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Fundamental principles of microbial community assembly in groundwater and the application of microbiological data for ecological groundwater monitoring

Lucas Fillinger

in memoriam
Wilfred F. M. Röling
(December 9, 1966 – September 25, 2015)

Contents

Summary

Groundwater aquifers are unique but so far little explored ecosystems that are characterized by environmental conditions that distinguish them from most other non-subsurface habitats, such as the lack of light, low nutrient concentrations, low temperatures, and little variation in environmental conditions over time. Microbial communities lie at the heart of key biogeochemical processes in these ecosystems like the turnover of carbon and other nutrients, mineral cycling, or pollutant degradation. Due to its vast size, the groundwater-saturated zones of the terrestrial subsurface are the largest freshwater ecosystem on Earth, and accommodate a large share of the global microbial biomass. In addition, groundwater is the most abundant source of liquid freshwater worldwide. The quality of this vital resource in large part hinges on the activity of groundwater organisms and microbial communities in particular. Despite the relevance of groundwater ecosystems for society, and the importance of microbial communities in these ecosystems, the processes that shape microbial community composition in groundwater environments are not well understood. Moreover, groundwater has historically been regarded mainly as a resource rather than an ecosystem that deserves to be protected. Even though this view has started to change—leading to the recognition of the ecosystem status of groundwater in environmental policies in different parts of the world—ecological criteria are still widely ignored in routine groundwater monitoring in practice. The reason for this mainly lies in the lack of practically applicable ecological assessment tools suitable for groundwater ecosystems. This thesis aims at shedding light on the processes that drive microbial community assembly in groundwater aquifers, and introduces an approach that enables the incorporation of biological-ecological criteria based on microbiological data into groundwater monitoring schemes.

In the first part of this thesis, I applied theoretical frameworks from community ecology to investigate the impact of processes related to species sorting, dispersal, and drift on microbial community composition across geographically distinct aquifers. The factors that drive species sorting in these habitats were distinguished between selection due to local environmental conditions, and possible selection effects resulting from broad-scale region-specific factors, which may represent impacts of climate, geology, or historical events. While several studies have analyzed biogeographic distribution patterns of microbial communities across broad spatial scales, it has often remained unclear to what extent differences in community composition across different regions are caused by dispersal limitation or species sorting, and if species sorting is caused mainly by local

environmental conditions alone or additional broad-scale region-specific factors. This is especially true for microbial communities in groundwater environments, which have been understudied in this context relative to other non-subsurface habitats so far. To tackle these questions, we analyzed microbial community composition based on exact 16S rRNA amplicon sequence variants (ASVs) from four geographically separated aquifers located in different regions along a latitudinal transect of ∼700 km across Germany. Using a combination of variation partitioning and ecological null models revealed that differences in microbial community composition were mainly the product of species sorting imposed by local environmental conditions, and to a smaller but still significant extent dispersal limitation and drift across regions. Only ∼23 % of the total variation in microbial community composition remained unexplained, possibly due to underestimated effects of dispersal limitation among local communities within regions and temporal drift. However, no evidence was found for species sorting due to region-specific factors independent of local environmental conditions.

The second part of my thesis addresses the processes that drive microbial community assembly on groundwater sediments, as they typically account for the bulk of the microbial biomass and activity in groundwater environments, and thus are expected to play a particularly important part in these ecosystems. Although previous studies could show that the composition of sediment-attached communities can differ significantly from planktonic communities in the surrounding groundwater, the processes that give rise to these differences are not well understood. In order to unravel these processes, we followed the microbial colonization of initially sterile sediments in *in situ* microcosms that were exposed to groundwater for almost one year at two distant but hydrologically connected sites of a shallow porous aquifer. Our results revealed intriguing similarities between the microbial community succession on the newly-colonized sediments and succession patterns previously observed for the assembly of biofilms in other more dynamic aquatic environments, indicating that the assembly of microbial communities on surfaces may be governed by similar underlying mechanisms across a wide range of different habitats. Null model simulations on spatiotemporally resolved 16S rRNA amplicon sequencing data further indicated selection of specific operational taxonomic units (OTUs) rather than random colonization as the main driver of community assembly. A small fraction of persistent OTUs that had established on the sediments during the first 115 days dominated the final communities, with combined relative abundances of 68 % to 85 %, suggesting a key role of these early-colonizing organisms for community assembly and succession during the colonization of the sediments. Overall, the results of this study suggest that differences between sediment-attached and planktonic communities in groundwater en-

vironments are not the result of purely stochastic events, but that sediment surfaces select for specific groups of microorganisms that assemble over time in a reproducible, non-random way.

In the third part, I introduce a simple, inexpensive approach that enables ecological groundwater monitoring based on three microbiological parameters that can be easily integrated into existing routine monitoring protocols: prokaryotic cell density (D) measured by flow cytometry; microbial activity (A) measured as prokaryotic intracellular ATP concentrations using a simple cell lysis-luminescence assay; and, as an optional parameter, the bioavailable carbon (C) measured as the concentration of assimilable organic carbon in a simple batch growth assay. To explore the potential of this approach, we analyzed data for three case studies of different disturbances representing some of the main threats to groundwater ecosystems, that is organic contamination with hydrocarbons, surface water intrusion, and agricultural land use. For all three cases, disturbed samples could be reliably distinguished from undisturbed samples based on a single index value obtained from multivariate outlier analyses of the microbial variables (i.e. robust Mahalanobis distances). We could show that this multivariate data analysis approach allowed for a significantly more sensitive and reliable detection of disturbed samples compared to separate univariate outlier analyses of the measured variables. Furthermore, a comparison of non-contaminated aquifers from nine different regions across Germany revealed distinct multivariate signatures along the three microbial variables, which should be considered when applying our approach in practice by analyzing data on a suitable regional scale. In essence, our approach offers a practical tool for the detection of disturbances of groundwater ecosystems based on easy-to-analyze microbial parameters, which can be seamlessly extended in the future by additional parameters to further increase the sensitivity as well as flexibility of the analysis.

All in all, this thesis contributes to a better understanding of the fundamental ecology of microbial communities in groundwater environments, and additionally presents application-oriented research that led to the development of a practical approach that enables the implementation of ecological criteria in routine groundwater monitoring, which will allow for a more informed and sustainable management of vital groundwater ecosystems.

Zusammenfassung

Grundwasser ist ein einzigartiges, jedoch bislang wenig erforschtes Ökosystem, das sich durch Umweltbedingungen auszeichnet, die sich deutlich von anderen, oberirdischen Lebensräumen unterscheiden. Hierzu gehören das Fehlen von Licht, niedrige Nährstoffkonzentrationen und Temperaturen sowie geringe zeitliche Schwankungen der abiotischen Bedingungen. Mikrobielle Gemeinschaften bilden die Basis wichtiger biogeochemischer Prozesse in diesen Ökosystemen, wie beispielsweise Nährstoffkreisläufe oder Schadstoffabbau. Auf Grund ihrer ausgedehnten Ausmaße ist die grundwassergesättigte Zone der größte aquatische, limnische Lebensraum der Erde und beherrbergt einen Großteil der globalen mikrobiellen Biomasse. Des Weiteren ist Grundwasser die weltweit größte Quelle flüssigen Süßwassers. Die Aktivität von Grundwasserorganismen, und mikrobiellen Gemeinschaften im Speziellen, ist ausschlaggebend für die Qualität dieser lebenswichtigen Ressource. Trotz der hohen Relevanz von Grundwasserökosystemen und der darin lebenden mikrobiellen Gemeinschaften, gibt es derzeit noch wenig wissenschaftliche Erkenntnisse zu den Mechanismen, welche die Zusammensetzung dieser Gemeinschaften im Grundwasser bestimmen. Auf gesellschaftlich-politischer Ebene wurde Grundwasser über lange Zeit hauptsächlich als Ressource und nicht als schützenwertes Ökosystem wahrgenommen. Zwar ist diesbezüglich seit einigen Jahren ein Umdenken zu verzeichnen, das in einzelnen Ländern bereits zur gesetzlichen Anerkennung von Grundwasser als Ökosystem in Umweltrichtlinien geführt hat, dennoch werden ökologische Kriterien derzeit kaum in der routinemäßigen Überwachung von Grundwasser berücksichtigt, da es bisher an geeigneten Methoden für deren Erfassung mangelt. In dieser Dissertation werden die Prozesse, welche die Zusammensetzung mikrobieller Gemeinschaften im Grundwasser beeinflussen, beleuchtet. Zusätzlich wird ein Ansatz vorgestellt, der es ermöglicht, biologisch-ökologische Kriterien in die Routineüberwachung von Grundwasser zu integrieren.

Im ersten Teil dieser Arbeit wurden, unter Berücksichtigung theoretischer Ansätze aus der Gemeinschaftsökologie, die Einflüsse von Selektion (*species sorting*), Dispersion und stochastischem Drift auf die Zusammensetzung mikrobieller Gemeinschaften in verschiedenen, geografisch voneinander abgegrenzten, Grundwasserleitern untersucht. Die Faktoren, von denen Selektion ausgeht, wurden unterschieden in von lokalen Umweltbedingungen bestimmte Selektionsfaktoren und solchen, die sich möglicherweise aus übergeordneten, regional-spezifischen Faktoren ergeben, wie etwa Klima, Geologie, oder zeitlich zurückliegende Ereignisse. Biogeografische Verteilungsmuster mikrobieller Gemeinschaf-

ten über größere räumlich Distanzen waren bereits Gegenstand einiger vorangegangener Studien. Allerdings ist bislang weitestgehend unklar, in wie weit Unterschiede zwischen Gemeinschaften in unterschiedlichen Regionen durch Selektion bedingt, oder das Ergebnis von verminderter Dispersion sind und ob Selektion hauptsächlich von lokalen Umweltbedingungen bestimmt wird, oder zusätzlich von übergeordneten regional-spezifischen Faktoren ausgeht. Dies gilt besonders für mikrobielle Gemeinschaften im Grundwasser, die im Vergleich zu Gemeinschaften in anderen, oberirdischen Habitaten diesbezüglich noch weitestgehend unerforscht sind. Um Antworten auf diese offenen Fragen zu liefern, haben wir die Zusammensetzungen mikrobieller Gemeinschaften auf Basis exakter 16S rRNA Amplicon Sequence Variants (ASVs) zwischen vier geografisch voneinander abgetrennten Grundwasserleitern verglichen, die in unterschiedlichen Regionen entlang eines ∼700 km langen Nord-Süd-Transsekts durch Deutschland beprobt wurden. Mittels der Kombination von Varianzpartitionierung und ökologischen Nullmodellanalysen konnten wir zeigen, dass die Unterschiede zwischen Gemeinschaften aus verschiedenen Regionen hauptsächlich auf Selektion durch lokale Umweltbedingungen zurückzuführen waren, wobei verminderte Dispersion zwischen Regionen und Drift eine untergeordnete, aber dennoch signifikante Rolle spielten. Lediglich ∼23 % der gesamten Varianz in der Zusammensetzung der mikrobiellen Gemeinschaften konnte nicht zugeordnet werden, womöglich durch nicht erfasste Effekte von verminderter Dispersion zwischen lokalen Gemeinschaften innerhalb der einzelnen Regionen. Es wurden jedoch keine Anhaltspunkte gefunden, die auf Selektion durch übergeordnete, regional-spezifische, von lokalen Umweltbedingungen unabhängige Faktoren hindeuten.

Der zweite Teil meiner Dissertation beleuchtet die Prozesse, die für die Zusammensetzung mikrobieller Gemeinschaften auf Grundwassersedimenten verantwortlich sind. Diese Gemeinschaften machen in der Regel den Großteil der mikrobiellen Biomasse und Aktivität im Grundwasser aus, weshalb ihnen eine besondere Rolle innerhalb dieser Ökosysteme zugesprochen wird. In der Vergangenheit konnte bereits in mehreren Studien gezeigt werden, dass sich die Zusammensetzung der Gemeinschaften auf Sedimenten deutlich von der Zusammensetzung planktonischer Gemeinschaften im umgebenden Grundwasser unterscheiden kann. Die Ursache dieser Unterschiede ist jedoch bislang nicht genau bekannt. Um diese Ursachen näher zu ergründen, haben wir die Besiedelung von anfangs sterilen Sedimenten in *in situ* Mikrokosmen verfolgt, die über einen Zeitraum von fast einem Jahr an zwei räumlich getrennten aber dennoch hydrologisch miteinander verbundenen Standorten eines Grundwasserleiters inkubiert wurden. Unsere Ergebnisse haben bemerkenswerte Ähnlichkeiten zwischen dem Ablauf der Besiedelung der inkubierten Sedimente und in der Vergangenheit beobachteten Mustern bei der Bildung von Biofilmen in ande-

ren, dynamischeren aquatischen Öksystemen aufgezeigt. Dies deutet darauf hin, dass die Besiedelung von Oberflächen durch mikrobielle Gemeinschaften in unterschiedlichen Habitaten von ähnlichen grundlegenden Mechanismen bestimmt wird. Nullmodellanalysen auf Basis von räumlich und zeitlich aufgelösten 16S rRNA Gensequenzdaten deuteten an, dass die Besiedelung der Sedimente maßgeblich durch Selektion spezifischer Operational Taxonomic Units (OTUs) bestimmt wurde anstatt durch stochastische Besiedelungsprozesse. Am Ende der Inkubation wurden die Gemeinschaften von einem kleinen Anteil beständiger OTUs dominiert, die sich im Laufe der ersten 115 Tage auf den Sedimenten angesiedelt hatten und am Ende zusammen eine relative Abundanz von 68 % bis 85 % in den Gemeinschaften aufwiesen, was auf eine besondere Rolle dieser Organismen für die Besiedelung der Sedimente schließen lässt. Schlussfolgernd lässt sich aus den Ergebnissen dieser Studie ableiten, dass Unterschiede zwischen mikrobiellen Gemeinschaften auf Sedimenten und planktonischen Gemeinschaften in Grundwassersystemen wahrscheinlich nicht das Ergebnis von hauptsächlich stochastischen Besiedlungsprozessen sind, sondern dass Sedimente spezifische Gruppen von Mikroorganismen selektieren, die sich reproduzierbar und auf nicht zufällige Art und Weise auf den Sedimenten ansiedeln.

Im dritten Teil dieser Arbeit wird ein einfacher, kostengünstiger Ansatz zur ökologischen Bewertung von Grundwasser vorgestellt, der sich problemlos in bereits bestehende Protokolle zur Grundwasserüberwachung integrieren lässt. Dieser Ansatz stützt sich auf drei mikrobiologische Parameter: die mittels Durchflusszytometrie ermittelte prokaryotische Gesamtzellzahl (*cell density, D*); die mikrobielle Aktivität (*activity, A*), abgeleitet von der Konzentration des prokaryotischen intrazellulären ATPs, was mit Hilfe eines einfachen Zelllyse-Lumineszenzverfahrens bestimmt wird; und, als optionaler zusätzlicher Parameter, biologisch verfügbarer Kohlenstoff (*carbon, C*), der anhand der Konzentration des assimilierbaren organischen Kohlenstoffs in einem einfachen mikrobiologischen Wachstumsversuchs in Batchkulturen bestimmt werden kann. Der Ansatz wurde anhand von Datensätzen zu drei Fallstudien getestet, die repräsentativ für einige der Hauptgefährdungen für Grundwasserökosysteme stehen, nämlich organische Belastung mit Kohlenwasserstoffen, Oberflächenwassereintrag und landwirtschaftliche Landnutzung. Mit Hilfe statistischer multivariater Ausreißeranalysen, die auf robusten Schätzverfahren basieren (robuste Mahalanobis-Distanzen), konnten belastete Proben in allen drei Fallstudien zuverlässig von nichtbelasteten Proben unterschieden werden. Wir konnten zeigen, dass durch die gleichzeitige multivariate Analyse aller Parameter belastete Proben signifikant sensitiver und zuverlässiger angezeigt wurden als bei separaten univariaten Analysen der einzelnen Parameter. Des Weiteren ergab eine Analyse von Daten zu nichtbelasteten Grundwasserleitern aus unterschiedlichen Regionen Deutschlands, dass

sich die multivariaten Signaturen anhand der drei mikrobiologischen Parameter signifikant zwischen einzelnen Regionen unterscheiden können. Regionale Gegebenheiten sollten daher für die Datenerhebung und -analyse bei der praktischen Anwendung dieses Ansatzes berücksichtigt werden. Alles in allem bietet unser Ansatz eine praktische Lösung für das Aufspüren von Störungen in Grundwasserökosystemen anhand von einfach messbaren mikrobiologischen Parametern, die in Zukunft nahtlos um weitere Parameter erweitert werden können um die Sensitivität und Flexibilität der Analyse weiter zu erhöhen.

Im Ergebnis liefert diese Dissertation einen Beitrag zum besseren Verständnis der grundlegenden ökologischen Prozesse, die für die Zusammensetzung mikrobieller Gemeinschaften im Grundwasser verantwortlich sind. Darüberhinaus beinhaltet diese Arbeit das Ergebnis anwendungsorientierter Forschung, welche einen praktischen Ansatz für die ökologische Bewertung von Grundwasser hervorgebracht hat und damit ein nachhaltigeres und sachkundigeres Management lebenswichtiger Grundwasserökosysteme ermöglicht.

1 Introduction

1.1 Groundwater ecosystems: characteristics, microbial ecology, and relevance

The water-saturated zones of the terrestrial subsurface are among the least explored ecosystems on Earth, with unique features that set them apart from most other, non-subsurface habitats. The lack of light—and hence absence of primary production via photosynthesis—is certainly one of the most striking features that distinguish groundwater from other ecosystems such as soil, surface freshwater, or marine systems (apart from the deep sea) (Griebler and Lueders, 2009). As a consequence, groundwater ecosystems depend on inputs from soils and surface waters as main source of carbon and other essential nutrients like nitrogen and phosphate. The carbon pool in groundwater mainly consists of dissolved organic carbon (DOC) since particulate organic matter is largely removed by filtration as it seeps through overlaying zones before reaching the groundwater (Griebler and Lueders, 2009). Because the DOC is being degraded in this process, concentrations in the groundwater are usually 10 to 1000 times lower than for example in soils, typically ranging between 0.5 and 5 $\mathrm{mg\,L^{-1}}$ (Hancock et al., 2005; Goldscheider et al., 2006; Gooddy and Hinsby, 2009). Furthermore, since the degradation mainly attacks labile organic matter, a large fraction of the DOC in groundwater often consists of recalcitrant compounds, of which only a small part is readily bioavailable to microorganisms. Concentrations of this assimilable organic carbon (AOC) can be two to three orders of magnitude lower compared to the DOC (Gooddy and Hinsby, 2009). Next to low nutrient concentrations, relatively low temperatures as well as stable environmental conditions are another distinctive feature of groundwater environments compared to surface habitats. Although large geological, hydrological, and chemical variation may exist between different zones of an aquifer, the environmental conditions within each zone are often more stable, exhibiting little variation in pH, nutrient concentrations, and temperature over time (Hancock et al., 2005; Goldscheider et al., 2006; Griebler and Lueders, 2009).

Despite these seemingly harsh conditions, which have led researchers to consider groundwater even an extreme environment (Danielopol et al., 2000), these ecosystems are far from being lifeless deserts. Aquifers are ubiquitously colonized by diverse microbial communities, which mainly consist of heterotrophs and chemolithoautotrophs that are well adapted to these oligotrophic, stable conditions (Hancock et al., 2005; Griebler

and Lueders, 2009; Akob and Küsel, 2011; Smith et al., 2018). The majority of these organisms and the bulk of the microbial activity can usually be found attached to rock surfaces and sediment particles (Alfreider et al., 1997; Lehman et al., 2001; Lehman and O'Connell, 2002; Griebler et al., 2002; Zhou et al., 2012; Smith et al., 2018). This attached mode of life may have several advantages over a planktonic lifestyle, for instance by providing access to limiting nutrients and carbon adsorbed to mineral surfaces (Griebler and Lueders, 2009; Smith et al., 2018). Because of the energetic constraints and low temperatures, local prokaryotic cell densities and activity are often lower compared to other environments, typically ranging between 10^2 and 10^6 cells mL^{-1} groundwater and 10^4 and 10^8 cells cm^{-3} sediment (Griebler and Lueders, 2009; Akob and Küsel, 2011). However, due to its vast size, the terrestrial subsurface is estimated to harbor between 6 % and 40 % of the total global prokaryotic biomass, making it one of the largest microbial habitats on Earth, second probably only to marine systems (Griebler and Lueders, 2009; McMahon and Parnell, 2013; Magnabosco et al., 2018).

Microbial communities are key actors in groundwater ecosystems and beyond. Groundwater not only accommodates 'simple' prokaryotic organisms, but provided that oxygen is present, aquifers are inhabited by a diverse protozoan and metazoan fauna comprising several rare and endemic species which can only be found in these habitats (Danielopol et al., 2000; Hancock et al., 2005; Goldscheider et al., 2006; Griebler et al., 2014; Griebler and Avramov, 2015). Microorganisms serve as prey for many of these higher organisms and thus are a crucial component of groundwater food webs (Hancock et al., 2005; Foulquier et al., 2011a; Griebler et al., 2014; Griebler and Avramov, 2015; Hutchins et al., 2016). Moreover, microorganisms are the bedrock of key biogeochemical processes like the cycling of carbon and other nutrients. Since aquifers are open systems that are hydrologically connected to other aquatic and terrestrial ecosystems on the surface, the ecological importance of microbially catalyzed processes in groundwater extends beyond the confines of the subsurface (Goldscheider et al., 2006; Akob and Küsel, 2011; Griebler and Avramov, 2015; Smith et al., 2018).

Next to the ecological relevance, the functioning of groundwater ecosystems is also of direct concern to society. Out of the many services that societies obtain from groundwater (Griebler and Avramov, 2015; Griebler et al., 2019), the provision of clean freshwater is probably the one that most people would rank highest. About 95 % of the liquid freshwater on Earth is groundwater, making it the most important source of drinking and irrigation water worldwide. For example, in Europe 75 % of all the drinking water is produced from groundwater; worldwide, an estimated 2.5 billion people—about one third of the global population—solely rely on groundwater as source of freshwater. However,

the availability of this vital resource is increasingly diminished not only due to overexploitation, but also because of chemical and pathogenic contamination resulting from intensive agriculture, industrial activities, and insufficient wastewater treatment (United Nations World Water Assessment Programme, 2015). Microbial communities are the main drivers of contaminant degradation and furthermore play important roles in the elimination of pathogens, which makes them a crucial element of maintaining the quality of this vital resource (Griebler and Lueders, 2009; van Elsas et al., 2012; van Nevel et al., 2013; Feichtmayer et al., 2017).

Elucidating the mechanisms that shape microbial community composition would be an important step towards a better understanding of biogeochemical processes in groundwater ecosystems, which in turn can pave the way to a better prediction and protection of pivotal ecosystem services. It is widely accepted within the general field of ecology that biodiversity and community composition are determining factors for the functioning and stability of ecosystems (Loreau et al., 2001; Tilman et al., 2014), and microbial communities likely are no exception in that sense (Wallenstein and Hall, 2012; Stegen et al., 2018a). For instance, studies on soil could show that key functions related to carbon and nitrogen cycling are linked to microbial community diversity and composition (Wallenstein et al., 2010; Wagg et al., 2014), and furthermore that including information on microbial community composition can improve the accuracy of regression models to predict process rates of these functions (Strickland et al., 2009; Graham et al., 2016b). Moreover, the extent to which microbial communities are subject to species sorting and dispersal can affect rates of biogeochemical functions, and likely has a determining effect on the vulnerability of communities to perturbations (Dini-Andreote et al., 2015; Graham et al., 2016a; Graham and Stegen, 2017; Albright et al., 2019). However, despite the importance of groundwater microbial communities, our understanding of the ecological processes that shape their composition and diversity is still relatively limited compared to other environments (see Section 1.2). Historically, research on microbial communities in groundwater has mainly been conducted from the perspective of applied microbiology, focusing on issues like corrosion and plugging of pipes used for groundwater extraction, or bioremediation of contaminated sites, whereas fundamental ecological processes—especially in pristine, undisturbed aquifers—have only relatively recently started to be addressed with the emergence of groundwater ecology as a scientific discipline (Hancock et al., 2005; Griebler et al., 2014).

Viewing groundwater from an ecological perspective has not only driven scientific research in that area, but has also led to changes in environmental policies in several parts of the world, which now require the integration of ecological criteria in routine

groundwater monitoring to ensure a better protection of these ecosystems (Danielopol et al., 2007; Griebler et al., 2010). However, the lack of suitable ecological assessment tools has largely hindered the implementation of these policies in practice to date (see Section 1.3).

1.2 Ecological theory on microbial community assembly and knowledge gaps

1.2.1 Species sorting, dispersal, and drift

Understanding the processes that cause differences in microbial community composition across space has become one of the central questions in today's field of microbial ecology (Lindström and Langenheder, 2012; Nemergut et al., 2013; Antwis et al., 2017; Zhou and Ning, 2017). Different theoretical concepts have been developed, which share overlapping perspectives on the processes that determine the assembly of local communities from a common regional species pool, which can be broadly categorized as selection processes—also referred to as species sorting or environmental filtering—and processes related to species dispersal and random drift due to stochastic extinction, migration, or speciation events (Vellend, 2010; Chase et al., 2011; Leibold and Chase, 2018) (Fig. 1.2.1). The species sorting view assumes that species from a regional species pool assemble into local communities according to environmental conditions, determined by abiotic and biotic factors, which select for distinct sets of species that are able to thrive and compete under these conditions. Accordingly, communities at different locations with similar environmental conditions are expected to be composed of similar species, provided that species can disperse freely to track environmental gradients and reach their preferred environment. Thus, from this perspective, differences in community composition are expected to be strongly linked to differences in environmental conditions, while spatial distance between communities is supposed to have little effect (Leibold et al., 2004). However, community composition can be uncoupled from environmental conditions by processes that determine species dispersal. Apart from species sorting, differences between communities can also arise due to dispersal limitation, in which case the impeded exchange of species in combination with random drift causes communities to diverge over time (Chase and Myers, 2011). Additionally, dispersal limitation no longer allows species to track environmental gradients and reach locations with their preferred environment, causing differences in community composition to be predominantly associated with spatial distance between communities rather than differences in environmental conditions.

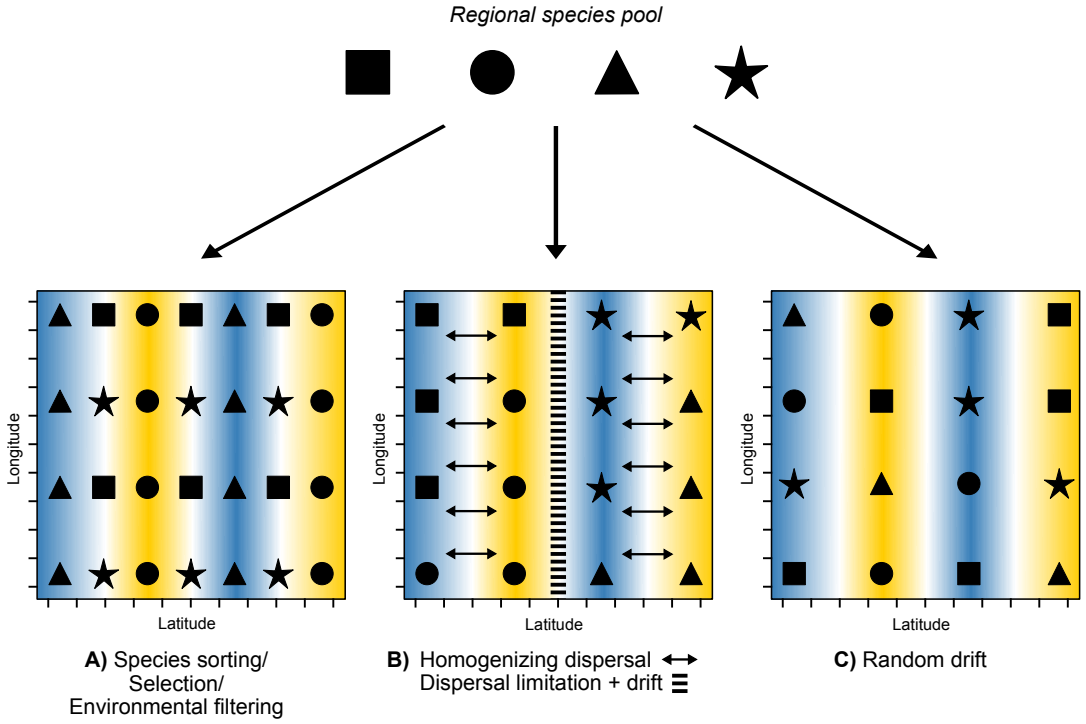

Figure 1.2.1: Schematic illustration of processes that affect local community assembly from a regional species pool. Species are represented by different shapes, environmental conditions are shown as color gradient, and spatial location is indicated by arbitrary measures of latitude and longitude. ***A***) Assembly purely driven by species sorting. Species are sorted into local communities according to environmental conditions independent of spatial distance, such that the blue and yellow environments are always occupied by triangle and circle species, respectively, while square and star species are exclusively found at the interface. ***B***) Community assembly driven by homogenizing dispersal and dispersal limitation. Strong dispersal (represented by bidirectional arrows) homogenizes local communities across space, whereas dispersal limitation (represented by the dashed line) impedes species exchange between local communities. In both cases, the distribution of species is uncoupled from their preferred environmental conditions shown in ***A***. ***C***) Community assembly purely driven by random drift causing local communities to be random subsets of the regional species pool regardless of spatial distance or environmental conditions.

On the other hand, high similarities between communities can arise—and species sorting be overruled—under conditions with high dispersal rates, which homogenize local communities and allow species to occur even under unfavorable environmental condi-

tions, if dispersal rates are sufficient to compensate for species extinction caused by the unfavorable conditions (Leibold et al., 2004).

These theoretical frameworks have their origins in classical community ecology dealing with the distribution of macroscopic organisms such as animals or plants and have only recently started to be considered in microbial ecology. The reasons for this are twofold: first, microbial ecology is mainly a descendant of microbiology, which traditionally takes reductionist experimental approaches to decipher physiological mechanisms rather than trying to understand ecological processes that shape community diversity; second, it has long been impossible to quantitatively assess microbial diversity in nature using classical culture-dependent techniques (Prosser et al., 2007; Barberán et al., 2014). Only the relatively recent advent of molecular tools such as DNA fingerprinting and later on sequencing has enabled microbial ecologists to measure microbial diversity with a sufficient resolution and conduct studies from the perspective of ecological theory, which had not been possible to this extent until then (Lindström and Langenheder, 2012; Barberán et al., 2014).

Before the necessary molecular tools were available, the historically most widely held view on the spatial distribution of microorganisms was essentially a species sorting perspective, as famously stated by Baas Becking in his hypothesis that "*everything is everywhere, but the environment selects*" (Baas Becking, 1934). The basic assumption of this tenet is that microorganisms are not subject to dispersal limitation and drift, because their small body size, fast growth, and large population size facilitate ubiquitous dispersal and prevent local extinction. Therefore, the distribution of microorganisms was supposed not to be limited by spatial distance, but only by environmental conditions that allow some microorganisms with certain traits to colonize a given environment, while excluding others that lack the necessary traits to thrive under these conditions. Directly opposed to this fundamentally selection-oriented perspective stands Hubbell's neutral theory, which assumes that traits of trophically similar organisms are irrelevant to the success of colonizing a given environment, but that this success is purely determined by stochastic species arrival in a local community through dispersal and random drift (Hubbell, 2001). Consequently, according to this theory, local community composition can be predicted simply from average abundances of species in a region, without taking environmental conditions into account. Taking this perspective, more recent studies have started to challenge the classical view of microbial community assembly being exclusively driven by selection, showing that purely neutral models can explain observed diversity patterns of microbial communities with surprising accuracy (Sloan et al., 2006; Woodcock et al., 2007; Ofiţeru et al., 2010).

It is important to note that those studies did not deny the role of species sorting completely, but rather pointed out that also dispersal processes and drift can have important impacts on the assembly of microbial communities. Thus, in between the two extremes of a purely selection-oriented view on the one hand and a purely neutral view on the other, there has now been growing consent that both types of processes play a role, as recently captured in a rephrased version of the Baas Becking hypothesis by van der Gast (2013): "*Some things are everywhere and some things are not. Sometimes the environment selects and sometimes it doesn't*". Albeit arguably not as appealing and elegant as the original version, this quote pinpoints the current knowledge and focus of research on microbial community assembly. It is now widely recognized that both species sorting and processes related to dispersal and drift act simultaneously, and that the contribution of each process is context-dependent—as highlighted by the word "*sometimes*"—and may shift over the course of community succession (see Section 1.2.2), with changing environmental conditions, and with the spatial scale at which communities are being investigated (Martiny et al., 2006, 2011; Hanson et al., 2012; Lindström and Langenheder, 2012; Wang et al., 2013; Zhou et al., 2014; Dini-Andreote et al., 2015; Langenheder and Lindström, 2019). Therefore, rather than trying to find evidence in support of one hypothesis or the other, the focus has changed towards understanding the relative importance of these processes with respect to environmental conditions and habitat types.

A common pattern that has emerged from studies and meta-analyses that have compared microbial community assembly mechanisms across habitats is that species sorting appears to be the dominating process in most cases (Hanson et al., 2012; Lindström and Langenheder, 2012; Wang et al., 2013; Langenheder and Lindström, 2019). However, this dominant effect is not a general rule as it depends to a large degree on the interplay between environmental conditions and spatial scale. Langenheder and Lindström (2019) have provided a conceptual synthesis of this interplay as shown in Figure 1.2.2. Generally, the effect of species sorting is expected to be weak relative to processes affecting species dispersal and drift in stable, homogeneous environments due to the low variation between local environmental conditions (Ofiţeru et al., 2010; Zhou et al., 2013; Wang et al., 2013). In this case, the role of dispersal, dispersal limitation, and drift is then largely determined by the spatial distance between communities. Homogenizing dispersal is expected to predominately operate over short distances, whereas the likelihood of a dominating effect of drift and dispersal limitation increases as distances become larger. The exact spatial scale at which these processes gain importance, however, depends on habitat-specific factors that determine the connectivity between communities. For example, homogenizing dispersal may only have a significant impact in the micrometer

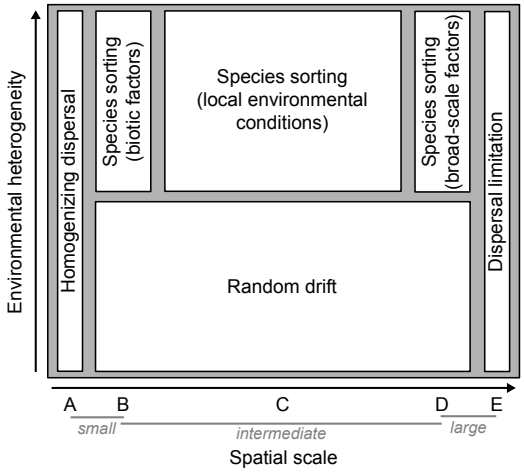

Figure 1.2.2: Dominance of community assembly processes in relation to spatial scale and environmental heterogeneity. Spatial scale is given in arbitrary units increasing from A to E; the exact units may vary depending on habitat characteristics as explained in the main text. Also note that although the changes between dominating processes are shown in discrete steps along the two axes, in reality they can be expected to overlap and change continuously. Processes related to dispersal and drift are expected to dominate if differences between local environmental conditions are small; the importance of homogenizing dispersal decreases with spatial distance in favor of random drift and dispersal limitation at intermediate and large distances, respectively. The importance of species sorting increases with increasing environmental heterogeneity, but may still be overruled by homogenizing dispersal and dispersal limitation, respectively, at the outer extremes of the spatial scale. At short to intermediate distances, biotic factors like species interactions are expected to be the main mediators of species sorting. At intermediate distances, local environmental conditions gain importance, whereas at larger distances, broad-scale factors like climate or geology may impose species sorting, in addition to selection events in the past having left a permanent imprint on community composition. (*Modified from Langenheder and Lindström*, 2019).

to centimeter range in less fluid habitats like soils and other fine-grained media, or in stagnant water bodies where microorganisms primarily disperse via active movement or passive diffusion. However, its effect could extend over large scales of several (tens or hundreds of) meters, for instance in aquatic environments with strong water currents that facilitate long-range passive transport. By the same token, dispersal limitation may operate significantly already at the scale of a few meters or only at large geographic dis-

tances of hundreds or thousands of kilometers. Pure random drift is expected to mainly drive differences in community composition at intermediate spatial scales where dispersal is low enough to avoid homogenization of communities but still sufficient not to be limiting (Langenheder and Lindström, 2019).

