface. The bacterial communities attached to granite, magnetite and apatite mineral surfaces were practically identical, as shown with a double-centered principal component analysis (Figure 3- 6). The eukaryotic community separated this mineral surface more decisively from the communities of other minerals (Figure 3- 6). Nonetheless, colonization or weathering was low, as determined by examining SEM images. Some possible reasons for low leaching activity could be that microbes taking up soluble PO_4^{-3} from a solution could shift the solution's equilibrium and prevent apatite dissolution. Also, phosphate levels in the groundwater and bulk water within the mesocosm were at the limit of detection (Table 3- 2), which might have hindered such a shift. Furthermore, the removal of Ca^{2+} from the solution by microbially mediated precipitation of Ca-oxalate phases can also promote apatite dissolution (Welch *et al.*, 2002). However, calcium oxalate crystals were not observed on the electron microscopy images indicating no such process.

The number of substrates used and the activity and diversity of apatite detached cells in the Biolog assay was lower compared to the communities removed from the magnetite and granite surfaces. The activity measured in the assay leveled off or fell after 100 hours, indicating substrate depletion. The substrate guilds responsible for the highest activity were amines, followed by carboxylic and acetic acids and finally carbohydrates. The absence of activity related to amino acids of the apatite community was surprising, since several bacteria are known to readily utilize amino acids, such as carbon and nitrogen sources under nutrient limiting conditions (Sepers, 1984, Egli, 2010).

In essence, an indication that apatite exposed to low temperature groundwater at circum-neutral pH and at low nutrient concentrations can lift phosphate limitations or cause increases in carbon usage activity was not provided compared to other mineral surfaces.

Magnetite

Several bacteria, such as *Shewanella* spp., can couple the oxidation of organic carbon to the reduction of amorphous Fe(III) oxyhydroxide and crystalline Fe(III) oxides (Lovley, 1991, Kostka & Nealson, 1995). Magnetite reduction clearly resulted in electron flow and growth of groundwater microbes in an anaerobic environment (Kostka & Nealson, 1995, Roh *et al.*, 2006). In the microcosms investigated here, oxygen concentrations in the groundwater and the lack of significant biofilm formation, leading to oxygen depletion, obviously impaired anaerobic processes. Bacterial community composition was similar to those of apatite and granite surfaces attached communities. The high number of sulfur-oxidizing *Sulfuritalea* spp. could indicate a leaching of sulfide minerals. However, these taxa have also been associated with magnetotactics (Dziuba *et al.*, 2013) and sulfur-oxidation (Sakaguchi *et al.*, 1993), and are capable of magnetite formation. Yet, biomineralization on any magnetite mineral surface was not observed here. Still, microbial populations attached to magnetite surfaces were the most active and diverse, in terms of substrate use. The breadth of substrates converted was much higher, especially compared to all other mineral surface communities. A preference for amines was exhibited after 60 h and was almost twice as high as for other substrate guilds. Carboxylic and acetic acids followed the use of amines and, most interestingly, by amino acids, which showed only minor contributions to the activity in all other mineral observations. Potentially, this high activity could be related to the more diverse eukaryotic community detected, which is capable of increasing carbon substrate utilization. Mycorrhizal fungi are especially known to impact mineral weathering (Gadd & Raven, 2010).

The microscopic images taken from the magnetite minerals showed clusters of microbial cells, which might facilitate the cooperation between cells, but no direct evidence for such interactions can be provided. Thus, even though magnetite was clearly not relevant as an electron acceptor, this reactive surface managed to select for the most functionally diverse microbiota, although this was not apparent on the structural level. Although biofilm communities taken from the magnetite surfaces were almost identical to those communities attached to apatite and granite, the microbes associated with magnetite appeared the most diverse (Table 3- 4 & Table 3- 5), arguably increasing the breadth of substrate use.

Pyrite

The sulfide mineral exposed to the indigenous groundwater community represented a mix of sphalerite (ZnS), pyrite (FeS_2), and silicates. These surfaces were the only potential electron donors and were also most dissimilar to the other minerals. A wealth of literature (Edwards *et al.*,

1999, Rodríguez *et al.*, 2003, Li *et al.*, 2013) on the colonization and leaching of pyrite minerals is available. Several acidophilic microorganisms such as *Acidithiobacillus ferrooxidans*, *Thiobacillus thiooxidans*, *Leptospirillum ferrooxidans* or *Thiobacillus ferrooxidans* were found to be capable of oxidizing sulfide and ferrous iron from mineral surfaces (Johnson, 1998). The leaching of pyrite involves intermediate oxidation stages, e.g. thiosulfate and polythionates (Johnson, 2001, Sand *et al.*, 2001), which are further oxidized to sulfate and also elemental sulfur as a byproduct. In our experiment, the common acidophilic bacteria were not observed. Community diversity and cell numbers on pyrite were the highest of all exposed surfaces, but activity in the heterotrophic metabolic assay was low. A possible explanation could be that the lack of substratum after transfer deprived the community of its lithotrophic electron donor, and that alternative heterotrophic capacities were not developed. Even though no EM images of the mineral surfaces at the start of the experiment were provided, weathering patterns of pyrite have been analysed in detail (Liu *et al.*, 2003). A clear indication of whether heavy weathering of the sulfide mineral was due to abiotic or biotic processes is not possible (Figure 3- 7). In previous work, it was differentiated between a direct bacterial attack of the mineral surface and an indirect attack by ferric iron (Fe^{3+}) in solution regenerated from planktonic bacteria (Konishi *et al.*, 1992, Rodriguez *et al.*, 2003). Abiotic dissolution of zinc sulfide by ferric sulfate, where ferric ions oxidize the sphalerite to form zinc and ferrous ions in solution and elemental sulfur, has also been reported (Fowler & Crundwell, 1999). The ferrous ions formed in this bioleaching reaction can be oxidized by microbes to ferric ions, accelerating the leaching process (Sand *et al.*, 2001, Li *et al.*, 2013). Given that we did not detect any specialized, acidophilic bacteria, one has to consider abiotic weathering as well as bioleaching by a mixed microbial community as prevalent mechanisms.

Microbes most closely affiliated to heterotrophic *Zoogloea* spp. (*Betaproteobacteria*) were dominant on the pyrite surfaces. The appearance of *Zoogloea* spp. in pyrite tailings has been reported (Kalin *et al.*, 2005) They are capable of producing acidophilic polysaccharides to accumulate metals (Norberg & Rydin, 1984). *Zoogloea* spp. were also found in greater abundance in the box water, which indicates their potential for dispersal and detachment from surfaces.

Taken together, pyrite as a source of electron donors was the only surface to cause the expected selective effects on attached microbiomes. An indicator for the selectivity of pyrite was the low bacterial diversity observed on these minerals. Moreover, the biofilm community was not capable of utilizing carbon substrates, at least in the absence of the substratum. It appears that under heavy energy limitations, electron donors rather than putative nutrient release select

groundwater microbes. This provides a new perspective on the attached growth of microbes in energy- and nutrient-limited aquifers.

Granite

The granite coupons exposed in this experiment supposedly represented the least reactive surface, providing neither limiting nutrients, electron acceptors nor electron donors. Evidence for the colonization and even weathering of granite has been reported (Song *et al.*, 2007). Different bacterial communities have been linked to specific granite minerals showing correlation between OTUs and chemical composition (Gleeson *et al.*, 2006). In my experiment, the similarity in community composition between granite, apatite and magnetite indicated a lack of specific preference of groundwater bacterial communities towards any of these surfaces. Previously, it was concluded that pH changes during microbial growth could stimulate silicate mineral dissolution over longer timescales (Wu *et al.*, 2008), so that the duration of the experiment may still have been too short. Nonetheless, ongoing colonization would most likely rather be driven by species interactions than by nutrient supply. Interestingly the community attached to granite coupons was relatively active in the Biolog plates. The high similarity of the microbial communities grown on granite and apatite emphasize the notion that nutrient limitations were not alleviated by attached growth. Thus, the granite biofilms served as an important control to exclude any selective patterns for apatite and magnetite, but substantiated the observed selectivity and role of pyrite in this system.

4.1.2 Planktonic Microbes in Groundwater and the Mesocosm

Planktonic microorganisms in groundwater can be seen as part of a regional metacommunity, connecting and shaping local communities through processes of adhesion, colonization and dispersal (Leibold *et al.*, 2004, Besemer *et al.*, 2012). The community sampled within the mesocosm was certainly a mixture of detached microbes from the surfaces as well as inflowing groundwater.

Substrate utilization assays (Preston-Mafham *et al.*, 2002) provide information on the range and diversity of compounds potentially used by a community. Oligotrophic conditions in groundwater can be survived by utilizing a range of carbon substrates at the same time (Egli, 2010). The low activity of planktonic cells in the Biolog assay was due to low cell numbers, but the range of substrates used exceeded that of attached communities. Thus, planktonic cells seem to experience even more oligotrophic conditions in groundwater, which is consistent with the concept of the biofilm mode of life providing essential benefits for microbes.

