universität wien

DISSERTATION | DOCTORAL THESIS

Titel | Title

Vulnerability and response of groundwater microbial communities in alpine regions to anthropogenic impacts and climate change

verfasst von | submitted by

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angestrebter akademischer Grad | in partial fulfilment of the requirements for the degree of

Doktorin der Naturwissenschaften (Dr.rer.nat.)

Wien | Vienna, 2025

Studienkennzahl lt. Studienblatt | Degree programme code as it appears on the student record sheet:	UA 796 605 299
Dissertationsgebiet lt. Studienblatt | Field of study as it appears on the student record sheet:	Environmental Sciences
Betreut von | Supervisor:	Univ.-Prof. Mag. Dr. Christian Griebler

Acknowledgements

I would like to sincerely thank my supervisor, Christian, and co-supervisors Henrik, and Fede. Above all, I thank my supervisor Christian for the last years and beyond. Thank you for showing me sampling routines, how exciting the world under our feet really is, taking me places and offer many opportunities for collaborations, without the integrative nature of this thesis would not have been possible, and properly nurtured. As such, I want to thank all our partners at University of Graz, and University of Natural Resources and Life Sciences, such as Johannes Haas, Steffen Birk, and Christine Stumpp for their collaboration and providing hydrological, and water stable isotope know-how and subsequent analyses and never missing a chance for fruitful discussions. Sincere thanks also to goes out to Fede, and Eva for taking the time to provide valuable input and methodological knowledge for additional data generation, writing of the publications and being my go-to people to chat on Fungi.

I also really want to thank everyone in the working group, especially Clemens, Constanze, Grit and Hubert Kraill for never hesitating to help out, and providing support may it be work related or private. I never felt left alone, and I always loved going to work and seeing your faces. Thank you for the good atmosphere and for putting up with my shenanigans, and my forgetfulness. I also want to thank my parents, who never expected or pushed me to pursue any of this, but who are proud of me and support me no matter what I'm doing in life. I want them to know, that even when it is sometimes hard to have the brain that I have, I wouldn't want it any other way. If you know, you know.

I also want to thank my friends and all the important people in my life, especially Emanuel, Julia, Claudia, and Jasmin. You make life worthwhile and fun and provided me with an extra laughter when I really needed it and are my ride or die since forever. You can always count on me, same as I can always count on you.

Last but not least, to Henrik, whom I have known already from my time during my Master's in Sweden, I cannot express enough my gratitude for your continued support and believing in me since day one. Without you, I do not think I would have started the adventure which would lead me to writing these lines.

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Summary

This doctoral thesis focuses on understanding the vulnerability, response, diversity, distribution, and ecological roles of microbial and fungal communities in groundwater, with an emphasis on alpine regions. Prokaryotic and eukaryotic communities are ubiquitous and play a crucial role in the biogeochemical cycling of nutrients and energy in the Earth's subsurface aquatic ecosystems. Alpine regions are characterized by a unique, diverse, and complex environment, being particularly vulnerable to anthropogenic influences and shifts towards higher temperatures and altered hydrological dynamics.

The study area covered an altitudinal and landscape gradient in the Mur river valley, Austria, comprising diverse habitats from alpine regions to urban and lowland areas with diverse regional climatic and land use influences. Groundwater temperatures along the major surface water body intersecting the groundwater aquifers, the river Mur, were consistently lower than in the river, but with notable seasonal and spatial variations. The thesis overall underscores that river and groundwater ecosystems are biologically distinct, but with varying levels of connectivity. Groundwater aquifers in alpine regions were more connected to natural water sources such as glacial melt via the river, while urban and arable lowlands exhibited significant human impact, particularly in terms of heat, and nutrient and salt loads. In groundwater, prokaryotic diversity exceeded that of river systems, particularly in alpine regions, where isolated hydrological conditions and habitat complexity contributed to community assembly. However, in aquifers downstream the river environmental conditions were prominent drivers. Fungal diversity in groundwater, though lower than in the river Mur, followed a less pronounced spatial trend, with particular genera associated with nutrient input into the naturally energy-poor groundwater. The influence of local landscape characteristics such as climate, local land use, and bedrock lithology on shaping fungal communities in groundwater was highlighted. In terms pf productivity, the river Mur was overall more productive than groundwater, exhibiting higher prokaryotic activity, carbon input through terrestrial coupling, but also fungal diversity.

The urban groundwater around the city of Graz experienced significant warming, evapoconcentration of nutrients during extended dry periods, and low microbial, and fungal diversity due to anthropogenic influences such as organic and inorganic pollution from e.g., road runoff, but also increased evapotranspiration. Altogether, the climate is much dryer in the urban area and downstream. In the agriculturally intensively used lowland, groundwater was characterized by increased temperatures, disproportionately high nitrate levels and evaporation rates, influenced by, e.g., irrigation practices, and increased surface sealing through industrial and transportation facilities.

In addition, this thesis presents tools and methods for assessing the microbiological-ecological status of groundwater and underscored the importance of considering various factors, such as biological aspects, when evaluating groundwater health status and puts emphasis on having pristine reference datasets available.

Zusammenfassung

Diese Doktorarbeit befasst sich mit dem Verständnis der Anfälligkeit, der Reaktion, der Vielfalt, der Verteilung und der ökologischen Rolle von mikrobiellen und pilzlichen Gemeinschaften im Grundwasser, wobei der Schwerpunkt auf alpinen Regionen liegt. Prokaryotische und eukaryotische Gemeinschaften sind allgegenwärtig und spielen eine entscheidende Rolle im biogeochemischen Kreislauf von Nährstoffen und Energie in den unterirdischen aquatischen Ökosystemen der Erde. Alpine Regionen zeichnen sich durch eine einzigartige, vielfältige und komplexe Umwelt aus, die besonders anfällig für anthropogene Einflüsse und Verschiebungen hin zu höheren Temperaturen und veränderter hydrologischer Dynamik ist.

Das Untersuchungsgebiet erstreckt sich über einen Höhen- und Landschaftsgradienten im Murtal, Österreich, und umfasst verschiedene Lebensräume von alpinen Regionen bis hin zu städtischen und Flachlandgebieten mit unterschiedlichen regionalen Klima- und Landnutzungseinflüssen. Die Grundwassertemperaturen entlang des wichtigsten Oberflächenwasserkörpers, welcher die Grundwasserleiter durchschneidet, der Mur, waren durchweg niedriger als in der Mur, allerdings mit bemerkenswerten saisonalen und räumlichen Schwankungen. Insgesamt unterstreicht die Arbeit, dass Fluss- und Grundwasserökosysteme biologisch unterschiedlich sind, jedoch in unterschiedlichem Maße miteinander verbunden sind. Grundwasserleiter in alpinen Regionen waren über den Fluss stärker mit natürlichen Wasserquellen wie der Gletscherschmelze verbunden, während städtische und ackerbaulich genutzte Niederungen erhebliche menschliche Einflüsse aufwiesen, insbesondere in Bezug auf Wärme, Nährstoff- und Salzbelastung. Im Grundwasser war die prokaryotische Vielfalt größer als in Flusssystemen, insbesondere in alpinen Regionen, wo isolierte hydrologische Bedingungen und die Komplexität der Lebensräume zur Bildung von Gemeinschaften beitrugen. In den Grundwasserleitern flussabwärts waren jedoch die Umweltbedingungen des Flusses ausschlaggebend. Die Pilzvielfalt im Grundwasser war zwar geringer als in der Mur, folgte aber einem weniger ausgeprägten räumlichen Trend, wobei bestimmte Gattungen mit dem Nährstoffeintrag in das von Natur aus energiearme Grundwasser in Verbindung gebracht wurden. Der Einfluss lokaler Landschaftscharakteristika wie Klima, lokale Landnutzung und Gesteinsbeschaffenheit auf die Bildung von Pilzgemeinschaften im Grundwasser wurde hervorgehoben. Was die Produktivität betrifft, so war die Mur insgesamt produktiver als das Grundwasser und wies eine höhere prokaryotische Aktivität, einen höheren Kohlenstoffeintrag durch terrestrische Kopplung, aber auch eine größere Pilzvielfalt auf.