The relative contribution of dispersal to microbial community assembly diminishes in favor of species sorting as differences between environmental conditions become larger (Fig. 1.2.2). Biotic factors involving species interactions—antagonistic as well as synergistic—have been suggested to be a key element of species sorting, especially at small spatial scales in the range of centimeters or smaller, provided that dispersal is not too strong to cause community homogenization (Raynaud and Nunan, 2014; Konopka et al., 2015; Cordero and Datta, 2016). At intermediate distances, the effect of biotic interactions decreases and local environmental conditions defined for example by pH, temperature, or nutrient concentrations become the main drivers of species sorting (Hanson et al., 2012; Cordero and Datta, 2016; Langenheder and Lindström, 2019). However, even in heterogeneous environments where a dominating effect of selection processes becomes more likely, dispersal and drift can still prevail in certain situations. For example, perturbations (i.e. strong or sudden changes in environmental conditions that exceed the range of fluctuation to which native species are adapted) can inactivate part of a community and thereby leave open niches, leading to reduced competition and hence increasing the chance of randomly dispersed species to establish in a community (Shade et al., 2012; Ferrenberg et al., 2013; Zhou et al., 2014; Fukami, 2015; Zhang et al., 2016). Moreover, conditions with high biomass production have been suggested to weaken species sorting, presumably by offering a larger niche space and thus favoring random colonization (Chase, 2010; Zhou et al., 2014; Ren et al., 2017), although contrary observations have also been reported indicating opposite effects (Langenheder et al., 2012). Overall, while biological productivity does seem to influence community assembly, the direction of these effects is still little understood and might vary depending on habitat type (Langenheder and Lindström, 2019).

In addition to local environmental conditions, broad-scale region-specific factors can become an important part of species sorting over large spatial distances spanning hundreds to thousands of kilometers across different regions. These factors may comprise features like climate, geology, or land use, but also past environmental conditions that may have left a lasting imprint on contemporary community structure (Martiny et al., 2006; Andersson et al., 2014; Fukami, 2015; Stegen et al., 2016b; Vass and Langenheder, 2017; Rummens et al., 2018; Svoboda et al., 2018). However, a common problem of studies that have investigated changes in microbial community composition over large

spatial scales is that distance decay relationships (i.e. a significant decrease in community similarity with increasing spatial distance, while controlling for the effect of differences in local environmental conditions) can be caused both by dispersal limitation over large spatial distances as well as species sorting due to broad-scale regional factors (Leibold et al., 2010; Hanson et al., 2012; Wang et al., 2013). Therefore, it is often unclear to what extent adaptation to these broad-scale factors, relative to dispersal limitation and drift, contributes to differences between communities across regions. Moreover, the potential effect of broad-scale factors on local community composition within regions, on top of dispersal and selection imposed by local environmental conditions, is not well understood (Ricklefs, 2008; Heino et al., 2017).

Several studies have investigated differences in microbial community composition and the underlying assembly processes over different spatial scales in various habitats including soil, marine environments, and freshwater systems like ponds, streams, and lakes (for reviews see Hanson et al., 2012; Lindström and Langenheder, 2012; Langenheder and Lindström, 2019). However, the majority of studies so far have focused on local communities within a region, while only a few studies have explicitly investigated communities at larger spatial scales across regions (e.g. Martiny et al., 2011; Almasia et al., 2016; Comte et al., 2016; O'Brien et al., 2016; Ma et al., 2017; Hassell et al., 2018; Power et al., 2018; Shi et al., 2018). Strikingly, research on groundwater environments is largely underrepresented in this context compared to surface habitats. Although recent studies have addressed the impacts of changes in environmental conditions and the relative contributions of dispersal, drift, and species sorting on microbial community composition in groundwater (Stegen et al., 2012, 2013, 2015; Shabarova et al., 2014; Beaton et al., 2016; Savio et al., 2019) and the hyporheic zone (i.e. groundwater-surface water mixing zones) (Graham et al., 2016a, 2017; Stegen et al., 2016a, 2018b), they mainly focused on differences between local communities within a single aquifer, whereas studies that compared communities across aquifers from different regions are scarce (Ben Maamar et al., 2015; Danczak et al., 2018). Consequently, the factors and processes that determine microbial community composition in these characteristically stable, low-productivity environments have not been fully unraveled. Considering the importance of groundwater as a microbial habitat and the crucial role of microbial communities in these ecosystems and beyond, this is a critical knowledge gap in microbial ecology, which I set out to address in the first part of this thesis (see Section 1.4.1).

1.2.2 Microbial community assembly and succession during surface colonization

As briefly alluded to in the previous section, the relative contribution of species sorting to community assembly may shift over the course of community succession in newly-colonized environments (Tilman, 2004; Langenheder and Székely, 2011). At the initial stage of colonization, the arrival of species in a new environment is often driven by stochastic dispersal (Tilman, 2004; Ferrenberg et al., 2013; Dini-Andreote et al., 2015), which can override effects of species sorting in homogeneous environments with little variation between local environmental conditions as explained above. However, once established, resident species can affect the establishment of newly-arriving species (positively or negatively) over the course of the subsequent succession, either directly through species interactions, or indirectly by modifying their local environment (Nemergut et al., 2013; Fukami, 2015), for example by depleting oxygen and thereby changing redox conditions. Thus, the order and timing of species arrival, albeit initially stochastic, can affect subsequent species sorting events that determine the composition and functioning of the final community, which is commonly known as priority effect (Fargione et al., 2003; Tilman, 2004; Fukami et al., 2010; Peay et al., 2012; Tan et al., 2012; Nemergut et al., 2013; Rummens et al., 2018; Svoboda et al., 2018).

One example of the initial colonization of new environments is the formation of biofilms on initially empty surfaces. A general conceptual model that summarizes the successional stages during the development of biofilms has been described by Jackson (2003) (Fig. 1.2.3). According to this model, initially empty surfaces offer ample space and resources to facilitate the establishment of diverse microorganisms, resulting in a rapid increase in species richness and diversity that is fueled by the dispersal of newly-arriving species from the species pool in the overlaying water phase. The steady arrival of new species eventually leads to niche depletion and growing competition between established and newly-arriving species, which more and more suppresses the increase in species richness. As the competition intensifies, less competitive species are lost from the community, resulting in a decline of species richness after the initial stage of community assembly. However, as the biofilm matures further and becomes more heterogeneous, new niches are created that enable specialized species to establish (e.g. anoxic pockets permitting the growth of anaerobes), which may again lead to an increase in species richness and diversity in the mature biofilm. Although Woodcock and Sloan (2017) could demonstrate that these changes in species richness and diversity can be predicted by a purely neutral model based on stochastic birth-death-immigration events akin to

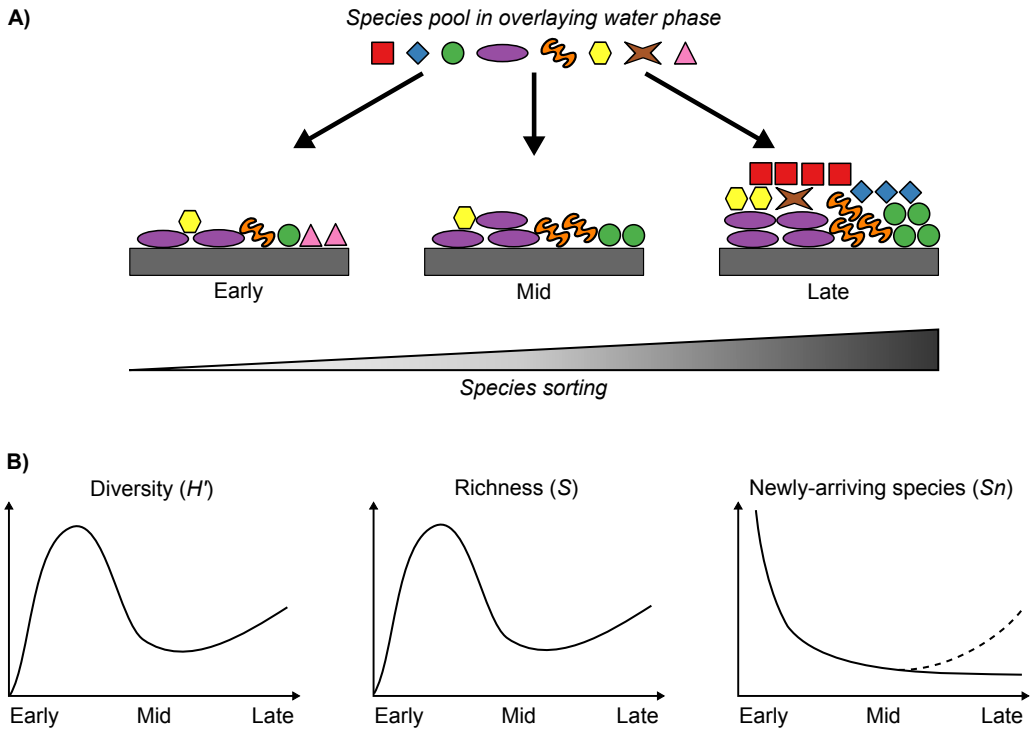

Figure 1.2.3: Microbial community succession during biofilm development. ***A***) Schematic illustration of the colonization of an initially empty surface (shown as gray rectangles) by species from an overlaying water phase over the successional stages of biofilm development, and accompanying changes in the relative importance of species sorting. Different species are represented by colored shapes. The ramp represents the increasing importance of species sorting as the biofilm develops. ***B***) Expected changes in species diversity, richness, and the fraction of newly-arriving species in the biofilm over the course of the succession (*Modified from Jackson,* 2003).

Hubbell's neutral theory (Hubbell, 2001), empirical studies suggest that the assembly of biofilm communities is often driven by a shift from initially stochastic assembly towards selection-driven succession at the later stages, for instance caused by species interactions or growing niche space due to increasing chemical heterogeneity of the biofilm (Martiny et al., 2003; Lyautey et al., 2005; Battin et al., 2016; Veach et al., 2016; Brislawn et al., 2019).

In contrast to typically studied biofilms in environments like surface waters, which form dense, spatially coherent, heterogeneous structures that can reach a thickness of several hundred micrometers (Battin et al., 2016), microbial communities attached to rock and sediment surfaces in groundwater environments usually occur as small, patchily

distributed microcolonies that consist of only a few cells (Schmidt et al., 2017; Smith et al., 2018). Nevertheless, they represent the bulk of the microbial biomass and activity in groundwater environments, and thus can be expected to play a particularly important role in these ecosystems (Alfreider et al., 1997; Lehman et al., 2001; Lehman and O'Connell, 2002; Griebler et al., 2002; Zhou et al., 2012; Smith et al., 2018). Even though previous studies have repeatedly shown that the composition of sediment-attached communities can differ substantially from planktonic communities in the surrounding groundwater (e.g. Zhou et al., 2012; Flynn et al., 2013; Hug et al., 2015), the ecological processes that give rise to these differences during community assembly and succession have largely remained in the dark so far. Thus, it is unclear whether sediment-attached communities assemble initially from random subsets of the planktonic species pool and subsequently diverge from planktonic communities through temporal drift, or whether sediment surfaces select for distinct suites of microorganisms.

To date, most of the studies on ecological processes behind the assembly of microbial communities in groundwater environments have focused on planktonic communities suspended in the groundwater (Stegen et al., 2012, 2013; Beaton et al., 2016; Danczak et al., 2018), while sediment-attached communities have received less attention and were mainly investigated in the hyporheic zone (Graham et al., 2016a, 2017; Stegen et al., 2016b). In these studies, the assembly of planktonic communities generally tended to be more influenced by dispersal and drift, as well as species sorting imposed by local hydrochemical conditions, compared to sediment-attached communities, which showed a relatively stronger effect of species sorting albeit mostly unrelated to hydrochemistry, but supposedly exerted by mineralogy. However, the groundwater-surface water mixing in the hyporheic zone creates a much more dynamic environment with higher nutrient concentrations that promote microbial activity and productivity relative to groundwater in the absence of surface water inputs (Hancock et al., 2005). Overall, it is unknown whether the assembly of sediment-attached communities in comparatively more stable and energy-poor groundwater environments follows the same trends as observed for communities in the hyporheic zone or typical biofilms in other aquatic environments as described above. The research presented in the second part my thesis aimed at shedding light on this open question (see Section 1.4.2).

1.3 Ecological monitoring of groundwater ecosystems

Fundamental research on the ecology of groundwater environments, like the aspects covered in the previous sections, is essential for a better understanding of the functioning

and vulnerability of these ecosystems, which in turn provides crucial information to advise policy makers in drafting environmental regulations. Over the past two decades, scientific advances in the field of groundwater ecology have stimulated a paradigm shift in environmental policy. While legislators in the past used to regard groundwater first and foremost as a commodity, it is now more and more recognized as a unique ecosystem that merits protection (Danielopol et al., 2007; Griebler et al., 2010). Certainly, socioeconomic aspects have played a large part in this development, as legislators have become increasingly aware of the strong link between ecosystem health and the quality of groundwater as an invaluable resource, as well as the importance of groundwater for the integrity of other ecosystems on the surface. In addition, there are also important and valid cultural-ethical arguments that deserve consideration, such as the responsibility of society to protect rare organisms in these subsurface habitats and to preserve them for future generations (Danielopol et al., 2000, 2007; Hancock et al., 2005; Goldscheider et al., 2006; Griebler et al., 2014; Griebler and Avramov, 2015). Nevertheless, to date, conventional groundwater monitoring has focused almost exclusively on physicochemical parameters (and hygienic indicators such as the presence of pathogens), which however do not provide information on the ecological or biological state of the groundwater. However, several advantages could be gained from ecological assessments during routine monitoring. Not only would it allow for a better protection of these unique ecosystems, it would also enable a more sustainable and informed ecosystem management, thereby helping maintain valuable ecosystem services such as the provision of clean freshwater (Griebler et al., 2014; Griebler and Avramov, 2015). Moreover, considering that the indigenous organisms have adapted to the characteristically stable environmental conditions in groundwater aquifers, they can be expected to react sensitively to various disturbances, some of which might not be detected by conventional monitoring of physicochemical parameters alone. Therefore, the sensitivity of groundwater monitoring could be increased by amending routine monitoring protocols with ecological-biological parameters.

In order to give incentives for the implementation of ecological criteria in groundwater monitoring, several countries have passed environmental directives demanding the consideration of parameters that allow for an ecological assessment of groundwater, in addition to the conventional physicochemical measurements. Examples of such directives can be found in Switzerland (SWPO, 1998) and Australia (NSW-SGDEP, 2002; EPA, 2003), and plans for the implementation of similar frameworks have furthermore existed in the European Union since 2006 (EU-GWD, 2006). However, these directives largely remain ambiguous about specific parameters that should be considered and ac-

cording benchmarks. To quote one representative example, the Swiss Water Protection Ordinance states that:"*The biotic community of underground waters shall: a) be close to nature and appropriate to the location; b) be specific to unpolluted or only slightly polluted waters.*" (SWPO, 1998; status of June 1, 2018). This ambiguity is attributed to the fact that, first, potential bioindicators like groundwater fauna communities are often highly specific to a given location, and second, there is still limited knowledge about such potential indicator organisms, which both complicate the definition of specific, universally applicable ecological monitoring criteria. Therefore, these directives collectively call for research striving to "*provide better criteria for ensuring groundwater ecosystem quality*" as stated for instance in the EU Groundwater Directive (EU-GWD, 2006).

The lack of suitable, universally applicable monitoring criteria has largely hampered the routine implementation of ecological groundwater monitoring in practice to date, although progress has recently been made in that direction (e.g. Korbel and Hose 2011; 2017). While ecological assessment criteria based on sets of indicator organisms have already been firmly integrated into routine monitoring of surface water ecosystems (e.g. EU-WFD, 2000), these approaches are not compatible with groundwater due to the characteristic environmental conditions outlined above (Section 1.1) and the specific repertoire of organisms (e.g. lack of algae, macrophytes, and often vertebrates) in these environments (Steube et al., 2009). Consequently, this raises the demand for new monitoring tools that are specifically tailored to groundwater ecosystems.

To be integrated into routine monitoring protocols, such tools should fulfill certain criteria:

1. The targeted parameters need to be easy and inexpensive to measure.
2. They need to be sensitive to a wide range of disturbances and globally applicable across different hydrogeological conditions.
3. The data need to be simple to analyze and interpret.
4. Ideally, samples for measuring ecological parameters should be obtainable along with samples for typical physicochemical analyses, without the need for additional sampling efforts.

Several studies have suggested the use of groundwater fauna as bioindicators for the detection of disturbances of groundwater ecosystems (Malard et al., 1996; Mösslacher, 1998; Hahn, 2006; Schmidt et al., 2007; Bork et al., 2009; Stein et al., 2010; Marmonier et al., 2013, 2018). However, the notoriously sparse and locally often highly heterogeneous distribution of groundwater fauna, as well as their absence under anoxic conditions,

complicates data collection and interpretation for these types of indicators, and furthermore restricts their applicability to oxic aquifers (Stein et al., 2010; Korbel and Hose, 2017).

Prokaryotic microorganisms on the other hand are ubiquitous in aquifers even under harsh conditions (Griebler and Lueders, 2009), making them ideal bioindicators for ecological monitoring in various environmental settings. The idea of using microorganisms for groundwater monitoring is not new (Brielmann et al., 2009; Pronk et al., 2009; Steube et al., 2009; Griebler et al., 2010; Stein et al., 2010; Foulquier et al., 2011b; van Driezum et al., 2018), but so far there are only a few examples of applicable frameworks that integrate microbial parameters into ecological assessment schemes. However, these approaches are either applicable only under certain conditions, for example requiring non-stagnant water in monitoring wells (Mermillod-Blondin et al., 2013), or rely on DNA sequencing-based approaches and elaborate bioinformatic analyses (Pearce et al., 2011). Although, as argued earlier (Section 1.2.1), these types of molecular tools have been essential for enhancing our fundamental understanding of microbial communities, to date they likely still exceed the expertise and financial resources of most local authorities and water suppliers responsible for conducting groundwater monitoring. We may speculate that rapidly declining sequencing costs and advances in automating sample preparation and data analysis pipelines might make these tools accessible to a broader range of users in the future, which may also open the doors to novel applications in routine groundwater monitoring. However, currently these methods are still too sophisticated and expensive for such applications to be feasible in practice.

To meet the demand for a universally applicable and at the same time user-friendly method that enables the consideration of biological-ecological criteria in groundwater monitoring, an approach was developed in the context of this thesis based on simple microbiological parameters that serve as indicators of the stability of groundwater ecosystems facing disturbances. The core parameters of this approach are microbial cell density measured as total porkaryotic cell counts by flow cytometry, and the activity displayed by those cells measured as intracellular ATP concentrations using a standardized commercially available kit. Furthermore, concentrations of AOC, as a measure for the energy available to the microorganisms, can be optionally included as an additional parameter. These parameters have already proved useful for monitoring the stability of drinking water distribution systems (FDHA, 2012; Vital et al., 2012; Lautenschlager et al., 2013; Vang et al., 2014; van Nevel et al., 2017), and have furthermore been shown to react readily to sudden disturbances in groundwater settings (Foulquier et al., 2011b; Mermillod-Blondin et al., 2013; Herzyk et al., 2017), strongly suggesting a great potential for their appli-

cation in groundwater monitoring. The developed approach integrates the signals of all measured variables in a multivariate analysis, and allows for a simple detection of disturbances based on a single value, which will be referred to as the D-A-(C) index, standing short for the parameters considered in the analysis (i.e. prokaryotic cell *density*, *activity*, and optionally concentrations of assimilable organic *carbon*). This approach will be illustrated in the third part of this thesis (see Section 1.4.3).

1.4 Outline and aims of this thesis

1.4.1 Ecological drivers of differences in microbial community composition across geographically distinct aquifers

The research presented in the first part of my thesis aimed at elucidating the ecological processes that determine microbial community composition in pristine groundwater ecosystems. Based on the ecological theory presented in Section 1.2.1, our goal was to establish the degree to which microbial community composition is shaped by species sorting imposed by local environmental conditions as well as possible broad-scale region-specific factors, and processes related to dispersal, dispersal limitation, and drift acting on communities within as well as across aquifers. To this end, we compared microbial community composition based on 16S rRNA amplicon sequence data in groundwater samples that were collected from four geographically distinct, shallow, porous aquifers, located in different catchment areas along a latitudinal transect of ∼700 km across Germany. We used a combination of variation partitioning and null model simulations to quantify the contributions of species sorting relative to dispersal and drift, and to identify the factors responsible for species sorting.

1.4.2 Assembly and succession of microbial communities during the colonization of groundwater sediments

The second part of my thesis addresses the processes that determine microbial community assembly on groundwater sediments and differences in community composition between sediments and groundwater. In this study, we set out to, first, investigate whether the assembly of sediment-attached communities in pristine groundwater environments can be explained by the general patterns observed for surface-attached biofilms in other habitats discussed in Section 1.2.2; second, study the importance of early colonizers for community succession during the colonization of the sediments; and third, see if the dominating effect of species sorting on community assembly that has been observed for sediment-attached

communities in the hyporheic zone also drives community assembly in comparatively more stable and energy-poor groundwater zones. To tackle these goals, we analyzed data from an incubation experiment with *in situ* microcosms that were filled with sterilized sediments and incubated in monitoring wells at two spatially separated but hydrologically connected sites of a pristine porous aquifer over a period of almost one year (Zhou et al., 2012). We used 16S rRNA amplicon sequence data to study changes in alpha and beta diversity of the sediment-attached communities incubated at the two sites as well as differences between sediment-attached and planktonic communities over the course of the succession. Null model simulations on the spatiotemporally resolved amplicon sequence data were used to explore the influence of species sorting, dispersal, and drift on community assembly and the differences between sediment-attached communities and planktonic communities in the surrounding groundwater.

1.4.3 Ecological groundwater monitoring based on microbiological parameters

Part three of this thesis illustrates the application of the approach for ecological groundwater monitoring described in Section 1.3 based on the combined analysis of prokaryotic cell density, intracellular ATP, and AOC concentrations using the D-A-(C) index. We tested the potential of the D-A-(C) index to indicate disturbances based on three case studies representing some of the main threats to groundwater ecosystems, that is 1) organic contamination with hydrocarbons; 2) surface water intrusion; and 3) disturbances related to agricultural land use. Furthermore, we explored the benefit of the simultaneous multivariate analysis of the microbiological parameters compared to separate univariate analyses of each individual parameter alone. In addition, we analyzed a large dataset comprising over 200 samples taken from nine non-contaminated aquifers located in different regions across Germany to investigate geographic differences in the microbial parameters as a first step towards the definition of monitoring benchmarks.

2 Materials and methods

2.1 Ecological drivers of differences in microbial community composition across geographically distinct aquifers

2.1.1 Sample collection

A total of 45 samples were collected on single sampling campaigns between spring 2016 and summer 2018 from four distinct unconfined, shallow, porous aquifers, mainly consisting of unconsolidated gravel and sand, located in four different regions across Germany (Fig. 2.1.1). Region NOR (n=12; September 2018; 53.72°N, 10.01°E) was located in Norderstedt near the city of Hamburg in the catchment of the Elbe River; region WUR (n=13; May 2016; 49.77°N, 9.93°E) was located in Würzburg in the Main River catchment; region AUG (n=12; June 2016; 48.25°N, 10.90°E) was located near Augsburg in the Lech River catchment; region MIT was located near Mittenwald (n=8; July 2018; 47.41°N, 11.26°E) at the foothills of the German Alps in the Isar River catchment. Groundwater from the sampled areas of all aquifers was classified as non-contaminated and is used for drinking water production in the respective regions. Prevalent types of land use in all four regions were forests, grasslands, and fallow agricultural land.

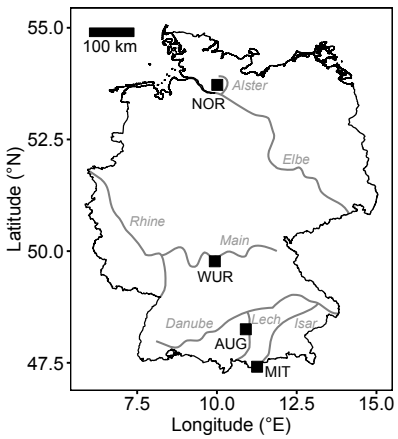

Figure 2.1.1: Schematic map of Germany. Locations of the investigated regions are shown as black squares; rivers are shown as grey lines. (*From Fillinger et al.,* 2019a).

Groundwater samples were collected from fully screened monitoring wells using a

MP1 submersible pump connected to an adjustable frequency converter to control the pumping rate (Eijkelkamp Soil & Water, Giesbeek, The Netherlands). The pump was lowered to half the depth of the water column inside the well. The collection of groundwater was done based national and international standard protocols (DIN, 1985; LUBW, 2013; EPA, 2017). In order to obtain a representative sample of the groundwater, monitoring wells were purged prior to sample collection to remove well water and water in the direct surrounding of the monitoring well by pre-pumping until two to three borehole volumes had been exchanged and physicochemical parameters (temperature, pH, electrical conductivity, and concentrations of dissolved oxygen) monitored by online measurements had stabilized. Groundwater pumping was done at the highest possible rate accepting a maximum drawdown of the groundwater table by ∼30 cm.

All containers were sterilized prior to the sampling and rinsed with sample water three times before sample collection. All samples were kept in the dark at 4 °C for transport to the lab and until further processing. Samples for DOC concentration measurements were collected in glass bottles that were baked at 450 °C for 4 h or soaked in 10 % w/v sodium persulfate overnight to eliminate residual carbon. To remove traces of particulate organic matter, DOC samples were passed through a 0.45 µm filter (Millex-HV; Merck-Millipore, Carrigtwohill, Ireland) rinsed once with sample water prior to sample collection, and were acidified on-site with HCl to a final pH ≤2. Samples for total prokaryotic cell counts were collected in sterile Falcon tubes and fixed with 2.5 % v/v glutardialdehyde (final concentration) immediately after sampling to stabilize and preserve cells. Samples for DNA extraction (5 L) were collected in autoclaved glass bottles or plastic containers rinsed three times with 1 M HCl followed by three washing steps with 80 % v/v ethanol; residual ethanol was allowed to evaporate from the containers overnight. Cells were collected on a 0.2 µm polycarbonate filter membrane (Merck-Millipore) within 48 h after sample collection and stored at −20 °C until DNA extraction.

2.1.2 Measurements of physicochemical parameters

To estimate local environmental conditions, we measured 13 physicochemical parameters for each groundwater sample in addition to total prokaryotic cell counts as a measure of microbial biomass (see Section 2.1.3). Electrical conductivity, pH, temperature, and concentrations of dissolved oxygen were measured online during sampling using field sensors (WTW, Weilheim, Germany). DOC concentrations were measured as non-purgeable organic carbon using high-temperature combustion of organic carbon at 680 °C and infrared detection of the resulting CO_2 on a TOC-V CPH Analyzer coupled to an ASI-V

autosampler (Shimadzu, Kyoto, Japan). Measurements of concentrations of major ions were carried out by the Research Unit Analytical BioGeoChemistry at Helmholtz Zentrum München. Inductively coupled plasma atomic emission spectrometry (ARCOS; Ametec-Spectro, Kleve, Germany) was used for the determination of cation concentrations (calcium (measured spectral element line: 183.801 nm), magnesium (279.079 nm), potassium (766.491 nm), and sodium (589.592 nm)) with radio frequency power set to 1400 W and argon as plasma gas at a flow rate of 15 L min^{-1}. Samples were introduced by a peristaltic pump connected to a micromist nebulizer with a cyclon spray chamber. Anion concentrations (chloride, nitrate, orthophosphate, sulfate) were determined by ion chromatography (Dionex ICS-1500; pre-column: Dionex AG4; analytical column: Dionex AS4; Thermo Scientific, Idstein, Germany) with Na_2CO_3 (1.8 mM) + $NaHCO_3$ (1.7 mM) as eluent at a flow rate of 1 mL min^{-1}.

2.1.3 Determination of total prokaryotic cell counts by flow cytometry

Total prokaryotic cell counts were determined using a FC500 CYTOMICS flow cytometer (Beckmann Coulter, Brea, CA, USA). To distinguish cells form abiotic particles and background noise, cells in 500 µL sample aliquots were stained with SYBR Green I fluorescent nucleic acid stain (Invitrogen, Darmstadt, Germany) at a ratio of 1:10 000 v/v followed by incubation for 13 min at 37 °C in the dark in an Eppendorf Thermomixer (Eppendorf, Hamburg, Germany). Gates for cell counting were set in dot plots of green fluorescence (recorded at 530 nm; FL1 channel) versus red fluorescence (610 nm; FL3 channel) (Hammes and Egli, 2005; Kötzsch et al., 2012; Zunabovic-Pichler et al., 2018). Excitation was achieved with a 488 nm argon ion laser. Instrument-specific filter gain settings were as follows: FL1: 569; FL3: 640; trigger threshold (set on FL1): 1. Data acquisition and analysis were done using the CYTOMICS FC500 CXP software (version 2.2; Beckman Coulter). Samples were measured with flow rate set to 'medium' for 60 sec. To determine the exact volume of measured sample, a 100 µL aliquot of a suspension of a known number of fluorescent reference beads (Trucount Tubes; Becton-Dickson, Franklin Lakes, NJ, USA) was added to each sample as internal standard. Samples were measured in duplicate.

2.1.4 DNA extraction

DNA was extracted from cells collected on membrane filters based on the protocol by Pilloni et al. (2012). Filters were cut into pieces of ∼5 mm × 5 mm and inserted into sterile 2 mL screw cap microcentrifuge tubes filled with ∼0.2 g of a 1:1 mixture of 0.1 mm

and 0.7 mm Zirconia/Silica beads (Roth, Karlsruhe, Germany). Cells were subjected to enzymatic lysis by adding 750 µL autoclaved and filter sterilized phosphate-Tris-NaCl (PTN) buffer (16.02 g L^{-1} Na_2HPO_4; 0.86 g L^{-1} NaH_2PO_4; 11.20 g L^{-1} Tris-HCl; 6.60 g L^{-1} Tris-Base; 1.46 g L^{-1} NaCl; pH 8; dissolved in Milli-Q water), 40 µL lysozyme (1.5×10^3 U mL^{-1} in 1x TE buffer), and 10 µL protein kinase K (1×10^6 U mL^{-1} in 1x TE buffer) (both Sigma Aldrich, Steinheim, Germany). After manual mixing, samples were incubated for 15 min at 37 °C in an Eppendorf Thermomixer. The enzymatic reaction was stopped by adding 100 µL 20 % w/v sodium dodecyl sulfate (Sigma Aldrich) followed by incubation for 15 min at 65 °C with shaking at 500 rpm. Following the enzymatic treatment, samples were mixed with 100 µL 25:24:1 phenol:chloroform:isoamly alcohol (PCIA; Sigma Aldrich; pH 8) and subjected to bead beating for 45 sec at 6.0 m sec^{-1} using a FastPrep-24 sample homogenizer (MP Biomedicals, Irvine, CA, USA). Samples were centrifuged for 5 min at 6000 × g and 4 °C in an Eppendorf 5417R table top centrifuge. 600 µL supernatant was transferred to a 2 mL Phase Lock Gel Heavy tube (Quantabio/QIAGEN, Beverly, MA, USA) and put on ice. The remaining sample was mixed with 300 µL PTN buffer followed by an additional bead beating step for 20 sec at 6.5 m sec^{-1} and centrifugation as before. Supernatants from both extraction steps were pooled and mixed with 1 volume (900 µL) PCIA followed by manual shaking and centrifugation for 4 min at 21 000 × g and 4 °C. 800 µL supernatant was transferred to a fresh Phase Lock Gel Heavy tube and mixed with 1 volume 24:1 chloroform:isoamyl alcohol (Sigma Aldrich) followed by centrifugation as before. 650 µL supernatant was transferred to a fresh 2 mL microcentrifuge tube and mixed with 2 volumes precipitation solution (30 % w/v polyethylene glycol 6000 (AppliChem, Darmstadt, Germany) plus 1.6 M NaCl). DNA was allowed to precipitate for ∼12 h at 4 °C. DNA was pelleted by centrifugation for 30 min at 21 000 × g and 21 °C. The supernatant was removed by pipetting and the DNA pellet was washed twice with ice-cold 70 % v/v ethanol in nuclease-free water with centrifugation (5 min; 21 000 × g; 4 °C) in between washing steps. The ethanol was removed by pipetting and the DNA pellet was allowed to air-dry for ∼5 min at room temperature. The final DNA pellet was resuspended in 30 µL EB buffer (QIAGEN, Hilden, Germany) and stored at −20 °C until further processing.

2.1.5 16S rRNA gene amplification and sequencing

DNA concentrations in raw extracts were determined using the Quant-iT PicoGreen ds-DNA Assay Kit (Invitrogen) according to the manufacturer's instructions. DNA extracts of all samples were diluted to an equal concentration of 1 ng $µL^{-1}$ with EB buffer and

used as template (1 µL) for PCR amplification of the V4 region of 16S rRNA genes using the primer pair 515FB (5'-GTGYCAGCMGCCGCGGTAA) (Parada et al., 2016) and 806RB (5'-GGACTACNVGGGTWTCTAAT) (Apprill et al., 2015) extended with Illumina adapters attached to the 5' end of each primer (forward: 5'-TCGTCGGCAGCGT CAGATGTGTATAAGAGACAG; reverse: 5'-GTCTCGTGGGCTCGGAGATGTGTA TAAGAGACAG). Each reaction (25 µL) contained 12.5 µL NEBNext High-Fidelity 2X PCR Master Mix (New England Biolabs, Ipswich, MA, USA), 3.75 µL 2 % w/v bovine serum albumin (Roche Diagnostics, Mannheim, Germany), 0.5 µL of each primer (10 µM), and 6.75 µL nuclease-free water. The amplification cycles were executed in an Eppendorf Mastercycler Epgradient. Initial denaturation was achieved at 98 °C for 30 sec followed by 25 amplification cycles (denaturation: 98 °C, 10 sec; primer annealing: 50 °C, 30 sec; elongation: 72 °C, 30 sec) and final elongation at 72 °C for 5 min. Each sample was amplified in independent triplicate reactions; triplicates were pooled after amplification. Pooled amplicons were purified using magnetic beads (AMPure-XP; Beckman Coulter) at a bead:sample ration of 0.8 and an incubation at room temperature for 5 min. After washing (two washing steps with 200 µL 80 % v/v ethanol) and air-drying (10 min, room temperature), amplicons were eluted from the beads with 30 µL EB buffer. Amplicon size and concentration were determined by capillary gel electrophoresis (Fragment Analyzer; Agilent Technologies, Santa Clara, CA, USA) using the DNF-473 Standard Sensitivity NGS Fragment Analysis Kit (Agilent Technologies). For each sample, 10 ng amplicons were used as template for index PCR using Illumina Nextera XT Index Kit v2 primers (Illumina, San Diego, CA, USA) according to the manufacturer's specifications and with the same polymerase as above. After purification and electrophoresis as above, barcoded amplicons were pooled in equimolar concentrations (4 nM) and used for paired-end sequencing (2×300 bp) on an Illumina MiSeq platform. Sequencing was carried out by the Research Unit Comparative Microbiome Analysis at Helmholtz Zentrum München.