4.1.3 General synthesis

In summary, selection pressure was not exerted by the apatite, granite and magnetite surfaces to the colonizing biofilm communities. This rejects our initial hypothesis that nutrient limitation (phosphate limitation) can cause biofilm community shifts in natural oligotrophic, low-temperature aquifers. It is likely that the attached taxa were simply exhibiting the best colonization abilities and were ubiquitous in the seeding community favoring their dispersal. In contrast, the sulfide mineral and its oxidation altered the community composition effectively. The community was obviously impacted by the attachment and weathering of the pyrite/sphalerite mineral. This is a new perspective of electron donor limitations being the central factor for selective surface colonization, in contrast to nutrient limitations, which obviously did not impact biofilm composition.

The sampled biofilms consisted of groundwater taxa and microbes adapted to oligotrophic conditions. The majority of previous studies on mineral weathering have focused on dissolution in conditions which are uncharacteristic for shallow groundwater bodies. In most aquifers, low temperatures, lack of energy sources, low nutrient levels, and relatively unreactive mineral surfaces hinder the growth of microbes or even microbial aggregates. Here, however, I show that electron donor limitation overrides the effect of nutrient-limitations and operates as a selective force in the colonization of minerals.

4.2 Microbial Biofilms in a Mineral Spring Cavern Dominated by Methane and Iodine

4.2.1 Methane as a Driver of Biofilm Formation

The central questions and hypotheses posed in this field study focus on the sources of biofilm growth and the microbial communities inhabiting and producing the biofilm in the Sulzbrunn spring cavern. The microbial biofilm community described herein impresses by its sheer biomass and the massive amount of extracellular polymeric substances it produces. The energy source allowing such accumulations was identified primarily as methane in this thesis. Other caves with inputs of methane have been described (Kumaresan *et al.*, 2014) but in this cavern, hydrogen sulfide appeared to be the primary energy source (Sarbu *et al.*, 1996). So far, the described biofilm system is the first evidence of such methane fuelled biofilm growth.

The isotopic signature of the outgassing methane identifies it as thermogenic (Figure 4- 2) (Whiticar, 1999), excluding recent microbial production. Although the stable isotope values of the freeze-dried biofilms were integrative over the whole community, gradients of distinct carbon inputs were identified. Interestingly, the only sample with a signal of seeping methane was found at the bottom of the wall dominating biofilms, which are usually submerged. A clear gradient towards the ceiling was shown (Figure 3- 8). The carbon isotope signature of the biofilms at the ceiling was comparable to soil leachate carbon from C_3 plants (Kohn, 2010), substantiating this as an additional source of carbon in the cave. This appears legitimate, since the cave water is also a mixture consisting of ~¾ seepage water, according to the estimations from the mixing of water stable isotopes and chlorine. The input of organic carbon in the form of dissolved organic matter from the overlaying pedosphere is common in caves (Sarbu *et al.*, 1996). Never before, however, has spatial localization of cave biofilms been identified as such an important discriminant between biosphere and geosphere carbon inputs.

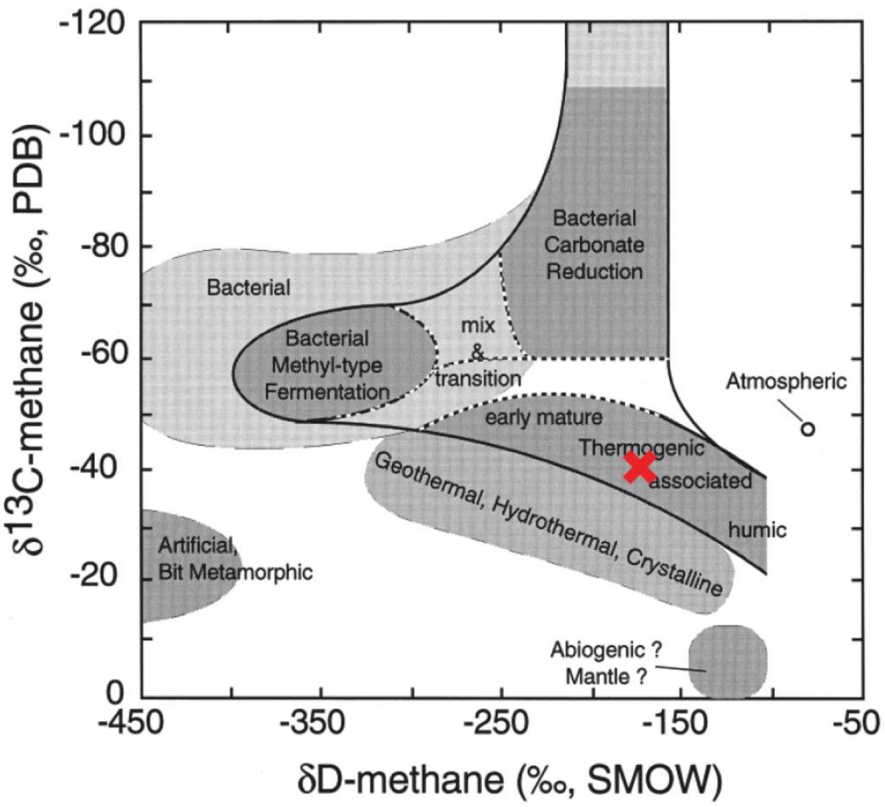


Figure 4- 1 The classification of the origin of methane, based on the isotopic signature of carbon and hydrogen atoms. The red cross indicates the reported values for methane from the Sulzbrunn Cave. Taken from Whiticar (1999)

Furthermore, the $\delta^{15}N$ signature of the respective biofilm communities provided information about local N partitioning. A close link between nitrogen cycling and methane oxidation has been described recently. Microbial consortia dominated by *Methylococcaceae* and *Methylophilaceae* linked to methane oxidation coupled to denitrification (Kalyuhznaya *et al.*, 2009, Liu *et al.*, 2014) and nitrite-driven oxidation of methane in *Methylomirabilis oxyfera* (Raghoebarsing *et al.*, 2006) are just two examples. *Methylophilaceae* were markedly represented in lower wall biofilms where $\delta^{15}N$ partitioning was the most pronounced (Figure 3-8). A direct link between the appearance of *Methylococcaceae* and *Methylophilaceae* was not supported by network analysis.

The physicochemical analysis of the upwelling mineral water displayed elevated values of ions resulting in high specific conductivity of the water, conforming to previous measurements (Table 3- 2). The water showed low oxygen concentrations, making the mixing of anoxic deep formation water with aerated meteoric water on the way up to the spring cavern likely. The circum-neutral pH of the groundwater differentiates this system from most other high biomass

subsurface systems, where biofilm snottites have been reported mostly in acidic environments (Bond *et al.*, 2000, Macalady *et al.*, 2007).

4.2.2 Massive Production of Extracellular Polymers

A central concern of this study was determining the factors initiating and influencing the production of the massive EPS matrix. The overproduction of EPS can be hypothesized to provide several advantages to biofilm communities. The most likely explanation for the high production of EPS is suggested by the nutrient composition within biofilms. A high carbon to nitrogen ratio has been identified as a proxy for biopolymer production (Linton, 1990). This situation was eminent in the ceiling biofilms, which also exhibited the highest biomass. The microbes appeared to have easy excess to carbon and energy, but other essential nutrients for growth seemed lacking. The matrix provides an electron sink for the microbes and also a storage capacity for reduced carbon. Methanotrophs using the ribulose monophosphat pathway (RuMP), called type I methanotrophs (Hanson & Hanson, 1996), such as *Methylococcaceae* and *Crenotrichaceae*, profit from an energetically favorable production of extracellular polysaccharides (Linton, 1990). Additionally, they can shunt toxic formaldehyde produced in the first step of methane oxidation. Nonetheless, these taxa were more abundant in the wall biofilms where exopolymer production was not as apparent as on the ceiling. The higher rRNA gene abundance of lower wall biofilms and the higher levels of nitrogen and phosphorous could indicate higher growth rates and less exudation of polysaccharides by these microbes.

Certainly, the formation of EPS provides ideal conditions for cooperative behavior. Physicochemical gradients in the biofilms are likely to create a multitude of microniches (Costerton *et al.*, 1994). Although an oxygen profile from within the snottites cannot be provided here, oxygen depletion within several hundred micrometers has been reported (De Beer *et al.*, 1994) and appears plausible for biofilms from a few millimeters up to a centimeter in thickness (Ziegler *et al.*, 2013). Although measurements of assumed oxygen gradients in the cavern remain to be done, several micro-aerophilic to anoxic environments are likely to exist in the cave.

The importance of methane as an energy source was supported by the fact that *pmoA* coding genes were detected in all samples, also indicating the availability of oxygen for lower wall biofilms. Almost all abundant taxa found within the cave biofilms were directly or indirectly linked to methylotrophy (Chistoserdova *et al.*, 2009, Chistoserdova & Lidstrom, 2013). This was also emphasized by the ubiquitous presence of the *mxaF* gene in all samples. While type II methanotrophs belonging to the *Alphaproteobacteria* can be found within the *Beijerinckiaceae*, they

were only of minor importance in lower biofilms. Instead, type I methanotrophs were common in those samples.