Das urbane Grundwasser unterhalb der Stadt Graz erlebte eine signifikante Erwärmung, eine Evapokonzentration von Nährstoffen während asugedehnter Trockenperioden und eine geringe

mikrobielle und pilzliche Diversität, die auf anthropogene Einflüsse wie organische und anorganische Verschmutzung, z. B. durch Straßenabflüsse, aber auch auf eine erhöhte Evapotranspiration zurückzuführen ist. Insgesamt ist das Klima im Stadtgebiet und flussabwärts im Alpenvorland deutlich trockener. Im landwirtschaftlich intensiv genutzten Tiefland war das Grundwasser durch erhöhte Temperaturen, überproportional hohe Nitratwerte und Verdunstungsraten, beeinflusst z.B. durch Bewässerungspraktiken, und erhöhte Oberflächenabflüsse gekennzeichnet.

Darüber hinaus werden in dieser Arbeit Instrumente und Methoden zur Bewertung des mikrobiologisch-ökologischen Zustands des Grundwassers vorgestellt und es wird unterstrichen, wie wichtig es ist, bei der Bewertung des Gesundheitszustands des Grundwassers verschiedene Faktoren, wie z. B. biologische Aspekte, zu berücksichtigen, und dass es massgeblich ist, über unbelastete Referenzdaten zu verfügen.

Introduction

The groundwater ecosystem and its importance in mountainous regions

Groundwater refers to the vast underground water stored within the continental boundary (e.g., soil, sediments, and rocks) for years or even decades. These nonphotic habitat is among the largest, but most poorly understood ecosystems on Earth. The water bearing matrix (i.e., the aquifer) can be located directly below the permeable soil cover (i.e., unconfined groundwater) or isolated by layers of impermeable material (i.e., confined groundwater) and made up of various types of rocks, such as sandstone, gravel, or limestone that have high porosity and permeability. Groundwater can also be divided into several different types based on its origin and structure (i.e., karstified, fissured, and unconsolidated/alluvial aquifers), or depth (shallow and deep, often confined aquifers). Shallow aquifers are situated close to the surface and are typically found in areas with permeable land surface. Deep aquifers are located at a greater depth that are more difficult to access, in areas with dense rock formations such as sandstone, or granite with relatively slow water circulation (Griebler and Mösslacher, 2003). Consequently, shallow groundwater is more vulnerable to contamination from human activities, where contaminants can easily seep into the shallow aquifer, often leading to ecosystem degradation. However, shallow groundwater also constitutes the most important source of freshwater for human communities. Globally, around 50% of potable water is supplied by groundwater (Mukherjee et al., 2021).

Of all freshwater available on Earth, 30% is stored as groundwater, which makes it the most abundant water resource on the planet. In contrast to surface water, it is preferably used as a source of drinking water supply. Groundwater is usually of higher quality due to its movement and subsequent filtration through the subsurface during which it is purified, and suspended particles and contaminants are removed. These can be pathogens, organic- and inorganic contaminants, such as pesticides, petroleum products, heavy metals, nitrates, or chlorides. It is also better protected from possible pollution, as the subsurface can provide a natural barrier between groundwater and the surface environment and naturally acts as a buffer, storing large volumes of water. Moreover, it is not as subject to seasonal and perennial fluctuations, as groundwater is usually recharged and distributed evenly from areas of surplus to depleted regions, and from aquifer to aquifer in interconnected aquifer systems.

However, groundwater does not exist isolated and can be intricately and dynamically connected to the surface and surface freshwater (e.g., rivers, streams, lakes, soil moisture) as a quantitatively important component of the hydrological cycle. Ongoing hydrological interactions are often complex, diverse, and nonlinear (Treidel et al., 2011), and infiltrating water is chemically altered along its flow path. Nutrients and other elements (e.g., nitrogen, phosphorus, potassium) are redistributed, concentrated, or diluted

depending on individual exchange processes and their extents. For example, groundwater can slowly infiltrate into the subsurface through precipitation and then re-enter surface water in the form of springs. It can also directly discharge into surface water, and streams can re-enter the ground and become groundwater. Groundwater is sustained through various sources. First, it can be recharged from surface water, such as rivers, lakes, or wetlands during periods of high flow, caused by e.g., heavy precipitation, or snow melting. Second, it can be replenished by infiltrating saline water from the ocean, which occurs in coastal areas where the groundwater table is close to the surface and the groundwater matrix is permeable. Groundwater salinization can for instance be further driven by increased groundwater withdrawal (e.g., through irrigation), aquifer mixing, and groundwater pollution by improper management of drainage systems, industrial discharges, mining operations, or improper waste disposal from landfills. Third, precipitation can directly infiltrate into the subsurface and recharge the groundwater ecosystem.

Additionally, there are processes and fluxes taking place between the groundwater and the atmosphere such as evaporation, or evapotranspiration, as well as interactions between groundwater and the soil. These support climate, soil moisture, and consequently plant growth, which transpire water back into the atmosphere. Their extents are often small but can have a large local impact on nutrient and element cycling, as well as maintaining streamflow during periods of low flow (Mukherjee et al., 2021). Depending on how quickly and extensively new water is supplied to the subsurface, one speaks of a high or low groundwater recharge rate. This in turn depends on the climatic conditions, the soil properties, the depth of the groundwater, and the properties of the surface of the Earth (Griebler and Mösslacher, 2003). Consequently, the recharge and depletion of groundwater is significantly influenced by global climate change, anthropogenic degradation, and alteration of the surface environment, as well as the amount of groundwater withdrawal (e.g., changes in air temperature, evapotranspiration, land use, surface sealing). This can lead to alterations not only in groundwater quantity, but also in quality especially due to pollution by agricultural practices, wastewater, industrial usage, and the input of pollutants of emerging concern (e.g., pesticides, pharmaceuticals, micro- and nanoparticles).

Mountainous areas cover around 70% of Austrian territory and are typically rich in water. They comprise glaciers, rivers, streams, lakes, artificial reservoirs (i.e., dams) and groundwater, and therefore play a key-role in the country's water cycle. Alpine river catchments are highly dynamic due to their various subsurface flow patterns. They are also structurally complex systems caused by a high spatiotemporal variability in bedrock topography, elevation gradients, climate, and geology (Figure 1).