2.1.6 Sequence data processing

Sequence data were processed in R (R Core Team, 2018) using the 'DADA2' package (version 1.10.1) for the inference of exact amplicon sequence variants (ASVs) (Callahan et al., 2016a). The concept of ASVs is a relatively novel alternative to the conventional approach of clustering sequences into operational taxonomic units (OTUs). Instead of grouping sequences above a certain similarity threshold into OTUs (usually >97 % similarity), the DADA2 algorithm makes use of the quality scores generated during sequencing and sequence abundances in the dataset, to build an error model that is subsequently

used to determine whether nucleotide mismatches between sequences are likely due to biological variation or mere sequencing errors. As such, this method in theory allows to distinguish sequences that differ by as little as a single nucleotide, thereby covering more of the biological diversity within a sample compared to the OTU approach. As an additional benefit, ASVs generated this way overcome problems associated with the comparability of OTUs across datasets (Callahan et al., 2016a). Although the rationale behind this approach is appealing, this method was mainly chosen for practical reasons for this study. Furthermore, it is still controversial whether the use of ASVs inferred from 16S rRNA amplicon sequence data actually provides significantly more accurate estimates of microbial community diversity and composition compared to the traditional OTU-based approach (e.g. see Glassman and Martiny, 2018). Therefore, for the purpose of this thesis, I will assume that the use of either ASVs or OTUs does not affect the ecological inferences drawn from the data.

Quality filtering, merging of paired-end reads, inference of ASVs, and chimera removal was done using the DADA2 workflow as described in Callahan et al. (2016b) with slight modifications. Truncation length during quality trimming was set to 280 bp and 200 bp for forward and reverse reads, respectively, after primer trimming. Negative controls (i.e. PCR reactions without added sample DNA as template) were excluded from the error model building step to infer error rates from the sequence data. ASVs were inferred across all samples using pseudo-pooling, which prevents discarding ASVs based on the occurrence in a single sample, if it is represented by at least two error-free reads in at least two samples in the full dataset. ASVs found in negative controls or with a sequence length $<$261 bp were discarded. In addition, to facilitate downstream processing and reduce sparsity of the data, ASVs with an abundance $<$0.001 % across all samples were removed. Taxonomic assignment was done using the online implementation of IDTAXA (Murali et al., 2018) by mapping ASV sequences against the SILVA SSU reference database (release 132; Quast et al., 2013) with a 50 % confidence threshold. ASVs that were classified as mitochondria or chloroplasts were discarded, as well as ASVs that could neither be classified as bacteria nor archaea. The final ASV table contained 9153 ASVs; abundances were rarefied to 6281 reads per sample, which was the lowest number of reads observed in a single sample. To infer phylogenetic relationships, ASV sequences were aligned using the 'DECIPHER' package (version 2.10.1; Wright et al., 2015); the sequence alignment was passed on to FastTree (Price et al., 2009) for building a midpoint-rooted phylogenetic tree. Sequence data are publicly available at the NCBI Sequence Read Archive (accession no. SRP191753).

2.1.7 Data analysis

All analyses were done in R. ASV richness and Faith's phylogenetic diversity (PD) were calculated using the 'picante' package (version 1.7; Kembel et al., 2010). Differences in microbial community composition were analyzed based on β-mean nearest taxon distance (β-MNTD), which is the mean phylogenetic distance of taxa in one community to their closest relatives in another community. Thus, β-MNTD focuses on short phylogenetic distances, that is the tips of a phylogenetic tree, indicating turnover of lineages that have diverged relatively recently in evolutionary history. We additionally used β-mean pairwise distance (β-MPD), which is the overall mean phylogenetic distance between taxa in two communities and thus also captures deeper phylogenetic distances, indicating turnover of deeper branching phylogenetic lineages (Fine and Kembel, 2011; Liu et al., 2017). β-MNTD and β-MPD were calculated with abundance weighting using the functions 'comdistnt' and 'comdist', respectively, of the 'picante' package. Differences in microbial community composition were illustrated by non-metric multidimensional scaling (NMDS) using the 'metaMDS' function of the 'vegan' package (version 2.5.3; Oksanen et al., 2018a).

Environmental variables were standardized to z-scores for all analyses. Variables containing censored data, that is values below the detection limit (nitrate: $<0.131\,\mathrm{mg\,L^{-1}}$; orthophosphate: $<22.2\,\mathrm{\mu g\,L^{-1}}$), were handled according to Helsel (2011) using rank-transformation with tied ranks for values below the detection limit. Overall environmental differences were calculated as standardized Euclidean distances between samples considering all measured variables. Permutational analysis of multivariate dispersion (PERMDISP; Anderson, 2006) was used for pairwise tests of differences between regions in microbial community beta diversity, environmental differences, and spatial distance between sampling locations, respectively, using the 'betadisper' function ('vegan') with 10 000 permutations. Differences in ASV richness were assessed using Kruskal-Wallis non-parametric analysis of variance with Dunn's mean rank sum tests for pairwise comparisons and Holm correction for multiple testing.

We used the null model approach described by Stegen et al. (2012, 2013) to study the contributions of species sorting, dispersal processes, and drift on community turnover within as well as across regions. This approach is based on two steps: under the assumption that phylogenetic similarity between closely related taxa approximates ecological similarity, the strength of species sorting is evaluated in the first step based on the β-nearest taxon index (β-NTI). β-NTI is the standardized effect size of β-MNTD, which measures how much the observed difference between a pair of communities differs from

a null distribution of β-MNTD calculated with randomized phylogenetic relationships. For this randomization, taxa labels and abundances are repeatedly shuffled across the tips of a phylogenetic tree. As such, β-NTI indicates whether the observed phylogenetic differences between two communities are as large, larger, or smaller than expected given the differences in taxa abundances between the communities. Values of β-NTI < -2 (β-NTI $> +2$) indicate departures from the mean of the null distribution by more than two standard deviations, which means that two communities are composed of taxa that are phylogenetically significantly more (less) closely related than expected under the null distribution, suggesting selection of similar (different) taxa in both communities (referred to as homogeneous and variable selection, respectively, *sensu* Dini-Andreote et al. (2015) and Stegen et al. (2015)). Absolute values of β-NTI <2 indicate no significant deviation from the null distribution, suggesting that processes other than species sorting are responsible for observed differences in community composition, that is dispersal, dispersal limitation, and drift (a graphical example of the calculation of β-NTI is given in Fig. 2.1.2).

In this case, the RC_{bray} index is used in the second step of the null model approach to identify the nature of these alternative processes. Because phylogeny is assumed to be irrelevant to the chance of taxa being subject to dispersal, dispersal limitation, or random drift, RC_{bray} does not consider phylogenetic relationships to calculate differences between communities, but only uses information on taxa occurrence and abundance. RC_{bray} is an extension of the Raup-Crick metric (Chase et al., 2011), which was modified by Stegen et al. (2013) to not only consider presence-absence of taxa, but also take into account abundances. Thus, while the original metric is based on Jaccard dissimilarity, RC_{bray} uses Bray-Curtis dissimilarity instead. The procedure starts by randomly assembling local communities from taxa found in the dataset. To this end, single individuals of each taxon are drawn into communities, until the empirically observed taxon richness in each community is reached. The probability of drawing an individual of a given taxon into a community is proportional to the number of communities occupied by that taxon. At this point, each taxon is represented by a single individual (in our case sequence read) in each community. Subsequently, further individuals are added to each community, but only for taxa that were drawn into a given community in the previous step. Here, the probability of drawing additional individuals of a taxon is proportional to that taxon's abundance observed across all communities in the dataset. This probabilistic assembly simulates community assembly under the assumption of pure random drift acting alone, akin to Hubbell's neutral theory (Hubbell, 2001). For each pair of communities that is to be compared, both communities are repeatedly assembled following the above

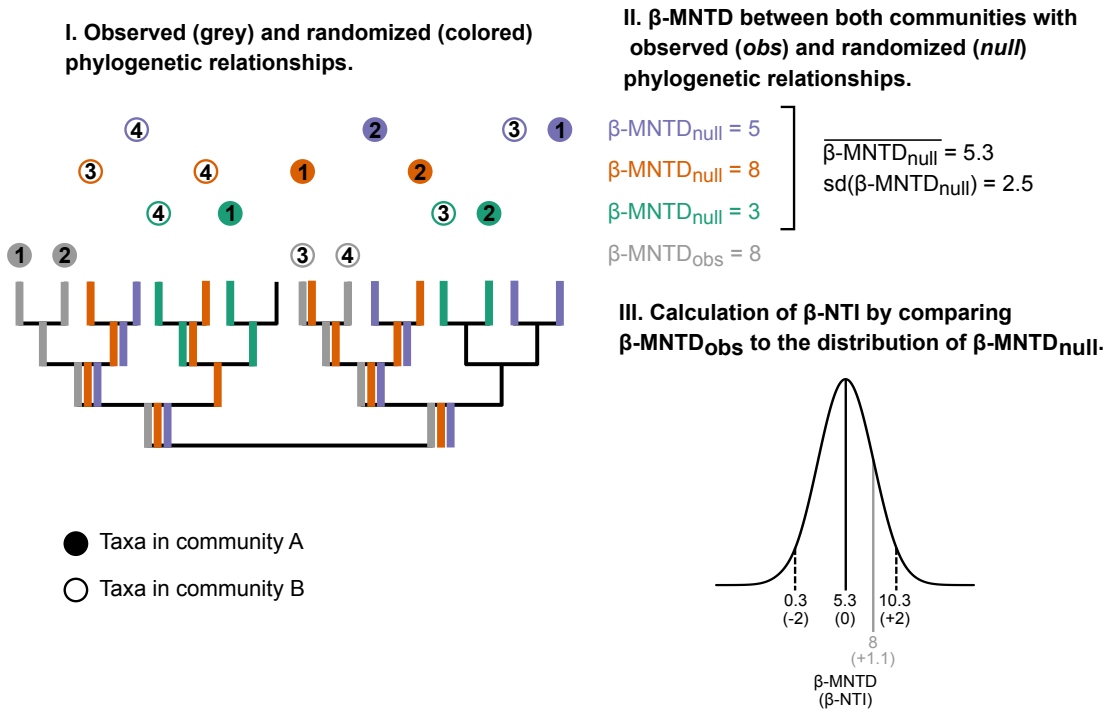

Figure 2.1.2: Schematic illustration of the calculation of β-NTI. The example compares two communities, each containing two taxa. Community A (closed symbols) contains taxa 1 and 2; community B (open symbols) contains taxa 3 and 4. For simplicity, the lengths of all branches in the phylogenetic tree as well as taxa abundances are assumed to be equal, all having a value of 1. The observed phylogenetic relationships are shown in grey in panel ***I***. The mean branch length connecting taxa in community A to their closest relatives in community B (and vice versa) gives the observed β-MNTD which in this case equals 8. To generate a null distribution, β-MNTD is calculated multiple times (in this example 3x) with randomized phylogenetic relationships shown in color in panel ***I*** and ***II***, where each color represents a single iteration. For example, after the first randomization (shown in green), taxon 1 in community A is separated by 4 branch lengths from its closest relative in B, which now is taxon 4; taxon 2 and its closest relative in community B (now taxon 3) are separated by 2 branch lengths. Thus, β-MNTD after the first randomization equals 3. Multiple iterations of this randomization yield the null distribution of β-MNTD to which the observed β-MNTD is compared as shown in panel ***III***. In this case, the null distribution has a mean value of 5.3 and a standard deviation of 2.5. The observed β-MNTD lies 1.1 standard deviations away from the mean of the null distribution, thus β-NTI =1.1, which falls within the thresholds of −2 and +2. Accordingly, the taxa in community A and B are as closely related as expected by chance, suggesting no significant effect of species sorting. (*Modified from Stegen et al.,* 2012).

procedure. The Bray-Curtis dissimilarity between both communities is calculated after each iteration to generate a null distribution of dissimilarities that are expected if random drift was alone responsible for differences between the two communities. As above for β-NTI, the empirically observed Bray-Curtis dissimilarity between the pair of communities is compared to this null distribution to obtain RC_{bray}. Values of RC_{bray} < -0.95 ($> +0.95$) indicate significant departures from the null distribution, such that two communities share more (less) taxa than expected by chance, which is interpreted as homogenizing dispersal (dispersal limitation in combination with drift) being responsible for the observed difference between a pair of communities. Absolute values of RC_{bray} <0.95 indicate no significant departure from the null distribution, which means that two communities share as many taxa as expected by chance, pointing towards random drift acting alone. β-NTI and RC_{bray} were calculated as in Stegen et al. (2012, 2013) with 999 randomizations. For the analyses within regions, β-NTI and RC_{bray} were calculated for each region separately based on null distributions that only considered ASVs found within a given region. For the analysis across regions, β-NTI and RC_{bray} were calculated across all samples with ASVs found in the full dataset.

As alluded to above, the ecological inference drawn from β-NTI regarding the influence of species sorting on differences between communities is based on the assumption that phylogenetic similarity between taxa across short phylogenetic distances approximates ecological similarity. This requires that phylogenetic distance between taxa correlates positively with differences in environmental optima (i.e. environmental optima have a phylogenetic signal). We tested this assumption for our dataset using Mantel correlograms as done by others (Wang et al., 2013; Dini-Andreote et al., 2015). Differences in environmental optima between ASVs were estimated as standardized Euclidean distances between relative abundance-weighted means for environmental variables that were shown to have a significant effect on microbial community composition by distance-based redundancy analysis (db-RDA; see below) (Stegen et al., 2012; Dini-Andreote et al., 2015). The phylogenetic signal was evaluated at phylogenetic distance steps of 2 % of the maximum phylogenetic distance with Mantel correlograms using Pearson correlation and 999 permutations for significance testing; p-values were adjusted for multiple testing using progressive Holm correction (function 'mantel.correlog'; 'vegan'). The analysis was done for each region separately only considering ASVs found within a given region, as well as with ASVs found across regions in the full dataset. For the latter, we randomly selected 4500 ASVs similar to Dini-Andreote et al. (2015), since an analysis comprising all 9153 ASVs was computationally unfeasible. In all cases, significant positive correlations were found mainly over short phylogenetic distances (12 % to 18 % of the maximum phyloge-

netic distance; Fig. S1.1), confirming that the assumption of a phylogenetic signal was met for our dataset.

We used variation partitioning based on db-RDA to examine the relative importance of local environmental conditions, spatial distance with regions, and region identity on differences in microbial community composition, and the degree to which these components were responsible for species sorting. Regular redundancy analysis (RDA) is a linear modeling approach that can be thought of as multiple linear regression with multiple response variables contained in a site-by-species matrix, where each response variable is the abundance of a species across samples/sites, which is modeled in relation to sets of explanatory variables such as environmental or spatial variables. db-RDA is an extension of this method that can handle any type of dissimilarity matrix as input and tries to model dissimilarities between samples in response to sets of explanatory variables (Legendre and Anderson, 1999). Variation partitioning allows to dissect the explained variation in the response matrix between individual explanatory variables, or sets thereof (Legendre, 2008). Suppose the aim was to model the response of a site-by-species matrix Y with respect to a set of environmental and spatial explanatory variables E and S, respectively, using RDA. To this end, individual RDA models are computed containing each set of explanatory variables separately, as well as a full model containing both sets. For each model, the adjusted R^2 is calculated, which is the fraction of explained variation adjusted for the number of variables included in the model. The adjusted R^2 of the full model $Y \sim E + S$ is the sum of the variation in Y explained by E alone ($[a]$), the variation explained by S alone ($[c]$), and the variation shared between E and S ($[b]$), which represents the effect of spatially structured environmental variables. Thus, $R^2_{Y\sim E+S} = [a + b + c]$. The adjusted R^2 of the individual models ($Y \sim E$ and $Y \sim S$) still contain the variation explained by the shared effects between E and S ($[b]$). Hence $R^2_{Y\sim E} = [a+b]$ and $R^2_{Y\sim S} = [b+c]$. To calculate the fraction of explained variation only accounted for by the individual effects of E and S, respectively controlling for the effect of the other (i.e. $[a]$ and $[c]$), their shared effect $[b]$ is calculated from the adjusted R^2 of the full model and both individual models: $R^2_{Y\sim E\cap S} = [b] = [a+b] + [b+c] - [a+b+c]$. Subsequently, $[a]$ and $[c]$ are computed by subtracting the calculated fraction $[b]$ from the adjusted R^2 of both individual models, that is $R^2_{Y\sim E|S} = [a] = [a + b] - [b]$ and $R^2_{Y\sim S|E} = [c] = [b + c] - [b]$. The fractions calculated this way can yield negative values for the adjusted R^2, which however merely indicates that the given fraction explains less of the variation than expected for a random variable. Hence, negative adjusted R^2 values for individual fractions are interpreted as 0. Finally, the residual fraction representing the variation in Y that remained unexplained in the model is calculated by subtracting

the variation explained by the full model from 1: $Res = 1 - [a + b + c]$ (Legendre, 2008).

To study the effect on differences in microbial community composition, we used β-MNTD and β-MPD as response matrices in the db-RDA models, respectively. Local environmental conditions were represented by standardized environmental variables. To reduce variance inflation, collinear environmental variables (electrical conductivity, sodium, calcium, magnesium, chloride, and sulfate concentrations) were replaced by the first principal component resulting from a principal component analysis (PCA) of these six variables (referred to as ionPC1). IonPC1 was significantly positively correlated with all six variables and explained 74 % of the variance. Environmental variables were selected by forward selection using the adjusted R^2 of a full db-RDA model containing all environmental variables as stopping criterion (Blanchet et al., 2008) (function 'ordiR2step' with 10 000 permutations; 'vegan'). Calculation of variance inflation factors (VIF) (function 'vif.cca'; 'vegan') confirmed low degrees of redundancy among the selected variables in all models (all VIF <2). The marginal significance of each selected environmental variable was assessed using permutation tests (function 'anova.cca' with 10 000 permutations; 'vegan').

Spatial distances within and across regions were included as separate components in the db-RDA models following the approach used by Declerck et al. (2011). Spatial distance between sampling locations within regions were represented by Moran's eigenvector maps (MEMs). MEMs are sets of orthogonal vectors derived from principal coordinate analysis on Euclidean geographic distances between connected sites, where individual vectors represent distances between sites at different spatial scales (Dray et al., 2006). Converting a Euclidean geographic distance matrix into sets of MEM vectors enables the inclusion of spatial distances over different scales as explanatory variables in statistical models like (db-)RDA. However, this approach does not work well if there are large gaps between sties, as is the case between unconnected sites located in different regions. Declerck et al. (2011) solved this problem by representing large spatial distances across regions as a dummy-coded variable matrix, and computing a staggered MEM matrix, where MEM vectors are arranged in blocks such that each block represents the spatial variation between locations within a given region, while locations from different regions are assigned a value of 0. The MEM matrix was constructed using the 'create.dbMEM.model' function in the 'adespatial' package (version 0.3-2; Dray et al., 2018). Permutation tests were used as above to assess the overall significance of each component (i.e. region identity, MEM matrix, and the set of selected environmental variables) in individual db-RDA models and only significant components were subsequently used for variation partitioning (function 'varpart'; 'vegan').

To study the effect on selection processes, we repeated the db-RDA and subsequent variation partitioning including the same three explanatory components as above, but this time using the β-NTI matrix calculated across all samples as response matrix. The rationale behind this approach is that changes in β-NTI should only result from species sorting, because the effects of processes related to dispersal and drift are accounted for in the null distribution by maintaining species abundances within samples during the randomization of phylogenetic relationships (Stegen et al., 2013; Wang et al., 2013). Accordingly, the fraction of variation in β-NTI explained by variables used to estimate local environmental conditions indicates that these variables impose species sorting, whereas a significant effect of spatial distance or region identity would indicate selection by spatially structured unmeasured environmental variables or broad-scale region-specific factors, respectively, rather than dispersal limitation. Since db-RDA requires only positive distance values, β-NTI was scaled to range between 0 and 1 as in Stegen et al. (2013).

2.2 Assembly and succession of microbial communities during the colonization of groundwater sediments

2.2.1 Site description, experimental setup, and origin of the raw data

The field experiment, collection of the raw data, DNA extraction, 16S rRNA gene amplification, and sequencing for this study were carried out within the PhD project of Dr. Yuxiang Zhou (Zhou, 2013) and are briefly outlined here for the sake of completeness.

The field experiment was conducted over a period of 347 days from March 2010 until February 2011 with intermediate sampling campaigns in May (day 49), July (day 115) and December (day 263). The area with the two monitoring wells used for the incubation of *in situ* microcosms was located at the foothills of the German Alps in the upper Isar River Valley close to the village of Mittenwald (Fig. 2.2.1). The wells were installed in a pristine, shallow, porous aquifer composed of quaternary sediment mainly consisting of gravel and coarse sand. Well MIT052 was located on a mountain pasture in the forested Riedboden nature reserve ~400 m away from the nearby river; well MIT039 was located ~2 km away from MIT052 in proximity to the village with a distance of ~240 m to the river (for a detailed site description, see Zhou et al., 2012).

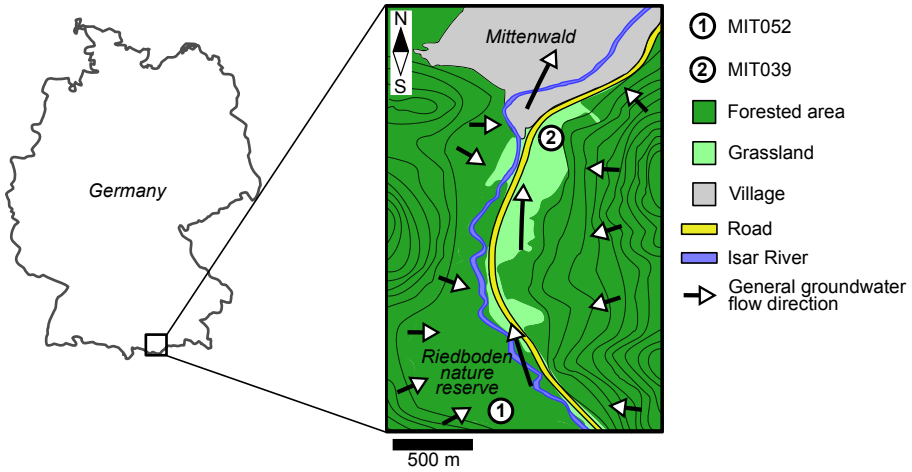

Figure 2.2.1: Schematic illustration of the main geographical features of the study site, the locations of the two monitoring wells, and general groundwater flow directions. (*From Fillinger et al.,* 2019c).

Sediments for the *in situ* microcosms were taken from the Isar river that drains the aquifer. Sediments were sieved (0.2 mm to 0.63 mm) and packed into perforated polyethylene columns with a mesh size of 1 mm to 2 mm. Sediment columns were submerged in

deionized water and sterilized by autoclaving five times at 121 °C for 30 min. After each autoclaving step, the sediments were rinsed with and again submerged in fresh deionized water. The columns were stored at 4 °C submerged in sterile water until the start of the incubation experiment. Replicate sediment columns were incubated in each well; duplicate columns were sampled destructively at each sampling campaign. Samples for DNA extraction were put on dry ice for transport to the lab, and were stored at −20 °C until DNA extraction using the protocol described by Anneser et al. (2010). For the comparison of sediment-attached versus planktonic microbial communities, cells from 5 L groundwater obtained by pumping as described in Section 2.1.1 were collected on a 0.2 µm polycarbonate filter on-site; filters were transported and stored as done with the sediment columns. For measurements of total prokaryotic cell counts, 0.5 mL groundwater (or 0.5 cm^3) was fixed on-site with glutardialdehyde as described in Section 2.1.1; fixed samples were stored in the dark at 4 °C until further processing. Cells were stained with SYBR Green I as above (Section 2.1.3) and subsequently counted using a LSR II flow cytometer (Becton Dickson, Heidelberg, Germany) according to Bayer et al. (2016). Physicochemical parameters of the groundwater (pH, temperature, electrical conductivity, and concentrations of DOC, AOC, dissolved oxygen, orthophosphate, sulfate, nitrate, chloride, potassium, sodium, calcium, and magnesium) were measured as described by Zhou et al. (2012) (Fig. S2.1).

PCR amplification (28 cycles) and subsequent bidirectional 454-pyrosequencing of 16S rRNA gene fragments was done according to Pilloni et al. (2011) using the primers Ba27f (5'-AGAGTTTGATCMTGGCTCAG) and Ba519r (5'-TATTACCGCGGCKGC TG) (Lane, 1991) extended with sequencing adapters and multiplex barcodes. Each of the sample duplicates was amplified again in duplicate; after amplification, all replicates of a given sample were combined, followed by purification using magnetic beads (AMPure-XP; Beckman Coulter) according to the manufacturer's instructions. After purification, DNA concentrations were determined using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen). Barcoded amplicons from all samples were pooled in equimolar amounts before sequencing on a 454 GS FLX pyrosequencer using Titanium chemistry (Roche, Penzberg, Germany).

2.2.2 Sequence data processing

Sequence data were processed in QIIME (version 1.9.0; Caporaso et al., 2010). Demultiplexing and quality filtering (min./max. sequence length: 250/600 bp; primer mismatches and barcode errors: 0; min. quality score: 25; quality score window size:

50 bp) was done using the 'split_libraries.py' command. Chimera filtering was done by mapping reads against the SILVA SSU reference data base (release 128; Quast et al., 2013) using the 'identify_chimeric_seqs.py' command with usearch61 as detection method. After quality and chimera filtering, the average number of combined forward and reverse reads per sample was 5709 with an average length of 388 bp. OTUs were clustered by uclust against the SILVA SSU reference database at 97 % similarity using the 'pick_open_reference_otus.py' command. To reduce data sparsity, OTUs with a combined abundance of <0.01 % across all samples were removed, in addition to OTUs classified as chloroplasts, leaving a total of 910 OTUs in the final OTU table. The total number of reads per sample was rarefied to 2045, which was the lowest number of reads observed for a single sample. A midpoint-rooted phylogenetic tree was constructed from the alignment of OTU reference sequences using FastTree (Price et al., 2009). Sequence data have been deposited in the NCBI Sequence Read Archive (accession no. SRP139256).

2.2.3 Data analysis

All analyses were done in R. Alpha diversity (OTU richness (S) and Shannon diveristy (H')) was calculated using the 'vegan' package (version 2.5-2; Oksanen et al., 2018b). The number of newly-arriving OTUs (Sn) in sediment samples was defined as the number of OTUs that displayed an abundance >0 % in a community for the first time at a given time point. Phylogenetic beta diversity was assessed based on abundance-weighted β-MNTD calculated as in Section 2.1.7. Differences in microbial community composition across time, space, and community type (i.e. sediment-attached and planktonic) were illustrated by NMDS as above (Section 2.1.7). To test for the effect of physicochemical parameters on changes in community composition of sediment-attached and planktonic communities, respectively, physicochemical variables were standardized to z-scores before fitting to the NMDS ordination using the 'envfit' function of the 'vegan' package with 10 000 permutations stratified within community types. Permutational multivariate analysis of variance (PERMANOVA; Anderson, 2001) was used to estimate the marginal effects of each of the three categorical variables community type, sampling time point, and site location, respectively controlling for the effects of the other two variables, using the 'adonis2' function with 10 000 permutations ('vegan'). For the identification of key organisms that were responsible for the differences between community types, similarity percentage (SIMPER) analysis (function 'simper' with 1000 permutations; 'vegan') was used on relative OTU abundances summarized at genus level. We applied parti-

tioning of beta diversity according to Baselga (2012), to estimate the extent to which sediment-attached communities were subsets of the planktonic species pool, and to assess the degree of temporal community turnover in the sediment-attached communities over the course of the succession. This approach is based on the additive partitioning of incidence-based Jaccard dissimilarity between two communities into a nestedness and a turnover component. A high contribution of nestedness to the total dissimilarity indicates that two communities are subsets of each other and that differences are caused by differences in species richness, that is gain or loss of species in one community relative to another. On the other hand, a high contribution of turnover indicates little overlap in species composition, such that species in one community have been replaced by other species in the other community. Beta diversity partitioning was done using the 'betapart' package (Baselga and Orme, 2012).

We calculated β-NTI and RC_{bray} from null model simulations as explained above (Section 2.1.7) to assess the impact of species sorting, dispersal, and drift on differences in community composition 1) across space between the two sites within community types and time points; 2) between sediment-attached and planktonic communities within sites and time points; and 3) between consecutive time points within community types and sites. For the calculation of null distributions, OTUs from the entire dataset were considered. The assumption of a significant phylogenetic signal was tested separately for sediment-attached and planktonic communities, respectively, using Mantel correlograms as above (Section 2.1.7). Significant positive correlations were found over short phylogenetic distances (2 % to 7 % of the maximum phylogenetic distance; Fig. S2.2), confirming that the assumption of a phylogenetic signal was met. To test for the effect of changes in physicochemical conditions on species sorting, Mantel correlations based on Spearman's rank correlation were calculated between the β-NTI matrix and individual Euclidean distances calculated for each physicochemical variable separately. Mantel tests were computed using the 'mantel' function ('vegan') with 10 000 permutations for significance testing.

2.3 Ecological groundwater monitoring based on microbiological parameters

2.3.1 Origin of the data and description of the disturbance case studies

The data for this study originated from different research projects and collaborations, and were collected at different sites located across Germany (Fig. 2.3.1).

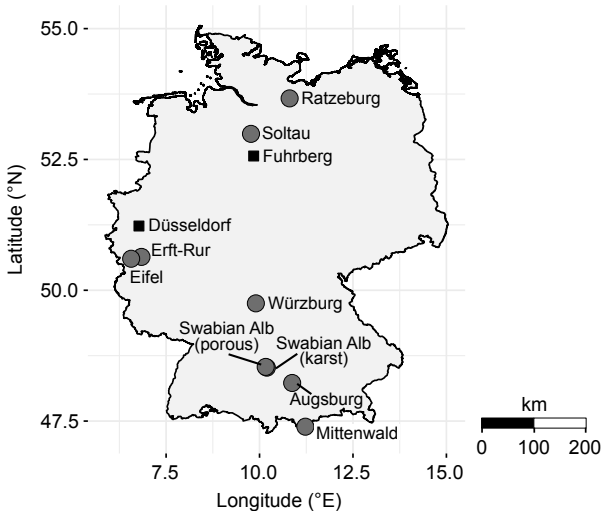

Figure 2.3.1: Schematic map with the locations of the different regions considered in this study. Black squares represent the two regions where data were collected for the disturbance case studies (data for the organic contamination and the surface water intrusion case study were collected in Düsseldorf; data for disturbances related to different types of land use were collected in Fuhrberg). Grey dots represent the nine regions that were analyzed for geographic differences in D-A-(C) signatures of non-contaminated aquifers. (*From Fillinger et al.,* 2019b).

For the first disturbance case study, we simulated a severe organic contamination event by combining data from a non-contaminated site in the city of Düsseldorf with data from a nearby former coal gasification site in Düsseldorf-Flingern that has a long history of organic contamination with aromatic hydrocarbons (BTEX and polyaromatics). Here, samples were collected across the vertical profile of the contaminant plume using peristaltic pumps connected to the ports of multilevel wells. Out of a total pool of 65 contaminated samples that were collected during different sampling campaigns in 2005, 2006 and 2007 (see Anneser et al., 2008; 2010; Jobelius et al., 2011), data from 30 randomly selected samples were combined with data from 81 non-contaminated samples.

The data for these non-contaminated samples were taken from a total pool of 108 samples that formed the basis for the second case study regarding the impact of surface water intrusion. These samples were collected between January and October 2018 from an alluvial aquifer at the bank of the Rhine River in Düsseldorf at a site where drinking water is produced via river bank filtration. At this site, groundwater monitoring wells were located along a transect perpendicular to the river shore at different distances to the river: sets of three wells each were situated close to the river at distances of about 2 m and 20 m, respectively; this area of the aquifer receives river water under normal conditions due to river bank filtration. Another set of three wells and one single well were located approximately 60 m and 75 m away from the river, respectively, in an area which usually does not come into contact with the river water but is recharged from landside groundwater. However, high water levels of the Rhine River from January until March 2018 led to an increased intrusion of river water into the aquifer at wells close to the river, as well as surface water intrusion in the more distant areas, which was revealed by stable water isotope signatures (Fig. S3.1).

For the third case study regarding the impact of different types of land use, we analyzed data collected in Fuhrberg near the city of Hannover, where areas with different types of land use could be found close to each other. A total of 29 samples were collected between spring and summer 2018, comprising 9 samples from a forest, and 10 samples from a grassland and an agricultural land, respectively.

For assessing differences in D-A-(C) signatures between regions, we analyzed data from non-contaminated aquifers in nine different regions across Germany (Fig. 2.3.1). Non-contaminated groundwater in this context is defined here as shallow groundwater that, although being exposed to a multitude of potential anthropogenic impacts (e.g. waste water, agriculture, or urban run-off), did not display significantly elevated concentrations of concerning chemicals or metals at the time of sampling (mainly based on thresholds set for drinking water). Samples were collected in each region at least during two sampling campaigns between the years 2007 and 2017. An in-depth description of the individual regions would go beyond the scope of this study; instead, a summary of key information is given in Table 2.3.1.

Except for the samples from the organically contaminated site as mentioned above, groundwater was collected as described in Section 2.1.1. Samples for measurements of total prokaryotic cell counts were collected as above (Section 2.1.1). Samples for ATP measurements were collected in autoclaved glass bottles. Glassware for the collection of AOC samples was baked at 450 °C for 4 h prior to sampling and closed with plastic caps with Teflon inlays that were treated with 10 % w/v sodium persulfate at 60 °C for 1 h to

Table 2.3.1: Overview of sample sizes, aquifer characteristics, and sampling time points for the non-contaminated aquifers that were analyzed for differences in D-A-(C) signatures between regions. (*From Fillinger et al.,* 2019b).