Fingerprinting *pmoA* amplicons provided basic additional information on the diversity and structure of the methanotrophic communities. Surprisingly, the diversity was lower at the bottom compared to ceiling biofilms, indicating a more specialized community. Several pyrotag reads were affiliated to *Crenotrichaceae*, as identified via the database published by Werner *et al.* (2011), but not recognized with other databases (e.g. Greengenes (DeSantis *et al.*, 2006), SILVA (Quast *et al.*, 2013)). *Crenotrix* spp. have been characterized as filamentous, sheated *Gammaproteobacteria* found in groundwater and slow running water at low organic matter and Fe^{2+} concentrations, but frequently encrusted with iron or manganese oxides (Harder, 1919, Stoecker *et al.*, 2006). The appearance of *Crenotrix* spp. in caves is well documented (Provencio & Polyak, 2001, Engel, 2010) but in this study its presence was not confirmed visually by microscopic examination. Therefore, several 16S sequences were blasted using NCBI. *Methylosarcina* spp., *Methylosoma* spp. and *Methylobacter* spp. were found to be closely related to the reads previously recognized as *Crenotrichaceae* in the sample libraries. *Methylosarcina* spp. have been described (Wise *et al.*, 2001) as slime forming bacteria found in diverse habitats, such as rice paddy soil (Qiu *et al.*, 2008), lakes (Kalyuzhnaya *et al.*, 2005) and landfills (Wise *et al.*, 2001) consuming methane and methanol as their sole carbon sources. In essence, the methylotrophs in this system are likely to drive EPS production using methane and methylhalides as energy sources.

4.2.3 Biofilm Elemental Speciation

4.2.3.1 Iron and Sulfur

An elemental analysis of biofilms revealed interesting distribution patterns for sulfur and iron, both of which were elevated along the wall. Massive biofilm growth in caves has previously been attributed to the presence of sulfide, but low sulfate levels in the water (Table 3- 2) and moderate sulfur levels in the biofilms (Table 3- 8) do not suggest a central role of sulfide oxidation within this cave. The high amount (up to 106 mg g^{-1}) of iron especially in biofilms, indicates an influence of the microbial communities. Certainly, iron was heavily enriched in biofilms compared to the spring water. An interaction of iron and with extracellular polymer matrices (e.g. regulation of EPS production, bioleaching) has previously been shown (Banin *et al.*, 2005, Sand & Gehrke, 2006). Cells with increased amounts of iron and glucuronic acids within the EPS matrix displayed higher respiratory activity (Sand & Gehrke, 2006). Wall biofilms displayed less EPS production compared to ceiling biofilms and were visibly pervaded with iron precipitates (Figure 4-3). The larger area around the cave is known to feature iron ore deposits (Heim, 1919)

so that iron rich geological formations could be the reason for the high iron concentrations. The elevated iron concentrations along the wall might be due to leaching into the biofilms and might play a role in regulating methylotrophic processes, especially under oxygen limitation (Beal *et al.*, 2009).

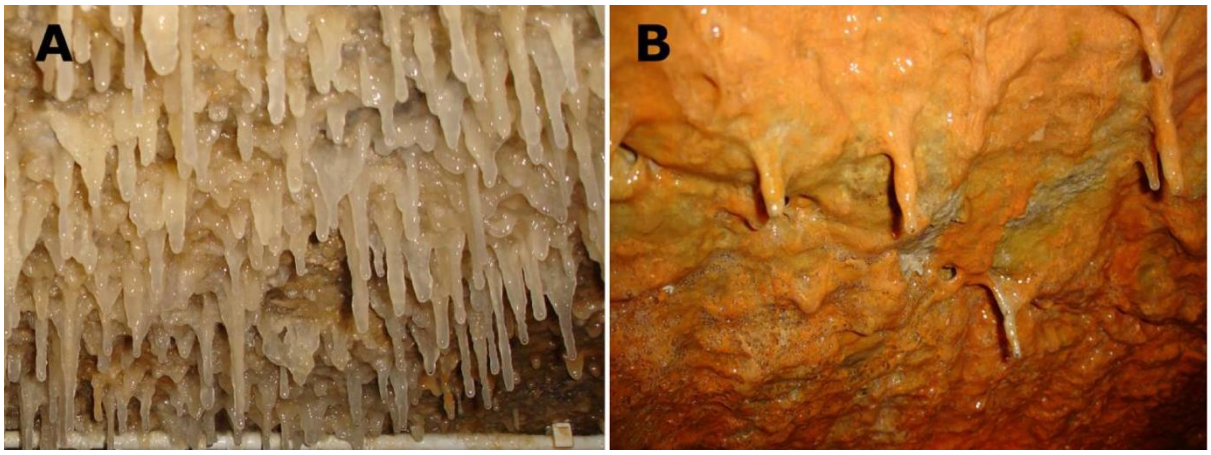


Figure 4- 2 A Ceiling and **B** wall biofilms displaying different morphology and coloration. Gas formation is visible in the left corner of the wall biofilm image. (Photo: Karwautz)

4.2.3.2 Iodine

The relatively high amount of iodine in spring water and also biofilm samples warrants further attention. Iodine in high concentrations is generally found only in marine environments, but iodine has also been detected in lakes, streams and groundwater ecosystems (Whitehead, 1984, Fuge & Johnson, 1986). Iodine is a biophilic element enriched in fluids of high hydrocarbon content (Moran *et al.*, 1998) and sorption to organic matter and minerals (e.g. ferric oxides and hydroxides) has been demonstrated (Couture & Seitz, 1983, Whitehead, 1984). An enhanced corrosion of iron has also been related to iodide oxidizing bacteria (Wakai *et al.*, 2014). Apart from geological settings (faults), methyl iodide (CH_3I) has been attributed to be responsible for the transfer of iodine from the ocean to land (Whitehead, 1984). In aquatic systems, it was demonstrated that dissolved iodine is mainly available as iodide (I^-), iodate (IO_3^-) and organic iodine (Figure 4-3)(Tsunogai & Sase, 1969, Elderfield & Truesdale, 1980, Jickells *et al.*, 1988, Muramatsu & Wedepohl, 1998, Wong & Cheng, 1998, Chapman & Truesdale, 2011). The distribution of iodine in groundwater is described as a spatially and temporally dynamic system (Voutchkova *et al.*, 2014). Radioiodines (^{129}I and ^{131}I) in particular, which are generated in nuclear energy facilities, are of major concern to human and ecosystem health (Snyder & Fehn, 2004, Zhang *et al.*, 2013).

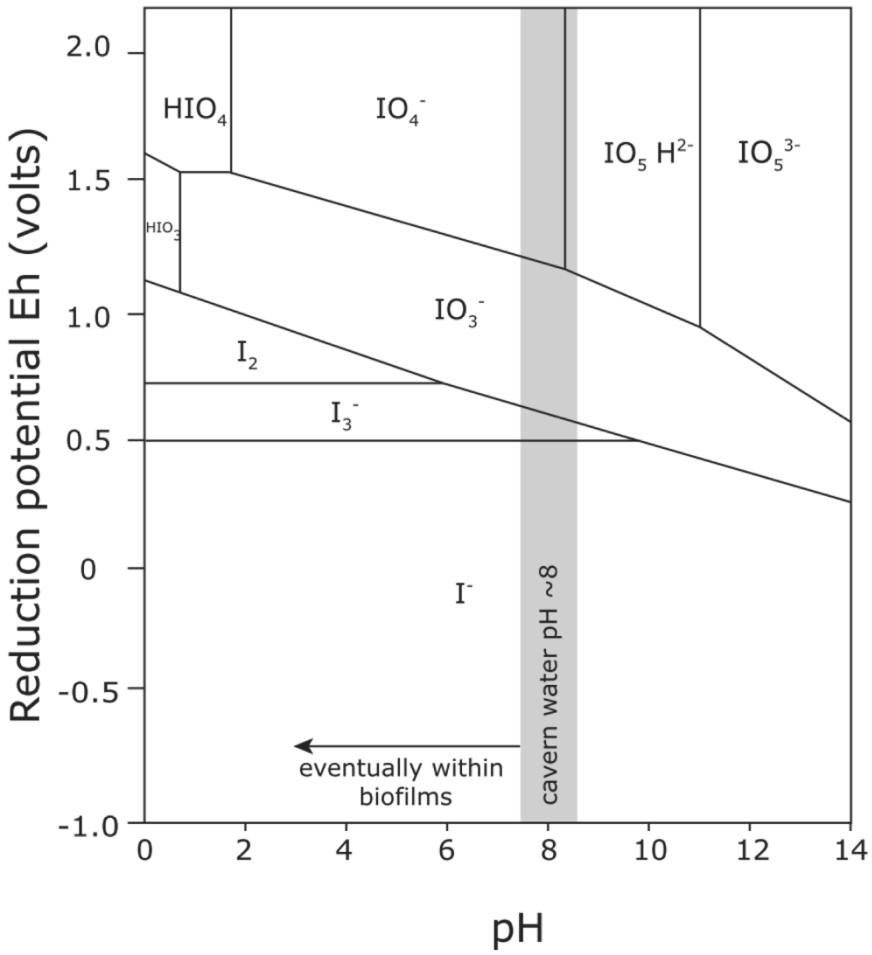


Figure 4- 3 Iodine speciation at 25 °C in relation to the pH and reduction potential of the environment. Under reducing conditions, aqueous iodine usually occurs as iodide (I^-), while in more oxic environments, iodine is present as iodate (IO_3^-). Taken from Whitehead (1984).