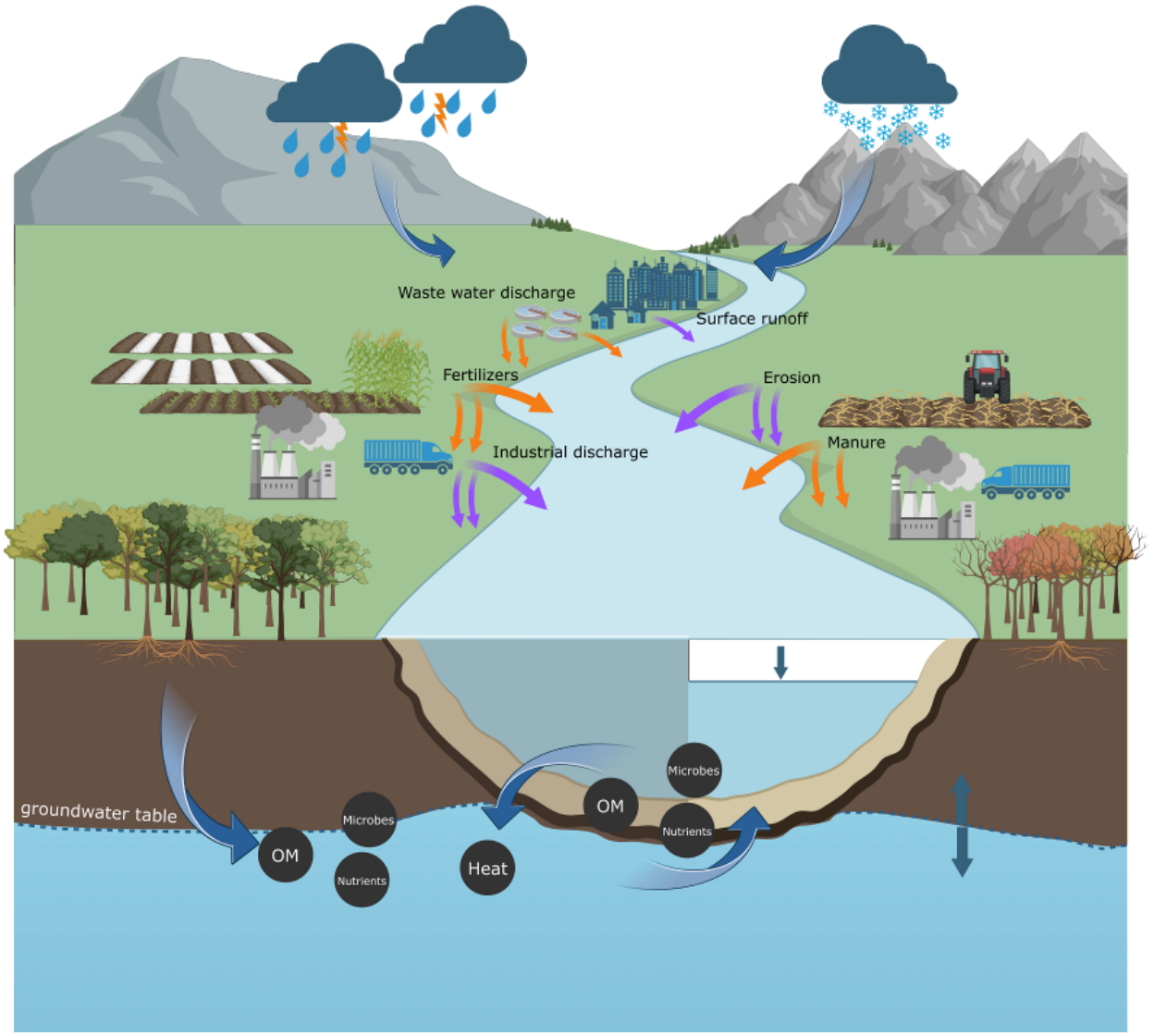

Figure 1 Schematic illustration of groundwater characteristics created using Biorender (www.biorender.com) along the alpine river Mur valley in Austria, depicting changing land cover, and climatic conditions and their various biotic, and abiotic stressors, as well as potential interaction pathways between the river, soil and groundwater.

Mountainous river catchments are typically smaller than lowland counterparts and are sustained by glacial meltwater, groundwater discharge at springs, precipitation and surface runoff (Karwautz et al., 2022a; Michelon et al., 2023). Besides, erosional processes, which are predicted to rise within the near geological future, shape the catchments drainage basin geometry (Winterberg and Willett, 2019). This means that future river networks and dependent freshwater ecosystems (e.g., groundwater) will influence alpine topography and vice versa.

However, high habitat heterogeneity in mountainous areas and limited hydrological data and models available (Cochand et al., 2019; Thornton et al., 2022) pose key challenges in studying aquatic alpine systems. This big knowledge gap is contrasted by a high biodiversity of often endemic, and endangered species (Bundi, 2010; Perrigo et al., 2020) and a disproportionally high vulnerability of alpine regions to

global warming (Beniston, 2003; Niedrist, 2023). Increasing temperatures and extreme hydro-meteorological events will have lasting effects on hydrological patterns (e.g., groundwater recharge) and groundwater ecology. However, the short-term, and long-term consequences of fluctuation rates and their variability across aquifers on the stability and function of alpine aquatic ecosystems are unclear (Chen et al., 2018). However, global warming is already leading to an increase in severe floods and droughts, especially in the Southern alpine region, which is most affected by a decrease in precipitation (Gobiet et al., 2014). Anthropogenic activities (e.g., recreational, tourism, hydropower production) pose an additional threat to aquatic alpine ecosystems, often leading to landscape devolution, threatening local habitat integrity by altering erosion processes, sediment transport and local biodiversity (Beniston et al., 2018; Frossard et al., 2023). Therefore, groundwater research in alpine regions is of utmost importance (Hayashi, 2020), and is a key element to understanding future water availability and ecosystem changes throughout the water cycle (Kløve et al., 2014).

Energy and nutrient availability in groundwater

Unlike surface water, groundwater is in constant darkness and therefore lacks any kind of vegetation and other photosynthetic organisms. The only exception are the portals in karstic systems that are sometimes reached by light, or the entrance of springs, or caves, which form a transitional state between the under- and above ground world. Aquifers are perceived as a relatively stable system that are different in their physico-chemical conditions to surface water ecosystems. However, shallow groundwater conditions can be quite dynamic. Stability is only given at certain depths, where influencing factors such as prevailing climatic conditions, topography or the composition of the soil are not acting on the groundwater anymore (Griebler and Mösslacher, 2003).

Due to the lack of sunlight the groundwater's temperature regime is relatively unchanging. As photosynthesis is missing as a source of primary production, groundwater environments are poor in available energy, making them an overall oligotrophic system (Hofmann and Griebler, 2018; Overholt et al., 2022). This means, that the supply of organic matter (OM), and nutrients originating from the surface is critical to the survival of groundwater organisms (Gibert et al., 2013). Since most of the OM (particulate, and dissolved OM) cannot be directly assimilated by higher level consumers (e.g., invertebrate species), it acts as the basis of the food web by first being converted into microbial biomass (Hartnett, 2018).

Nutrient, and organic carbon (OC) concentrations in groundwater are generally a result of several factors: (1) the rock type, (2) environmental, and climatic conditions, such as temperature, pressure, flow rate, and (3) flow conditions, such as groundwater matrix residence time (Gibert et al., 2013). Natural sources of OC are mainly of terrestrial origin, such as plant debris and soil leachate that are washed into the aquifer

by rain, snow melting, or infiltrating surface water. Moreover, it can originate from microbial decomposition in overlaying soil layers, or plant root exudates that leach into the groundwater. Higher levels of nutrients, and minerals can naturally be found in geogenic-contaminated groundwater (e.g., high-salinity, high As, Fe, Mn, F, and iodine levels) (Jia, 2021). Mostly, these higher concentrations are due to the characteristics of the geological sources, or deposition of atmospheric nitrogen, and nitrogen fixation by microbes (Jin et al., 2015). However, elevated quantities of nutrients and minerals are very often the result of anthropogenic activities, which can additionally promote the release of these minerals into the groundwater environment (e.g., well pumping, introduction of anthropogenic OC) (Schreiber, 2021). Additionally, agricultural practices can increase nitrate levels with the application of nitrogen-based fertilizers, manure, as well as municipal-, and industrial wastewater discharge into groundwater. Aquifers contaminated with high levels of nitrate are problematic, as they can lead to serious health problems in humans. Moreover, its discharge into surface water ecosystems can result in eutrophication of these environments ultimately affecting biodiversity, ecosystem function and productivity (Richa et al., 2022; Zhao et al., 2022). Therefore, the World Health Organization (WHO) has set an upper limit of 50 mg L^{-1} nitrate in drinking water (WHO, 2016). Besides, the infiltration of organic fertilizers, such as manure and compost, or leachate from landfills, waste disposal sites, or petroleum storage tanks can result in unnaturally high levels of OC with detrimental consequences for the oligotrophic groundwater ecosystem.