Region	n[a]	Unique wells[a]	Aquifer type	Dominating aquifer material	Dominating redox condition	Approximate span of the sampled area (km)	Groundwater temperature (°C)[b]	pH[b]	Electrical conductivity ($\mu S\,cm^{-1}$)[b]	Sampling campaigns
Ratzeburg	12 (6)	6 (6)	porous	gravel, fine sand	hypoxic-anoxic	3.5	9.6 ± 0.4	7.0 ± 0.6	595 ± 266	spring & autumn 2009
Soltau	33 (16)	17 (16)	porous	gravel, fine sand	hypoxic-anoxic	5	8.7 ± 1.6	6.9 ± 0.9	413 ± 128	spring & autumn 2009
Erft-Rur	20 (10)	10 (10)	porous	gravel, fine sand	oxic	20	10.8 ± 1.6	6.7 ± 0.2	964 ± 278	spring & autumn 2007, 2009
Eifel	22 (11)	11 (11)	fractured	sandstone	oxic	5	10.3 ± 0.5	7.2 ± 0.2	690 ± 236	spring & autumn 2009
Würzburg	40 (29)	16 (15)	porous	gravel, fine sand	oxic	0.5	13.7 ± 2.8	7.2 ± 0.2	972 ± 174	spring & autumn 2016; winter 2017
Swabian Alb	27 (13)	14 (13)	porous	coarse gravel	oxic	20	10.4 ± 0.9	7.1 ± 0.1	768 ± 96	spring & autumn 2007, 2009
Swabian Alb	31 (15)	16 (15)	karst	limestone	oxic	20	10.8 ± 1.0	7.1 ± 0.1	686 ± 47	spring & autumn 2007, 2009
Augsburg	23 (15)	12 (10)	porous	gravel, fine sand	oxic	12	11.2 ± 1.0	7.3 ± 0.2	603 ± 80	spring & autumn 2016
Mittenwald	25 (8)	14 (8)	porous	gravel, coarse sand	oxic	3.5	7.2 ± 0.8	7.8 ± 0.2	344 ± 60	spring & autumn 2009
Total dataset	233 (123)	116 (104)								

[a]AOC data were not available for all samples; the value within parentheses gives the number of samples and unique wells, respectively, for which AOC data were available.

[b]Mean ± standard deviation calculated across all samples.

remove traces of organic carbon. Samples were stored in the dark at 4 °C for transport to the lab and until analysis (within 48 h after sampling).

2.3.2 Determination of the D-A-(C) parameters

Total prokaryotic cell counts were determined by flow cytometry as in Section 2.1.3.

ATP was measured using the BacTiter-Glo Microbial Cell Viability Assay kit (Promega, Madison, WI, USA) based on the method by Hammes et al. (2010) with slight modifications. The measurement is a luminescence assay based on the ATP-dependent oxidation of luciferin catalyzed by luciferase. The kit offers a single-step sample preparation protocol by combining the bacterial cell lysis buffer and the luciferase enzymes in a single reagent. Samples and the BacTiter-Glo reagent, prepared according to the manufacturer's instructions, were pre-warmed separately at 38 °C for at least 2 min before mixing 1 mL sample with 50 µL reagent. The mixture was incubated at 38 °C for 1 min. Following the incubation, luminescence was measured on a GloMax 20/20 Luminometer (Promega). Concentrations were determined by comparison with external ATP standards dissolved in ATP-free water (both Fisher Scientific, Waltham, MA, USA), using ATP-free water as blank. To correct for the contribution of extracellular ATP in the samples to the measured luminescence signal, the measurements were performed on an unfiltered sample fraction, representing the total ATP concentration in the sample, and additionally on a fraction of the same sample that was passed through a 0.1 µm polyvinylidene fluoride Millex syringe filter (Merck, Kenilworth, NJ, USA) before sample preparation to remove cells, thus containing only extracellular ATP. The concentration of intracellular ATP was then calculated by subtracting the extracellular concentration from the total (Hammes et al., 2010). All measurements were carried out in triplicate.

AOC concentrations were determined indirectly with a batch growth assay based on the method by Hammes and Egli (2005). The assay quantifies prokaryotic cell growth of a natural microbial community in a water sample on the bioavailable carbon present in that sample during an incubation under *in situ* conditions until stationary phase (in our case over a period of 30 days). To this end, a sample was diluted 10:1 with filter-sterilized groundwater from the same site. The filtered groundwater served as carbon source to initiate batch growth of the cells derived from the unfiltered sample. Samples for the determination of total prokaryotic cell counts were taken immediately at the beginning of the incubation and at the end. The cells that were produced during the incubation were used as a measure for the amount organic carbon that was assimilated into microbial biomass. Thus, the AOC concentration was calculated from the average

net growth determined from triplicate incubations by converting the number of produced cells into carbon with a constant factor of 20 fg organic C cell^{-1} (Griebler et al., 2002). For the incubation, 80 mL diluted groundwater was incubated in 100 mL glass bottles at *in situ* groundwater temperature in the dark with shaking at 100 rpm. Cell numbers were measured as total prokaryotic cell counts by flow cytometry. All glassware used for the incubations were treated to remove traces of organic carbon as above.

2.3.3 Determination of hydrochemical parameters and isotope ratios

Groundwater temperature, pH, electrical conductivity, and oxygen concentrations were measured on-site using field sensors (WTW, Weilheim, Germany). DOC Concentrations were measured in samples passed through a 0.45 µm filter (VWR, Radnor, PA, USA) that was rinsed three times with ultra-pure water prior to sample filtration to remove residual carbon. Samples were filtered on-site and subsequently acidified with 20 mM HCl (final concentration). DOC was determined by high-temperature (680 °C) catalytic oxidation on a Shimadzu TOC-LCPH Analyzer equipped with a non-dispersive infrared detector. Nitrate concentrations were measured by ion chromatography at GBA Group laboratories (GBA Group, Gesellschaft für Bioanalytik mbH, Hamburg, Germany). Stable isotope ratios of oxygen ($^{18}O/^{16}O$) were determined using a PICARRO L2130-i Isotopic Water Analyzer (PICARRO, Santa Clara, CA, USA). $\delta^{18}O$ ratios were measured in samples via equilibration with CO_2 at 18 °C for 5 h under constant shaking. $\delta^{18}O$ ratios were determined relative to internal standards that were calibrated against V-SMOW, V-GESP, and V-SLAP standards (International Atomic Energy Agency, Vienna, Austria). All measurements were performed at least in duplicate.

2.3.4 Data analysis

In order to detect disturbances in the three case studies, we combined the signals of the D-A-(C) variables in a multivariate analysis as schematically shown in Figure 2.3.2. Under normal undisturbed conditions, samples are expected to display a certain distribution in a multivariate space along these variables. For multivariate normally distributed data, a cloud of samples arrange in an elliptical form, which is defined by the mean values of the variables forming the center of the ellipse, the variances of the individual variables, and the covariance between them, determining the shape and slope of the ellipse. Disturbances that cause severe changes in one or more of these variables increase the distance of affected samples to the center of the ellipse beyond the range of distances expected due to random variation. In other words, samples affected by a disturbance can

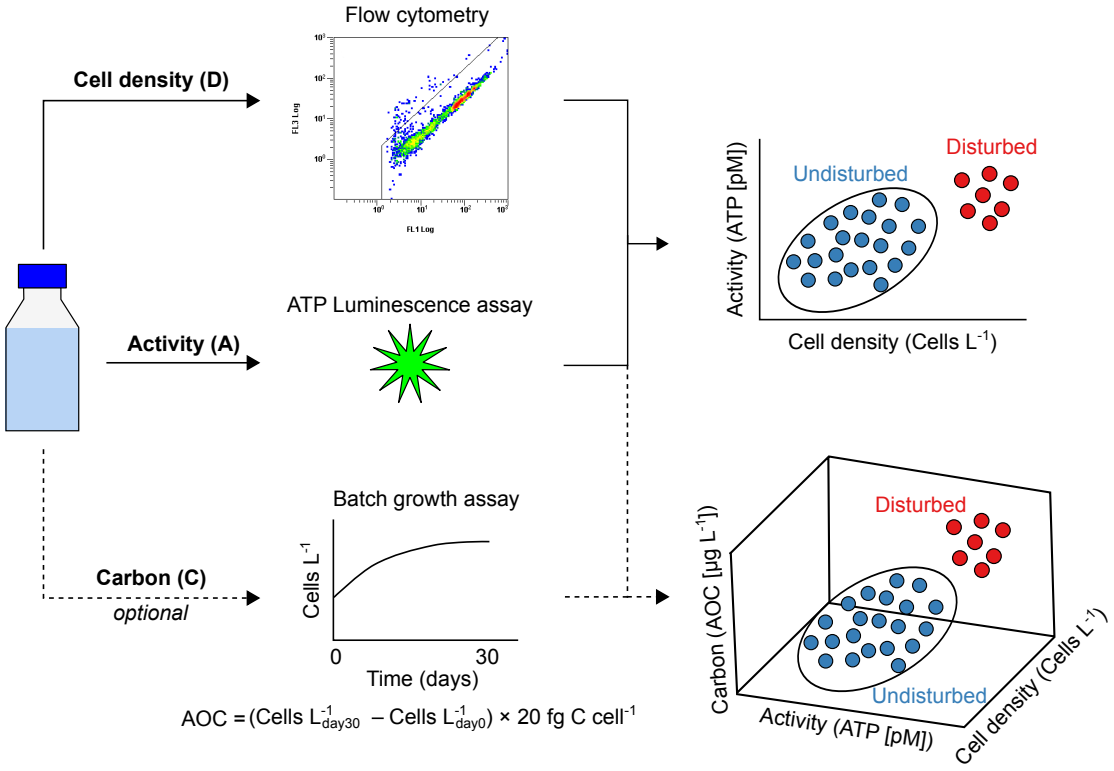

Figure 2.3.2: Schematic overview of the D-A-(C) workflow. The measured variables are combined in a multivariate analysis to detect disturbances. Samples affected by a disturbance are identified as multivariate outliers that deviate from the distribution of undisturbed samples along the measured variables. The D-A-(C) index is used to distinguish significant multivariate outliers from random variation in the data as explained in the main text. (*From Fillinger et al.*, 2019b)

be considered outliers. The distance of a single sample to the center is calculated as the Mahalanobis distance according to Equation 2.3.1:

$$MD_i = \sqrt{(X_i - \mu)' \times S^{-1} \times (X_i - \mu)}, \tag{2.3.1}$$

where X_i is a vector with the values of the individual variables for a single sample, and μ and S^{-1} are the vector with the variable means and the inverse of the covariance matrix, respectively, calculated from the full data. This distance is unitless and indicates how far a given sample lies away from the center of a multivariate distribution. Hence it can be considered a multidimensional extension of univariate z-scores (Manly, 1994). The

Mahalanobis distance takes values of the square root of a chi-squared distribution with as many degrees of freedom as there are variables in the analysis (e.g. $df = 3$ when all three D-A-C variables are considered in the analysis). This information is used to set thresholds to distinguish significant outliers from random variation in the data. For example, 97.5 % of the values of a chi-squared distribution with three degrees of freedom are <9.35. Thus, a sample with a Mahalanobis distance above the critical value of $\sqrt{9.35}$ would be declared an outlier at a 0.975 confidence level and consequently be interpreted as a sign of a disturbance (a simple numerical example of the calculation of Mahalanobis distances is given in Supporting Information SI.3 along with Fig. S3.4).

Problems arise, however, if Mahalanobis distances are calculated based on the raw data directly, since the presence of already a small fraction of outliers in the dataset can severely distort the estimates of μ and S. Therefore, we used the Fast-MCD algorithm, which can provide reliable robust estimates of μ and S in datasets contaminated with outliers, and calculated robust Mahalanobis distances based on these estimates (Rousseeuw and Van Driessen, 1999; Hubert and Debruyne, 2010). Briefly, the algorithm searches for a subset of samples of size h ($h = \frac{n+p+1}{2}$, where n is the number of samples in the dataset and p the number of variables) with the smallest determinant of S, that is with the smallest dispersion in the multivariate space, and subsequently calculates μ and S based on these h samples. We will refer to the final robust Mahalanobis distances as the D-A or D-A-C index, respectively, depending on the variables included in the analysis. A sample was declared an outlier at a 0.975 confidence level if its calculated index value exceeded the square root of the 0.975 quantile of a chi-squared distribution with the according degrees of freedom as described above (Rousseeuw and Van Driessen, 1999).

The strength of this multivariate approach is that it not only takes into account the variance of each variable, but also possible correlations (in the form of covariance) between these variables, which are not taken into account in separate univariate analyses of the individual variables (Manly, 1994). To illustrate this strength, we compared the multivariate D-A-(C) approach to a univariate method for outlier detection. For this univariate approach, separate thresholds were set for each variable based on the median and the median absolute deviation (MAD). This approach was chosen because the median and the MAD provide more robust estimates of the center and the dispersion of data, respectively, even in the presence of outliers, compared to the mean and standard deviation similar to what has been explained above. Using this approach, a sample was considered an outlier if it fell outside the range of $median \pm 2.5 \times MAD$ as recommended by Leys et al. (2013).

To assess differences in D-A-(C) signatures between regions, that is differences be-

tween the covariance matrices and variable means, we again applied the Fast-MCD algorithm to the data from the non-contaminated aquifers sampled at the different regions across Germany (Fig. 2.3.1). Box's M test was used to test for differences in covariance matrices (i.e. robust estimates of S) between regions. Differences in means for each variable were assessed using Welch's ANOVA followed by the corresponding Games-Howell post-hoc test for pairwise comparisons to assess differences between individual regions. Outliers for a given region that were detected based on the robust Mahalanobis distances were not considered for the analysis of differences in means.

All analyses were done in R. Variables were $\log_{10}$-transformed for all analyses to achieve normality. Normality was confirmed using the Shapiro-Wilk test implemented in the 'stats' package and its multivariate extension to test for multivariate normality implemented in the 'mvnormtest' package (version 0.1-9; Jarek, 2012). Robust estimates of μ and S and robust Mahalanobis distances with additional adjustment for sample size according to Pison et al. (2002) were obtained using the 'covMcd' function of the 'robustbase' package (version 0.93-3; Maechler et al., 2018). Confidence ellipses (two-dimensional) and ellipsoids (three-dimensional) were calculated at a 0.975 confidence level based on the robust estimates of μ and S using the 'ellipse' function of the 'ellipse' package (version 0.4.1; Murdoch and Chow, 2018) and the 'ellipse3d' function of the 'rgl' package (version 0.99.16; Adler and Murdoch, 2018), respectively. Box's M tests were performed using a modified version of the according function from the 'biotools' package (da Silva et al., 2017) with the modification that the robust estimates of S were used as input instead of the raw data. Welch's ANOVA was carried out using the 'oneway.test' function ('stats' package); Games-Howell tests were performed using the 'userfriendlyscience' package (version 0.7.2; Peters et al., 2018).

3 Results

3.1 Ecological drivers of differences in microbial community composition across geographically distinct aquifers

3.1.1 Differences in microbial community composition and environmental conditions within and across regions

Analyses of differences in environmental conditions and microbial community composition by PCA and NMDS based on β-MNTD, respectively, revealed distinct clustering of samples by region with little overlap of samples from different regions (Fig. 3.1.1). In terms of environmental conditions, regions along the North-South transect were mainly separated along the second PCA axis, mostly influenced by differences in pH and concentrations of oxygen, potassium, and DOC. Samples from the WUR region additionally separated from the other three regions along the first PCA axis, mostly influenced by temperature, electrical conductivity, and concentrations of various ions (summary statistics of individual environmental variables are listed in Table S1.1).

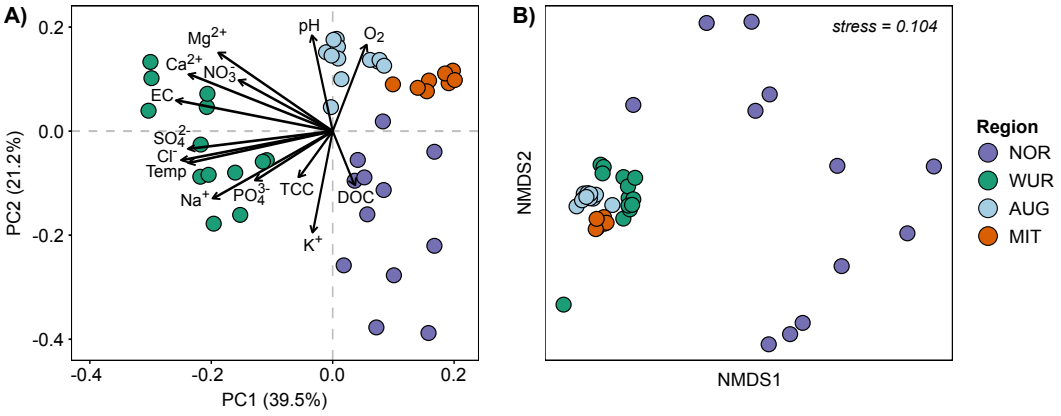

Figure 3.1.1: ***A***) PCA showing differences in local environmental conditions (O_2: dissolved oxygen; DOC: dissolved organic carbon; K^+: potassium; TCC: total prokaryotic cell counts; PO_4^{3-}: orthophosphate; Na^+: sodium; $Temp$: temperature; Cl^-: chloride; SO_4^{2-}: sulfate; EC: electrical conductivity; Ca^{2+}: calcium; NO_3^-: nitrate; Mg^{2+}: magnesium). ***B***) NMDS showing differences in microbial community composition based on abundance-weighted β-MNTD. (*From Fillinger et al.,* 2019a).

In contrast to the turnover of closely related ASVs across regions indicated by β-

MNTD, clustering of communities was weaker when differences in community composition were assessed across broader phylogenetic scales based on β-MPD (Fig. S1.2). Congruently, while >65 % of the ASVs were exclusively detected within a single region, the majority of higher taxonomic groups from phylum to genus level (∼69 % to 73 %) were observed across more than one region, further corroborating that differences between regions were mainly caused by turnover of related ASVs within broader clades such as genus or family. Regardless of the taxonomic level, taxa that occurred in more than one region also showed higher average relative abundances suggesting that local communities were dominated by more widespread taxa (Fig. S1.3). The most dominant taxonomic groups in all four regions were *Alpha-*, *Delta-*, and *Gammaproteobacteria*, in addition to *Bacteroidia*, *Actinobacteria*, and taxonomically unclassified bacteria (Fig. S1.4). Despite the dominance of these classes, community evenness calculated at the ASV level was high in all regions, with Pielou's index values ranging between 0.8 and 0.9 on average (Fig. S1.5). Accordingly, average relative abundances of the most dominant individual ASVs within a single region were relatively low ranging between 0.6 % and 6 %. These dominant ASVs were predominantly found within the families *Burkholderiaceae*, *Caulobacteraceae*, *Pseudomonadaceae*, and *Rhodocyclaceae* in the WUR, AUG, and MIT regions, and *Thiovulaceae*, *Gallionellaceae* as well as members of the *Thaumarchaeota* in the NOR region (Table S1.2).

Comparing the dispersion of samples in the PCA and NMDS analyses shown in Figure 3.1.1 suggested that regions with larger differences in environmental conditions also displayed larger differences in microbial community composition. This was confirmed by significant differences of within-region environmental heterogeneity and differences in community composition, respectively, revealed by pairwise PERMDISP tests (Fig. 3.1.2). Also in this case, patterns of observed differences in community composition based on β-MNTD matched the patterns of differences in environmental conditions better than β-MPD (Fig. S1.6).

Since we had to rely on access to pre-installed monitoring wells during the sampling campaigns, it was unfortunately not possible to obtain samples from each region with the same spatial coverage. However, these differences in spatial coverage did not seem to have biased the estimates of ASV richness (Faith's PD showed the same pattern as richness, Fig. S1.7), differences in microbial community composition, or environmental differences. For example, even though region WUR had the smallest spatial coverage, it displayed the second highest alpha and beta diversity estimates as well as the second largest environmental differences (Fig. 3.1.2).

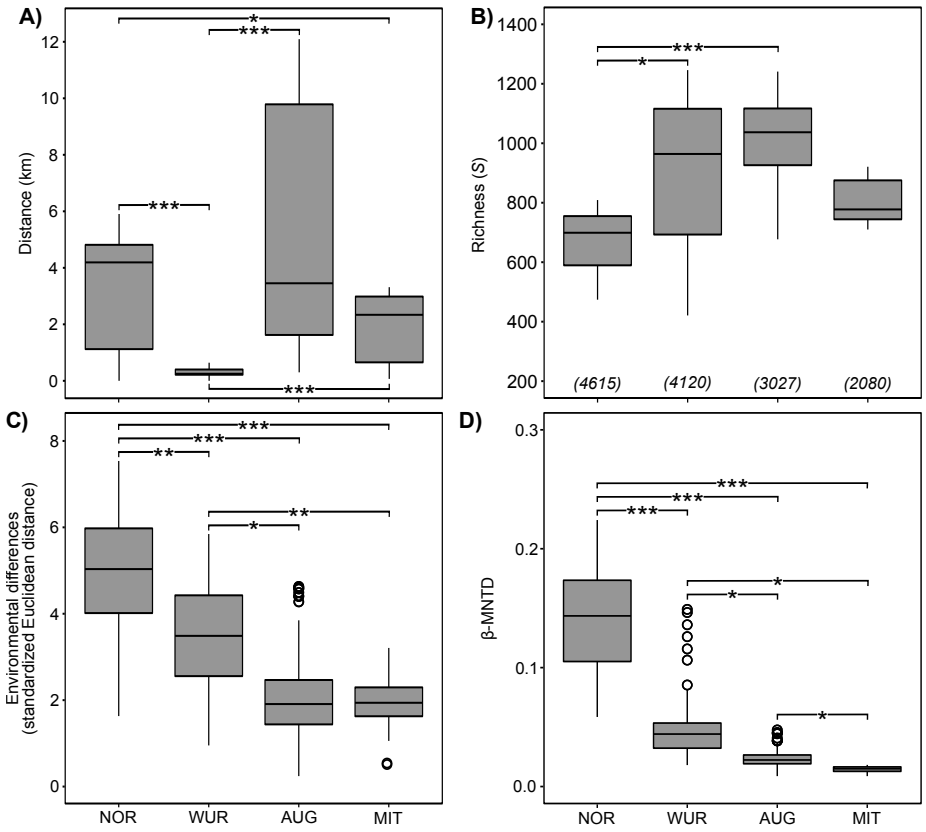

Figure 3.1.2: ***A***) Spatial distance between sites within regions. ***B***) ASV richness within regions (total number of ASVs within each region is given in parentheses). ***C***) Differences in local environmental conditions (standardized Euclidean distance considering all environmental variables) within regions. ***D***) Differences in microbial community composition (abundance-weighted β-MNTD) within regions. Asterisks indicate significant differences inferred from PERMDISP tests (10 000 permutations) (***A***, ***C***, ***D***) and Dunn's rank sum tests (***B***) (*p<0.05; **p<0.01; ***p<0.001). Note: we chose to display distances on their original scales as distances to group centroids obtained from PERMDISP revealed the same patterns as shown in ***A***, ***C***, and ***D***. (*From Fillinger et al.,* 2019a).

3.1.2 Effect of species sorting and dispersal processes on community turnover inferred from null models

When evaluated within the individual regions as well as for pairwise comparisons of communities across regions, median β-NTI values were not significantly different from the null expectation, except for the NOR region, thus indicating no significant effect of species

sorting on community assembly on average. However, the distributions of β-NTI in all regions as well as for comparisons across regions were strongly positively skewed (Fig. 3.1.3). Calculations of the fractions of pairwise community comparisons indicative of the

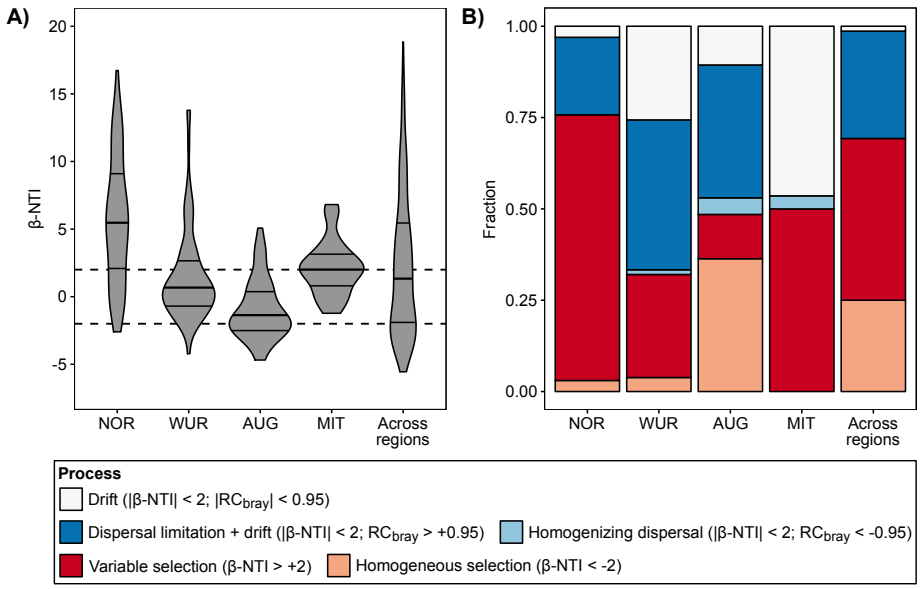

Figure 3.1.3: ***A***) Distribution of β-NTI values for pairwise community comparisons. Dashed lines indicate the range of β-NTI under the null expectation of no significant effects of species sorting (|β-NTI|<2). Solid lines within violins represent quartiles (1st, median, 3rd). ***B***) Contribution of individual assembly processes to observed differences in microbial community composition derived from null models according to Stegen et al. (2013). Null models were run for each region separately; for the analysis across regions, null models were run on the full dataset, and only results for pairs of communities from different regions are shown. (*From Fillinger et al.,* 2019a).

different turnover processes showed that the contribution of species sorting to the observed differences between communities varied for each region between 32 % (WUR) and 75 % (NOR) (Fig. 3.1.3). In most cases, variable selection was the dominating process, indicating that communities were more different than expected by chance, except for the AUG region, where homogenous selection was the dominating selection process, suggesting that communities were more similar than expected. The fractions not accounted for by selection processes were largely dominated by dispersal limitation and drift in most cases, or drift acting alone in the MIT region. Across regions, variable selection was the dominating process, accounting for 69 % of the observed differences between communi-

ties, while the remaining fraction was indicated to have resulted almost exclusively from dispersal limitation and drift.

3.1.3 Variation partitioning of differences in microbial community composition and changes in species sorting

We applied db-RDA and variation partitioning to identify environmental variables that shaped microbial community composition (β-MNTD), and to dissect the individual contributions of these variables relative to spatial distance within regions and region identity (Table 3.1.1). Contradictory to the null model results for the individual regions that hinted at dispersal limitation, spatial distance between sites within regions represented by MEMs did not have a significant effect on differences in microbial community composition in an individual db-RDA model (adjusted R^2=−0.03; p=0.916) and were therefore not considered for variation partitioning. In contrast, region identity and variables representing local environmental conditions (pH, ionPC1, and concentrations of dissolved oxygen, orthophosphate, and DOC) together explained 77 % of the variation in community composition, of which the majority (i.e. ∼41 %) was shared between both components. The effect of environmental variables alone was still significant after controlling for region identity (pH, dissolved oxygen) and explained ∼27 % of the variation, whereas region identity alone explained only ∼9 % after controlling for the effect of environmental variables. In contrast to the results obtained for β-MNTD, <7 % of the total variation could be explained for β-MPD, and the individual fractions explained by local environmental conditions and region identity were almost equally low (∼2 %) (Table S1.3). Hence, together with the results described above, differences in community composition both in response to local environmental and regional differences were best reflected by turnover across short phylogenetic distances represented by β-MNTD compared to turnover across broader phylogenetic scales captured by β-MPD.

Given that region identity alone explained a significant amount of the variation in community composition, we further explored to which extent this variation was due to dispersal limitation or caused by species sorting either imposed by local environmental conditions or region-specific factors. To this end, we used variation partitioning as above, only this time using the β-NTI matrix calculated for the full dataset as response matrix in the db-RDA (Table 3.1.1). As in the analysis above, spatial distance between sites within regions, which would reflect the contribution of spatially-structured unmeasured environmental variables, did not have a significant effect (adjusted R^2=−0.04; p=0.996), whereas region identity together with local environmental conditions (pH and concen-

Table 3.1.1: Partition of variation in microbial community composition (abundance-weighted β-MNTD) and species sorting (β-NTI) between local environmental conditions (*Env*; significant environmental variables are listed in the right-most column) and region identity (*Reg*). *Env+Reg* represents the total variation explained by both components; *Env|Reg* (*Reg|Env*) represents the marginal fraction of variation explained by each component after controlling for the other; *Env∩Reg* represents the fraction of explained variation shared between both components. The explained variation is given as adjusted R^2. Significance of each component and individual variables was tested using 10 000 permutations (note: significance of *Env∩Reg* cannot be tested). Spatial distance between sites within regions represented by MEMs was not significant in either case (adj. R^2=0, p>0.9) and was therefore not included in the analyses. (*From Fillinger et al.,* 2019a).

Response matrix	Component	df	Adj. R^2	p	Significant variables (p<0.05)
β-MNTD	Env	5	0.6772	0.0001	pH, O_2, ionPC1*, PO_4^{3-}, DOC
	Reg	3	0.4972	0.0001	Dummy-coded region identity
	Env+Reg	8	0.7691	0.0001	
	Env∩Reg	0	0.4053		
	Env|Reg	5	0.2719	0.0001	pH, O_2
	Reg|Env	3	0.0919	0.0001	
	Residuals	36	0.2309		
β-NTI	Env	5	0.6618	0.0001	pH, O_2, PO_4^{3-}, NO_3^-, DOC
	Reg	3	0.3747	0.0001	Dummy-coded region identity
	Env+Reg	8	0.6238	0.0001	
	Env∩Reg	0	0.4127		
	Env|Reg	5	0.2492	0.0001	pH, O_2, DOC
	Reg|Env	3	−0.0380	0.0022	
	Residuals	36	0.3762		

*Principal component representing 74 % of the variance in electrical conductivity and concentrations of sodium, calcium, magnesium, chloride, and sulfate (all positively correlated with ionPC1; see Section 2.1.7).

trations of dissolved oxygen, orthophosphate, nitrate, and DOC) explained ∼62 % of the variation in β-NTI. However, the effect of region identity was strongly tied to the effect of local environmental conditions, such that the variation explained by region identity alone dropped to zero after controlling for the effect of environmental variables (note

that negative adjusted R^2 although significant is interpreted as zero (Legendre, 2008)). In contrast, environmental variables alone were still significant (pH, dissolved oxygen, DOC) and explained almost 25 % of the variation in β-NTI after controlling for region identity. About 38 % of the variation was unexplained, representing regionally and spatially unstructured, unmeasured factors that imposed species sorting.

3.2 Assembly and succession of microbial communities during the colonization of groundwater sediments

3.2.1 Temporal dynamics of microbial cell numbers and alpha diversity of sediment-attached and planktonic microbial communities

Despite the spatial distance between the two sites, the microbial communities that developed on the initially sterile sediments followed identical trends in alpha diversity and cell numbers (Fig. 3.2.1). Already after the first 49 days, cell numbers of sediment-attached microbial communities at both sites had reached a plateau of $\sim 10^7$ cells cm^{-3} of sediment, followed by a slight decline for the remaining time of the experiment. Although cell densities of the sediment-attached communities stayed more or less constant, noticeable changes still occurred in the communities as indicated by OTU richness and diversity, which steadily increased by about 50 % and 25 %, respectively, from May until December, followed by a decline of both parameters in February. The changes in cell numbers and alpha diversity observed for the newly colonized sediments appeared to be independent from the changes that occurred in the planktonic microbial communities suspended in the surrounding groundwater, which were more variable over time and less comparable between the two sites. Moreover, microbial cell numbers measured in the groundwater were about one to two orders of magnitude lower compared to the sediments.

3.2.2 Establishment and persistence of newly-arriving OTUs in sediment-attached microbial communities

To assess the impact of early colonizers on microbial community succession, we looked at the number of newly-arriving OTUs (i.e OTUs that showed an abundance of >0 % for the first time at a given time point) that had entered the developing sediment-attached communities at each time point over the course of the experiment (Fig. 3.2.2). At both sites, the percentage of newly-arriving OTUs declined over time, showing that the majority of OTUs had established on the sediments during the initial phase of the incubation of the *in situ* microcosms. Despite this declining trend, the fraction of newly-arriving OTUs relative to the total OTU richness at the end of the incubation was still relatively high with ∼15 % to 20 %. However, looking at the changes in the cumulative relative abundances of the newly-arriving OTUs over time revealed that OTUs that had arrived towards the later stages only accounted for a relatively small fraction of the final communities. Even though the cumulative relative abundances of OTUs that had

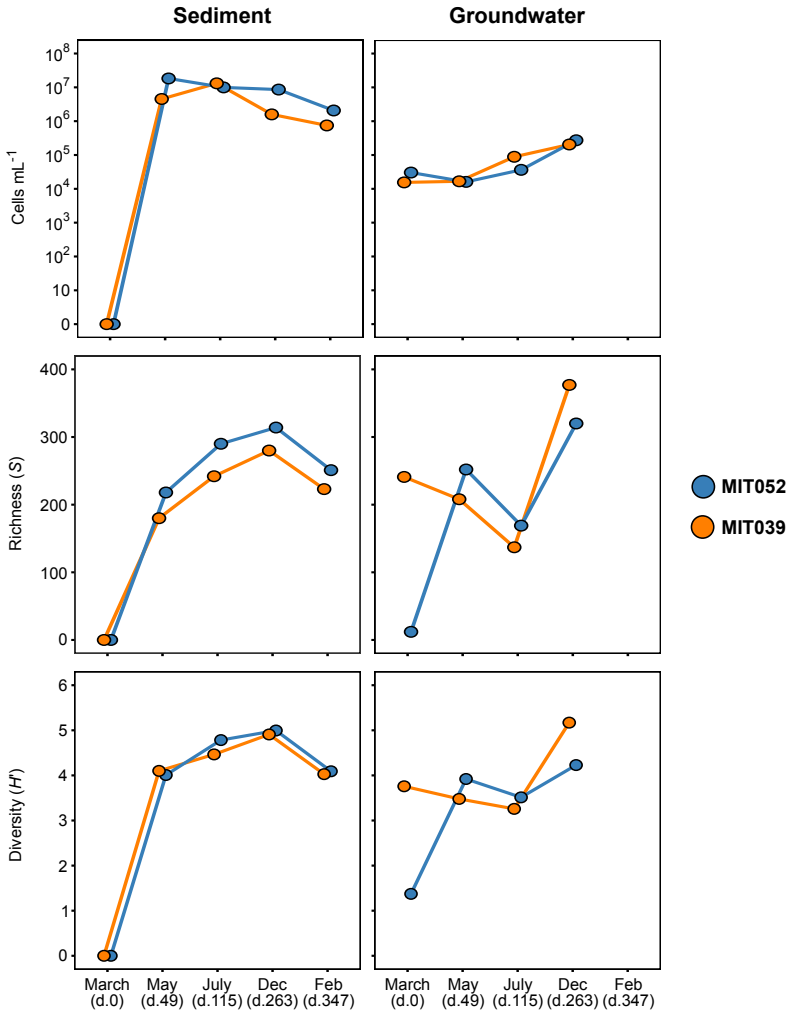

Figure 3.2.1: Temporal dynamics of microbial cell numbers and alpha diversity for sediment-attached and planktonic microbial communities at MIT052 and MIT039, respectively. Microbial cell numbers are given as cells per mL or the equivalent volume of sediment (cm^3). The time in days for each time point is given in parentheses. (*From Fillinger et al.,* 2019c).

established in the communities within the first 49 days steadily declined over time, these OTUs still made up 36 % and 47 % of the final communities at MIT052 and MIT039, respectively. At MIT052, these OTUs together with those that emerged at the following time point in July comprised the majority of the final community at the end of the incubation (together 85 %), while OTUs that arrived at the final time point accounted for only 5 %. At MIT039, OTUs that had arrived at the first two time points made up for

68 % of the final community, whereas OTUs that had arrived in December and February comprised 12 % and 20 %, respectively.