Several microbial processes involved in iodine cycling, such as the uptake and accumulation of iodine, the reduction of iodate, or the oxidation and volatilization of iodide, have been described (Malcolm & Price, 1984, Councell *et al.*, 1997, Fuse *et al.*, 2003, Amachi *et al.*, 2007b, Amachi, 2008, Arakawa *et al.*, 2012). Here, a cycling of different iodine species between spatially segregated cavern compartments is proposed. A link between iodine and methane cycling would be of considerable interest. Pathways that could be relevant in this system would be first, the production and oxidation of methyl halides, and second, a redox reaction involving methane oxidation and iodate reduction which would present a new mechanism for methane oxidation. No final evidence for these processes can be provided at this stage because the full iodine speciation awaits further investigation. The possible pathways are discussed below.

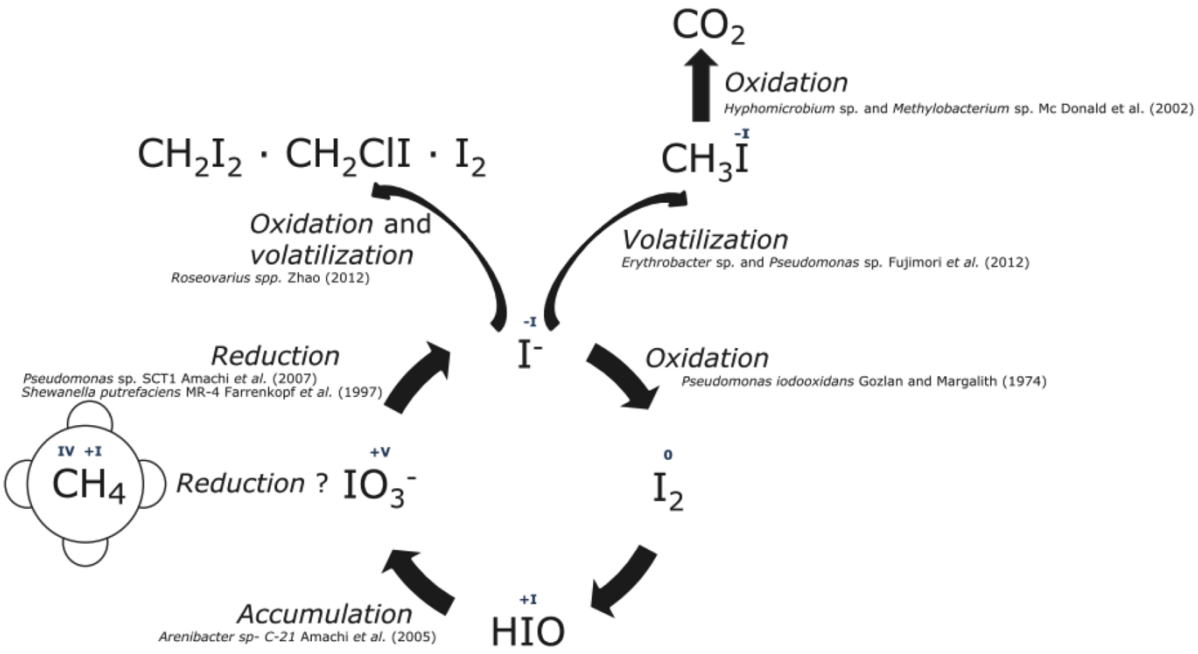


Figure 4- 4 Iodine cycle Iodine species and relevant bacterial taxa involved in the processing thereof. Modified from Amachi (2008)

4.2.3.2.1 Iodomethane (CH_3I) production and utilization

Iodomethane, or methyl iodide, is a mutagenic volatile halocarbon and actually the most abundant halogenated hydrocarbon in the atmosphere, being mainly produced by kelp (*Laminaria*) (Carpenter *et al.*, 2000), *Cyanobacteria* (Hughes *et al.*, 2008) and microbial aggregates of marine and terrestrial origin (Amachi *et al.*, 2001, Amachi *et al.*, 2003, Fujimori *et al.*, 2012). Attachment and aggregation of microbial communities seems to enhance the production of iodomethane (Asare *et al.*, 2012).

The production of methyl halides is likely to be wide-spread in marine microorganisms (Amachi *et al.*, 2001), and *Erythrobacteriaceae* have been particularly shown to do so (Fujimori *et al.*, 2012). The high numbers of *Erythrobacteriaceae* especially at the lower wall (Figure 3- 14), could be indicative of such a process occuring in the cave biofilm.

In the presence of an equimolar mixture of iodide, bromide and chloride, methyl halides were formed by a fungal culture in a ratio of 27:5:1, emphasizing the affinity of the fungal methylating system (Harper & Kennedy, 1986). However, no respective data is available for bacterial communities to date. Although a quantitative detection of methyl iodide or other methyl halides in the cavern atmosphere was not feasible, the first qualitative evidence was in fact generated in the course of this thesis and these measurements be continued.

Methyl halides can be co-oxidized by organisms containing methane-, ammonia- or toluene monooxygenases (Han & Semrau, 2000, Duddleston *et al.*, 2002, McDonald *et al.*, 2002, Goodwin *et al.*, 2005). Furthermore, a specific pathway for the oxidation of methyl halides was found in several microorganisms represented by taxa of the *Roseobacter* clade, *Hyphomicrobium chloromethanicum*, *Methylobacterium chloromethanicum*, *Aminobacter* sp. IMB-1, and others (Vannelli *et al.*, 1999, Amachi *et al.*, 2001, McAnulla *et al.*, 2001a, McAnulla *et al.*, 2001b, McDonald *et al.*, 2002, Amachi *et al.*, 2003, Schäfer *et al.*, 2007). The gene *cmuA* codes for a methyltransferase which carries out the first step of halomethane degradation, and was used as an indicator in this study (Woodall *et al.*, 2001, Schäfer *et al.*, 2005). Like the potential methyl halide producing organisms, this gene, suggestive of these specialized methylotrophs, was preferentially found on lower sections of the wall. Other possible methyl halide degraders such as *Hyphomicrobium* sp. (McAnulla *et al.*, 2001b) were mainly found in ceiling biofilms, but others e.g. *Rhodobacteraceae* (Miller *et al.*, 2004) were most abundant at the bottom of the walls, with a decreasing gradient toward the top (Figure 3- 14). A potential cooperation of *Erythrobacteraceae* and *Rhodobacteraceae* as indicated by the co-occurrence network graph (Figure 3- 16) should be further elucidated.
So far, the *cmuA*-encoded enzyme system has only been linked to the degradation of gaseous methyl chloride and methyl bromide but not of methyl iodide. It has been argued that this degradation pathway is primarily linked to energy gain rather than to carbon assimilation or a detoxification reaction (Cox *et al.*, 2012, Halsey *et al.*, 2012). These findings further support the newly proposed perspective of methane and iodomethane as an energy source but not a carbon source in biofilms. The presence of such a highly specific pathway could provide a selective advantage on a small scale, given the close proximity of the formation of methyl halides e.g. co-localized methyl halide producers and degraders in the biofilm matrix.