Bioavailability of carbon and nutrient sources, such as dissolved organic matter (DOM) as part of the overall OC pool, is an important aspect in OM processing and decomposition in groundwater. It determines how readily OM is taken up and broken down by microbes at the base of the food web (e.g., *Bacteria*, *Archaea*, *Fungi*). This indeed depends on several conditions, such as the redox-potential and chemical composition of the groundwater, the presence of other nutrients or compounds that alter microbial growth rates, as well as the type of microbial taxa present (Schwab et al., 2017). Hence, the reactivity of DOM can change over time as a result of microbial processing, degree of surface photodegradation, and input of autochthonous DOM from local groundwater sources such as chemolithoautotrophic *Bacteria* (Griebler and Mösslacher, 2003; McDonough et al., 2022). DOM is a key component in the biogeochemical cycling in groundwater and is composed of a diverse mixture of dissolved organic carbon (DOC), nitrogen, phosphorus, and sulfur in the form of carbohydrates, lipids, amino acids, as well as humic substances, among other compounds. Humic components of DOM are commonly associated with plant and soil material and contain highly aromatic or hydrophobic molecules (e.g., fulvic-, and humic acids, or phenols) that are structurally complex, and chemically stable. Since most microorganisms lack the necessary metabolic degradation pathways, refractory (i.e., unreactive) DOM can

accumulate and persist in the groundwater environment over a long period of time. Very recalcitrant (i.e., stable and unreactive) DOM is usually found in deeper aquifers, as the labile fraction was already degraded (McDonough et al., 2022). Even at these great depths, DOM is still diverse and the main support for the microbial life in groundwater (Osterholz et al., 2022; Saito et al., 2023). Most DOC (i.e., the carbon-based fraction of DOM) in groundwater is aged and recalcitrant, however, when groundwater conditions are reducing and due to the lack of photodegradation, a substantial fraction of microbially derived carbon compounds can accumulate in groundwater. Upon resurfacing, such as through irrigation, this biolabile fraction is then rapidly mineralized, a process that can increase greenhouse gas emissions (e.g., CH_4, CO_2) to the atmosphere (McDonough et al., 2022).

Moreover, groundwater itself acts a vast storage for inorganic carbon (dissolved carbon dioxide, carbonate, and bicarbonate), that is derived from atmospheric CO_2, degradation of OM, mineral weathering and leaching of carbon from rocks, as well as degassing (Sveinbjörnsdóttir et al., 2020). The inorganic carbon sequestrated in groundwater can find its way into adjacent aquatic ecosystems by flushing, especially in tidal ecosystems such as mangrove forests, or salt marsh soils (Correa et al., 2022). Groundwater is also often found to be oversaturated not only with CO_2, but with other dissolved greenhouse gases, such as methane, or nitrous oxide. Therefore, land use practices that practice irrigation, such as agriculture, can be an overall source of greenhouse gas emission from groundwater that is not negligible (McGill et al., 2018).

Groundwater organisms on a micro-scale

An intact biogeochemical cycle is important for maintaining the overall health and productivity of the groundwater ecosystem, as well as preserving the pristineness of groundwater as a resource. This is on one hand dependent on the hydrology driving the physico-chemical conditions, as well as quality and quantity of elements, and compounds available in the groundwater system. However, their transfer between the biotic and abiotic world has another main player: groundwater organisms. These encompass a large diversity of microeukaryotic and prokaryotic life, such as *Fungi*, *Protista*, *Bacteria*, *Archaea*, but also viruses (Fillinger et al., 2019b; Retter and Nawaz, 2022; Schweichhart et al., 2022), and metazoans (e.g., invertebrates, amphibians) (Malard et al., 2023).

Many organisms, particularly in the deeper subsurface, have historically received little to no attention, as they are not associated with animals (e.g., humans, livestock) or plants (e.g., crops) and research in these environments is traditionally costly and logistically challenging. Yet, they hold evolutionary and ecological significance and may contain novel lineages with distinctive physiology, phenotype, or metabolic functions that remain unknown to science (Griebler et al., 2014). We have long known that groundwater

provides a unique environment for microorganisms (Balkwill et al., 1997; Zhang et al., 2022). However, molecular advances (e.g., molecular analyses based on ribosomal RNA gene sequencing) within the last decades have revealed groundwater to be the richest habitat on Earth in terms of microbial biomass and biodiversity, harboring a large functional repertoire (Bar-On et al., 2018; Groult et al., 2023; Malard et al., 2023; Ruff et al., 2023). Life's biogeographical distribution in the subsurface is currently thought to be largely determined by depth and associated factors such as temperature and pressure, as well as by stochastic processes like advection, which is driven by fluid-filled fissures (Anderson, 2021; Meyer et al., 2022). Along a vertical gradient, shallow groundwater - particularly where it remains well-connected to the surface - supports a disproportionally rich and diverse array of microorganisms. Despite these advances, many aspects of microbial life, such as community formation (e.g., environmental filtering, dispersal limitation), function, and spatiotemporal dynamics in near-surface groundwater remain poorly understood and will be investigated in more detail in this thesis.

From an evolutionary perspective, the water-filled subsurface also provides an interesting insight into the formation and development of early life (Ivarsson et al., 2020) as essential requirements, such as favorable redox and temperature conditions are natural characteristics of groundwater. This is accompanied by a physical separation from the land surface, which is traditionally exposed to more unstable, harsh, and hostile conditions, including intense radiation (Trevors, 2002). It is therefore to no surprise that evolutionary ancient, and deeply branching lineages of microorganisms (e.g., DPANN archaea, bacterial candidate phyla radiation (CPR)) and microeukaryotes (e.g., chytrids) are frequently recovered from the deep subsurface (Bell et al., 2022; Castelle et al., 2015; Castelle and Banfield, 2018; Ivarsson et al., 2018; Sohlberg et al., 2015). Since these organisms are notoriously difficult to isolate and cultivate, progress in filling gaps in the tree of life has only been possible through advancing molecular methods (Garritano et al., 2022; Mikryukov et al., 2023; Reji et al., 2022) and many lineages are known only from sequencing data of, for instance, deep groundwater environments (Grossart et al., 2016; Nilsson et al., 2023; Quandt et al., 2023).

On the other hand, most of the microbial groups recovered from unconfined, more shallow groundwater have previously been described from soils and surface freshwater environments (Fillinger et al., 2023; Herrmann et al., 2020; Karwautz and Griebler, 2022). The majority are heterotrophs, with a smaller but still substantial share of chemolithoautotrophic microorganisms (i.e., biomass production by fixation of inorganic carbon), known to support complex trophic interactions in groundwater (Herrmann et al., 2020; Hutchins et al., 2016). Heterotrophic prokaryotes and microeukaryotes obtain their energy and nutrients by consuming OM. They play a vital role in the cycling of carbon and other elements in the groundwater ecosystem by saprotrophy, predation, or in association with other living organisms. Cycling of matter

extends through various trophic levels, but knowledge about interactions and the transfer of energy in groundwater food webs is limited, especially on the micro scale (Karwautz et al., 2022b; Lee et al., 2006; Schmidt et al., 2017). However, the food web in groundwater is likely detritus-based since growth rates are low and autochthonous primary production is limited.