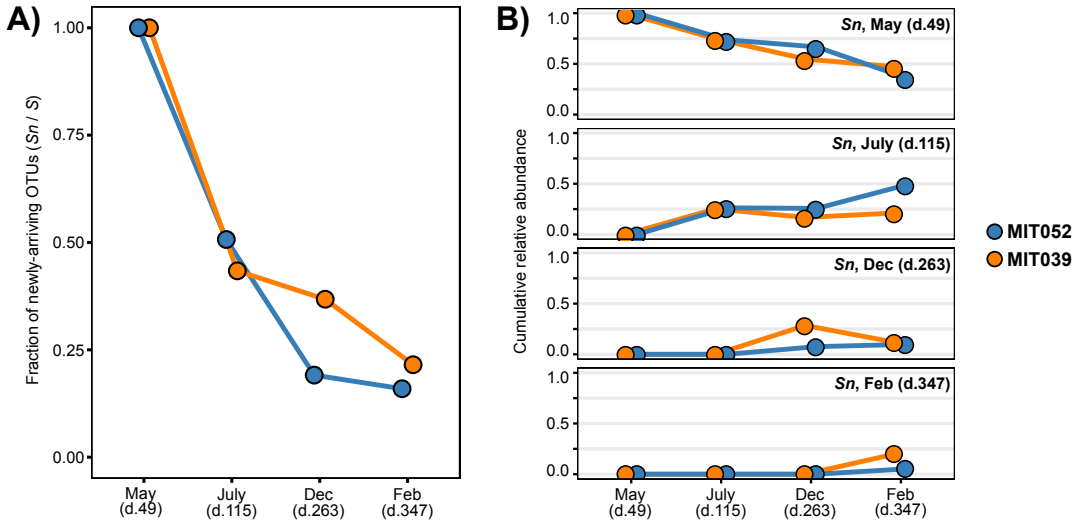

Figure 3.2.2: ***A***) Changes over time in the fraction of newly-arriving OTUs (*Sn*) in sediment-attached microbial communities. ***B***) Changes over time in the cumulative relative abundance of newly-arriving OTUs that first occurred at the time point indicated in the upper right corner of each graph. (*From Fillinger et al.,* 2019c).

Although these observations suggest the dominance of early-colonizer OTUs in the final communities, a closer look at how many of these OTUs actually persisted until the final time point showed that only ∼12 % of newly-arriving OTUs from each time point were still present in the final communities (data not shown). Looking at the taxonomies of the OTUs that persisted until the end of the incubation again revealed highly similar patterns for both sites (Fig. S2.3). At each time point, the most dominant groups were OTUs affiliated with *Comamonadaceae*, mainly *Aquabacterium* and *Polaromonas* spp., in addition to *Oxalobacteraceae*, mainly consisting of *Duganella*, *Massilia*, and *Undibacterium* spp., as well as *Pseudomonas* spp. and diverse *Caulobacteraceae* and *Sphingomonadaceae*.

3.2.3 Microbial community composition and beta diversity

Similar to the alpha diversity patterns, the microbial communities on the newly colonized sediments displayed comparable compositions at the two sites (Fig. 3.2.3). Especially during the initial phase of the incubation in May, sediment-attached communities at

both sites were dominated by *Oxalobacteraceae* as well as *Comamonadaceae* and smaller fractions of *Flavobacteriaceae* and *Caulobacteraceae*. Over the further course of the incubation, these taxonomic groups were in part replaced mainly by increasing numbers of *Comamonadaceae* (mostly *Aquabacterium* spp.), *Pseudomonadaceae*, *Nocardiaceae*, and *Rhodocyclaceae* especially at MIT052, in addition to *Sphingomonadaceae*, uncultured *Deltaproteobacteria*, and *Moraxellaceae* at MIT039. Moreover, OTUs affiliated with diverse low-abundant families (<10 % in the entire dataset) gradually increased in abundance over time. In contrast, planktonic communities were mainly dominated by members of the *Rhodocyclaceae*, *Caulobacteraceae* (mainly *Curvibacter*, *Simplicispira*, and *Rhodoferax* spp.), and *Leptospiraceae*.

To get a better understanding of the organisms that were responsible for differences between sediment-attached and planktonic communities, we used SIMPER analysis across all samples on relative abundances of OTUs grouped at genus level. Interestingly, we found high agreement between the genera that significantly contributed to the observed dissimilarities between community types and taxa identified as successful, persistent colonizers in the analysis discussed above and shown in Figure S2.3. *Aquabacterium*, *Massilia*, and *Duganella* ranked among the genera with the highest individual contributions to the dissimilarity (together >15 %; all $p<0.002$) and were highly differentially abundant in sediment-attached communities, next to *Flavobacteria*, and uncultured members of the *Oxalobacteraceae* (Figure S2.4).

Changes in microbial community composition over time as well as differences between sediment-attached and planktonic communities in the groundwater were revealed by NMDS performed on abundance-weighted β-MNTD (Fig. 3.2.3). At all measured time points, sediment-attached and planktonic communities clustered separately from each other as reflected by the distinct separation of the two types of communities along the first NMDS axis. Changes in microbial community composition over time were reflected by the separation of samples along the second NMDS axis. PERMANOVA revealed that community type (i.e. sediment-attached vs. planktonic) explained most of the variance in β-MNTD between communities ($R^2=0.626$; $p=0.001$), followed by sampling time point ($R^2=0.104$; $p=0.001$), whereas site location was not significant ($R^2=0$; $p=1$), showing that communities across sites were similar within each community type and time point. However, temporal variation in community composition could not be related to changes in physicochemical conditions in the groundwater, as fitting environmental variables to the NMDS ordination with permutations stratified within community types did not reveal significant correlations between changes in community composition and any of the measured physicochemical variables (all $R^2<0.32$; all $p>0.1$).

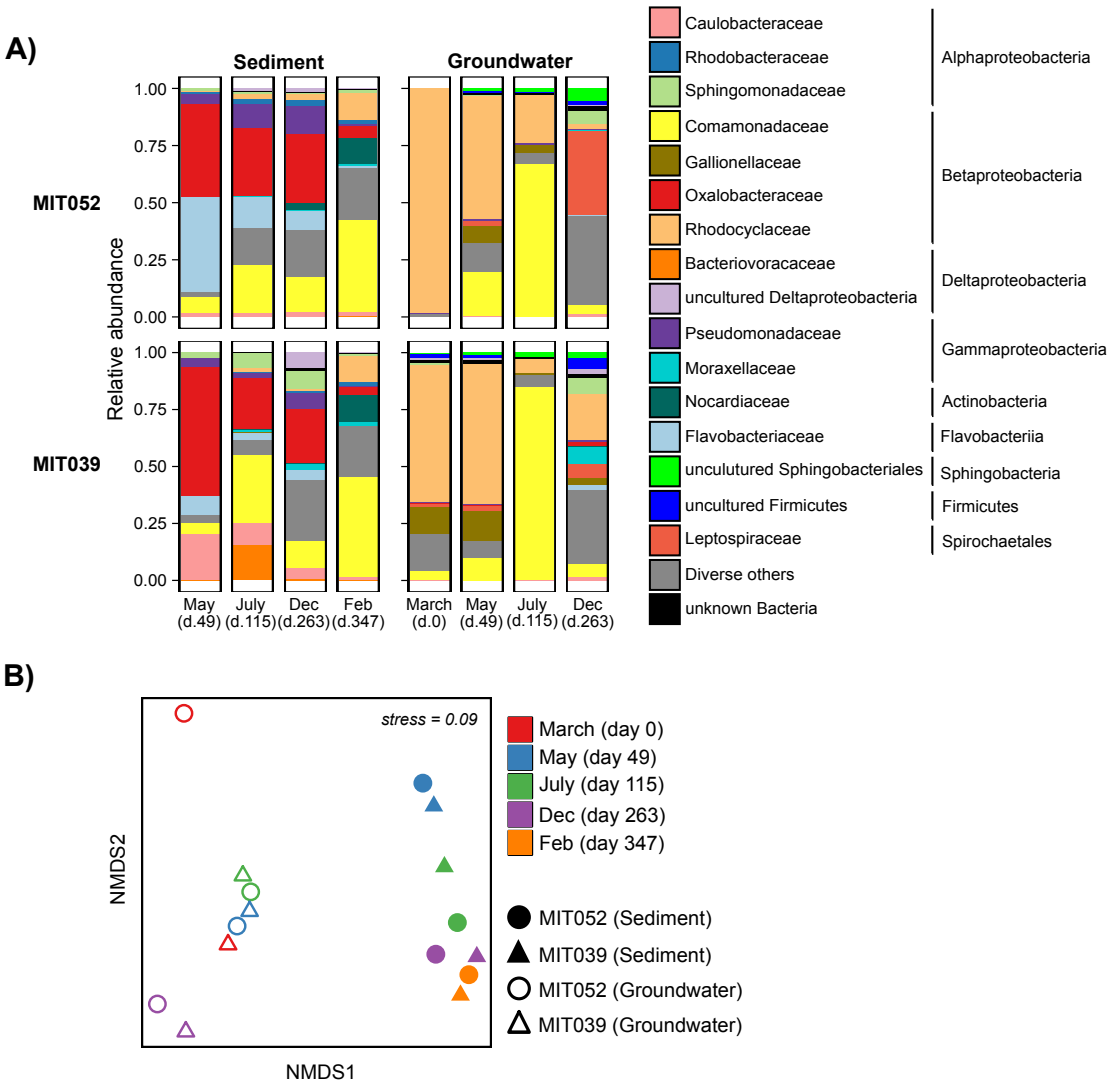

Figure 3.2.3: ***A***) Taxonomic microbial community composition of sediment-attached and planktonic communities in the groundwater per time point. Taxonomic groups were summarized at family level (based on taxonomic assignments using SILVA SSU reference database release 128). Families with an individual abundance of <10 % in the entire dataset were grouped as 'Diverse others' for clarity of display. ***B***) NMDS showing differences in microbial community composition based on abundance-weighted β-MNTD. (*From Fillinger et al.*, 2019c).

Since community type explained most of the variance in differences between communities, we applied beta diversity partitioning to identify the underlying causes of the

differences between sediment-attached and planktonic communities within sites and time points. The analysis showed that turnover (i.e. replacement of OTUs in one community relative to another) was the dominating process behind the differences between the two types of communities, accounting for >97 % of pairwise differences between communities for all time points (Fig. S2.5). Thus, both types of communities were to a large extent composed of distinct OTUs rather than being subsets of each other.

We applied the same approach to the dissimilarity between sediment-attached communities at different time points within sites to investigate the extent to which nestedness and turnover contributed to changes in community composition over the course of the sediment colonization. Also in this case, turnover dominated over nestedness in all comparisons, especially over long time scales (i.e. comparing communities between May and February; 95 % to 97 %), and with a slightly weaker effect over short time scales (i.e. comparing communities between consecutive time points; 74 % to 90 %) (Fig. S2.6).

3.2.4 Impact of species sorting, dispersal, and drift on community assembly and succession inferred from null models

We again applied the null model approached used above (Section 3.1.2) to study the role of species sorting, dispersal, and drift on three levels: first, spatial community turnover between the two sites within community types and time points; second, turnover between sediment-attached and planktonic communities within sites and time points; and third, temporal turnover between communities at consecutive time points within community types and sites (Fig. 3.2.4).

Species sorting and dispersal processes had different impacts on differences between communities across sites for sediment-attached and planktonic communities, respectively. For planktonic communities, pairwise comparisons between sites for each time point resulted in β-NTI values that were not significantly different from the null expectation, indicating that differences between communities were not the result of species sorting. Pairwise comparisons based on RC_{bray} hinted at homogenizing dispersal as the dominating process (all $RC_{bray}=-1$; data not shown), except for December, where dispersal limitation in combination with dirft was indicated to be responsible for differences between planktonic communities at the two sites ($RC_{bray}=+0.99$; data not shown). In contrast to the lack of significant effects of species sorting on planktonic communities suspended in the groundwater, pairwise comparisons of sediment-attached communities clearly tended towards homogeneous selection (β-NTI< -2; Fig. 3.2.4) as main cause of the similarities between sediment-attached communities from the two sites. The only ex-

ception was observed for July, where β-NTI was not significant, but a slightly significant deviation of RC_{bray} from the null expectation hinted at dispersal limitation together with drift to be responsible for the observed differences (RC_{bray}=+0.97; data not shown).

Since the sediments had to be colonized by microorganisms that were recruited from the surrounding groundwater, even though beta diversity partitioning revealed little overlap between these two types of communities as described above, we tested for the effect of species sorting on the assembly of sediment-attached communities from planktonic microorganisms suspended in the groundwater. The differences between the communities on the newly colonized sediments and the planktonic communities at each site were indicated not be caused by species sorting during the first 115 days of the incubation (|β-NTI|<2; Fig. 3.2.4). Instead, pairwise comparisons based on RC_{bray} pointed towards dispersal limitation acting together with drift as processes responsible for the observed differences (all RC_{bray}=+1; data not shown). This trend changed at the later stage in December after 263 days. At this time point significantly positive values of β-NTI indicated variable selection of phylogenetically distinct OTUs in sediment-attached communities compared to the microorganisms in the surrounding groundwater.

Unlike the trends observed for spatial community turnover, the influence of species sorting, dispersal, and drift on changes in community composition over time was much more variable and no clear trends could be observed. Although species sorting appeared to have played a role, this effect mostly did not occur consistently at both sites for neither sediment-attached nor planktonic communities (Fig. 3.2.4).

We used Mantel correlation analysis to investigate whether changes in physicochemical parameters in the groundwater (Fig. S2.1) had an effect on changes in species sorting based on β-NTI. Similar to the lack of correlations between environmental variables and differences in community composition mentioned above (Section 3.2.3), we did not find significant correlations for planktonic (all |Spearman's ρ|<0.34; p>0.08) or sediment attached communities (all |Spearman's ρ|<0.27; p>0.1).

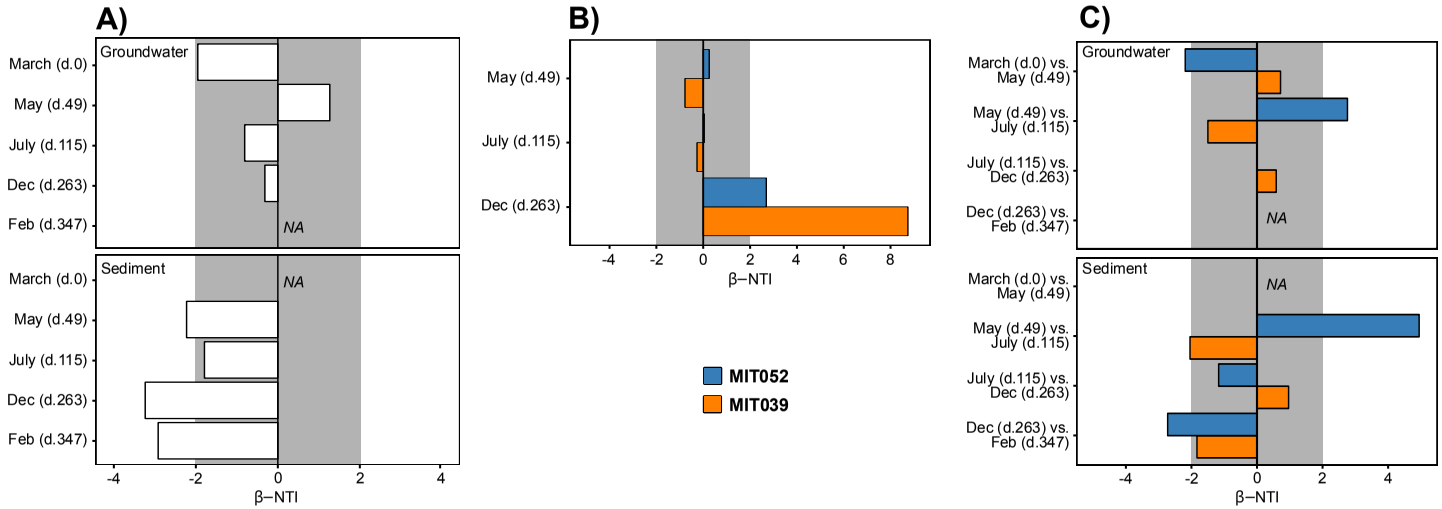

Figure 3.2.4: Values for β-NTI inferred from pairwise microbial community comparisons. The range of β-NTI expected under the null expectation of no significant effect of species sorting is shaded in grey. ***A***) Spatial turnover of planktonic communities in the groundwater and sediment-attached communities, respectively, between sites within time points. ***B***) Community turnover between planktonic and sediment-attached communities within sites per time point (note: the bar corresponding to the comparison of communities at MIT052 in July is not visible; β-NTI=0.04). ***C***) Temporal turnover of plankotnic and sediment-attached communities, respectively, between consecutive time points within sites (note: the bar for the comparison of July vs. December for planktonic communities at MIT052 in not visible; β-NTI=−0.005). NA: data not available. (*From Fillinger et al.,* 2019c).

3.3 Ecological groundwater monitoring based on microbiological parameters

3.3.1 Using the D-A-(C) index to detect disturbances

Case study 1: organic contamination

For simplicity, and for reasons that will be discussed in Section 4.3, the analysis was based only on the variables cell density and activity to introduce how disturbances can be detected based on the identification of outliers. All contaminated samples were correctly identified as outliers by the multivariate approach using the D-A index, which exceeded the critical value of the chi-squared distribution for all contaminated samples (Fig. 3.3.1). In general, the contaminated samples showed higher activity levels and cell densities compared to the non-contaminated samples. However, if both variables were considered separately in univariate analyses, only 83 % of the contaminated samples would have been correctly identified as outliers based on cell density; based on activity, the fraction would have been even lower with only 53 %. Even if the outcomes of the two separate univariate analyses were to be considered together, still 17 % of the contaminated samples would not have been detected as outliers. This is apparent from the bivariate plot where a group of contaminated samples clearly deviates from the distribution of non-contaminated samples indicated by the confidence ellipse, but still falls within the univariate thresholds for cell density and activity (Fig. 3.3.1). In fact, by looking at the bivariate plot it is becomes clear that, in principle, any sample that would lie to the top left or the bottom right of the confidence ellipse would not be detected as an outlier using the univariate approach if it still fell within the range defined by the univariate thresholds, even though it would clearly deviate from the distribution of the rest of the data along the two variables. This distribution is not only shaped by the variances of the variables but also by their mutual covariance, which is ignored with the univariate approach. Thus, by making use of the additional information about covariance, the multivariate approach fully exploits the information gained from the two variables. Thereby it is able to provide a more accurate picture about the available data and accordingly allows for a more accurate identification of outliers.

To assess the impact of the fraction of contaminated samples in the dataset on the ability of the two approaches to detect those contaminated samples as outliers, we repeated the analysis from above with datasets containing different fractions of contaminated samples, ranging from 10 % to 60 %. For each fraction, we constructed 100 datasets with a total of 70 samples that were randomly selected from the non-contaminated site

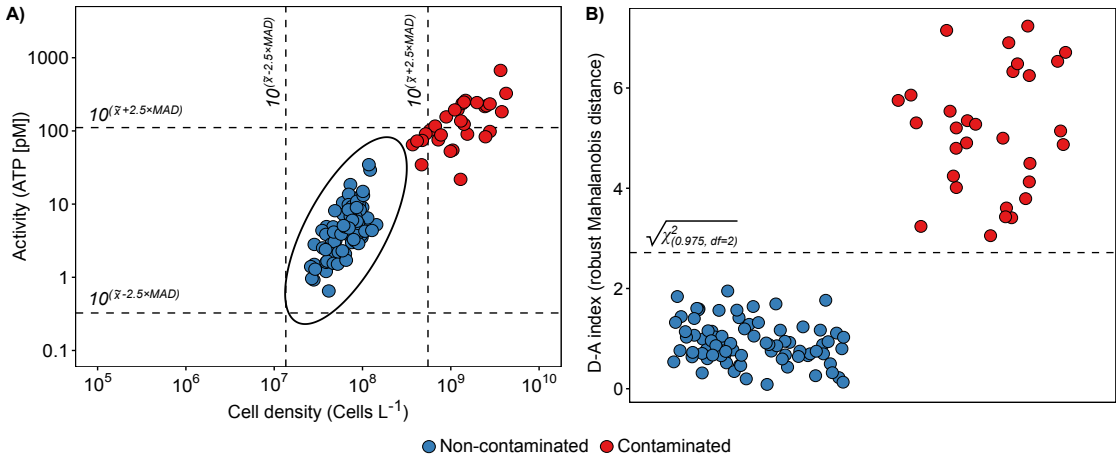

Figure 3.3.1: Detection of disturbances related to organic contamination based on microbial cell density and activity. ***A***) Bivariate plot showing the distribution of samples along the variables cell density (measured as prokaryotic cells L^{-1}) and activity (measured as prokaryotic intracellular ATP concentrations). Dashed lines represent the univariate thresholds calculated as $median \pm 2.5 \times MAD$ for each variable. The ellipse shows the 0.975 confidence ellipse calculated from the robust estimates of the center and covariance matrix of the data (note: all calculations were done on $\log_{10}$-transformed data; values were converted back to the original scale to allow for a more intuitive representation of the data). ***B***) D-A index values (robust Mahalanobis distances) for the samples shown in ***A***. The dashed line represents the critical value of the chi-squared distribution at a 0.975 confidence level with two degrees of freedom. (*From Fillinger et al.,* 2019b).

and the hydrocarbon-contaminated site, respectively. For each dataset, we calculated the average fraction of contaminated samples that were correctly identified as outliers using the univariate approach, where a sample was declared an outlier if it fell outside the thresholds of $median \pm 2.5 \times MAD$ for either of the two variables, and the multivariate approach, respectively (Fig. 3.3.2). In datasets that contained 10 % contaminated samples, both approaches were equally reliable, detecting >99 % of the contaminated samples as outliers (Welch's t-test, t_{99} =1.42, p=0.16). However, already at 20 % contaminated samples, the multivariate approach detected the contaminated samples significantly more reliably (98.5 %) compared to the univariate approach (93.7 %) (Welch's t-test, t_{140} =6.21, p$\ll$0.0001), and was able to correctly identify >96 % of the contaminated samples as outliers up to a fraction of 30 % contaminated samples in the dataset, above which also the multivariate approach started to noticeably lose its reliability.

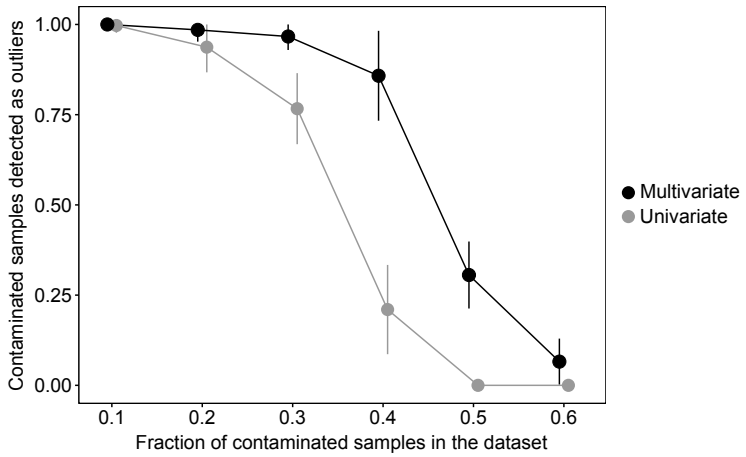

Figure 3.3.2: Comparison of the ability to identify contaminated samples as outliers depending on the fraction of contaminated samples in the dataset between the multivariate approach using the D-A index (robust Mahalanobis distances) and the univariate approach based on separate thresholds of $median \pm 2.5 \times MAD$ for cell density and activity, respectively. Each data point shows the average fraction of contaminated samples correctly identified as outliers calculated from 100 randomly assembled datasets each containing 70 samples in total with different fractions of contaminated samples. Error bars show the standard deviation of the averages. (*From Fillinger et al.,* 2019b).

Case study 2: surface water intrusion

As for the previous example, the analysis was based only on the variables cell density and activity. Out of the total of 108 samples, nine samples were identified as outliers using the multivariate approach as indicated by the D-A index for these nine samples, which exceeded the critical value of the chi-squared distribution at a 0.975 confidence level (Fig. 3.3.3). The presence of these outliers coincided with the period of the increased surface water intrusion, and seemed to reflect the spatiotemporal impact of this event as revealed by stable isotope signatures of the groundwater (Fig. S3.1). In January, at the early stage of the event, the effect of the surface water intrusion was only apparent close to the river as indicated by the outlier from a well at a distance of 2 m from the river. However, at the later stage in March, when the surface water had reached the areas more distant from the river, outliers were also detected 60 m and 75 m away from the river. After the surface water had receded, the system appeared to have recovered from the disturbance. The only exception was a single well 60 m away from the river that was still identified as an outlier in April, June, and October, which, however, did not seem to be

directly related to the surface water intrusion (Fig. S3.1).

Similar to the results for the first disturbance case study, the multivariate approach detected outliers more sensitively compared to the univariate approach. Only four samples that were identified as outliers in the multivariate analysis also fell outside the range defined by the $median \pm 2.5 \times MAD$ for cell density as well as activity if both were considered separately. If the information for both variables were to be combined, still one outlier would not have been detected, which was however detected by the multivariate approach.

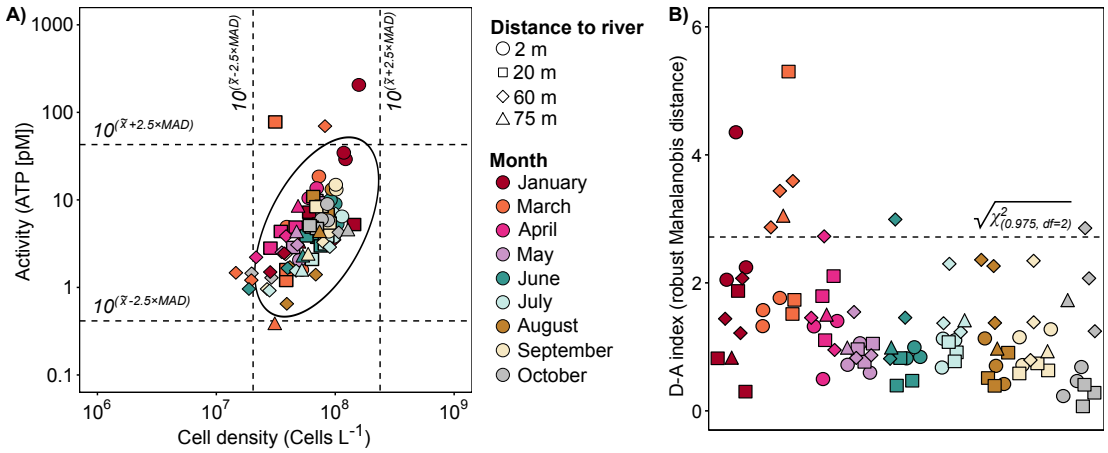

Figure 3.3.3: Detection of disturbances related to surface water intrusion based on microbial cell density and activity. ***A***) Bivariate plot showing the distribution of samples along the variables cell density (measured as prokaryotic cells L^{-1}) and activity (measured as prokaryotic intracellular ATP concentrations). Dashed lines represent the univariate thresholds calculated as $median \pm 2.5 \times MAD$ for each variable. The ellipse shows the 0.975 confidence ellipse calculated from the robust estimates of the center and covariance matrix of the data (note: all calculations were done on $\log_{10}$-transformed data; values were converted back to the original scale to allow for a more intuitive representation of the data). ***B***) D-A index values (robust Mahalanobis distances) for the samples shown in ***A***. The dashed line represents the critical value of the chi-squared distribution at a 0.975 confidence level with two degrees of freedom. (*From Fillinger et al.,* 2019b).

Case study 3: impact of different types of land use

Considering only cell density and activity in the multivariate analysis, no significant outliers were detected for any of the three types of land use, although the D-A index

value of a single sample from the agricultural land was close to the critical value of the chi-squared distribution (Fig. 3.3.4). However, if AOC concentrations were additionally

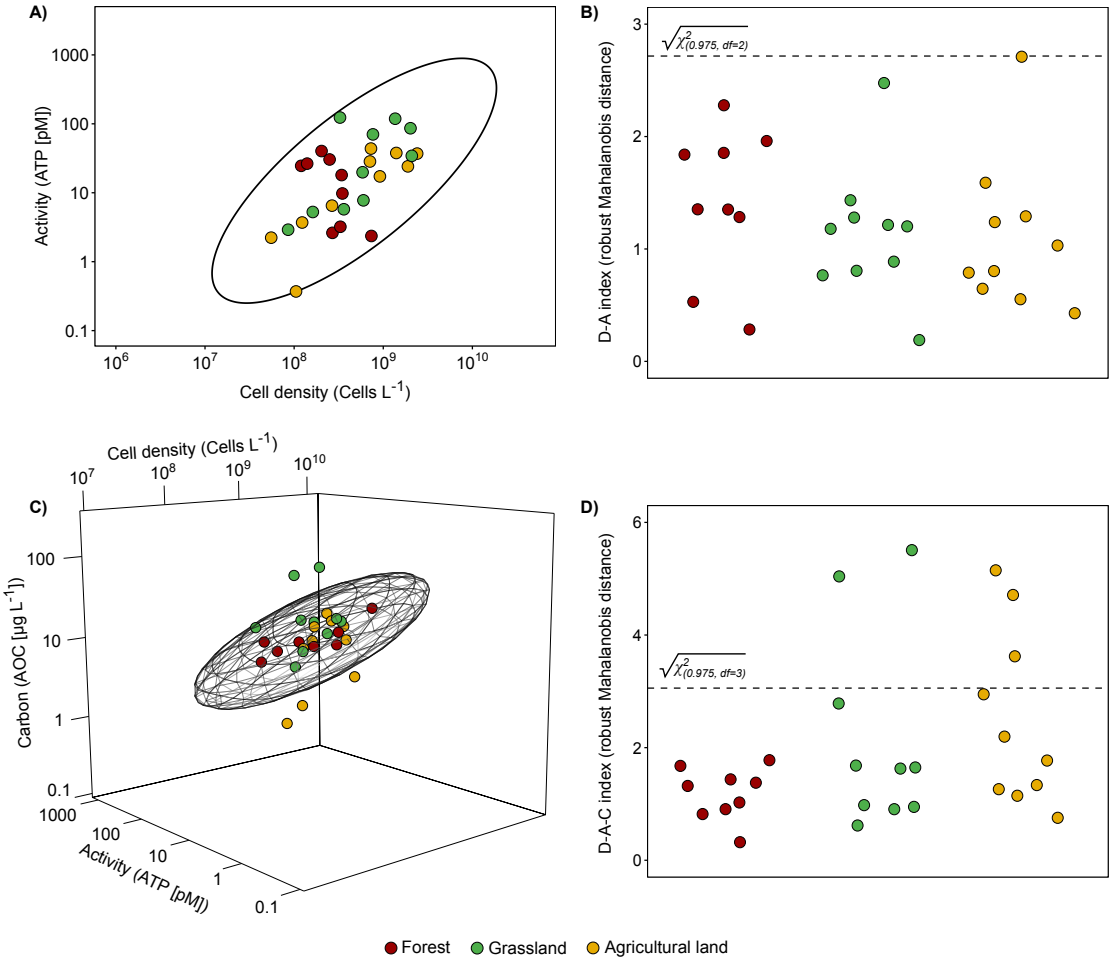

Figure 3.3.4: Detection of disturbances related to different types of land use based on microbial cell density and activity, and additionally AOC. ***A*** and ***C***) Distribution of samples along the variables cell density (measured as prokaryotic cells L^{-1}) and activity (measured as prokaryotic intracellular ATP concentrations), and additionally carbon (measured as AOC concentrations) (***C***), for the different types of land use with 0.975 confidence ellipse (***A***)/ellipsoid (***C***) calculated from the robust estimates of the center and covariance matrix of the data. ***B*** and ***D***) D-A-(C) index values (robust Mahalanobis distances) of the samples shown in ***A*** and ***C***, respectively. Dashed lines represent the critical values of the chi-squared distribution at a 0.975 confidence level with two and three degrees of freedom in ***B*** and ***D***, respectively. (*From Fillinger et al.,* 2019b).

included in the analysis, five outliers could be clearly identified based on the D-A-C index, three of which derived from the agricultural land, showing lower AOC concentrations than the majority of the samples, and two from the grassland, which showed higher AOC concentrations. Similar to the first two disturbance case studies, these outliers would not have been detected based on separate univariate analyses (except for one sample from the agricultural land, which fell below the lower threshold for activity; Fig. S3.2). The hydrochemical data revealed that the three outlier samples from the agricultural land displayed exceptionally high nitrate concentrations ($>100\,\mathrm{mg\,L^{-1}}$) as well as unusually high dissolved oxygen concentrations for the region, and were among the samples with the lowest pH in the entire dataset (Fig. S3.3). For the two outliers from the grassland, the chemical data revealed unusually high concentrations of dissolved organic carbon, corresponding to the high AOC concentrations for these samples, as well as relatively low pH values (Fig. S3.3).

3.3.2 Regional differences in D-A-(C) signatures

For simplicity of display, the signatures of the nine different regions are shown in Figure 3.3.5 only based on the two variables cell density and activity. The signature of each region is defined by the center (i.e. mean values of each variable) and the covariance matrix, which both are graphically captured by the confidence ellipse. Already based on visual comparison of the confidence ellipses, clear differences were apparent between each region and the full dataset as well as between the individual regions, both in terms of variable means and covariances. Significant differences in covariance matrices between regions were confirmed using Box's M test on the robust estimates of the covariance matrices (χ^2_{24} =76.5, p$\ll$0.0001). For the comparisons of variable means, only samples were considered that were not indicated to be outliers for a given region based on their robust Mahalanobis distances at a confidence level of 0.975. As seen for the covariance matrices, significant differences were also found for the means of cell density (Welch's ANOVA, $F_{8;75.5}$ =44.3, p$\ll$0.0001) and activity (Welch's ANOVA, $F_{8;75.8}$ =14.0, p$\ll$0.0001). Unfortunately, AOC concentrations were not available for all samples. We repeated the analyses only considering regions for which at least 10 samples were available with data for all three D-A-C variables (Table 2.3.1). The results were in agreement with the previous analyses, showing significant differences between covariance matrices (Box's M, χ^2_{36} =133.8, p$\ll$0.0001) and average AOC levels (Welch's ANOVA, $F_{6;27.5}$ =12.9, p$\ll$0.0001).