4.2.3.2.2 Iodide (I^-) oxidation and accumulation

The oxidation of iodide to iodine (I_2), or to hypoiodous acid (HIO) by haloperoxidases, has been observed in brown algae and bacteria (Küpper *et al.*, 1998, Amachi *et al.*, 2007a). Iodine is then intracellularly stored as iodide or and might act as an antioxidant (Küpper *et al.*, 2008). Iodide accumulation in bacteria was first shown in microbial communities of marine sediments (Amachi *et al.*, 2005a) where *Flavobacteraceae* showed relatively high uptake. Previous investigations examining aquifer communities and groundwater conditions have shown lower uptake rates but additional taxa (within the *Betaproteobacteria* - closely related to *Ralstonia* spp., the *Firmicutes* - closely related to *Bacillus* spp., the *Actinobacteria* – closely related to *Streptomyces* spp. and *Bacteriodetes*) are also capable of iodide accumulation (Li *et al.*, 2011). The maximum iodide

concentration in bacterial cells was reported to be 30 μg g^{-1} cells (Amachi *et al.*, 2005a), which is comparably low to what was found in the cave biofilms where the total iodine was estimated to be 480 ± 180 μg g^{-1} dry biomass. These relatively high values of iodine in the biofilms (Table 3-8) were not significantly (R^2 = 0.39, p=0.07) related to the total carbon content, although such a link has been established previously in surface sediments (Malcolm & Price, 1984).
Iodide oxidation is an energetically favorable process but so far there is no evidence that bacteria can actually capitalize on this reaction:

$$4\,I^- + O_2 + 4\,H^+ \rightarrow 2\,I_2 + 2\,H_2O \qquad \Delta G^{\circ\prime}\ \text{-56 kJ/reaction}$$

The oxidation of iodide has been examined in *Alphaproteobacteria* related to *Roseovarius* spp., *Rhodothalassium* spp. and *Kordiimonas* spp. (Amachi *et al.*, 2005b, Arakawa *et al.*, 2012, Wakai *et al.*, 2014). The responsible peroxidases were found only extracellularly, making energy conservation impossible. The microorganisms were also able to generate organic iodine species (CH_2I_2, CH_2ClI). Iodine (I_2) from iodide oxidizing bacteria could act as an inhibitor towards other species, providing an advantage over iodine-sensitive taxa (Zhao *et al.*, 2013). Elemental iodine and hypoiodous acid are biocidals (Koch, 1881, Chang & Morris, 1953, Chang, 1958, Brion & Silverstein, 1999), exerting their effect through their chemical property as strong oxidants. In comparison to chlorine, its disinfection ability is neither decisively pH–related nor does the presence of organic or inorganic nitrogenous substances interfere (Chang & Morris, 1953). Bacteria appear more sensitive to iodine compared to viruses (Brion & Silverstein, 1999) and protozoa (Chang, 1958). Exposure to 0.2 ppm iodine for 10 minutes was shown to be sufficient to kill enteric bacteria (Gottardi, 2001). Nevertheless, the iodine resistance of bacteria e.g. *Pseudomonas alclaigenes* is well established (Favero & Drake, 1966).
At present, the high abundance of unclassified alphaproteobacterial sequences found in the cave cannot be directly linked to iodide oxidation. However, ongoing enrichments with added iodide and also the detection of further gene markers (Arakawa *et al.*, 2012) might identify novel iodide-oxidizing bacteria.

4.2.3.2.3 Iodate (IO_3^-) reduction

Iodate is expected to be stable under oxidized conditions (Figure 4- 3). Iodate is known to preferentially sorb to surfaces compared to iodide. The oxidation of compounds using iodate as an electron acceptor has been demonstrated for marine phytoplankton and bacteria (Councell *et al.*, 1997, Farrenkopf *et al.*, 1997, Amachi *et al.*, 2007b, Chance *et al.*, 2007). The reduction of iodate to iodide might also be catalyzed by nitrate reducing bacteria (Tsunogai & Sase, 1969).

Sulfur- and iron reducing bacteria (*Desulfovibrio desulfuricans* and *Shewanella oneidensis*) were reported to reduce iodate anaerobically in experimental setups, but mechanisms remained unclear (Councell *et al.*, 1997, Farrenkopf *et al.*, 1997). Dissimilatory iodate reduction has indeed been demonstrated for *Pseudomonas* sp. strain SCT growing on iodate as the sole electron acceptor(Amachi *et al.*, 2007b). *Pseudomonas* sp. strain SCT is most closely related to denitrifying bacteria *P. stutzeri* (Lehmann & Neumann, 1896) and chlorate-reducing *P. chloritidismutans* (Wolterink *et al.*, 2002) but does not grow on chlorate. The *Pseudomonadaceae* found in the Sulzbrunn biofilms were phylogenetically distant from these two. Still, a potential oxidation of methane under anaerobic conditions using iodate as an electron acceptor would be thermodynamically feasible.

$$3\,CH_4 + 4\,IO_3^- \rightarrow 3\,CO_2 + I^- + 6\,H_2O \qquad \Delta G^{\circ\prime} \text{ -1022 kJ/mol } CH_4$$

Representatives of anaerobic nitrate and nitrite-driven methane oxidation (*Methylomirabilis oxyfera*) were also present in small numbers in the samples. It is not known if they could grow on iodate but they do contain an iodide peroxidase, which allows for the oxidation of iodide (Ettwig *et al.*, 2010). Iodine was measured as the total iodine in water and freeze-dried biofilm samples, therefore the available data does not allow for differentiation between different oxidation states. Iodine geochemistry is rather complex, as under relevant environmental conditions several physical and oxidation states exist (Kaplan *et al.*, 2013). Hydrolysis of iodine and iodide in water results in a multitude of possible compounds (Table 4- 1). Under conditions prevalent in aquifers, only the –I, 0, and +V valence states are common (Fuge & Johnson, 1986). Iodate is assumed to be stable under oxic conditions and was found to be the prevalent chemical form in oxic seawater (Tsunogai & Sase, 1969). On the contrary, iodide is the dominant form in anoxic seawater (Chapman, 1983) and marine sediments (Muramatsu *et al.*, 2007). Elemental iodine (I_2) is readily oxidized in alkaline solution to iodate (IO_3^-), while under acidic conditions, the oxidation of I_2 to IO_3^- requires a high redox potential (Figure 4- 3), making this process highly unlikely. In 1969, Tsunogai & Sase postulated that the reduction of iodate to iodide (E_0 + 0.67 V, pH 7) is more difficult than the reduction of chlorate to chloride (E_0 + 1.03 V, pH 7) but is thermodynamically more favorable than the reduction of nitrate to nitrite (E_0 + 0.43 V, pH 7). However, the referred standard redox potential for the IO_3^-/I^- couple is vastly different from what was reported by Amachi (2008) of +1.54 V. Nonetheless, the reduction of iodate was shown in several independent studies and should be included in further examinations of the Sulzbrunn cave system, especially regarding the oxidation of methane.

Table 4- 1 The main reactions of iodine and iodide hydrolization in water. Taken from Kaplan *et al.* (2013)

Chemical reaction	Equilibrium constant
$I_{2(g)} = I_{2(aq)}$	$\log K_{20\,°C} = 0.65$
$I_{2(aq)} + I^- = I_3^-$	$\log K_{20\,°C} = 2.89$
$I_{2(aq)} + H_2O = H^+ + I^- + HIO$	$\log K_{20\,°C} = -12.49$
$I_{2(aq)} + 2\,H_2O = 2H_2OI^+$	$\log K_{20\,°C} = -10.80$
$I^- + H^+ = HI$	$\log K_{20\,°C} = 0$
$2\,I^- = I_{2(aq)} + 2\,e^-$	$\log K_{20\,°C} = -21.33$
$I^- + H_2O = HIO_{(aq)} + H^+ + 2\,e^-$	$\log K_{20\,°C} = -33.81$
$I^- + 3\,H_2O = IO_3^- + 6\,H^+ + 6\,e^-$	$\log K_{20\,°C} = -113.31$

4.2.4 Biodiversity in Biofilms

The considerable diversity observed in cave biofilms and sediments was surprising and is likely to be due to the mixing of different source communities and diverse carbon and energy resources. The appearance of several bacterial taxa known to thrive in anoxic (*Syntrophobacteraceae)*, saline (*Sinobacteraceae*) or deep subsurface habitats as well as the presence of classic soil microbiota (*Verrucomicrobia*) reflected this situation. The influence of the cave's atmosphere composition and distribution of elements along vertical and horizontal gradients on the bacterial community was observed. Further analysis of microgradients within the EPS matrix could help to describe the realized niches for specific taxa. Niche partitioning was shown to shape microbial communities involved in the cycling of methane (Bodelier *et al.*, 2013). In the same study, the role of less abundant microbial constituents was stressed. The interactions within the biofilms between community taxa and their environment are certainly manifold (Little *et al.*, 2008). Different nutritional strategies (generalists, specialists), as well as organic and inorganic electron donors, are likely to ease competition for resources. The oxidation of methyl halides, which can presumably be utilized by most methylotrophs, but also allow for the emergence of a specific *cmuA* gene containing population would provide such an example. At the same time, exclusion could take place by chemical warfare via the exudation of iodine, which itself might enhance microbial diversity (Lenski & Riley, 2002). However, it is most likely that mutualistic and cooperative behavior sets the tone in these microenvironments. The production of massive EPS and putative steep gradients of substrates and metabolites (Stewart & Franklin, 2008, Ziegler *et al.*, 2009) within these structures certainly provide the basis for the observed diversity.

4.2.5 Using Network Analysis to Explore Co-Occurrence Patterns in Microbial Cave Communities

The described co-occurrence of bacterial taxa taken over all samples represents a first approach towards revealing key community members and possible interaction partners in biofilms. The relatively low sample size (n=14) certainly hinders the integration of community dynamics. As a next step, a greater data set including *Archaea* and eukaryotic community members is crucial. Nevertheless, the visual examination of the co-occurrence network certainly provided useful information on biofilm community assembly. The highly linked central part of the network dominated by putative methylotrophic taxa highlighted the importance of this lifestyle. The additional taxa split into typical soil microbes, as well as further potential methylotrophs dominated by alphaproteobacterial lineages. These primary insights describe a diverse bacterial network containing several interconnected methylotrophic taxa.