The bulk of groundwater microbial biodiversity and biomass is comprising microorganisms - various members of *Bacteria*, and *Archaea*. Prokaryotic (i.e., *Bacteria* and *Archaea*) organisms play a key role in groundwater ecosystem functioning through their metabolic plasticity, complex genetic repertoire, short generation times and fast reproduction rates (Smith et al., 2018; Soares et al., 2023). The majority of prokaryotic communities in groundwater are attached to subsurface sediments as small patchy colonies rather than large biofilms (Griebler et al., 2022). They are ubiquitous, rapid, and fierce degraders of structurally diverse organic compounds. Simple organics (i.e., low molecular weight) are preferably degraded by prokaryotes, but some members of *Archaea* have been shown to potentially break down complex organic compounds known to accumulate in groundwater (Lazar et al., 2016). In groundwater, prokaryotes are well characterized compared to microeukaryotes (e.g., protists, fungi) and important players in various key metabolic processes such as denitrification, nitrification, sulfate-, and iron reduction, or methanogenesis by hydrolysis of organic substances (Purkamo et al., 2018). Along a depth gradient, most microorganisms are well adapted to low oxygen or anoxic conditions with limited energy available to them. Consequently, taxa capable of utilizing alternative, less thermodynamically favorable electron acceptors have shown to be more prevalent in groundwater with increasing depth (Flynn et al., 2013). Prokaryotes have evolved various stress response mechanisms, such as heat shock proteins, DNA repair systems, and osmoregulation, enabling them to survive and proliferate under harsh conditions. Cooperative behaviors, facilitated by quorum sensing, and dormancy strategies, including spore formation, allow them to persist through periods of change and environmental stress. In groundwater, the most abundant lineages of *Bacteria* encompass mainly *Proteobacteria* (*α*-, *β*-, and *γ*-*Proteobacteria*), but also *Actinobacteria*, *Bacteroidota*, and *Firmicutes* (Yan et al., 2020; Zelaya et al., 2019; Zhong et al., 2023). Besides, some members of *Archaea* are regularly found in groundwater and comprise lineages such as *Woesearchaeota*, *Crenarchaeota*, and *Thaumarchaeota* (Groult et al., 2022; Lazar et al., 2017).

Despite their huge diversity, prokaryotic productivity is usually low in groundwater, often 10 – 1000-fold lower than in soil, or surface freshwater ecosystems (Griebler and Lueders, 2009). Therefore, higher microbial activity and cell densities potentially serve as a proxy for a certain degree of ecosystem disturbance, such as an increased input of allochthonous resources (e.g., fertilizers, terrestrial OM) (Fillinger et al., 2019a; Khan et al., 2018). These changes, however, might not always be linear as bacterial activity is also shaped by morpho-physiological traits that adapt in response to environmental changes or

heightened stress. Dormancy and miniaturization are important life strategies in groundwater and are a consequence of prioritizing cell maintenance over growth (Cavicchioli et al., 2019). However, whether increased ecosystem productivity directly responds to groundwater disturbance and how it furthermore influences prokaryotic biodiversity remains to be evaluated (Griebler et al., 2014; Porter et al., 2009). Recently, microbial abundance and activity have been integrated into a simple tool to advance the assessment of groundwater health and ecological status in a fast and cost efficient manner (Fillinger et al., 2019a). In this thesis, we will apply this tool in order to test its universal applicability as a proxy of the microbiological-ecological status of groundwater in alpine regions. In addition, we will assess whether there is a causal relationship with ecological status, as derived from the tool, and microbial biodiversity. This will be an important step in advancing monitoring methods beyond chemical assessment of groundwater aquifers.

Since fungi were believed to be strictly aerobic and evolutionary linked to terrestrial environments, they have been notoriously overlooked in the water bearing subsurface (Saitoh et al., 2019). It is true that many fungal lineages display a high dependency on surface interactions both in terms of hosts, and energy (Peay et al., 2016; Wang et al., 2022). However, recent studies have shown that fungi play fundamental roles in the groundwater ecosystem through their widespread anaerobic metabolism (Drake and Ivarsson, 2018; Hoque and Fritscher, 2023; Müller et al., 2012), trophic relationships (e.g., symbiosis, parasitism, predation) (Bengtson et al., 2014; Galindo et al., 2018; Grabner et al., 2020), and evolutionary traits such as flagellated unicellular life stages and hyphae formation (Rojas-Jimenez et al., 2017; Voigt et al., 2021). The evolutionary success of fungi stems from their lower nutrient demand, the production of a wide array of unspecific enzymes capable of breaking down manyfold substrates (including complex, low-quality OM such as lignin, cellulose or chitin), as well as their large phenotypic (e.g., dimorphism) and nutritional mode variation (Fabian et al., 2017; Naranjo-Ortiz and Gabaldón, 2020). By translocating nutrients over broader distances through their hyphal network (i.e., their hyphae working as ”living pipes”), fungi are able to colonize the groundwater matrix and distribute nutrients and carbon between different redox zones (Watkinson et al., 2015). This facilitates the rapid mobilization of metals and other elements (e.g., Ca, Si, Na, K) through weathering and mineralization processes (Fomina et al., 2006; Ivarsson et al., 2018). While complex hyphal growth is typically associated with more derived fungal lineages that evolved upon land colonization, some fossil evidence suggests that mycelial networks represent an ancestral morphological state in subsurface environments (Ivarsson et al., 2020). In fact, three-dimensional mycelial growth would be the optimal strategy for maximizing surface area to nutrient uptake in an oligotrophic environment with limited pore space (Ivarsson et al., 2020). However, fossil characterization based on morphology can be ambiguous, challenging evolutionary hypotheses based on

such records (Kanaparthi et al., 2024). Since fungi produce spores for reproduction and dispersal (e.g., active and passive), they can survive and distribute over large time spans and distances and readily proliferate when conditions become suitable. While less susceptible to environmental conditions compared to other microorganisms, some fungal taxa have repeatedly been shown to be useful bioindicators in surface freshwater ecosystems (DiLeo et al., 2010; Sutcliffe et al., 2018), while potential relationships between anthropogenic pollution and fungal pathogens have also been suggested (Pietryczuk et al., 2018).

Due to their high nutritional quality, fungal spores and mycelia may also serve as potential grazing material for extant invertebrate species, or substrates for bacterial colonization thereby supporting life in groundwater (Kagami et al., 2014). Interactions between fungi and prokaryotic organisms have shown to be widespread in the subsurface (Meyer et al., 2022; Nuppunen-Puputti et al., 2021; Wang et al., 2022). While not well characterized, they potentially encompass several known positive (e.g., mutualistic, commensalistic), and negative (e.g., antagonistic, parasitic) associations (Ekendahl et al., 2003; Grabner et al., 2020; Karwautz and Griebler, 2022; Sapir et al., 2014). For instance, prokaryotic biofilms are known to support groundwater fungal communities by providing easily accessible mono- and polysaccharides (Reitner et al., 2006). On the other hand, fungi are initial colonizers and degraders of plant debris and animal matter, and thus essential in providing small carbon compounds to lower trophic members of the food web. They do this through their external digestion, serving as important electron donors, as well as providing growth substrate for extant microorganisms. Additionally, some fungal lineages can produce hydrogen through anaerobic respiration of complex OM and thereby fuel autotrophic microbial processes and metabolic functioning in the subsurface (Drake and Ivarsson, 2018; Ruff et al., 2023). Moreover, the diversity of parasitic, endosymbiotic, or hyper parasitic fungi (e.g., microsporidians, cryptomycetes) and their extent in controlling groundwater populations (e.g., protists, invertebrates, other fungi), is largely untapped (Grabner et al., 2020). These inter-specie interactions represent important, but largely unexplored mechanisms underlying evolution, adaptation, and speciation of groundwater organisms and communities.