Additional pairwise comparisons of means for each variable revealed that although

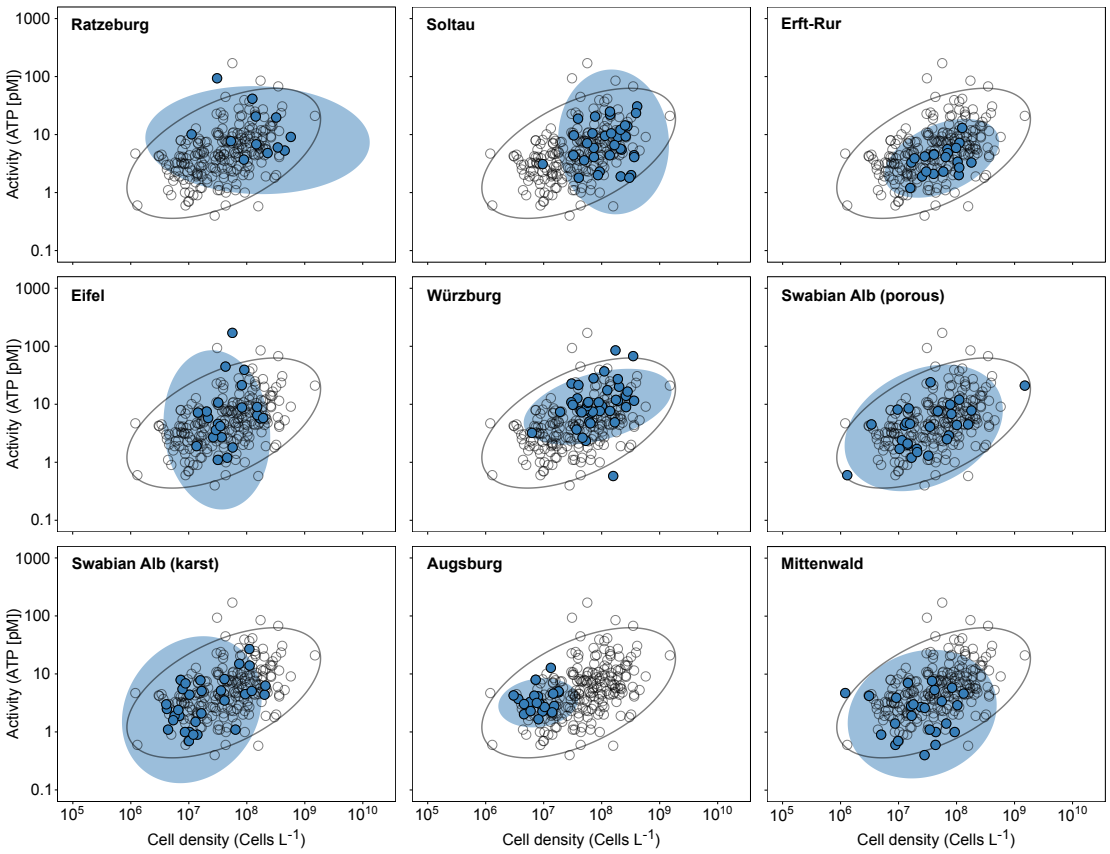

Figure 3.3.5: Signatures along the variables cell density and activity of the nine non-contaminated aquifers at the regions indicated in the upper left corner of each plot. Open symbols represent the full dataset comprising all nine regions and the corresponding 0.975 confidence ellipse calculated from the robust estimates of the center and covariance matrix of the data; colored symbols represent data for an individual region (note: all calculations were done on $\log_{10}$-transformed data; values were converted back to the original scale to allow for a more intuitive representation of the data). (*From Fillinger et al.,* 2019b).

the overall differences were significant, differences between pairs of regions were relatively small, mainly occurring within one order of magnitude for each variable (Fig. 3.3.6; summary statistics for each region are given in Table S3.1). Moreover, even though there were significant correlations between the D-A-C variables considering the full dataset (Pearson's $r_{cell\ density\text{-}activity}$=0.463, p$\ll$0.0001; $r_{activity\text{-}carbon}$=−0.285, p=0.008; not significant: $r_{activity\text{-}carbon}$=0.191, p=0.08), the pairwise comparisons further showed that these variables are not mutually redundant, as significant differences between two re-

gions in one variable did not necessarily coincide with significant differences in another variable for a given pair of regions.

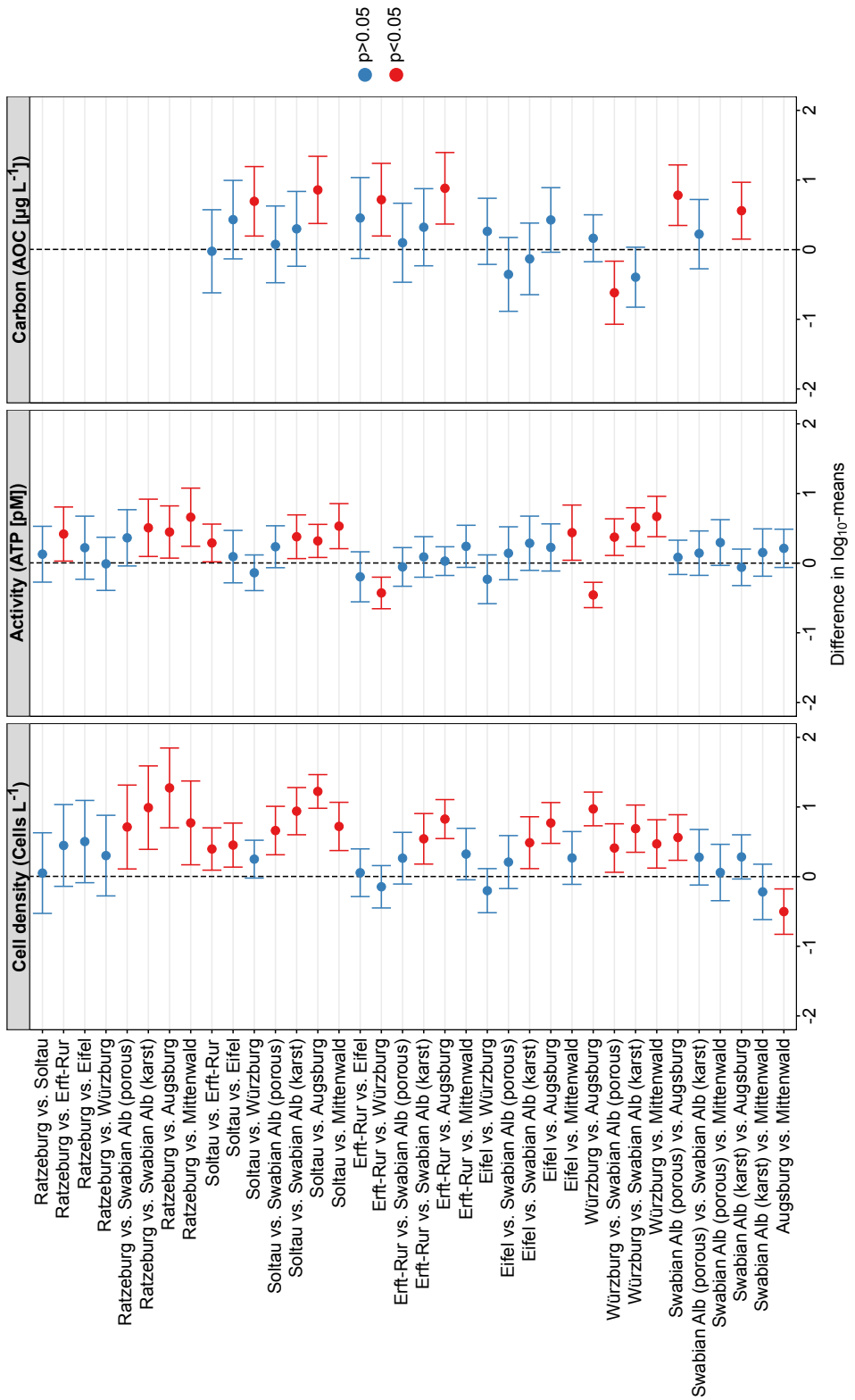

Figure 3.3.6: Multiple pairwise comparisons using Games-Howell post-hoc tests for differences in each D-A-C variable between non-contaminated aquifers at different regions studied across Germany. Points show the size of the difference in $\log_{10}$-means between two regions (a difference of 1 shown here represents a difference of one order of magnitude on the original scale); error bars represent 0.95 confidence intervals of the differences. Red symbols indicate significant differences ($p<0.05$) between two regions. (*From Fillinger et al.*, 2019b).

4 Discussion

4.1 Ecological drivers of differences in microbial community composition across geographically distinct aquifers

The aim of this study was to establish the relative contributions of processes that cause variation in microbial community composition in groundwater environments across distinct aquifers located in different regions. We hypothesized that variation in community composition can be due to species sorting imposed by local environmental conditions measured at the time of sampling, and potential broad-scale region-specific factors like climate, geology or historical events, in addition to processes related to dispersal and drift within as well as across regions. Our analyses showed that differences in local environmental conditions were well reflected by differences in microbial community composition within regions. This observation points towards the influence of species sorting, where stronger environmental gradients within a region are predicted to increase niche diversity, and hence cause different species to sort into local communities along these environmental gradients (Langenheder and Lindström, 2019).

The results obtained from the null models only partially agreed with this observation. On the one hand, the different degrees to which selection was indicated to be responsible for the differences in community composition in the NOR region compared to the AUG and MIT region did match the observed differences in environmental heterogeneity for these regions. This would support the hypothesis outlined above that stronger environmental gradients increase the influence of species sorting. On the other hand, contradictory results were found for the WUR region, which showed the second largest environmental differences, but exhibited the lowest contribution of selection. However, it has to be mentioned that parts of the aquifer in the WUR region are artificially recharged with treated river water during the summer months (i.e. May to October) but not during the rest of the year. The samples for this study were collected at the early stage about two weeks after the start of the annual infiltration period, which may have constituted a perturbation to the microbial communities. It has been shown that random colonization through dispersal and drift can gain importance on community assembly in disturbed environments (Ferrenberg et al., 2013; Zhou et al., 2014; Fukami, 2015; Langenheder and Lindström, 2019), which could explain the relatively low contribution of species sorting in the WUR region. Furthermore, the null models indicated relatively strong contributions of dispersal limitation acting alongside drift, especially in the WUR and AUG region.

Although comparable results have been obtained in previous studies on microbial community assembly in groundwater environments (Stegen et al., 2013; Beaton et al., 2016; Graham et al., 2017), this is at odds with our observation that spatial distance within regions did not have a significant effect on differences in community composition in the db-RDA.

Such apparently conflicting results between distance-based regression approaches and ecological null models have previously been reported by Langenheder et al. (2017) in a study on community assembly in lake biofilms. There are two possible explanations for these observations. One is that the inferences drawn from the null models might be an oversimplification of the actual ecological processes that shape microbial communities. The approach by Stegen et al. (2012, 2013) assumes that species sorting causes stronger or weaker phylogenetic community turnover than expected by chance (i.e. significant values for β-NTI). The basic assumption that phylogenetic relatedness tends to approximate ecological similarity between microbial taxa has been confirmed by previous studies (Peay et al., 2012; Stegen et al., 2012; Tan et al., 2012; Wang et al., 2013; Dini-Andreote et al., 2015; Martiny et al., 2015; Liu et al., 2017), and was further suggested by a significant phylogenetic signal of environmental differences between ASVs in our dataset (Fig. S1.1). In the light of these findings, inferring the effect of selection from phylogenetic community turnover seems valid. However, it is also known that certain microbial traits are phylogenetically not well conserved (Martiny et al., 2015), and therefore selection involving such traits would not be reflected by phylogenetic turnover metrics like β-NTI, but could still result in higher than expected community turnover reflected by RC_{bray}, which does not consider phylogenetic relationships. Thus, a significant deviation from the null expectation of RC_{bray} could still be the result of selection processes even if phylogenetic community turnover does not deviate from the null expectation of β-NTI (Langenheder et al., 2017).

Alternatively, it is possible that mere spatial distance does not appropriately reflect actual groundwater flow paths via which microorganisms may disperse in porous aquifers (Freimann et al., 2015; Schmidt et al., 2017; Smith et al., 2018). In this case, differences in community composition would not necessarily correlate with spatial distance even if dispersal was limited between local communities. For our study, this seems to be the more likely explanation, as we did not find significant correlations between changes in RC_{bray} and differences in environmental conditions within the individual regions (based on Mantel correlation tests with 10 000 permutations; all $p>0.05$; data not shown). This suggests that we may have underestimated the effect of dispersal limitation between local communities in the db-RDA, although we cannot fully rule out that selection involving

phylogenetically non-conserved traits may have played a role as well.

Even though we might have missed the variation in community composition caused by dispersal limitation between local communities, the majority of the total variation in community composition evaluated across all regions (>75 %) could still be explained by local environmental conditions and region identity. Variation partitioning of β-MNTD revealed a larger marginal effect of local environmental conditions (27 %) compared to the effect of region identity (9 %). This strongly indicates that microbial communities were shaped by these local environmental conditions, whereas dispersal limitation between regions, and potential species sorting due to region-specific factors, only played a secondary albeit still significant role. This was furthermore supported by the large contribution of selection to differences in community composition across regions inferred from the null models, similar to findings reported by Danczak et al. (2018). It is worth noting that the four different regions in our study were sampled at different time points due to logistic constraints, although all sampling campaigns were conducted roughly in the same season, that is late spring and summer. Still, the variation in community composition possibly caused by temporal drift independent of environmental conditions, in addition to possibly undetected effects of dispersal limitation within regions, which both would be represented by the residual fraction of unexplained variation, was relatively small (∼23 %).

Interestingly, the effect of environmental conditions and region identity were mainly reflected by turnover of closest relatives between communities, that is turnover over short phylogenetic distances measured as β-MNTD, but not by turnover of deeper branching phylogenetic lineages measured as β-MPD. Responses of microbial communities to environmental conditions have previously been shown to affect turnover across short phylogenetic distances, which indicate evolutionary relatively recent adaptations (Wang et al., 2013; Liu et al., 2017). Deep phylogenetic distances on the other hand capture more distant evolutionary events (Fine and Kembel, 2011), which we hypothesized may include region-specific adaptations or evolutionary origins of phylogenetic clades within regions (Ricklefs, 2006). This, however, was not the case as >93 % of the variation in β-MPD could not be explained by region identity and local environmental conditions, which both had equally minuscule individual effects.

The dominance of species sorting by local environmental conditions is in line with the meta-analysis by Hanson et al. (2012), who compared studies on microbial communities across various habitats and spatial scales and found that environmental conditions explained most of the variation in microbial community composition in the majority of analyzed studies. Similar conclusions were drawn from a literature review by Lindström

and Langenheder (2012). Additional evidence for the importance of local environmental conditions on microbial community structure in groundwater environments in particular was provided by Ben Maamar et al. (2015) who reported similarities in microbial community composition in relation to similar environmental conditions across three unconnected fractured groundwater aquifers, as well as by other studies on single aquifers within a region (Stegen et al., 2013; Beaton et al., 2016; Graham et al., 2017). Nevertheless, region identity still explained a significant fraction of the variation in community composition after controlling for local environmental conditions in our study, comparable to previous studies that compared microbial community composition over broad spatial scales in various aquatic and terrestrial habitats (Souffreau et al., 2015; Power et al., 2018; Plassart et al., 2019), or similar examples from studies on larger organisms (Declerck et al., 2011; Viana et al., 2016; Heino et al., 2017). However, in these studies it largely remained unclear whether such large-scale distance decay relationships were the result of dispersal limitation across regions or selection by regionally structured factors. Using the standardized effect size of differences in community composition obtained from null models like β-NTI in addition to raw metrics like β-MNTD allows making such a distinction, because β-NTI quantifies the degree to which the phylogenetic turnover between two communities is stronger than expected given the observed differences in species richness, occupancy, and abundance caused by dispersal and drift (Stegen et al., 2013; Wang et al., 2013). By partitioning the variation in β-NTI between region identity and local environmental conditions, we could show that environmental conditions, both explained by measured variables and by unmeasured, spatially unstructured variables represented by the residual fraction, explained most of the variation in selection, whereas region identity alone did not have a significant effect. Combined with the results obtained for β-MNTD, this leads to the conclusion that the variation in β-MNTD explained by region identity was mainly due to dispersal limitation and drift across regions rather than species sorting imposed by broad-scale regional factors.

4.2 Assembly and succession of microbial communities during the colonization of groundwater sediments

The objective of this study was to shed light on the processes that determine microbial community assembly on groundwater sediments, and to compare patterns of microbial community succession to those previously observed for biofilms in other aquatic habitats or sediment-attached communities in comparatively more dynamic surface water-groundwater mixing zones. Our study showed that the changes in alpha diversity over the course of the colonization of initially sterile sediments followed similar trends as those predicted by the conceptual model for the formation of biofilms on empty surfaces outlined by Jackson (2003) (Fig. 1.2.3). According to this model, the early stage of biofilm development is characterized by large niche space offered by empty surfaces that allows for the establishment of diverse microorganisms, resulting in a steady increase in community richness and diversity. This increase subsequently levels off and eventually declines due to niche depletion and the loss of less competitive species as the biofilm grows over the course of the succession. However, at the final stage, the mature biofilm becomes increasingly spatially and chemically heterogeneous, which opens new niches for specialized species to thrive, thereby fueling a renewed increase in alpha diversity.

Our results were in agreement with these predicted trends, with the exception that we did not observe an increase in species richness and diversity towards the final stage of the incubation. However, the framework by Jackson (2003) was conceptualized for biofilms in resource-rich, high-productivity environments like activated sludge, wetlands, and lakes. Although we cannot rule out that alpha diversity may have increased again with a prolonged incubation time, we may hypothesize that diverse, specialized niches that develop in mature, spatially heterogeneous biofilms may not form to such an extent in the small, patchily distributed microcolonies that typically colonized groundwater sediments (Schmidt et al., 2017; Smith et al., 2018). Hence, total niche space in such microcolonies might be smaller compared to mature biofilms in other environments, similar to what Graham et al. (2016a) have proposed for sediments in the hyporheic zone. Moreover, although the general pattern of decreasing fractions of newly-arriving OTUs could also be observed from our data, reflecting the saturation of niche space according to Jackson's biofilm model (2003), we noticed that the fraction of these OTUs at the end of the incubation was still 5 to 10 times higher compared to findings on biofilms in other environments (e.g. Brislawn et al., 2019). These deviations of our results from assembly patterns of biofilms, together with the findings made for sediment-attached communities in the hyproheic zone (Graham et al., 2016a), could point towards important differences

in ecological niche structures between biofilms in resource-rich non-subsurface environments and sediment-attached microbial communities in the typically more energy-poor subsurface.

Considering the abundance changes of newly-arriving OTUs over time, our data showed that OTUs colonizing the sediments during the early stage of community assembly (i.e. the first 49 to 115 days) largely dominated the final communities at the end of the incubation. However, at the same time, these OTUs represented only a small fraction of newly-arriving OTUs that were found at each time point. This was furthermore reflected by the large dominance of turnover over nestedness between successional stages in the sediment-attached communities inferred from beta diversity partitioning, showing that the majority of OTUs that had established in the communities at a given time point were not very persistent, but were in fact replaced to a large extent by other OTUs over the course of the succession. Therefore, in agreement with findings made by Brislawn et al. (2019), the mere timing of OTU arrival did not seem to be a determining factor for the final community structure. Instead, the consistent dominance of specific taxa among the few OTUs that were persistent—mainly genera within the *Oxalobacteraceae*, *Comamonadaceae*, *Caulobacteraceae*, and *Sphingomonadaceae*, in addition to *Pseudomonas* spp.—suggests the involvement of certain traits that enable these taxa to sustainably colonize and thrive on sediment surfaces.

Interestingly, we found the same genera as the ones listed above among the most important contributors to differences between sediment-attached and planktonic communities inferred from SIMPER analysis, and to be highly differentially abundant in sediment-attached communities. The association of these organisms with biofilms and traits that facilitate surface colonization like motility or production of extracellular polysaccharides have been reported before for other environments (Kalmbach et al., 2000; Baldani et al., 2014; Bižić-Ionescu et al., 2015; Niederdorfer et al., 2016, 2017), supporting the hypothesis about their importance for the development of sediment-attached communities in our study. Over the course of the succession, these dominant OTUs may have facilitated the recruitment of other more diverse taxa that were observed towards the later stages of the colonization (Battin et al., 2007; Nemergut et al., 2013; Fukami, 2015).

Comparisons of differences in microbial community composition revealed that sediment-attached and planktonic communities, respectively, were similar across the two sampling locations at each time point. Using the null model approach by Stegen et al. (2012, 2013) suggested that different processes were responsible for the observed similarities. Whereas similarities between planktonic communities between the two locations were mainly driven by homogenizing dispersal, the high similarities between sediment-attached

communities at both sites were mostly indicated to be caused by species sorting selecting for similar OTUs in the two communities. We are aware that our study consisted of only a relatively limited number of observations, and therefore the results should be interpreted with the necessary caution. Nevertheless, our results fit observations on assembly processes for sediment-attached communities in the hyporheic zone (Graham et al., 2016a, 2017; Stegen et al., 2016b) as well as biofilms in surface water streams (Besemer et al., 2012; Veach et al., 2016), suggesting that species sorting not only plays a determining role in the assembly of surface-attached microbial communities in those more energy-rich environments, but also in pristine groundwater aquifers, despite the comparatively more oligotrophic conditions. However, we were unable to establish which factors may have been responsible for species sorting, as we did not find significant correlations between changes in β-NTI and any of the measured environmental parameters. Mineral composition has previously been demonstrated to be a driving factor for microbial community composition and assembly (Grösbacher et al., 2016; Stegen et al., 2016b; Jones and Bennett, 2017). Given that the *in situ* microcosms that were incubated at the two sites for our experiment were filled with sediment that originated from the same source, it is likely that identical sediment properties might have selected for the highly similar communities at the two sites.

In the same vein, assembly processes behind changes in the composition of sediment-attached communities were highly variable over time without a clearly discernible trend in favor of a single process, and without apparent associations to changing environmental conditions. This could suggest that the changes in community composition over time and the influence of species sorting were determined by unmeasured environmental variables (Stegen et al., 2013). Alternatively, the observed lack of correlations between changes in environmental conditions and the processes that determined community assembly can also hint at the impact of endogenous factors like species interactions (Konopka et al., 2015; Battin et al., 2016; Cordero and Datta, 2016). Recently, Danczak et al. (2018) could show that interaction network structures can affect assembly processes in pristine groundwater environments. Although our results showed that the succession of OTUs in sediment-attached communities was highly reproducible between the two sites, and that similarities between communities were mostly higher than expected by chance (β-NTI< -2), the compositions of the communities at both sites for each time point—and therefore possibly interaction networks—were not fully identical. Hence, variable patterns of processes that determined changes in community composition over time at each site might, at least in part, be attributed to potential differences in interaction networks within the communities between the two sites.

An additionally important factor for the assembly and succession of surface-attached communities in aquatic environments is the recruitment of species from the surrounding water phase (Battin et al., 2016). The establishment of recruited species in a biofilm community depends on both dispersal as well as interactions with already established species (Battin et al., 2007, 2016). Beta diversity partitioning showed that sediment-attached and planktonic communities were composed of distinct OTUs. We again used the null model approach to test to what extent species sorting and dispersal contributed to these differences. We found that over the first successional stages, the differences between sediment-attached and planktonic communities were driven by dispersal limitation acting alongside drift and later on shifted towards species sorting selecting for significantly different OTUs in both types of communities. The latter observation could be explained in the light of previous studies which have suggested that species with similar ecological niches as resident species have a lower chance of successfully invading a community than species that have less niche overlap with already established ones (Fargione et al., 2003; Tilman, 2004; Peay et al., 2012; Tan et al., 2012).

However, the processes that were indicated to have determined differences in community composition between groundwater and sediments at the earlier stages—that is, dispersal limitation in combination with drift as indicated by significantly positive values for RC_{bray}—were counterintuitive. Multiple causes could explain these unexpected results. For one, it has to be noted that the sediment microcosms were incubated in groundwater monitoring wells, which is a relatively artificial environment that is not necessarily fully representative of the actual groundwater in an aquifer. Consequently, communities found directly inside the well water may differ from the communities in the surrounding groundwater (Griebler et al., 2002; Korbel et al., 2017). In fact, previous analyses of our samples by T-RFLP fingerprinting did indeed reveal some differences between groundwater and well water microbial communities (Zhou et al., 2012). However, considering the relatively high groundwater flow velocities in the investigated area, and the fully screened monitoring wells that readily enable water exchange, dispersal limitation appears unlikely to have caused the observed differences between planktonic and sediment-attached communities in our study.

As discussed earlier (Section 4.1), apart from dispersal limitation, significantly positive values for RC_{bray} might also occur if differences between communities are the result of species sorting involving phylogenetically non-conserved traits. Revisiting the framework regarding the dominance of different assembly processes in relation to spatial scale presented in Figure 1.2.2, the processes that determined differences between sediment-attached and planktonic communities in this study can be expected to have operated

over small distances (on the scale of A to B shown in Fig. 1.2.2), where biotic factors are predicted to be the main drivers of species sorting. Strong biotic factors like competition between species can cause communities to be more different than expected by chance (Chase et al., 2011). We may hypothesize that competition involving phylogenetically non-conserved traits could have been responsible for the differences between sediment-attached and planktonic communities during the early stage of the colonization (i.e. that is the first 115 days), which would not necessarily result in a deviation from the null expectation in phylogenetic null models (i.e. β-NTI), but could still cause significantly positive values for RC_{bray}. Only at the later stage, when the communities on the sediments had matured further (i.e. after 263 days), phylogenetically more conserved traits may have gained importance as suggested by significantly positive values for β-NTI.

4.3 Ecological groundwater monitoring based on microbiological parameters

Despite the fact that policy makers in different parts of the world have started to acknowledge the ecosystem status of groundwater aquifers, and therefore no longer consider them merely as a storage of freshwater, ecological aspects still have received little attention in groundwater monitoring to date. One of the main reasons for the currently still lacking implementation of ecological criteria is certainly the lack of suitable tools that can be easily incorporated into already existing routine monitoring practices. The approach based on the microbiological parameters that are analyzed simultaneously using the D-A-(C) index as presented in this study was developed to fill this gap. Previous studies have analyzed responses of prokaryotic microorganisms to different disturbances in groundwater environments. For instance, increased microbial activity and growth have been reported in response to increased concentrations and fluxes of carbon caused by groundwater recharge with surface water (Foulquier et al., 2011b; Mermillod-Blondin et al., 2013; van Driezum et al., 2018) or suddenly occurring contamination with aromatic hydrocarbons (Herzyk et al., 2017). These general trends are in line with our observations for the first two disturbance case studies. However, in contrast to the monitoring of drinking water distribution systems (FDHA, 2012; van Nevel et al., 2017), most of the studies that have investigated these parameters in relation to different disturbances of groundwater ecosystems so far have mainly been descriptive and did not provide a practical framework of how these parameters could be integrated into monitoring protocols.

Our approach uses an integrated analysis of multiple microbiological variables to detect disturbances. Although it has been shown that for example microbial activity correlates positively with microbial cell numbers (Eydal and Pedersen, 2007; van der Wielen and van der Kooij, 2010; Foulquier et al., 2011b; van Nevel et al., 2017), a pattern that was also reflected in our data, these variables should not be regarded as mutually redundant. Our analyses showed that disturbances were not equally mirrored by these two variables, and furthermore, that significant differences between regions in one variable did not necessarily coincide with significant differences in another variable. It is crucial to realize that total prokaryotic cell counts are a measure of the total number of cells in a sample, while only the active fraction of these cells contributes to the activity measured as intracellular ATP concentrations. These two variables can be affected differently by different disturbances or over different time scales. For instance, it could be shown that microbial cell density can exhibit a delayed response to increased nutrient inputs (Foulquier et al., 2011b), while intracellular ATP concentrations can react almost

immediately (Hofmann and Griebler, 2018). Moreover, although such a pattern was not observed in our particular example of the surface water intrusion event, it is easy to imagine how surface water intrusion could result in an increase in microbial cell numbers without causing changes in activity. This would be the case if cells from the surface water were transported into the groundwater, but were inactivated by the characteristically lower nutrient levels in the groundwater compared to surface water. Hence, considering multiple variables can allow for a more nuanced approach to detect different types of disturbances.

We additionally demonstrated the advantages of a simultaneous, multivariate analysis of the considered microbiological variables compared to separate univariate analyses of each variable. The main advantage of the multivariate approach is that correlations between the variables are also taken into account, thereby capturing the actual distribution of samples along the measured variables, unlike univariate approaches where each variable is analyzed separately and thereby possible correlations are ignored (Manly, 1994). Our analyses showed that disturbances were not only detected more sensitively by the multivariate approach, but also that it was more robust by being able to detect disturbances more reliably in datasets with higher fractions of contaminated samples compared to the univariate approach. Moreover, using the D-A-(C) index (i.e. robust Mahalanobis distances) as indicator of disturbances offers an easy interpretation of the data based on a single value which integrates the signals from multiple variables. The power of this approach to detect anomalies (i.e. outliers) has already been successfully demonstrated in various contexts, ranging from finance to astronomy and biotechnology as well as quality monitoring of industrial manufacturing processes (Rousseeuw and Van Driessen, 1999; Cohen Freue et al., 2007; Hubert et al., 2017), which allows for a seamless transfer of this approach into the context of ecological monitoring.

As another advantage, the multivariate analysis approach can be easily extended by additional variables that may be identified in the future as sensitive indicators of disturbances without compromising the simplicity of the analysis, since the interpretation would still be based on a single index value regardless of the number of variables considered in the analysis. For example, additional information such as cell size distributions, numbers of phototrophic cells, ratios of live over dead cells, or ratios of cells with high nucleic acid content over cells with low nucleic acid content, can be directly derived from flow cytometry measurements used to determine cell densities (Hammes and Egli, 2010; Wang et al., 2010; Hammes et al., 2011; van Nevel et al., 2017). This enables a straightforward amendment of the analysis which may further increase the sensitivity of detecting disturbances.

We have illustrated how the analysis can be extended, and its sensitivity can be increased, by including additional variables based on the example of the third disturbance case study related to the impacts of different types of land use. In this example, disturbances related to agricultural land use that were not identified by only looking at cell density and activity could be identified by additionally including AOC concentrations in the analysis. Although a higher sensitivity was achieved in this particular example by amending the analysis with AOC, there are certain pitfalls associated with this variable that need to be considered. It is important to remember that AOC concentrations are derived indirectly from the increase in cell numbers in a sample over a certain amount of time during batch incubation under *in situ* groundwater conditions, that is in the dark and at the according temperature (Hammes and Egli, 2005). However, from our own experience, this measure is only applicable under certain conditions. For instance, we have frequently noticed that the cell density decreases over the course of the incubation resulting in calculated negative AOC concentrations. This is mainly the case for surface water samples, where phototrophic cells die and decay during the incubation in the dark, or for groundwater samples that are strongly affected by surface water and may have contained phototrophic cells. Moreover, we can think of cases where the presence of toxic compounds like heavy metals can lead to growth inhibition (Şengör et al., 2009). In such a case, the cell density in a sample would not increase, or only increase slightly, during the incubation, which would be falsely interpreted as low AOC concentrations. Similar situations would arise in cases where carbon is not the limiting resource, but the cell growth is constrained by the availability of other essential nutrients like nitrogen or phosphate (Hofmann and Griebler, 2018), or electron acceptors, as is often the case for organically contaminated groundwater (Meckenstock et al., 2015). In the light of these pitfalls, we recommend the use of AOC as an additional variable only for groundwater samples in absence of strong surface water impacts. Furthermore, protocols to measure AOC could be adjusted by amending samples with additional nitrogen and phosphate sources to overcome potential biases of AOC estimates caused by nutrient limitations (Trimbach et al., *unpublished*).

Certain shortcomings may also be encountered if only cell density and activity are considered. The disturbance case studies that we investigated to test our approach could all be roughly categorized as disturbances that affect organic carbon and nutrient levels in the groundwater. Even though we could clearly demonstrate that these disturbances can be detected by the multivariate analysis of prokaryotic cell density and activity, there are other potential impacts which might not be detected using this approach. One example could be groundwater contamination with micropollutants like pharmaceuticals

or pesticides, which has become a growing concern over the past years (Lapworth et al., 2012; Fenner et al., 2013). Since these compounds typically occur in groundwater only at concentrations of a few nanograms to micrograms per liter—often much lower than ambient AOC concentrations—they usually do not serve as substrate for microbial growth (Helbling, 2015) and, from a critical point of view, might not be expected to have toxic effects on microorganisms that would cause noticeable changes in intracellular ATP concentrations. Whether impacts caused by micropollutants can be detected at all based on microbiological parameters needs to be assessed in future research. However, in general, certain other drawbacks of our approach could be alleviated by incorporating additional variables in the analysis as discussed above.

Looking at the regional D-A-(C) signatures of non-contaminated aquifers across Germany, we noticed significant differences in terms of average levels of prokaryotic cell density, activity, and AOC, respectively, as well as covariance matrices. A detailed discussion of differences between individual regions would exceed the scope of this study. However, the main implication of these findings for groundwater monitoring is that these differences between regions need to be taken into account for data collection and interpretation by analyzing data on a suitable regional scale. Furthermore, our analysis revealed that disturbances can only be reliably detected up to a certain fraction of disturbed samples in the dataset. Therefore, in practice, a number of known reference monitoring wells should be routinely included in the analysis that are well protected against disturbances and provide a representative estimate of D-A-(C) signatures for a given region. A step towards the biogeographical classification of groundwater ecosystems has been made by Stein et al. (2012), who showed that the distribution of metazoan groundwater fauna across Germany does not agree with conventional classifications based on hydrogeology, geochemistry, or bioregions defined based on surface water fauna. In the light of these findings, we suggest that also the definition of suitable biogeographical boundaries for microbiological parameters should be guided by ecological criteria rather than being solely based on hydrogeology or geochemistry.

5 Conclusions and outlook

In this thesis, I set out to provide a better understanding of the processes that shape microbial community composition in groundwater environments, and to exploit the potential of data on these ubiquitously distributed microorganisms to meet the pressing demand for a monitoring scheme that allows for the ecological-biological assessment of groundwater ecosystems.

The first principal goal was to shed light on the yet little understood relative contributions of species sorting, dispersal, and random drift to microbial community assembly in pristine groundwater aquifers. This goal was tackled in two separate studies addressing distinct key objectives: first, to uncover the driving factors that impose species sorting by distinguishing between selection effects caused by local environmental conditions in the groundwater relative to potential broad-scale region-specific factors that may determine microbial community composition within different regions, and to differentiate between the effect of species sorting potentially caused by such broad-scale factors and dispersal limitation across regions; second, to establish to what extent species sorting determines microbial community assembly on groundwater sediments in particular, since sediment-attached communities are assumed to play a critical role in groundwater environments, given that they typically represent the majority of the microbial biomass in aquifers, and often display higher activity levels compared to planktonic communities in the surrounding groundwater (Alfreider et al., 1997; Lehman et al., 2001; Lehman and O'Connell, 2002; Griebler et al., 2002; Zhou et al., 2012; Smith et al., 2018).