4.2.6 A Conceptual Model of the Sulzbrunn Cavern System

A conceptual model of the cavern system (Figure 4- 5) combining the main results and considerations was established. The three main compartments, consisting of the mixed cavern water, the wall and ceiling biofilms, were all shown to be bioreactive but distinctive in their elemental and taxonomic composition. High gene copy numbers in the outflow indicate the importance of the groundwater filled basin for biogeochemical processes compared to other cavern habitats. The distribution of bacterial populations along the wall gradient is indicative of a shift of physicochemical conditions. Key taxonomic groups were identified for each compartment and the influx of water and methane was quantified. Outgassing methane and putative methyl halides were used as energy and electron sources but not as the main carbon source. In the absence of limiting nutrients (nitrogen, phosphorous), the microbes produce EPS to shunt off electrons creating a unique habitat. The mixotrophic growth of biofilms observed here is unusal for methylotrophs that are classically considered as autotrophic.

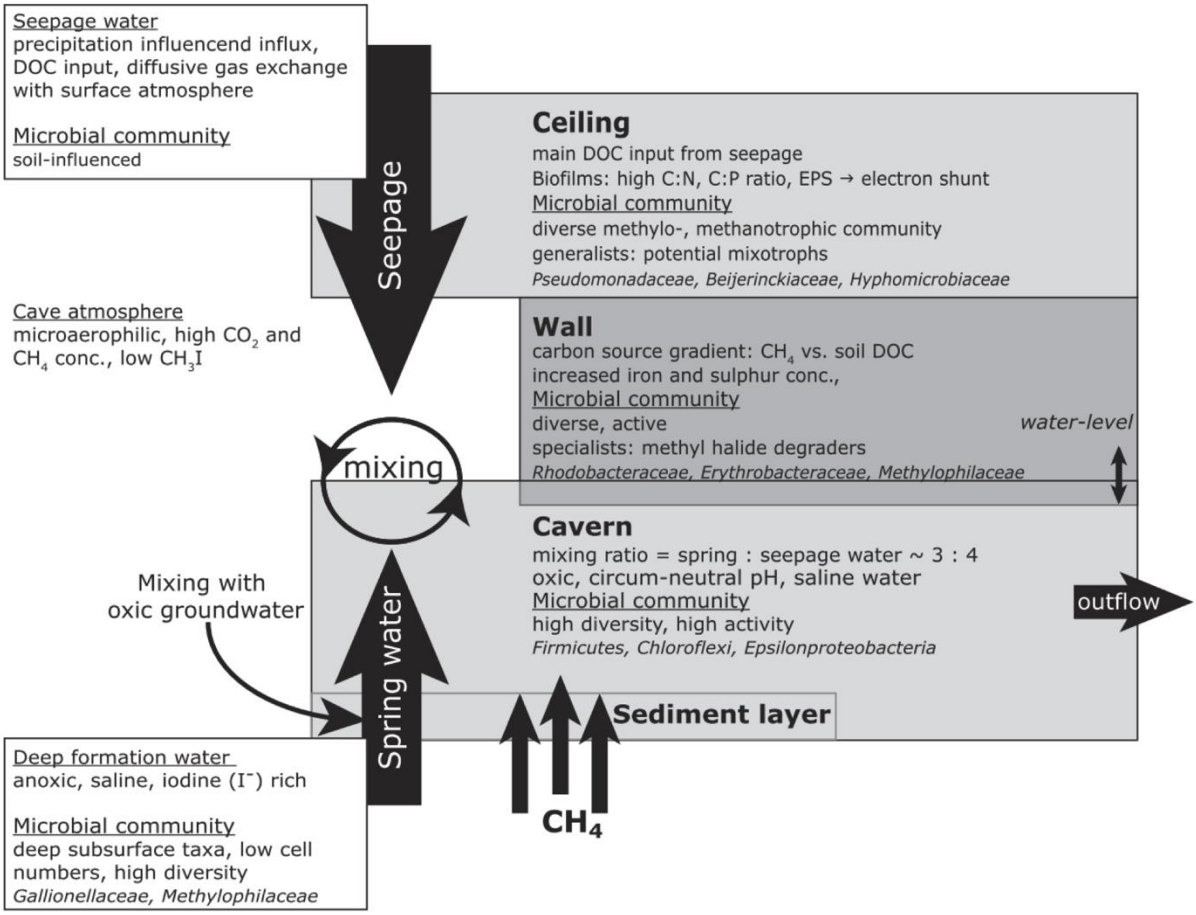


Figure 4- 5 Biofilm compartments, fluxes and populations within the cavern system

4.3 Drinking Water Biofilms

4.3.1 Well Populations and Variability

The last part of this thesis provides evidence for a distinct bacterial well community. A generally high level of diversity of bacterial communities in drinking water has been reported (Poitelon *et al.*, 2009, Lautenschlager *et al.*, 2013). The prevalence of *Proteobacteria* was not surprising in the investigated drinking water wells. *Proteobacteria*, and especially the *Alpha-*, *Beta-*, and *Gamma-*subclasses, have been identified as the predominant taxa in potable water and drinking water biofilms (Kalmbach *et al.*, 1997, Schmeisser *et al.*, 2003, Williams *et al.*, 2004). Members of the *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Planctomycetes*, as well as *Cyanobacteria* are also frequent constituents of these communities (Revetta *et al.*, 2010, Kahlisch *et al.*, 2012). Moreover, several unidentified candidate phyla (e.g. WS3 and TM7) were found in this study, confirming their general presence in potable water samples (Hwang *et al.*, 2012, Lautenschlager *et al.*, 2013). Microbial diversity is linked to a range of environmental and species interactions (Horner-Devine *et al.*, 2004). The paradox of finding high microbial diversity in oligotrophic systems has been reported but often related to disturbance regimes, which are negligible in groundwater ecosystems. Other drivers, such as bacteriophages or dispersal mechanisms, are more likely to play a crucial role in these habitats.

Several taxa representing relatively defined metabolic capacities were observed (e.g. *Nitrospira* spp.: nitrification, *Diaphorobacter* spp.: nitrification, denitrification (Khardenavis *et al.*, 2007), *Methyloversatilis sp*: methylotrophy). Ammonia oxidizing bacteria and nitrite oxidizing bacteria have been regularly observed in potable water (Lipponen *et al.*, 2002, Martiny *et al.*, 2005) and attributed to disinfection with chloramine. However, this treatment has never been applied to the drinking water wells investigated here, and points towards the influx of distinct nitrogen sources. Also, typical methylotrophic and methanotrophic taxa (e.g. *Methyloversatilis* spp., *Methylococcus* spp.) were present at low, but still sizable, read frequency (up to 5 %). Methane oxidizers are also capable of oxidizing ammonium and are often associated with denitrifying bacteria that can use simple carbon compounds released by the methanotrophs as substrates for denitrification reactions and for growth (Knowles, 2005)

Despite high hydraulic conductivity of the local aquifer, bacterial communities between wells differed in their diversity and structure. Potentially, this could have been related to the different usage routines and production intensities of the wells, as well as differences in sediment composition, even though water chemistry was very similar. While well 3 is in use throughout the

year, well 1 and well 2 are stagnant over several weeks and are then flushed to inhibit clogging. Constant flow might enhance the growth of more compact biofilms, while stagnant communities are more easily detached. Microbes in stagnant wells are likely to be more influenced by the well environment than the constantly used well which is continuously fed with transported microorganisms. Well clogging and its accompanied reduction of hydraulic conductivity has been attributed to the production of low solubility gases, precipitation and deposition of metals and $CaCO_3$, as well as the filtration of suspended particles (Ross *et al.*, 2001). Microorganisms, especially biofilms, play a crucial role in most of these processes.