To address questions regarding species diversity, distribution and community assembly of microbial, and microeukaryotic communities in groundwater, this thesis applies metabarcoding as a tool and places groundwater in contrast to a river ecosystem. So far, the majority of studies have drawn insights into these questions from rDNA-based approaches, potentially overestimating diversity. They do not enable us to distinguish active, dormant, or dead cell stages (Carini et al., 2016), which is relevant given the high prevalence of dormancy strategies among groundwater taxa (e.g., spore formation, metabolic shutdown, and ultra-small cell states). Targeting the active microbial fraction through RNA-based approaches,

thereby identifying metabolically active members, and comparing these results to the total community can provide crucial insights into community functioning, resilience to environmental change, and community reactivation upon groundwater disturbance. Moreover, the influence of spatiotemporal fluctuations in physicochemical and geochemical properties on taxonomic and phylogenetic diversity in groundwater remains largely understudied and less clear. In this thesis, these changes will be investigated in terms of both short-term (e.g., extreme hydrometeorological events) and longer-term (e.g., seasonal shifts) temporal variations, as well as spatial changes in geology and land use along an alpine to lowland gradient of a 300 km river stretch in the Mur valley, Austria.

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Chapter Overview

Chapter I

"Groundwater Microbial Communities in Times of Climate Change" is a publication authored by Retter, A., Karwautz, C., & Griebler, C. and included in the book "Climate Change and Microbial Ecology: Current Research and Future Trends" (2nd edition) edited by J. Marxsen and published by Caister Academic Press in the U.K. in 2020. The publication is a chapter in the book and is located on pages 421-450. The publication is available online at the DOI: https://doi.org/10.2436/im.v19i2.142243. The chapter discusses the impact of climate change on groundwater microbial communities, and the authors' own contribution to the research in this field was conceptualization, formal analysis, visualization and writing the original draft.

Chapter II

"From the mountain to the valley - drivers of groundwater prokaryotic communities along a alpine river corridor" is a publication authored by Retter, A., Brik, S., Haas, J., Stumpp, C., Hausmann, B., Griebler, C., Karwautz, C. and it is published in the Journal Microorganisms under the DOI https://doi.org/10.3390/microorganisms11030779. This paper examines the drivers of groundwater prokaryotic communities along an alpine river corridor. The authors' own contribution to the research in this field was conceptualization, formal analysis, visualization and writing the original draft.

Chapter III

"The Groundwater Mycobiome: Fungal Diversity in Terrestrial Aquifers" is a publication authored by Retter, A., & Nawaz, A. and included in the book "Encyclopedia of Inland Waters" (Second Edition) edited by Mehner, T., Tockner, K., and published by Elsevier in Oxford in 2022. The publication is a chapter in the book and is located on pages 385-396. The ISBN number of the book is 978-0-12-822041-2, and the article is avail-able online at the DOI: https://doi.org/10.1016/b978-0-12-819166-8.00032-3. The chapter discusses the mycobiome of groundwater, specifically the diversity of fungi in terrestrial aquifers. The authors' own contribution to the research in this field was conceptualization, formal analysis, visualization and writing the original draft.

Chapter IV

"Metabarcoding reveals ecologically distinct fungal assemblages in river and groundwater along an Austrian alpine to lowland gradient" is a publication authored by Retter, A., Griebler, C., Nilsson, H., Haas, J., Birk, S., Breyer, E., Baltar, F., and Karwautz, C. and it is published at FEMS Microbiology ecology under

the DOI: https://doi.org/10.1093/femsec/fiae139. The study aims to understand how environmental factors, such as landscape characteristics, hydrology and human activities, impact the distribution and diversity of fungal assemblages in the groundwater and adjacent river along an alpine river valley. The authors' own contribution to the research in this field was conceptualization, formal analysis, visualization and writing the original draft.

Chapter V

"Application of the D-A-(C) index as a simple tool for microbial-ecological characterization and assessment of groundwater ecosystems—a case study of the Mur river Valley, Austria" is a research article authored by Retter, A., Griebler, C., Haas, J., Birk, S., Stumpp, C., Brielmann, H., & Fillinger, L. and published in the journal "Österreichische Wasser- und Abfallwirtschaft" in 2021. The volume number is 73, issue number is 11-12, and the pages of the article are 455-467. The article is available online at the DOI: https://doi.org/10.1007/s00506-021-00799-5. The authors of the article present the D-A-(C) index as a simple tool for microbial-ecological characterization and assessment of groundwater ecosystems, and the article includes a case study of the Mur river Valley, Austria. The author's own contribution to the research was conceptualization, formal analysis, visualization, and writing the original draft.

Chapter I: Groundwater Microbial Communities in Times of Climate Change

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Retter, A., Karwautz, C., Griebler, C., 2020. Groundwater Microbial Communities in Times of Climate Change. In: Marxsen, J. (Ed.), Climate Change and Microbial Ecology: Current Research and Future Trends. Caister Academic Press, U.K., pp. 421–450.

Abstract

Climate change has a massive impact on the global water cycle. Subsurface ecosystems, the Earth's largest reservoir of liquid freshwater, currently experience a significant increase in temperature and serious consequences from extreme hydrological events. Extended droughts as well as heavy rains and floods have measurable impacts on groundwater quality and availability. In addition, the growing water demand puts increasing pressure on the already vulnerable groundwater ecosystems. Global change induces undesired dynamics in the typically nutrient and energy poor aquifers that are home to a diverse, and specialized microbiome and fauna. Current and future changes in subsurface environmental conditions, without doubt, alter the composition of communities, as well as important ecosystem functions such as the cycling of elements such as carbon and nitrogen. A key role is here, without doubt, with the microbes. Understanding the interplay of biotic and abiotic drivers in subterranean ecosystems is required to anticipate future effects of global and climate change to groundwater resources and habitats. This chapter summarizes potential threats to groundwater ecosystems with emphasis on climate change and the microbial world down below our feet in the water saturated subsurface.

Introduction

Groundwater ecosystems contain 97 % of the non-frozen freshwater resources and as such provide an important water supply for irrigation of agricultural land, industrial use (e.g. cooling agent), as well as for production of potable water. Worth mentioning, in Europe, around 50 – 70 % of all drinking water stems from groundwater (Zektser and Everett, 2004). Groundwater constitutes a major component of the

hydrological cycle, and sustains streams, lakes, and wetlands, many of which would not be perennial without the direct connection to an aquifer. Groundwater ecosystems are typically covered by vegetated soil and vadose sediment layers of varying thickness. Where the protective layers are thin and perforated (e.g. in mountainous areas and karstic rock) or even absent and where groundwater reaches land surface (e.g. wetlands and springs), the down below systems are highly vulnerable to disturbance from above. Moreover, due to the comparably long residence time of groundwater in the subsurface (Danielopol et al., 2003), its response time to external impacts can be delayed and sometimes masked by complex hydrologically patterns (Alley et al., 2002; Kløve et al., 2014). The subsurface is a naturally light deprived, and nutrient limited environment, hence the energy required for sustaining groundwater ecosystems is largely derived from the surface. In fact, the groundwater ecosystems balance is susceptible to several external influences. It is on one hand largely dependent on energy import from the surface, but on the other hand responding sensitively to increased input of organics and nutrients as well as various types of contaminants including heavy metals and heat. While in the past, groundwater pollution mainly resulted from source contaminations with deposited or spilled petroleum hydrocarbons and halogenated solvents as well as leaking landfills, modern impacts to groundwater ecosystems are numerous. A major threat still (and ongoing) comes from intensive land use in terms of agriculture and urbanization (Saccò et al., 2019a). Application of fertilizers and pesticides to agricultural land are a major issue besides surface sealing, down below infrastructure and geothermal energy use in big cities. Accelerated import of organic matter, nutrients and chemicals cause eutrophication as well as acute and chronic toxicological effects. In urban areas, a typical phenomenon is groundwater warming.