Our results showed that differences in microbial community composition across distinct aquifers from different geographic regions were mainly the product of species sorting imposed by local environmental conditions, with a relatively smaller—but still significant—contribution of dispersal limitation and drift across regions. However, we did not find evidence for significant selection effects caused by region-specific factors independent of local environmental conditions, represented by both measured and unmeasured environmental variables. Although species sorting also played a determining role in structuring local microbial communities within the individual regions, we found partially inconsistent results between distance-based regression analyses and ecological null models regarding the contribution of dispersal limitation and drift within regions. Hence, combining microbial community analyses with hydrological models to map groundwater flow paths, and to identify possible dispersal routes for microorganisms, will be important for future research to allow for more accurate estimates of the contribution of dispersal to

microbial community assembly in groundwater environments.

Furthermore, we could show that species sorting also plays a determining role for the assembly of microbial communities on groundwater sediments. Our analyses revealed that taxa in sediment-attached communities that developed in *in situ* microcosms were not simply a random subset of the taxa found in the surrounding groundwater. Thus, differences between sediment-attached and planktonic communities often reported for groundwater environments do not seem to be the result of purely stochastic arrival of taxa on the sediments and random drift. Instead, sediment surfaces appear to select for specific groups of microorganisms that assemble over time in a reproducible, non-random way. Moreover, the colonization of the sediments in several aspects followed general patterns that have also been described for the development of biofilms in other, comparatively more energy-rich, non-subsurface aquatic habitats, as well as the assembly of sediment-attached microbial communities in more dynamic hyporheic zones. This might indicate that the assembly of microbial communities on surfaces is governed by similar underlying mechanisms across a wide range of different habitats. Although we found that OTUs that had established on the initially sterile sediments during the early stage of the colonization dominated the final communities at the end of the experiment, the mere timing of OTU arrival was likely not a determining factor for the subsequent community succession, considering that the majority of these early-colonizing OTUs were not very persistent over time. Rather, traits associated with identified key taxa seemed to have been a more decisive factor for the persistence of these OTUs. However, the ecological processes behind the temporal succession of OTUs during the colonization still remained unclear and might be influenced by species interactions. In addition, we hypothesized that different traits with different degrees of phylogenetic conservation may have determined the establishment of OTUs in the developing sediment-attached communities from the surrounding groundwater at different stages of community development. A better understanding of these traits, and how they integrate into species interaction networks, will be an important aspect for future research. Computational modeling of microbial communities based on meta-omics data, albeit still in its infancy, offers a promising tool to unravel complex species interactions within microbial communities and thus could provide a means to illuminate these open questions (Faust and Raes, 2012; Hanemaaijer et al., 2015; Muller et al., 2018).

It is important to realize that the conclusions from these two studies were derived from 16S rRNA amplicon sequence data. Consequently, the effect of species sorting and dispersal on microbial community assembly inferred from those studies was limited to differences in community composition that could be resolved based on 16S rRNA

sequences. It has been shown that even closely related strains of the same species, with near identical 16S rRNA genes, can differ significantly in their ecological preferences and show distinct biogeographic distributions (Hahn et al., 2016; Larkin and Martiny, 2017; Chase et al., 2018; Choudoir and Buckley, 2018). Therefore, our results need to be interpreted with some caution, bearing in mind that the high degree of conservation of 16S rRNA genes may not have allowed for a complete differentiation between microbial ecotypes beyond the level of ASVs or OTUs, respectively, in the two studies. Advances in assembling high-quality draft genomes from metagenomic data could offer deeper insights into microbial community diversity at a higher resolution beyond the variation captured in 16S rRNA genes (Parks et al., 2017, 2018; Wilkins et al., 2019), and thus may deliver a more detailed picture of the processes that determine microbial community assembly in the future.

The dominating effect of species sorting on the assembly of microbial communities in groundwater environments indicated by our findings leads to the question about the implications for community functioning and stability. We may hypothesize that strong species sorting leads to communities that are composed of microorganisms which are well adapted to their local environment. As a consequence, these organisms might have to invest less energy into maintenance, leaving more energy available for the synthesis of enzymes that can catalyze biogeochemical processes. Therefore, communities primarily assembled by species sorting could be expected to catalyze these processes at higher rates as opposed to communities that are predominantly assembled by dispersal (Wallenstein and Hall, 2012; Graham and Stegen, 2017). However, at the same time, the strong adaptation to local environmental conditions could come at the cost of a low resistance to changing environmental conditions, which could cause inactivation and replacement of organisms in a community, and thereby disrupt community functioning, if the magnitude of the environmental changes exceeds the range of fluctuation to which these organisms have adapted (Shade et al., 2012; Graham and Stegen, 2017). Indeed, low resistance of groundwater microbial communities to perturbations and subsequent rapid replacement of resident taxa has been documented in previous studies (Zhou et al., 2014; Herzyk et al., 2017). However, whether the strong influence of species sorting presented in this thesis translates into high performance of groundwater microbial communities awaits detailed experimental testing in future research.

In addition to these fundamental ecological aspects, the second main goal of this thesis was the development of an assessment scheme for the ecological monitoring of groundwater ecosystems that is compatible with existing routine monitoring practices. Based on the presented case studies, we could show that prokaryotic cell density and activity

can be used as reliable and sensitive indicators of disturbances of groundwater ecosystems. These parameters can be easily integrated into existing groundwater monitoring schemes as they are simple and inexpensive to measure, and furthermore can be analyzed in samples that can be directly collected along with samples for the routine monitoring of conventional physicochemical parameters. Furthermore, we have demonstrated that the multivariate analysis of these variables using the D-A-(C) index not only allows for a higher sensitivity, but also increases the robustness of the analysis compared to separate univariate analyses of the individual variables. An even higher sensitivity might be achieved by including additional parameters in the analysis such as AOC concentrations. However, optimization of this particular parameter may be required under certain conditions to overcome biases associated with limitations of nutrients other than organic carbon. Further evaluation of our approach is underway to assess its potential as well as limitations to detect disturbances that go beyond the types of disturbances presented in the three case studies. However, in theory, potential limitations of our approach can be overcome by amending the analysis with additional parameters that might be identified as potent indicators of various types disturbances in the future. These parameters could be directly integrated in the analysis without compromising its simplicity, as the interpretation would still be based on a single index values (i.e. robust Mahalanobis distances) that integrates the signals of all variables included in the analysis. Significant differences in the multivariate D-A-(C) signatures of the microbial variables between regions stress the importance of analyzing data and selecting reference wells at a suitable regional scale, the definition of which should not only be guided by hydrogeology or geochemistry, but primarily by ecological criteria.

In summary, the results presented in this thesis contribute to a better understanding of the fundamental processes that shape microbial community composition in groundwater by revealing a strong effect of species sorting on microbial community assembly in these environments. Furthermore, a practical approach has been introduced that allows for the implementation of ecological criteria in routine groundwater monitoring that is increasingly required under modern environmental law in Europe and worldwide.

Supporting information

SI.1 Ecological drivers of differences in microbial community composition across geographically distinct aquifers

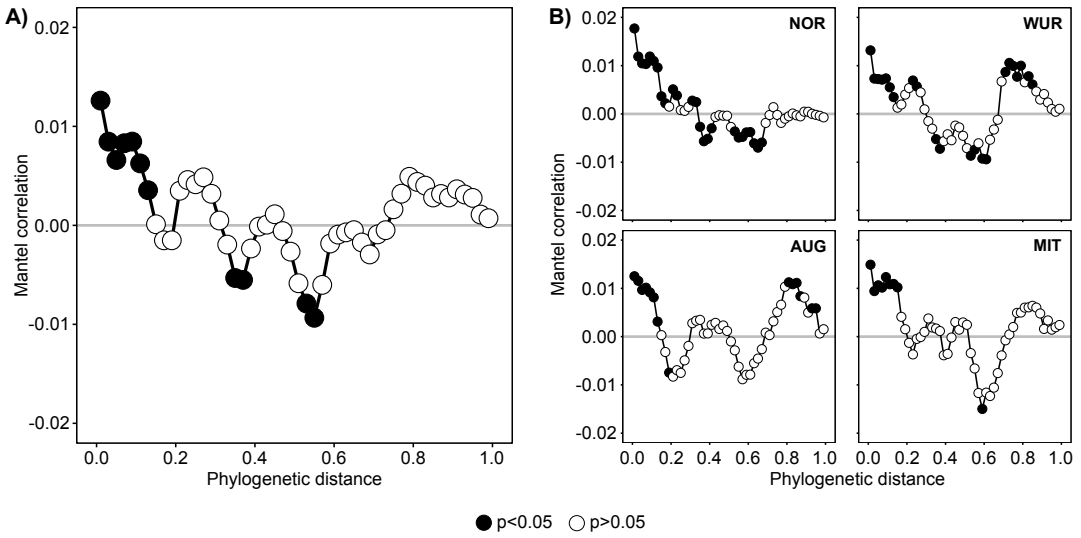

Figure S1.1: Test of the presence of a significant phylogenetic signal using Mantel correlograms. The correlograms show Pearson correlations between phylogenetic distance separating ASVs evaluated at distance class steps of 0.02 and Euclidean distances of ASV environmental optima, based on relative abundance-weighted means for pH, ionPC1, and concentrations of dissolved oxygen, orthophosphate, and DOC. Correlograms were computed for the full dataset (***A***) and each region separately (***B***). Solid symbols indicate significant correlations based on p-values obtained from 10 000 permutations after Holm correction for multiple testing (see Section 2.1.7 for detailed information). (*From Fillinger et al.,* 2019a).

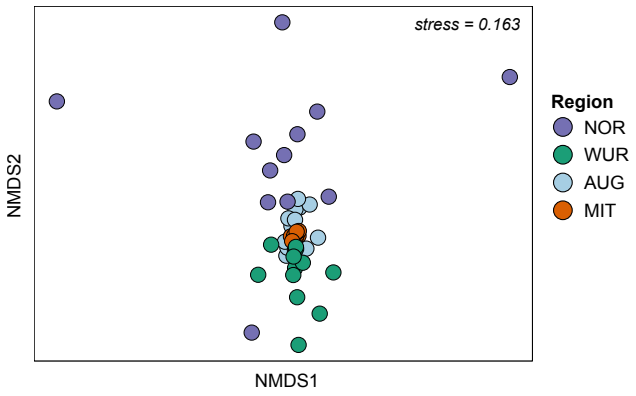

Figure S1.2: NMDS showing differences in community composition based on abundance-weighted β-MPD. (*From Fillinger et al.,* 2019a).

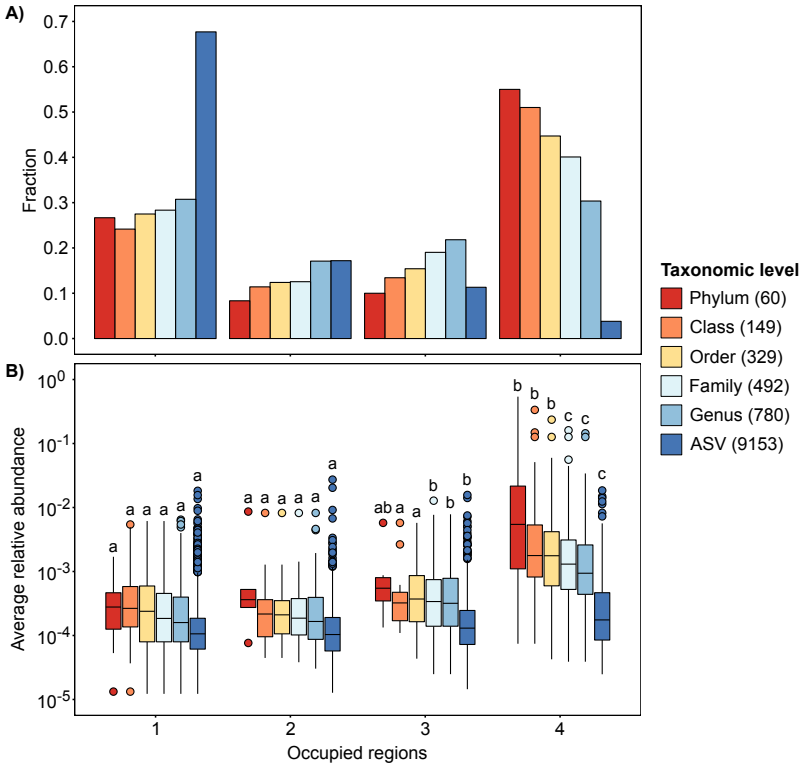

Figure S1.3: ***A***) Fraction of taxa found within only one region, or distributed across two, three, and all four regions, respectively. The total number of individual taxa within taxonomic levels is given in parentheses in the legend. ***B***) Average relative abundances of taxa with different levels of distribution. Relative abundances of taxa were averaged across all samples in which a given taxon was present. Differences between levels of distribution were evaluated within taxonomic groups by pairwise comparisons using Dunn's rank sum tests; significant differences ($p<0.05$) are indicated by different letter labels. (*From Fillinger et al.,* 2019a).

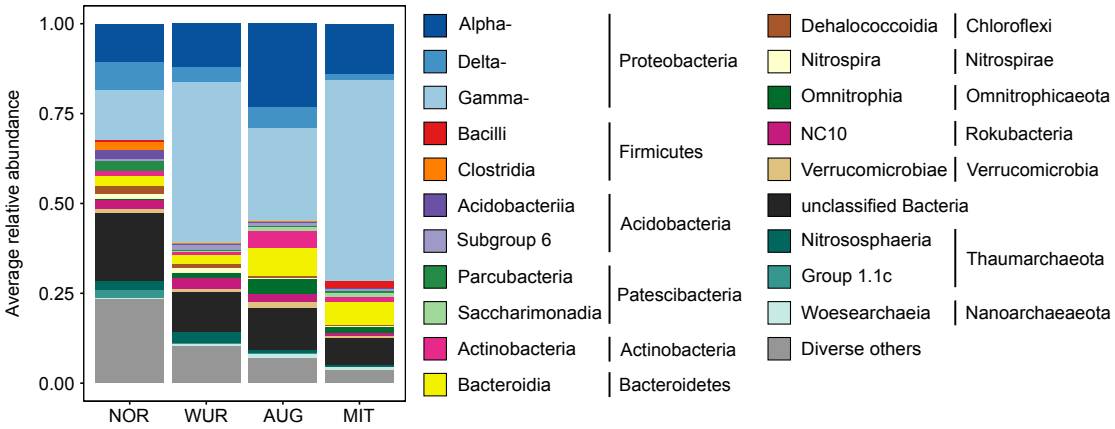

Figure S1.4: Taxonomic microbial community composition summarized at class level (based on taxonomic assignments using SILVA SSU reference database release 132). Relative abundances were averaged across samples within regions. Individual classes with an average relative abundance <2 % were grouped as 'Diverse others' for clarity of display. (*From Fillinger et al.*, 2019a).

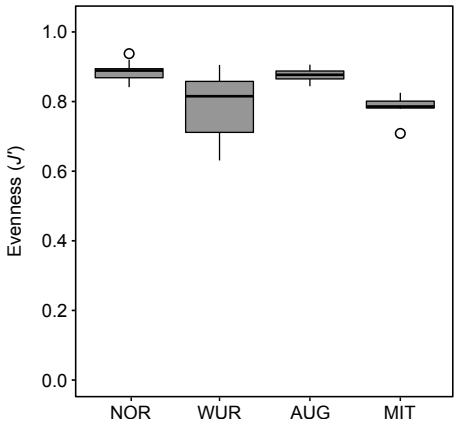

Figure S1.5: Community evenness (Pielou's *J'*) calculated based on ASVs within regions. (*From Fillinger et al.*, 2019a).

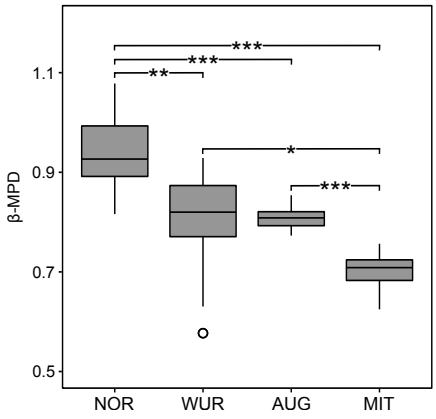

Figure S1.6: Differences in microbial community composition within regions based on abundance-weighted β-MPD. Asterisks indicate significant differences between regions inferred from PERMDISP tests (10 000 permutations; *p<0.05; **p<0.01; ***p<0.001). (*From Fillinger et al.,* 2019a).

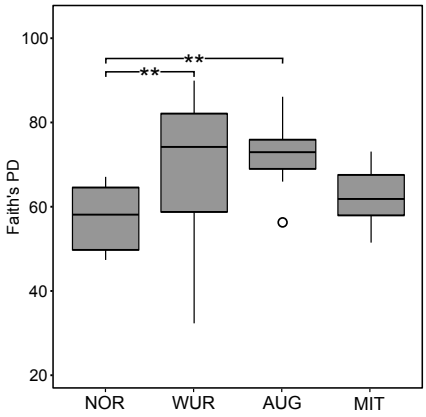

Figure S1.7: Faith's phylogenetic diversity within regions. Asterisks indicate significant differences inferred from Dunn's rank sum tests (p<0.01). (*From Fillinger et al.,* 2019a).

Table S1.1: Overview of sample size, sampling time point, region location, distances between monitoring wells within regions, and environmental variables given as mean ± standard deviation (minimum; maximum) calculated across all samples from a given region. Variables with censored data below the detection limit (nitrate and orthophosphate) are reported as concentrations corresponding to the ranks of the data within a region (minimum; 25^{th}; 50^{th}; 75^{th} percentile; maximum) according to Helsel (2011). (*From Fillinger et al.,* 2019a).

	NOR	WUR	AUG	MIT
n	12	13	12	8
Sampling time point	September 2018	May 2016	June 2016	July 2018
Location	53.72°N, 10.01°E	49.77°N, 9.93°E	48.25°N, 10.90°E	47.41°N, 11.26°E
Distance between wells (km)	3.1 ± 2.0 (0.001; 5.9)	0.3 ± 0.1 (0.001; 0.6)	5.3 ± 4.0 (0.3; 12.1)	1.8 ± 1.2 (0.07; 3.3)
pH	6.4 ± 1.4 (4.9; 8.2)	7.3 ± 0.1 (7.2; 7.4)	7.4 ± 0.1 (7.2; 7.6)	7.6 ± 0.2 (7.3; 7.8)
Temperature (°C)	10.7 ± 0.7 (9.9; 11.7)	14.0 ± 1.3 (12.2; 16.2)	10.4 ± 0.5 (9.5; 11.0)	8.8 ± 1.1 (7.2; 10.2)
Dissolved oxygen ($mg\,L^{-1}$)	4.0 ± 4.7 (0.4; 11.5)	5.9 ± 3.0 (0.5; 10.9)	9.3 ± 1.5 (5.7; 10.4)	8.8 ± 0.9 (7.1; 9.9)
DOC ($mg\,L^{-1}$)	4.1 ± 9.6 (0.5; 34.4)	0.9 ± 0.3 (0.6; 1.5)	0.8 ± 0.1 (0.7; 1.1)	0.9 ± 0.3 (0.7; 1.4)
Prokaryotic biomass ($cells\,mL^{-1}$)×10^4	7.7 ± 5.4 (1.0; 17.5)	5.6 ± 5.5 (2.1; 21.2)	4.4 ± 6.8 (0.5; 24.9)	1.5 ± 0.4 (0.9; 2.0)
Electrical conductivity ($\mu S\,cm^{-1}$)	316 ± 145 (104; 576)	908 ± 160 (653; 1135)	558 ± 55 (435; 611)	325 ± 96 (236; 506)
Sodium ($mg\,L^{-1}$)	16.7 ± 7.7 (4.9; 25.9)	24.5 ± 5.2 (17.0; 33.9)	10.8 ± 3.1 (6.8; 17.0)	2.2 ± 2.2 (0.3; 6.0)
Potassium ($mg\,L^{-1}$)	4.6 ± 3.9 (1.1; 13.2)	3.7 ± 1.7 (1.5; 5.9)	2.1 ± 0.5 (1.3; 3.2)	0.3 ± 0.2 (0.1; 0.7)
Magnesium ($mg\,L^{-1}$)	4.6 ± 2.1 (1.5; 8.3)	33.9 ± 6.8 (20.6; 43.1)	33.9 ± 4.0 (25.4; 39.1)	13.6 ± 6.7 (8.6; 27.3)
Calcium ($mg\,L^{-1}$)	33.7 ± 27.6 (5.3; 84.7)	142.2 ± 38.3 (92.0; 215.0)	97.9 ± 10.7 (75.6; 110.0)	46.0 ± 10.6 (34.8; 60.3)
Chloride ($mg\,L^{-1}$)	28.0 ± 21.6 (7.2; 88.2)	76.0 ± 14.1 (57.9; 104.0)	18.7 ± 5.8 (9.6; 27.2)	2.9 ± 2.8 (0.3; 7.5)
Sulfate ($mg\,L^{-1}$)	40.8 ± 25.5 (3.4; 95.6)	106.4 ± 55.5 (105.0; 257.0)	12.0 ± 3.0 (8.5; 19.2)	12.1 ± 14.1 (4.4; 45.6)
Nitrate ($mg\,L^{-1}$)	<0.1; <0.1; <0.1; 8.3; 25.2	9.0; 18.0; 20.0; 21.0; 29.0	11.0; 15.3; 20.5; 23.3; 36.0	1.8; 2.0; 2.1; 4.0; 4.7
Orthophosphate ($\mu g\,L^{-1}$)	<22.2; <22.2; <22.2; 38.5; 130.0	<22.2; 26.8; 45.4; 178.0; 507.0	all <22.2	<22.2; <22.2; <22.2; 24.4; 28.2

Table S1.2: Taxonomic assignments of the 10 most abundant individual ASVs with the highest average relative abundances within regions (taxonomic assignments are based on SILVA SSU reference database release 132; table continues on next page). (*From Fillinger et al.,* 2019a).

Region	ASV ID	Average relative abundance	Domain	Phylum	Class	Order	Family	Genus
NOR	ASV59	0.61 %	Bacteria	Epsilonbacteraeota	Campylobacteria	Campylobacterales	Thiovulaceae	Sulfurimonas
	ASV61	0.59 %	Archaea	Thaumarchaeota	Group 1.1c	unclassified	unclassified	unclassified
	ASV55	0.56 %	Bacteria	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Gallionellaceae	Gallionella
	ASV80	0.48 %	Archaea	Thaumarchaeota	Group 1.1c	unclassified	unclassified	unclassified
	ASV96	0.45 %	Bacteria	Proteobacteria	Gammaproteobacteria	Methylococcales	Methylomonaceae	Methylobacter
	ASV81	0.45 %	Bacteria	Epsilonbacteraeota	Campylobacteria	Campylobacterales	Thiovulaceae	Sulfurimonas
	ASV72	0.43 %	Bacteria	Rokubacteria NC10	unclassified	unclassified	unclassified	unclassified
	ASV88	0.43 %	Archaea	Thaumarchaeota	Nitrososphaeria	unclassified	unclassified	unclassified
	ASV138	0.41 %	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	unclassified
	ASV129	0.39 %	Bacteria	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Rhodocyclaceae	unclassified
WUR	ASV1	4.75 %	Bacteria	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Rhodocyclaceae	Ferribacterium
	ASV3	4.60 %	Bacteria	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Burkholderiaceae	unclassified
	ASV2	3.65 %	Bacteria	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Rhodocyclaceae	Ferribacterium
	ASV8	3.36 %	Bacteria	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Burkholderiaceae	unclassified
	ASV4	3.09 %	Bacteria	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Burkholderiaceae	unclassified
	ASV7	2.34 %	Bacteria	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Burkholderiaceae	unclassified
	ASV10	1.99 %	Bacteria	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Burkholderiaceae	unclassified
	ASV15	1.36 %	Bacteria	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Burkholderiaceae	unclassified
	ASV6	1.13 %	Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Caulobacter
	ASV21	1.01 %	Bacteria	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Burkholderiaceae	unclassified

Table S1.2 (continued from previous page).

Region	ASV ID	Average relative abundance	Domain	Phylum	Class	Order	Family	Genus
AUG	ASV6	2.96 %	Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Caulobacter
	ASV10	1.72 %	Bacteria	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Burkholderiaceae	unclassified
	ASV18	1.29 %	Bacteria	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Burkholderiaceae	unclassified
	ASV4	1.26 %	Bacteria	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Burkholderiaceae	unclassified
	ASV24	1.14 %	Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Brevundimonas
	ASV19	1.00 %	Bacteria	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Burkholderiaceae	unclassified
	ASV7	0.92 %	Bacteria	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Burkholderiaceae	unclassified
	ASV32	0.90 %	Bacteria	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Burkholderiaceae	unclassified
	ASV40	0.85 %	Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Brevundimonas
	ASV29	0.84 %	Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Phenylobacterium
MIT	ASV5	6.27 %	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
	ASV9	4.48 %	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
	ASV12	2.72 %	Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	Rheinheimera
	ASV14	2.38 %	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
	ASV1	2.13 %	Bacteria	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Rhodocyclaceae	Ferribacterium
	ASV16	1.98 %	Bacteria	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Burkholderiaceae	unclassified
	ASV11	1.89 %	Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Caulobacter
	ASV23	1.82 %	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Pseudoxanthomonas
	ASV22	1.71 %	Bacteria	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Burkholderiaceae	Janthinobacterium
	ASV26	1.70 %	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas

Table S1.3: Partition of variation in microbial community composition (abundance-weighted β-MPD) between local environmental conditions (*Env*; significant environmental variables are listed in the rightmost column) and region identity (*Reg*). *Env*+*Reg* represents the total variation explained by both components; *Env*|*Reg* (*Reg*|*Env*) represents the marginal fraction of variation explained by each component after controlling for the other; *Env*∩*Reg* represents the fraction of explained variation shared between both components. The explained variation is given as adjusted R^2. Significance of each component and individual variables was tested using 10 000 permutations (note: significance of *Env*∩*Reg* cannot be tested). Spatial distance between sites within regions represented by MEMs was not significant (adj. R^2=0, p=0.79) and was therefore not included in the analysis. (*From Fillinger et al.,* 2019a).

Response matrix	Component	df	Adj. R^2	p	Significant variables (p<0.05)
β-MPD	Env	5	0.0422	0.0001	pH, K^+, ionPC1*, NO_3^-, DOC
	Reg	3	0.0480	0.0001	Dummy-coded region identity
	Env+Reg	8	0.0683	0.0001	
	Env∩Reg	0	0.0220		
	Env|Reg	5	0.0203	0.0011	pH, NO_3^-
	Reg|Env	3	0.0261	0.0001	
	Residuals	36	0.9317		

*Principal component representing 74 % of the variance in electrical conductivity and concentrations of sodium, calcium, magnesium, chloride, and sulfate (all positively correlated with ionPC1; see Section 2.1.7).

SI.2 Assembly and succession of microbial communities during the colonization of groundwater sediments

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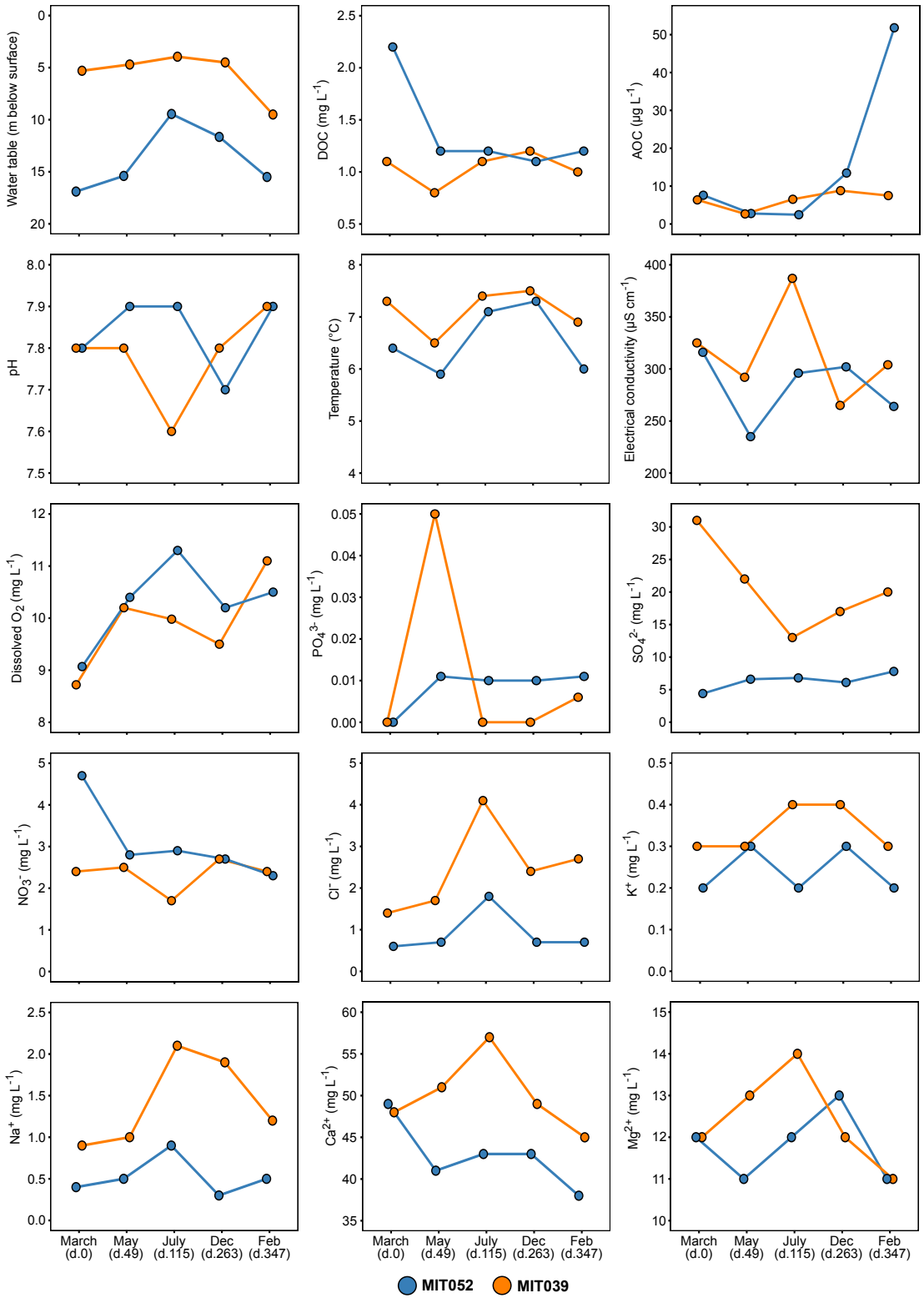

Figure S2.1: Changes in physicochemical parameters over time measured in the groundwater at the two sites. (*From Fillinger et al.*, 2019c).

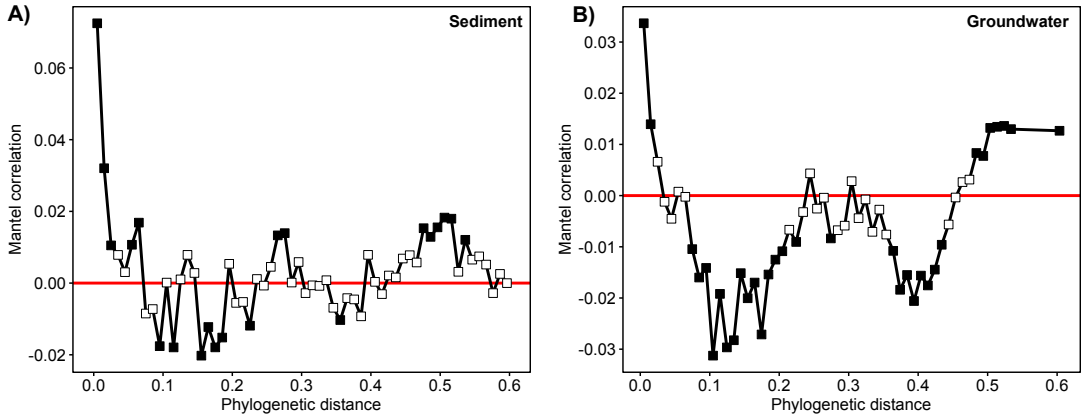

Figure S2.2: Test of the presence of a significant phylogenetic signal using Mantel correlograms. The correlograms show Pearson correlations between phylogenetic distance separating OTUs evaluated at distance class steps of 0.01 and Euclidean distances of OTU environmental optima, based on relative abundance-weighted means for environmental variables shown Figure S2.1. Correlograms were computed separately for OTUs found in sediment-attached (***A***) and planktonic (***B***) communities. Solid symbols indicate significant correlations based on p-values obtained from 10 000 permutations after Holm correction for multiple testing. (*From Fillinger et al.,* 2019c).

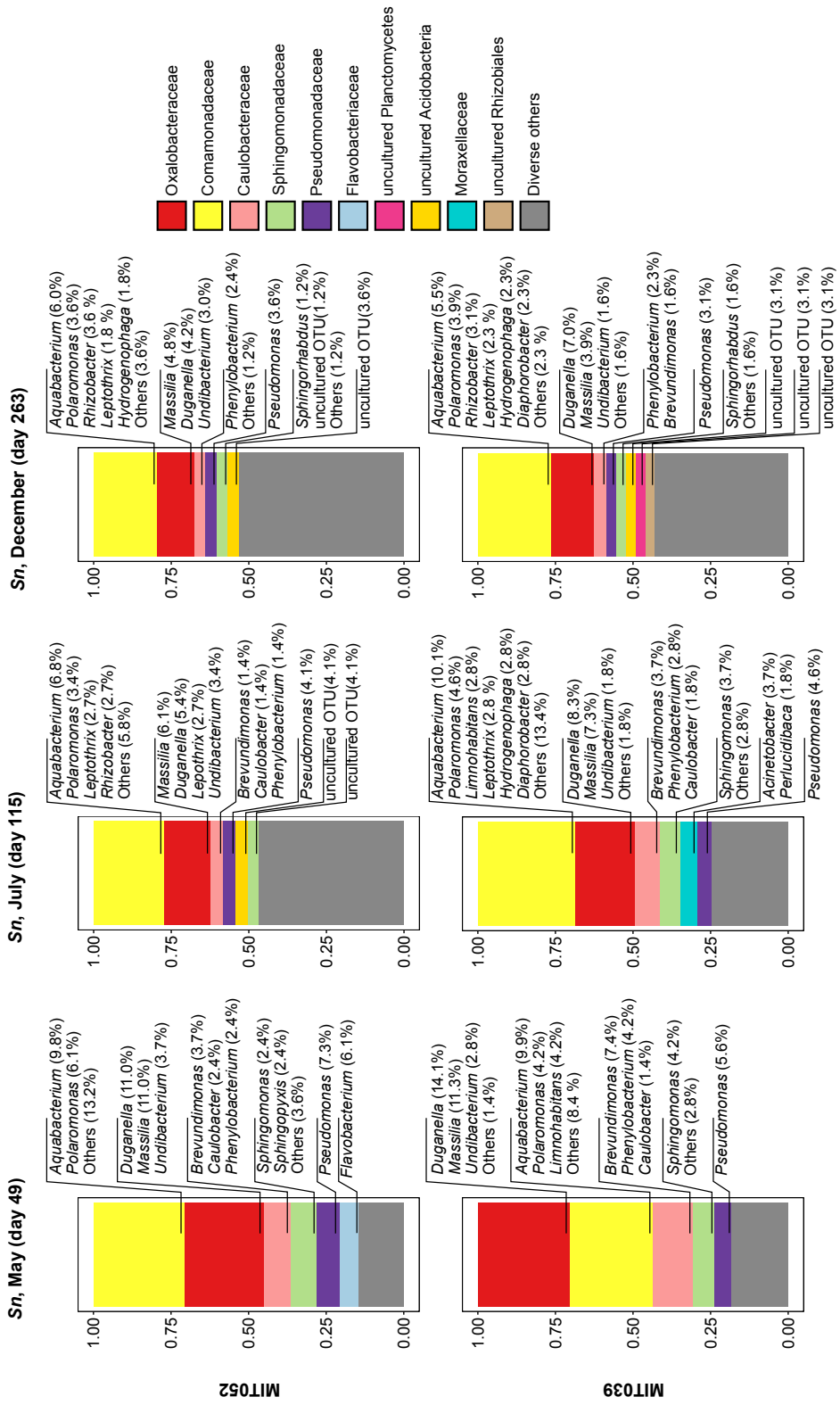

Figure S2.3: Occurrence frequencies of the most dominant taxonomic families and most dominant genera within those families among newly-arriving OTUs (*Sn*) in the sediment-attached communities that first occurred at the indicated time point and were still detected in the final communities at MIT052 and MIT039, respectively. Families with an individual occurrence frequency <3 % are grouped as 'Diverse others' for clarity of display. Bars representing each family are ordered by occurrence frequency in descending order from top to bottom. (*From Fillinger et al.*, 2019c).