Microbial biofilms and the production of extracellular polymeric substances (EPS) change the physicochemical properties of their local environment. Microbes in bulk water are more susceptible to the depletion of nutrients than biofilm residents (Boe-Hansen *et al.*, 2002). Taxa associated with considerable EPS production, such as *Arthrobacter* spp., *Cytophaga* spp., *Rhizobium* spp. and others, have been linked with bioclogging (Ross *et al.*, 2001). A noticeable number (4.3 %) of *Arthrobacter* spp. reads were found in well 2, and of *Cytophaga spp.* in the two other wells (2 % and 3.4 %, respectively). The influence of biofilms in the proximity to the wells on overall community structure and drinking water production can only be speculated upon. Observed differences in community composition between wells suggest that the sampled bulk water biota could consist of a mixture of 'background' aquifer microbes and dispersed site specific well communities. Site specific taxa could be identified by high variability in relative abundance between wells e.g. *Pseudomonas*, unclassified *Rhodospirillaceae*, *Legionella*, *Methyloversatilis*, *Acidovorax*. Taxa present in all wells in similar numbers are likely to be distributed by transportation or are common aquifer taxa, displaying a low impact in the principal component analysis e.g. *Gallionella.*

4.3.2 Restoration of Well 2 by Hydraulic Jetting

The ratio of potential well-specific biofilm bacteria in the effluent was expected to increase during physical removal via high pressure jetting. In the presented time series, several taxa were found at transiently increased abundance, suggesting their presence in the well vicinity. Strong fluctuations of taxa between different sampling time points indicate the high heterogeneity of communities in the well itself. Most notably, *Diaphorobacter* spp., *Nitrospira* spp., *Sphingobium* spp. and *Ralstonia* spp., were prevalently removed in the first 15 min. As they were less dominant in later time points (Figure 3- 16), these populations might be situated directly at the well–aquifer interface. At the third time point (45 min), the transient dominance of *Alkanindiges* populations were accompanied by *Janthinobacterium* spp. (Figure 3- 16), a typical soil bacterium known to form biofilms. *Janthinobacterium* spp. and *Ralstonia* spp. have both been previously reported for drinking

water systems (Schmeisser *et al.*, 2003, Ultee *et al.*, 2004, Kormas *et al.*, 2010). Both taxa are well known soil dwelling bacteria likely belonging to the constantly seeding community.
Although *Cyanobacteria* have also been repeatedly found in drinking water systems (Williams *et al.*, 2004, Kahlisch *et al.*, 2012), the appearance of cyanobacterial DNA at the end of the maintenance process was intriguing since there is no surface water body close by. After the sampling of drinking water with direct surface water influence, (Revetta *et al.*, 2010) argued that *Cyanobacteria* might survive in the dark. Several Bacillariophyta have been recognized as soil microorganisms that are prevalently found in recently unglaciated soils (Nemergut *et al.*, 2007). In another recent study, Hwang *et al.* (2012) also found high numbers of *Cyanobacteria* in chlorinated drinking water directly stemming from an aquifer. All of this taken together suggests that *Cyanobacteria* are able to survive (Kahlisch *et al.*, 2010) and spread in the subsurface, even when facing rather unsuitable conditions for their phototrophic lifestyle. Recently, the candidate phylum *Melainabacteria*, which appears to be closely related to *Cyanobacteria,* was found in aquifers living as obligate anaerobic fermenters (Di Rienzi *et al.*, 2013).
The cleaning procedure reduced bacterial diversity in drinking water considerably. It can be speculated that high pressure jetting actually reduced the diversity of microbial niches in the vicinity of the well previously established by microbial colonization, filtration and precipitation processes. Especially, the relative abundance of *Actinobacteria*–related reads decreased in each successive sample and was almost absent after two weeks. The dominance of *Betaproteobacteria* two weeks after cleaning could be a further indication for the reduction of biofilm bacteria, often belonging to the *Alpha-*, *Gamma-* and *Deltaproteobacteria* (Henne *et al.*, 2012). In contrast, the specific taxa (more abundant 2 weeks after cleaning) seem to represent the more mobile fraction of the aquifer microbes, amongst them 'typical' drinking water representatives such as *Rhodocyclus* spp., *Sphingobium* spp. or *Polaromonas* spp. (Loy *et al.*, 2005, Kämpfer *et al.*, 2006).
Lineages harboring potential pathogens of drinking water concern (i.e. *Legionellaceae*, *Pseudomonadaceae*, *Acinteobacter* spp.) reacted distinctly to hydraulic jetting. As mentioned above, the read abundance of *Pseudomonas* spp. decreased steadily during well restoration, and was almost absent after 2 weeks. This suggests that they were more a component of the attached microbiota in the well vicinity than in the aquifer itself. In this respect, a positive effect of hydraulic jetting on microbiological drinking water quality can be inferred. Still, given the ubiquity and versatility of *Pseudomonas* and also *Acinetobacter* in aquatic environments, conclusions on the impact of this purging on any hygienic parameters are not possible. In contrast, reads of the *Legionellaceae* were identified in all samples, but at decreased abundance during the actual purging event. This emphasizes the omnipresence of these taxa in oligotrophic drinking water

systems (Wullings *et al.*, 2011) but opposes their establishment in biofilms in the vicinity of the well. Also, the appearance of reads related to *Chryseobacterium* spp. (Kim *et al.*, 2008) during cleaning indicates its presence in the outer well sediments.

From an ecological perspective, the cleaning procedure has to be seen as a disturbance of the well ecosystem. The dynamic equilibrium model (Huston, 1979) predicts that in low productive environments where species have slow growth rates, infrequent disturbances are enough to promote invasion of the system. Thus, disturbances can permanently alter communities by decreasing slow growing species that are often excellent competitors (Mata *et al.*, 2013). Phylogenetically more diverse communities are less susceptible to invasion, which is linked to the more efficient use of resources by dissimilar communities (Jousset *et al.*, 2011).

The well microbiome presents a seed bank (Leibold *et al.*, 2004) dispersing cells to subsequent drinking water supply systems all the way to the tap. This implies that drinking water community characteristics are influenced by the size of the seeding community and the diversity of taxa therein, the spatial structure of the community and the rate of dispersal (Curtis & Sloan, 2004). After a disturbance, recolonization of such heterogeneous and oligotrophic habitats is difficult to predict. While 'niche-assembled communities' would predict the coexistence of species because of microbial niche differentiation, 'dispersal-assembled communities' are determined by the ability to disperse, settle and persist independently of coexisting microbes. Neutral theory (Hubbell, 2001) suggests that different species of a community are able to coexist because they reproduce, die, disperse or evolve with the same probability (Gilyarov, 2011). Resident taxa within aquifers are adapted to low substrate concentrations and are very likely to follow this assumption. Functionally similar but phylogenetically differing groups are found in all three wells. Several taxa which were found are frequently detected in drinking water and are therefore likely to be adapted to low nutrient conditions. Microbes are constantly passing through the well environment, many of which are organisms associated with soil, being potential colonizers of a habitat which shows distinct patterns in distribution as revealed by successive sampling of the maintenance process. Finding substantial differences between the purged samples indicates a heterogeneous distribution within biofilms throughout the surrounding well filter and the impact of high pressure jetting. Despite low nutrient conditions, microbial diversity was substantial and metabolic versatility can be inferred from taxonomic information. In synthesis, distinct well communities were found despite similar water chemistry. Also, high-pressure jetting proved effective in considerably reducing the microbial diversity.

5 Conclusions and Outlook

Microbial biofilms in groundwater ecosystems control several important processes and ecosystem services. The studies conducted here exhibit considerable novelty in biofilm communities in aquifers, showing that they can be found over a large span of physiologically, trophically and ecologically distinct systems.

The discussion of whether biofilms even exist in groundwater (Taylor & Jaffé, 1990, Baveye *et al.*, 1992) depends on the investigated system and the available energy, as well as on the definition of microbial biofilms. In this thesis, a biofilm is considered as a functionally defined consortium of microbial cells attached to a surface in an organized manner. Although the role of biofilms in the environment has been studied over the past 40 years, energy and nutrient limited aquatic systems have been more or less neglected. Thus this thesis contributes to the recently emerging paradigm shift in biofilm research from the 'classical' multilayer, monospecies biofilms towards more environmentally relevant, monolayer and multispecies systems (Karatan & Watnick, 2009). The situation in pristine aquifers where microbial cells need to budget their energy in order to survive calls for such a change in paradigm, and the production of extracellular polymers or signal molecules appears inefficient.

Whereas the interaction of microbial cells is emphasized in many studies of biofilms, cell-surface interactions are often largely ignored. The importance of the geological media to which microbes attach is highlighted in the first experiment described in this thesis. Biofilms were examined in an experimental mesocosm system, which allowed for a controlled and reproducible colonization of selected mineral coupons. The dissolution of minerals and the leaching of nutrients could both be potential drivers of biofilm formation. However, in contrast to our initial expectations, the experiment did not provide evidence for an alleviation of nutrient limitations by reactive mineral surfaces. Thus, this idea must be reconsidered for pristine aquifers, at least for the given hydrogeochemical setting. Still, the observation of discrete microbial assemblages on most exposed surfaces indicated a clear benefit of species interactions for attached microbes, even under growth-limiting conditions. Most surprisingly, the only biofilm community that was different from all of the other attached microbial biomass was found on the only surface which provided a potential electron donor. Although typical sulphide- and ferrous iron-oxidizing populations were not identified in the respective biofilms, the influence of increased energy availability was more than apparent. This indicates that the strict electron donor limitation in pristine aquifers has the potential to override all other potential benefits of attached growth, as well as that the capacity for lithotrophic electron donor use is potentially far more widespread

than currently perceived. In the future, this work should be extended towards combinatory effects of growth limitation relief, potentially even by considering resources provided by the aquifer matrix and the mobile water phase at the same time. This will greatly advance our understanding of the role of attached growth in oligotrophic groundwater systems.