Related to wastewater discharge, a long list of emerging pollutants including pharmaceuticals, anticorrosion agents, artificial sweeteners, drugs, or sanitary products comprise a future challenge not only for groundwater. Finally, the application of manure to arable land and the infiltration of wastewater influenced surface water occasionally lead to groundwater contamination with biological agents such as human pathogenic germs and viruses (Krauss and Griebler, 2011). Not surprisingly, also excessive water withdrawal constitutes a major threat to groundwater ecosystems. Said that, it is timely to evaluate the possible effects of climate change to the groundwater microbiome.

Climate change impacts on groundwater ecosystems

Abiotic effects of global warming

Climate change, and its consequences for global warming presents one of the biggest environmental and societal challenges of our time. The pace at which it is advancing varies across ecosystems in different parts of the world and is determined to a large extent by geographic location and atmospheric chemistry

e.g. greenhouse gas concentrations (Yvon-Durocher et al., 2010). The changes in seawater levels, climate, and environmental conditions alter the recharge, flow direction, storage, and discharge capacities of groundwater (Edmunds and Milne, 2001). Current climate change models predict more extreme weather events in the future that will directly impact the hydrological dynamics of groundwater (Alley at el., 2002). Figure 1 compiles available information on predicted changes in precipitation and groundwater recharge for the world's major climate zones.

Some consequences of climate change already appear globally (e.g. increase in mean air temperature) while others show up geographically distinct or act only on a local scale. Prominent effects include increasing frequency of extreme hydrological events, i.e. droughts and floods, and heavy rainfall, increasing evapo(transpi)ration rates, vertical expanding of the vadose zone, horizontal expanding of dry areas including modified soil properties, decline of groundwater tables, rising sea levels and salt water intrusion, loss of glaciers as water stores, among others (Fig. 2) (Bates et al., 2008; Treidel et al., 2011; Richts and Vrba, 2016; Bastin et al., 2017; Betts et al., 2018). And without doubt, global warming also affects groundwater temperatures on a regional to global scale (Fig. 3).

Currently, it is difficult to provide quantitative predictions to which extent climate change is modifying the frequency, and intensity of these events and how this directly, and indirectly feeds back on groundwater quantity and quality, and in consequence on microbial processes and the sequestration and cycling of matter in the subsurface. This leaves huge uncertainties to the full impact of climate change on groundwater ecosystems (Boulton, 2005). In fact, most recent studies indicate an overall deteriorating trend with respect to groundwater quantity and quality, with only few exceptions; i.e. increased groundwater recharge in northern regions of Europe and partly in regions where ice stocks and permafrost is melting (e.g. polar regions) or some dry and arid regions that may receive intensified rain falls (Fig. 1; Treidel et al., 2011).

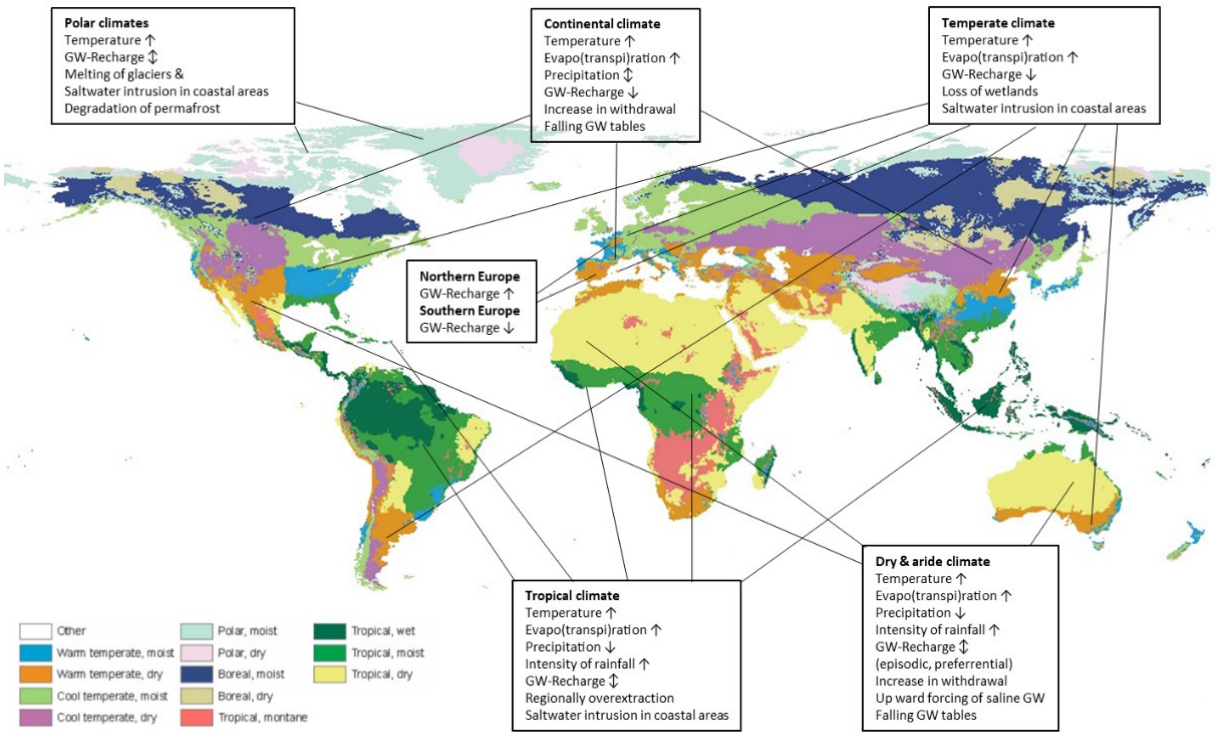

Figure 2 Major hydrological and meteorological effects with relation to groundwater quantity and quality caused by climate change. Information compiled mainly from Treidel et al. (2011). Map of world climate zones from https://ec.europa.eu/jrc/en/.

Groundwater aquifers, as well as connected surface ecosystems (i.e. groundwater associated aquatic ecosystems [GWAAES], and groundwater dependent terrestrial ecosystems [GWDTES]) are believed to be highly vulnerable to consequences of climate change and related pressures (Nathan and Evans, 2011; Barthel and Banzhaf, 2016; Jasperson et al., 2018). With the GWAAES and the GWDTES it is mainly quantitative issues, i.e. the temporal disconnection to groundwater, that matters besides consequences from altered groundwater quality that may impact the connected surface ecosystems. With groundwater ecosystems, the vulnerability is related to various frame conditions. First, their low productivity goes along with a low resilience to disturbance. Second, any negative impact that traced into an aquifer stays there for long, because water residence times in the subsurface may be orders of magnitude higher than in surface waters (Danielopol et al. 2003). Third, groundwater ecosystems are typically characterized by stable environmental conditions (e.g. temperature, water chemistry, oligotrophy) all year round. In such systems, minor disturbances can have major effects on the well-adapted communities therein and the processes they mediate.