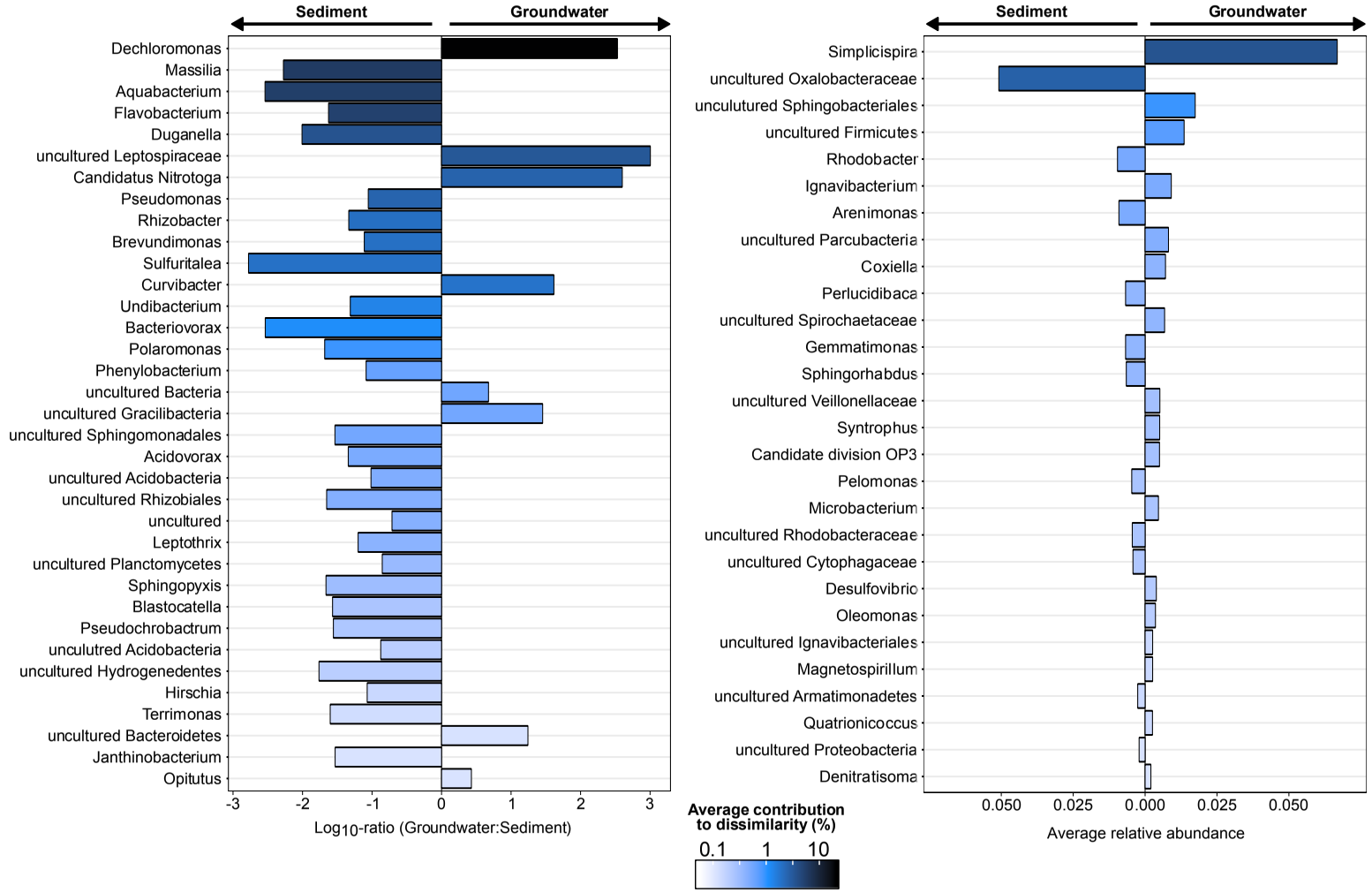

Figure S2.4: Differential abundances of genera that contributed most to the dissimilarity between sediment-attached and planktonic communities in the groundwater identified by SIMPER analysis. Only genera with the highest significant contribution to the dissimilarity are shown (>0.1 %; p<0.05). The average contribution of each displayed genus is indicated by the color intensity of the bars. The left panel shows $\log_{10}$-ratios of differential average abundances in planktonic communities over sediment-attached communities for genera found in both types of communities; the right panel shows average relative abundances of genera exclusively found in only one community type. (*From Fillinger et al.*, 2019c).

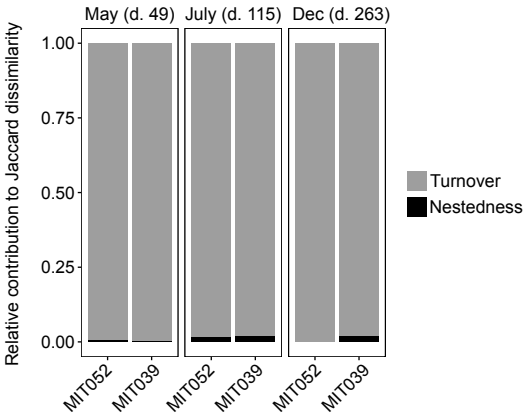

Figure S2.5: Beta diversity partitioning of differences between sediment-attached and planktonic communities showing the relative contributions of turnover and nestedness to the Jaccard dissimilarity between community types at each site per time point. (*From Fillinger et al.,* 2019c).

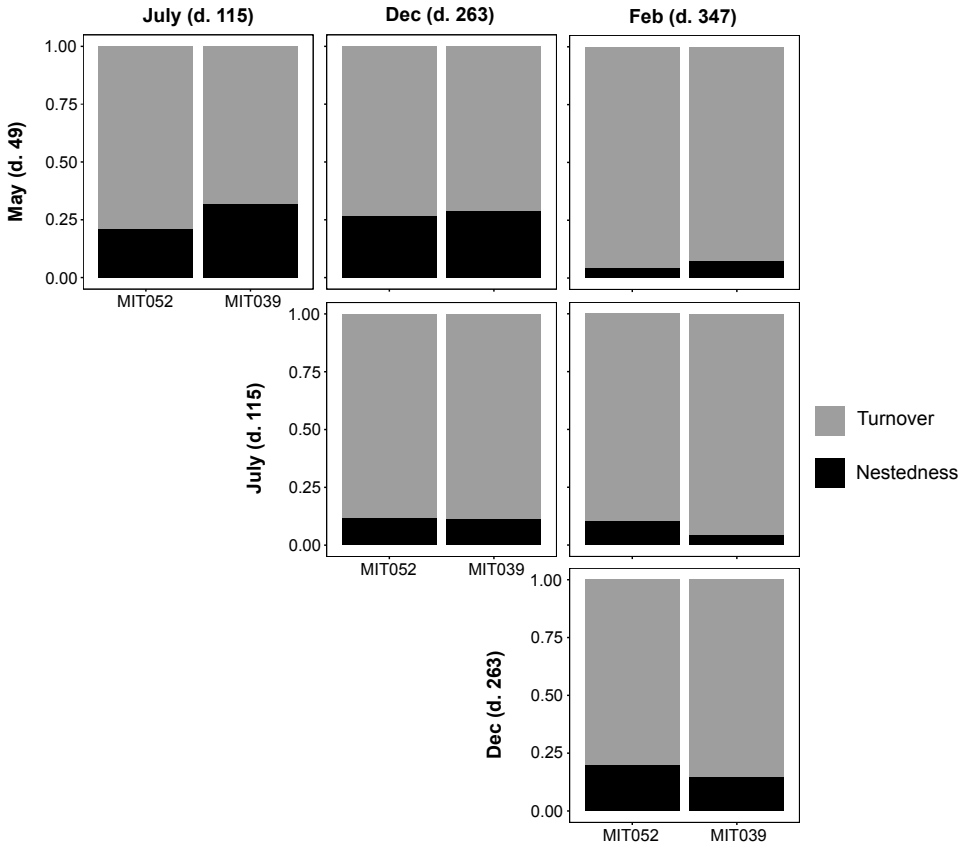

Figure S2.6: Beta diversity partitioning of differences between sediment-attached communities showing the relative contributions of turnover and nestedness to the Jaccard dissimilarity within sites across time points. (*From Fillinger et al.,* 2019c).

SI.3 Ecological groundwater monitoring based on microbiological parameters

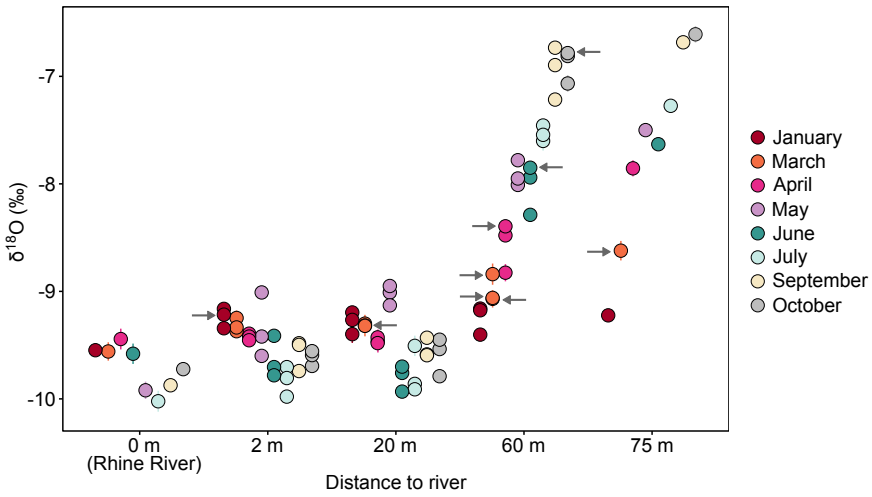

Figure S3.1: Stable oxygen isotope ratios at different time points for the surface water intrusion data (disturbance case study 2) measured in the Rhine River and groundwater monitoring wells located at different distances to the river. Grey arrows indicate samples identified as outliers based on the D-A index (see Section 3.3.1). No corresponding isotope data was available for data presented in the main text for August. (*From Fillinger et al.*, 2019b)

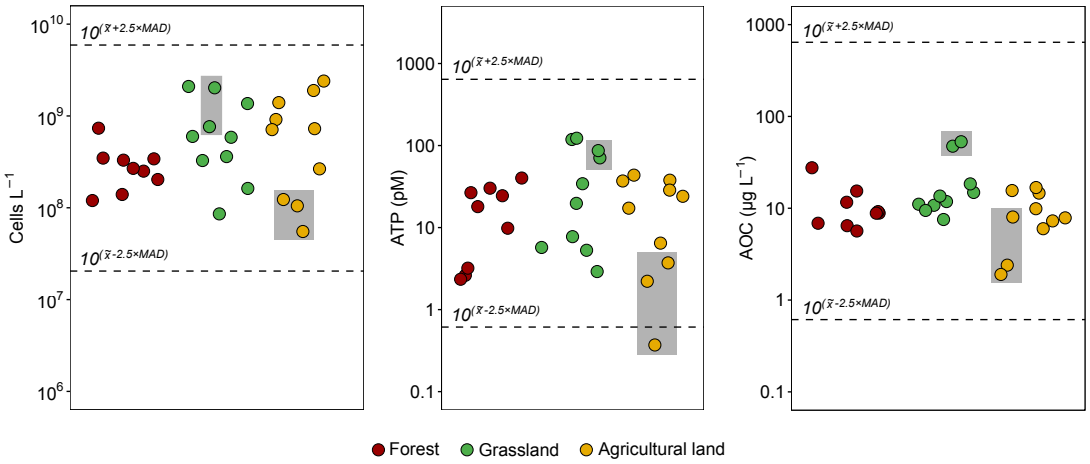

Figure S3.2: Separate univariate analyses of cell density (measured as prokaryotic cells L^{-1}), activity (measured as prokaryotic intracellular ATP concentrations), and carbon (measured as AOC concentrations) for different types of land use (disturbance case study 3). Dashed lines represent the univariate thresholds calculated as $median \pm 2.5 \times MAD$ for each variable. Samples identified as outliers based on the D-A-C index are highlighted with grey rectangles (see Section 3.3.1; note: all calculations were done on $\log_{10}$-transformed data; values were converted back to the original scale to allow for a more intuitive representation of the data). (*From Fillinger et al.,* 2019b)

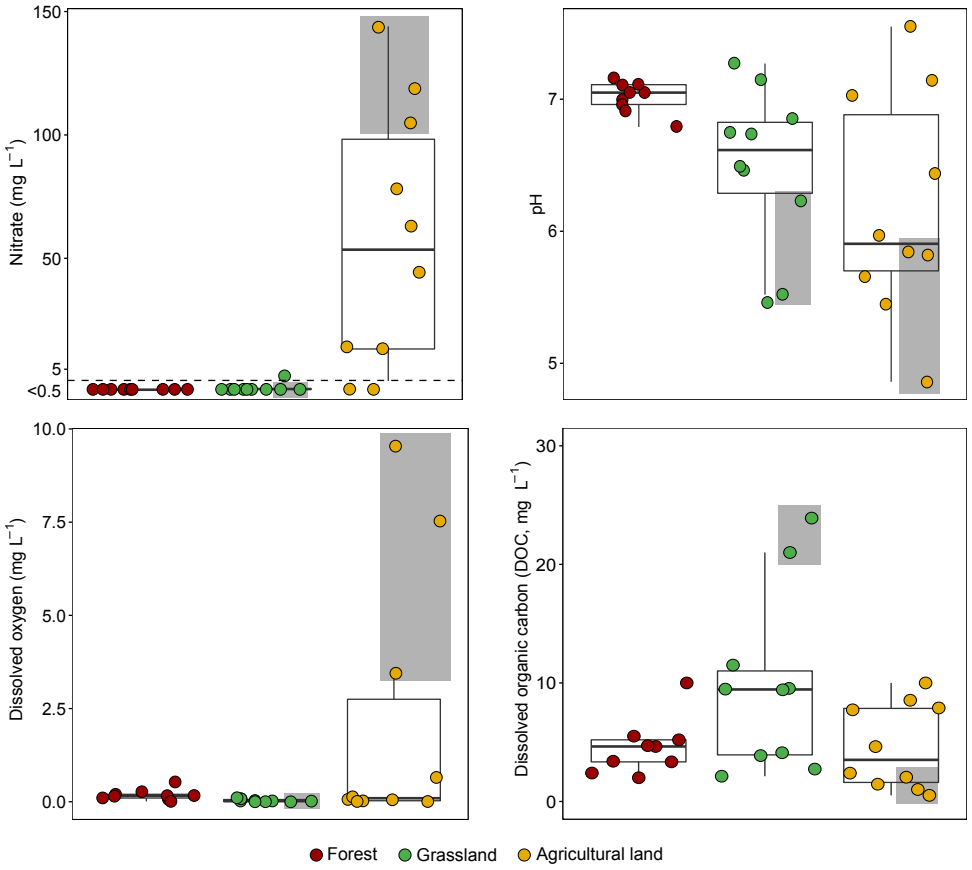

Figure S3.3: Boxplots of hydrochemical variables for different types of land use (disturbance case study 3). Samples identified as outliers based on the D-A-C index are highlighted with grey rectangles (see Section 3.3.1). For nitrate, the majority of samples exhibited concentrations below the detection limit ($<0.5\,\mathrm{mg\,L^{-1}}$) represented by the dashed line. (*From Fillinger et al.,* 2019b)

Table S3.1: Summary statistics of prokaryotic cell density, activity, and AOC concentration for non-contaminated aquifers sampled at the different regions across Germany (Fig. 2.3.1) given as geometric mean, minimum, maximum, and geometric standard deviation for each variable (all calculations were performed on $\log_{10}$-transformed data and values were converted back to the original scale). Only samples were considered that were not identified as outliers for the individual regions (or within the full dataset) based on the D-A-(C) index (see Section 3.3.2). (*From Fillinger et al.,* 2019b)

		Cell density (cells L^{-1})		Activity (ATP, pM)		Carbon (AOC, µg L^{-1})	
Region	n[a]	Mean (min; max) $\times 10^8$	SD	Mean (min; max)	SD	Mean (min; max)	SD
Ratzeburg	11	1.51 (0.11; 5.75)	3.07	9.31 (3.70; 41.40)	2.08	NA	NA
Soltau	32 (11)	1.34 (0.33; 4.09)	2.17	6.93 (1.80; 30.60)	2.33	12.97 (2.80; 63.50)	2.63
Erft-Rur	20 (7)	0.54 (0.16; 1.80)	2.08	3.56 (1.20; 13.00)	1.72	13.69 (5.80; 54.30)	2.12
Eifel	21 (7)	0.47 (0.14; 1.92)	2.23	5.59 (1.10; 44.70)	2.70	4.81 (2.40; 13.40)	1.96
Würzburg	37 (27)	0.75 (0.06; 3.62)	2.32	9.52 (2.34; 37.02)	1.90	2.62 (0.36; 10.67)	2.75
Swabian Alb (porous)	25 (10)	0.29 (0.03; 1.80)	2.77	4.03 (1.20; 24.00)	2.16	10.89 (2.50; 35.00)	2.23
Swabian Alb (karst)	28 (13)	0.15 (0.04; 1.22)	2.84	2.90 (0.70; 15.00)	2.42	6.52 (1.70; 26.50)	2.40
Augsburg	22 (11)	0.08 (0.03; 0.18)	1.62	3.33 (1.66; 7.98)	1.43	1.80 (0.79; 3.39)	1.66
Mittenwald	24	0.26 (0.03; 1.32)	2.69	2.04 (0.40; 7.60)	2.35	NA	NA
Total dataset	225 (116)	0.41 (0.01; 15.00)	3.61	4.73 (0.60; 41.40)	2.44	5.73 (0.18; 202.60)	3.38

[a]AOC data were not available for all samples; the value within parentheses indicates the number of samples for which AOC data were available.

Numerical example of the calculation of Mahalanobis distances

The analysis shown in Figure S3.4 is a fictive example of a dataset containing 20 undisturbed samples and a single disturbed sample. For simplicity, the example only deals with two variables representing $\log_{10}$-transformed prokaryotic cell density (D) and activity (A) similar to the data presented in the main text.

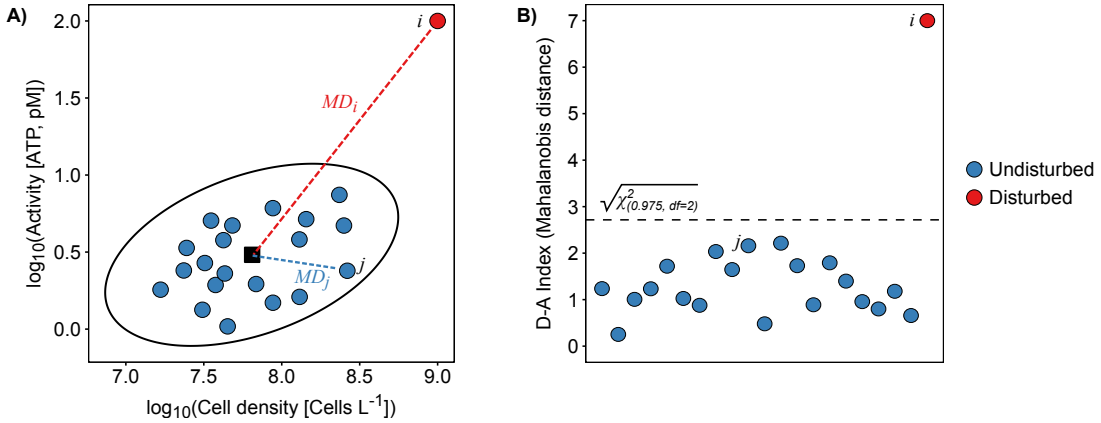

Figure S3.4: Example to illustrate the concept of Mahalanobis distances for multivariate outlier detection. ***A***) Distribution of samples along the variables cell density and activity. The center defined by the variable means is represented by the black square. The distances to the center for samples i and j are indicated by the dashed lines. ***B***) Mahalanobis distances calculated for the samples shown in ***A***. The dashed line represents the critical value of the chi-squared distribution at a 0.975 confidence level with two degrees of freedom. (*From Fillinger et al.,* 2019b)

Cell density and activity have mean values of 7.81 and 0.48, and variances of 0.12 and 0.05, respectively. The covariance between cell density and activity is 0.03. Hence, the vector μ defining the center of the group of samples and the covariance matrix S are as follows:

$$\mu = \begin{bmatrix} \overline{D} \\ \overline{A} \end{bmatrix} = \begin{bmatrix} 7.81 \\ 0.48 \end{bmatrix} \tag{S3.1}$$

$$S = \begin{bmatrix} var(D) & cov(D,A) \\ cov(D,A) & var(A) \end{bmatrix} = \begin{bmatrix} 0.12 & 0.03 \\ 0.03 & 0.05 \end{bmatrix}. \tag{S3.2}$$

Thus, the inverse of S is:

$$S^{-1} = \begin{bmatrix} 9.80 & -5.88 \\ -5.88 & 23.53 \end{bmatrix}. \tag{S3.3}$$

The values of cell density and activity for the disturbed sample i are 9.00 and 2.00, respectively. Hence the vector Xi giving the coordinates for that sample in the bivariate plot is:

$$X_i = \begin{bmatrix} D_i \\ A_i \end{bmatrix} = \begin{bmatrix} 9.00 \\ 2.00 \end{bmatrix}. \tag{S3.4}$$

Based on this the Mahalanobis distance for sample i is calculated as:

$$MD_i = \sqrt{(X_i - \mu)' \times S^{-1} \times (X_i - \mu)} \tag{S3.5}$$

$$= \sqrt{\begin{bmatrix} (9.00 - 7.81) & (2.00 - 0.48) \end{bmatrix} \times \begin{bmatrix} 9.80 & -5.88 \\ -5.88 & 23.53 \end{bmatrix} \times \begin{bmatrix} (9.00 - 7.81) \\ (2.00 - 0.48) \end{bmatrix}}$$

$$= 6.85.$$

Because the analysis comprised two variables, the critical value derived from the chi-squared distribution with two degrees of freedom at a 0.975 confidence level is:

$$\sqrt{\chi^2_{(0.975, df=2)}} = 2.72. \tag{S3.6}$$

Since the Mahalanobis distance of sample i exceeds this critical value, that sample is considered an outlier. For comparison, doing the same calculation for the undisturbed sample j with

$$X_j = \begin{bmatrix} D_j \\ A_j \end{bmatrix} = \begin{bmatrix} 8.42 \\ 0.38 \end{bmatrix} \tag{S3.7}$$

gives

$$MD_j = 2.14, \tag{S3.8}$$

which is smaller than the critical value of the chi-squared distribution and therefore sample j is not considered an outlier at a 0.975 confidence level.

Abbreviations

ANOVA	Analysis of variance
AOC	Assimilable organic carbon
ASV	Amplicon sequence variant
ATP	Adenosine tirphosphate
β-MNTD	β-mean nearest taxon distance (phylogenetic beta diversity metric)
β-MPD	β-mean pairwise distance (phylogenetic beta diversity metric)
β-NTI	β-nearest taxon index (standardized effect size of β-MNTD)
DNA	Deoxyribonucleic acid
Diversity (*H'*)	Shannon diversity (taxonomic alpha diversity metric)
DOC	Dissolved organic carbon
Evenness (*J'*)	Pielou's evenness (taxonomic alpha diversity metric)
Faith's PD	Faith's phlyogenetic diversity (phylogenetic alpha diversity metric)
MAD	Median absolute deviation
MEMs	Moran's eigenvector maps
NMDS	Non-metric multidimensional scaling
OTU	Operational taxonomic unit
PCA	Principal component analysis
PCIA	Phenol:chloroform:isoamyl alcohol
PCR	Polymerase chain reaction
Pearson's r	Pearson correlation coefficient
PERMANOVA	Permutational analysis of multivariate variance
PERMDISP	Permutational analysis of multivariate dispersion
PTN buffer	Phosphate-Tris-NaCl buffer
RC_{bray}	Extension of Raup-Crick metric using Bray-Curtis dissimilarity
(db-)RDA	(distance-based) Redundancy analysis
Richness (S)	Number of taxa in a community (taxonomic alpha diversity metric)
rRNA	Ribosomal ribonucleic acid
SIMPER analysis	Similarity percentage analysis
Spearman's ρ	Spearman's rank correlation coefficient
TE buffer	Tris-EDTA buffer
VIF	Variance inflation factor
v/v	volume-volume ratio
w/v	weight-volume ratio

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Publications and authorship clarification

Publications directly related to this thesis

Ecological drivers of differences in microbial community composition across geographically distinct aquifers

The research presented in the first part of this thesis has been published as:

> **Fillinger, L.**, Hug, K., and Griebler, C. Selection imposed by local environmental conditions drives differences in microbial community composition across geographically distinct groundwater aquifers. *FEMS Microbiology Ecology*, 95(11):fiz160. https://doi.org/10.1093/femsec/fiz160.

I conceptualized and wrote the manuscript. Field sampling campaigns for this study were conducted by Dr. Katrin Hug (KH), Prof. Dr. Christian Griebler (CG), and me. DNA extractions and chemical analyses of the samples were done by KH and me, and the Research Unit Analytical BioGeoChemistry at Helmholtz Zentrum München (HMGU) for ion measurements. Flow cytometry measurements were done by KH and Eleonora Lombardo. I prepared the raw DNA extracts for 16S rRNA amplicon sequencing. Sequencing was done at the HMGU Research Unit Comparative Microbiome Analysis. I performed the bioinformatic processing of the raw sequence data and all subsequent data analyses for the manuscript. I responded to the reviewers' comments and revised the manuscript accordingly after the initial submission.

Assembly and succession of microbial communities during the colonization of groundwater sediments

The research presented in the second part of this thesis has been published as:

> **Fillinger, L.**[1], Zhou, Y.[1], Kellermann, C., and Griebler, C. (2019). Non-random processes determine the colonization of groundwater sediments by microbial communities in a pristine porous aquifer. *Environmental Microbiology*, 21(1):327–342. https://doi.org/10.1111/1462-2920.14463.
>
> [1] *Shared first authorship.*

I conceptualized and wrote the manuscript. The raw data for the study were collected in a previous PhD project conducted by Dr. Yuxiang Zhou at HMGU between 2010 and 2011 (Zhou, 2013). Bioinformatic processing of the raw 16S rRNA amplicon sequence data and all subsequent analyses were done by me. I responded to the reviewers' comments and revised the manuscript accordingly after the initial submission.

Text passages, figures, and tables from this publication were used in the following sections (with permission by John Wiley & Sons, Inc.):
Summary, Sections 1.2.1, 1.2.2, 1.4.2, 2.2, 3.2, 4.2, Chapter 5, and Supporting Information SI.2.

Ecological groundwater monitoring based on microbiological parameters

The research presented in the third part of this thesis has been published as:

> **Fillinger, L.**, Hug, K., Trimbach, A. M., Wang, H., Kellermann, C., Meyer, A., Bendinger, B., and Griebler, C. (2019). The D-A-(C) index: a practical approach towards the microbiological-ecological monitoring of groundwater ecosystems. *Water Research*, 163:114902. https://doi.org/10.1016/j.watres.2019.114902.

The ecological monitoring approach presented in this study was developed by CG; I provided the statistical framework for the data analysis. I selected and analyzed the data to present and test the approach in the manuscript. I conceptualized and wrote the manuscript in consultation with CG and feedback from the other co-authors. I responded to the reviewers' comments and revised the manuscript accordingly after the initial submission. Data for the first disturbance case study were collected by He Wang (HW); data from Fuhrberg for the third case study were provided by Anne Trimbach (AT) and Dr. Bernd Bendinger (Hamburg University of Technology); data for the second case study and the non-contaminated groundwater were collected within different research projects that were conducted at HMGU between 2007 and 2018. I participated in five field sampling campaigns for this study between 2016 and 2018. Concentrations of AOC and ATP, as well as physicochemical parameters were determined by KH, HW, and AT. Isotope ratios were measured by Petra Seibel at HMGU.

Text passages, figures, and tables from this publication were used in the following sections (with permission by Elsevier, Ltd.):
Summary, Sections 1.3, 1.4.3, 2.1.1, 2.1.3, 2.3, 3.3, 4.3, Chapter 5, and Supporting Information SI.3.

Publications produced during this PhD project but not included in this thesis

Pilloni, G., Bayer, A., Ruth-Anneser, B., **Fillinger, L.**, Engel, M., Griebler, C., and Lueders, T. (2019). Dynamics of hydrology and anaerobic hydrocarbon degrader communities in a tar-oil contaminated aquifer. *Microorganisms*, 7(2):46. https://doi.org/10.3390/microorganisms7020046.

Griebler, C., Hug, K., **Fillinger, L.**, Meyer, A., and Avramov, M. (2018). Der B-A-E Index – Ein mikrobiologisch-ökologisches Konzept zur Bewertung und Überwachung von Grundwasser. *Hydrologie & Wasserbewirtschaftung*, 62(6):378–386. https://doi.org/10.5675/HyWa_2018.6_1.

Herzyk, A.[1], **Fillinger, L.**[1], Larentis, M., Qiu, S., Maloszewski, P., Hünniger, M., Schmidt, S. I., Stumpp, C., Marozava, S., Knappett, P. S. K., Elsner, M., Meckenstock, R., Lueders, T., and Griebler, C. (2017). Response and recovery of a pristine groundwater ecosystem impacted by toluene contamination – A mesoscale indoor aquifer experiment. *Journal of Contaminant Hydrology*, 207:17–30. https://doi.org/10.1016/j.jconhyd.2017.10.004.

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Publications not related to this PhD project

Röling, W. F. M., **Fillinger, L.**, and Nunes da Rocha, U. (2014). Analysis of the regulation of the rate of hydrocarbon and nutrient flow through microbial communities. In McGenity T., Timmis K., and Nogales B., editors, *Hydrocarbon and Lipid Microbiology Protocols*, pages 233–246. Springer, Berlin, Heidelberg. https://doi.org/10.1007/8623_2014_15.

Röling, W. F. M., **Fillinger, L.**, and Nunes da Rocha, U. (2014). Analysis of the hierarchical and metabolic regulation of flux through metabolic pathways. In McGenity T., Timmis K., and Nogales B., editors, *Hydrocarbon and Lipid Microbiology Protocols*, pages 245–258. Springer, Berlin, Heidelberg. https://doi.org/10.1007/8623_2014_6.

Bolhuis, H., **Fillinger, L.**, and Stal, L. J. (2013). Coastal microbial mat diversity along a natural salinity gradient. *PLOS ONE*, 8(5):e63166. https://doi.org/10.1371/journal.pone.0063166.

Conference contributions related to this PhD project

Fillinger, L., Hug, K., and Griebler, C. Species sorting as the main driver behind the biogeography of groundwater microbial communities in shallow porous aquifers. Poster presentation at *16th Symposium of Aquatic Microbial Ecology (SAME16)*, Potsdam, Germany, September 1–6, 2019.

Fillinger, L., Hug, K., and Griebler, C. Das B-A-E Konzept: Ein Ansatz zur mikrobiologisch-ökologischen Bewertung von Grundwasserökosystemen. Oral presentation at *34. Jahrestagung der Deutschen Gesellschaft für Limnologie e.V. (DGL)*, Kamp-Lintfort, Germany, September 10–14, 2018.

Fillinger, L., Zhou, Y., Kellermann, C., and Griebler, C. Non-random processes determine the colonization of groundwater sediments by microbial communities in a porous aquifer. Poster presentation at *17th International Symposium on Microbial Ecology (ISME17)*, Lepizig, Germany, August 12–17, 2018.

Curriculum vitae

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Education

- **2011–2013:** Master of Science, *Biomolecular Sciences* with specialization in *Molecular Cell Biology*, VU University Amsterdam, The Netherlands (graduation with distinction *cum laude*).
- **2007–2011:** Bachelor of Applied Science, *Biology and Medical Laboratory Research*, Stenden University of Applied Sciences, Emmen, The Netherlands.
- **1997–2006:** Secondary education with qualification for university entrance (*Abitur*), Ostendorf Gymnasium Lippstadt, Germany.

Research positions

- **SEP 2019– :** Research assistant at University of Vienna, Center of Functional Ecology, Department of Limnology & Bio-Oceanography.
  Topic: Groundwater microbial ecology. *Supervision by Prof. Dr. Christian Griebler.*
- **SEP 2016–SEP 2019:** PhD position at Helmholtz Zentrum München, Institute of Groundwater Ecology.
  Topic: Microbial community composition, assembly, and succession in groundwater ecosystems, and development of an approach towards ecological groundwater monitoring. *Supervision by Prof. Dr. Christian Griebler.*

- **SEP 2013–SEP 2016:** PhD position at VU University Amsterdam, Faculty of Earth and Life Sciences, Department of Molecular Cell Physiology.
  Topic: Metabolic interactions and community dynamics in anaerobic aromatic compound-degrading microbial communities. *Supervision by Dr. Wilfred F. M. Röling.*

- **FEB 2013–JUL 2013:** Internship at Helmholtz Zentrum München, Research Unit Microbe-Plant Interactions.
  Topic: Presence of quorum sensing-active compounds in plant extracts, and the effect of bacterial quorum sensing molecules on antioxidative stress responses in plants. *Supervision by Prof. Dr. Dr. Peter Schröder.*

- **FEB 2012–JUL 2012:** Internship at VU University Amsterdam, Faculty of Earth and Life Sciences, Department of Molecular Cell Physiology.
  Topic: Reductive dehalogenation of tetrachloroethylene (PCE) in anaerobic batch cultures of fermenting microbial communities. *Supervision by Dr. Wilfred F. M. Röling.*

- **AUG 2010–AUG 2011:** Internship at Royal Netherlands Institute for Sea Research (NIOZ), Department of Marine Microbiology, Yerseke, The Netherlands.
  Topic: Community composition of microbial mats along a coastal salinity gradient, and development of a method for RNA extraction from microbial mat samples. *Supervision by Dr. Henk Bolhuis.*