In the second part of this thesis a unique cave-biofilm system is described, which most likely is nutrient limited rather than energy limited. The mixing of geogenic methane and iodine inputs with biosphere seepage water allowed for an extraordinary growth of previously undescribed biofilms harbouring an unexpectedly diverse array of microbiota. The distribution of putative methylotrophic and methanotrophic taxa was characteristically linked to different patterns of carbon and nitrogen usage in biofilms. The relevance of these biofilms for putative methyl halide cycling is of considerable biochemical and ecological relevance. Also, the co-occurrence of taxa known to utilize a large range of substrates (generalists) and taxa realizing a highly specialised metabolism can be ideally studied in this complex cave system. The transport of substrates and metabolites, as well as the potential efflux of bactericidal iodine, can only be understood within a perspective of cooperation in a microbial network. The elucidation of these processes and respective key microbiota is subject to ongoing work, in which I will embark in a PostDoc project after completion of this PhD thesis.

The third study in this thesis investigated the importance of microbial biofilms in drinking water wells, one of the most critical groundwater habitats for human health. The management of drinking water facilities necessitates a clear understanding of the microbial community in the proximity of production wells. Distinct microbial well communities were characterized, which provided a reference status and allowed for the monitoring and evaluation of the impact of maintenance procedures. I show that within the proximate well area, microbes actually realize several niches for their attachment and dispersal. The subsequent in-depth analysis of specific taxa allowed for the identification of bacteria susceptible to high-pressure jetting, an opportunity never realized before for an active drinking water system. This, in turn, also allows for the identification of taxa which are resistant to this procedure, provides a seeding capacity for downstream microbial communities, and links such events to water quality and risk assessment.

In conclusion, this thesis covers an exceptional range of microbial biofilms in subsurface ecosystems. Their role in the turnover of organic and inorganic substrates, as well as a potential refuge for drinking water pathogens, is dissected for both energy-limited as well as nutrient-limited systems. These insights substantiate the largely neglected relevance of biofilms in groundwater ecosystems, which is an advance in our perspective of the functional diversity and biogeochemical fluxes in our societies' most important drinking water resource.

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Publications and Authorship Clarifications

Accepted and submitted

1. **Karwautz C** and Lueders T (2014) Impact of hydraulic well restoration on native bacterial communities in drinking water wells Microbes Environ. 2014;29(4):363-9. doi: 10.1264/jsme2.ME14035. Epub 2014 Oct 2.

Pending manuscripts

2. **Karwautz C**, Stöckl M, Kus G, Lueders, T. Massive methane-fueled microbial biofilms in an iodine-rich spring cavern. In preparation for *ISME J*.
3. **Karwautz C** and Lueders T. Mineral surfaces as controlling factor for attached growth and ecophysiology of biofilms in an oligotrophic aquifer. In preparation for *Geomicrobiol.*

ad 1) The accepted publication is based on the examination of biofilms in drinking water wells specified in the third hypothesis of the thesis. Tillmann Lueders planned the field study together with the author. The `Wasserverband Baldham` represented by Dr. Claus Ortner and Karl Seebauer provided access to all samples. The author took samples in cooperation with Katrin Hörmann, technical staff at Institut für Grundwasserökologie (IGÖ). Water chemical parameters were evaluated under guidance of Dr. Heike Brielmann (at that time PostDoc at the IGÖ) and Michael Stöckl (technical staff at the IGÖ). Marion Engel at the Research Unit Environmental Genomics (HMGU) was responsible for Pyrotag sequencing. The author did all data analysis and multivariate statistics. Graphics of the sampling site and the geological well profile were modified from the booklet `Wasserbeschaffungsverband Baldham: 1929 -1999 Dokumentation und Information. The hydrogeological map was taken from the Landesamt für Digitalisierung, Breitband und Vermessung (http://geoportal.bayern.de/bayernatlas). The author developed the manuscript draft. Tillmann Lueders revised and edited the manuscript.

ad 2) The field study was planned by the author and Tillmann Lueders. Franz Hösle (local cave attendant) provided access to the cavern and helped to prepare the sampling campaigns. In the first sampling campaign, Tillmann Lueders, Michael Stöckl and Franz Hösle took biofilm and water samples. The author and Michael Stöckl took further gas and water samples. Dr. Günter Kus (Landesamt für Umwelt) invited our team to investigate the cave and provided water chemical parameters recorded from 2011 to 2012. The author completed quantitative gas measurements. The author carried out compound-specific isotope analysis of the gas under guidance of Dr. Armin Meyer (PostDoc) and Michael Maier (PhD student) of the IGÖ Stable

Isotope Group. Dr. Bernhard Michalke of the Research Unit Analytical BioGeoChemistry (HMGU) was responsible for the elemental analysis of lyophilized biofilm samples. Harald Lowag (technical staff of the Stable Isotope Group, IGÖ) analyzed the carbon and nitrogen isotope composition of the biofilms. Michael Stöckl (IGÖ) acquired water chemical parameters describing the ion composition and dissolved organic carbon concentration of the cavern water. Petra Seibel (technical staff of the Hydrogeology group, IGÖ) conducted the water isotope measurements. The author was responsible for all molecular work, assisted by Katrin Hörmann in the sequencing workflow. The author handled the sequence data. The author cut biofilm cryosections under supervision of Elonore Samson (technical staff, Institute of Pathology, HMGU). Michael Rothballer (Abteilung Mikroben-Pflanzen Interaktion) provided know-how and several probes for staining and fluorescence in situ hybridization of samples, which was done by the author. Under the guidance of Nina Weber (Microbial Ecology Group, IGÖ) the author conducted cell number quantification via flow cytometry. The author performed the data analysis, multivariate statistics and graphical representation of the results. The author and Tillmann Lueders currently write a manuscript based on the massive methane-oxidizing cave biofilms.

ad 3) The experiment was planned by the author. The author designed the mesocosm box and Dr. Marko Hünniger (IGÖ) drew a dimensional sketch. Minerals were acquired from Wards Scientific, while coupons were cut and finished by Franziska Häuser, technical staff at the Geology Dept., LMU München. Evaluation of hydrological properties (porosity, flow velocity, tracer experiment) within the box was conducted by Marko Hünniger and the author. Water chemical parameters (ion concentration, dissolved organic carbon concentration) were measured by the author with help of Michael Stöckl. Molecular work was done by the author with help of Katrin Hörmann in pyrosequencing. The author performed the carbon utilization assay. Dr. Marianne Hanzlik at the TUM Garching, Fachgebeiet Elektronenmikroskopie, accomplished preparation and visualization of mineral surfaces for SEM. The author performed data analysis and multivariate statistics. The manuscript draft was prepared by the author and revised by Tillmann Lueders.

Abbreviations

^{12}C	stable carbon isotope with standard atomic weight of 12
16S rRNA	ribosomal RNA, small subunit (bacteria)
bp	base pairs
cmuA	corrinoid-binding / methyltransferase encoding gene
CSIA	compound specific stable isotope analysis
ddH2O	double-distilled water
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide
DOC	dissolved organic carbon
e.g.	exempli gratia
EA	Elemental analyzer
EC	electrolytic Conductivity, SI: siemens per meter [S/m]
EDTA	Ethylenediaminetetraacetic acid
EM	Electron microscopy
EPS	extracellular polymeric substances
et al.	et alii
FAM	Carboxyfluorescein
FISH	Fluorescence in situ hybridisation
H'	Shannon-Weaver diversity index
HMGU	Helmholtz Zentrum München Deutsches Forschungszentrum für Gesundheit und Umwelt
i.e.	id est
IAEA	International Atomic Energy Agency
ICP-MS	Inductively coupled plasma mass spectrometry
IGOE / IGÖ	Institute for Groundwater Ecology / Institut für Grundwasserökologie
IRMS-GC	Isotope-ratio mass spectrometry gas chromotography
IUPAC	International Union of Pure and Applied Chemistry
LfU	Landesamt für Umwelt
mxaF	methanol dehydrogenase encoding gene
MspI	restriction enzyme of *Moraxella* sp.
OTU	organizational taxonomic unit
PBS	Phosphate buffered saline
PC1	first principal component
PC2	second principal component
PCA	principal component analysis
PCR	polymerase chain reaction
PEG	polyethylene glycol
pmoA	particulate methane monooxygenase encoding gene
PIE	probability of interspecific encounter
PPiase	peptidylprolyl isomerise
qPCR	quantitative (=real time) polymerase chain reaction
RDP	Ribosomal Databse Project
spp.	species (plural)
TaqI	restriction enzyme of *Thermus aquaticus*
TIC	triphenyl tetrazolium chloride
TMAH	tetramethyl ammonium hydroxide
TOC	total organic carbon
T-RFLP	terminal restriction fragment length polymorphism
U	unit of enzyme activity [µmol min^{-1}]
w/v	weight/volume

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