Global warming is influencing the various subterranean habitats at different spatial and temporal scales. Depending on groundwater depth, changes in the annual mean air temperature will translate into the subsurface with a time lag of years to decades compared to surface habitats, and daily or yearly fluctuations are usually attenuated (Fig. 3) (Menberg et al., 2014; Mammola, 2019 and citations therein).

Accordingly, shallow groundwater aquifers are most exposed to external impacts, and are expected to respond more rapidly to changing temperatures than deeper aquifers. Especially in highly urbanized and agricultural areas and areas of low vegetation cover, shallow groundwater temperatures can be expected to rise even within a relatively short period of time (Henriksen and Kirkhusmo, 2000; Menberg et al., 2014; Kurylyk et al., 2015). Elevated subsurface temperatures directly alter the physical- and chemical properties of the groundwater, by lowering the solubility of gases (e.g. dissolved oxygen), as well as leading to a higher variation in dissolved organic carbon (DOC) concentrations, and increased mineralization rates (Bonte et al., 2013). Additionally, a change in temperature can alter the organic matter (OM) composition and quantity of above-ground OM sources (e.g., surface flow-paths, the riparian zone, soils) that subsequently enter the groundwater via seepage water (Moldovan et al., 2018; Griebler et al., 2016; Stegen et al., 2016).

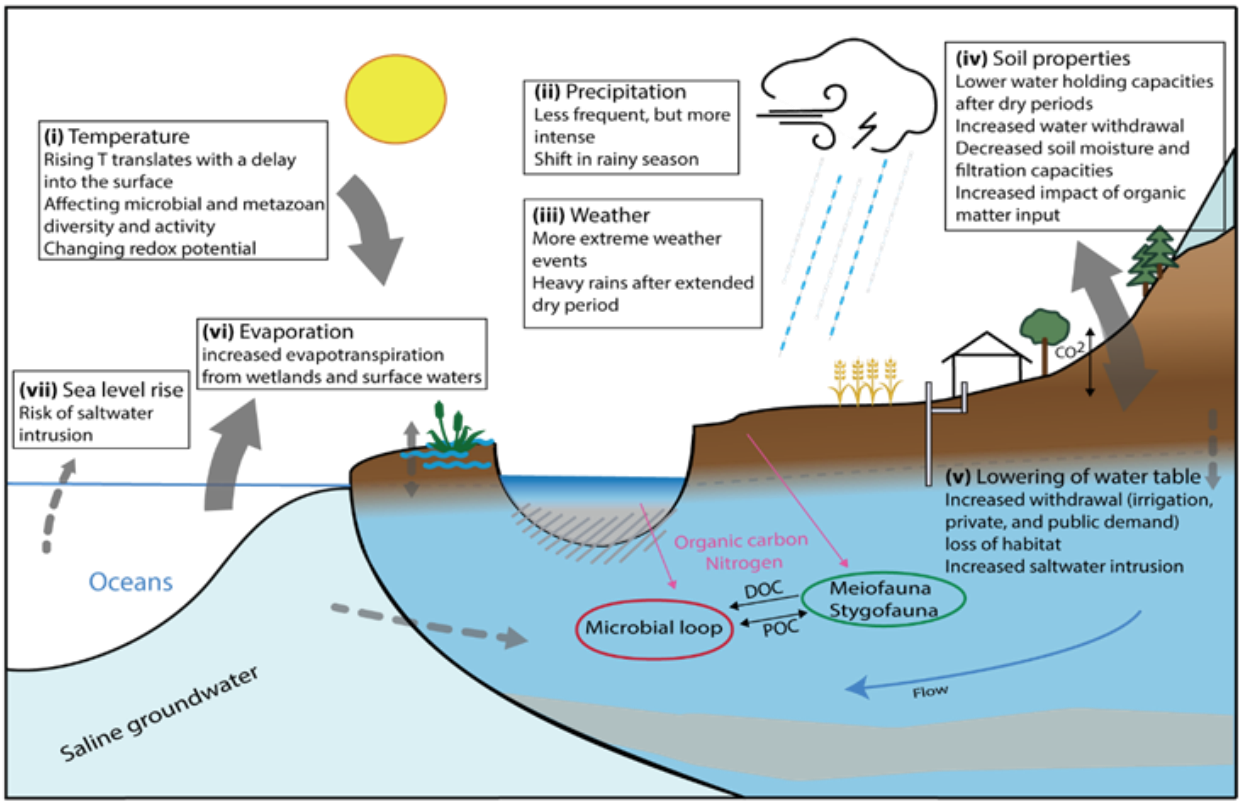

Figure 3 Cross-section scheme highlighting impacts of climate change on the hydrological cycle with regards to the groundwater ecosystems, water tables, physicochemical states, as well as groundwater communities and food webs.

Indeed, climate change may affect the composition and quantity of OM ultimately reaching the groundwater table. As depicted in Fig. 1 and 2, changes in spatiotemporal precipitation dynamics, soil properties and groundwater recharge initiate shifts in the vadose zone thickness, which in turn influences the passage of OM, nutrients, but also contaminants into the aquifer (Ward et al., 2017). Thus, the OM from the surface dynamically entering during recharge events potentially alters the composition and

activity of groundwater microbial communities on different temporal scales (Hofmann and Griebler, 2018; Benk et al., 2019). The same applies to allochthonous microorganisms introduced into the subsurface during recharge, possibly invading and re-assembling indigenous microbial communities, or even acting as a source for the groundwater microbiome (Fillinger et al., 2019; Yan et al., 2020).

The soil layer acts as a natural filter reducing or even impeding the seepage of contaminants from the surface into shallow groundwater. Extreme rainfall events have been linked to impaired groundwater quality and waterborne diseases (Ebi et al., 2017). Environmental factors such as the geographical region, land use or season do modify these effects (Guzman Herrador et al., 2015; Sonthiphand et al., 2019). Rain events can mobilize antibiotic resistant microbes from soils increasing their levels in riverine microbial communities and potentially groundwater (Di Cesare et al., 2017).

The groundwater is intricately linked to other components of the hydrological cycle through refined flow-, and recharge dynamics (Havril et al., 2018). Climate change related modifications in hydrological settings thus will in some areas lead to less, but more intense carbon, nutrient, as well as contaminant inputs into the groundwater (Kaushal et al., 2014). At the same time, an associated change in groundwater table depth cause the deterioration of groundwater and linked freshwater ecosystems. This is already leading to altered carbon source, sink, and carbon cycle feedback behavior in some parts of the world (Mander et al., 2015; Dhillon et al., 2019). Besides the fast disappearance of wetlands that has been going on for centuries, hydrological dynamics of GWDTEs are dramatically changing, affecting their size, and permanence. For instance, there is an ongoing trend of peatland desiccation (Swindles et al., 2019), as well as an increase of surface water areas elsewhere, for example high altitude lakes (Moore and Knowles, 1989; Zhang et al., 2017; Davidson et al., 2019), partly a leftover from disappearing glaciers.

Drought periods are expected to increase. Extended dry periods lead to desiccated surface soils which entails two main effects in terms of groundwater recharge. On one hand, dry soils have a decreased capacity to take up and hold water. In case of heavy precipitation, a large proportion of rainwater is thus directly lost from recharge by surface run-off. Simultaneously, dry cracks in desiccated soil allow some of the freshly precipitated water together with material collected at the surface to infiltrate fast via preferential flow paths, missing the natural attenuation during slow sediment passage. Thus, the decreased water retention capacity of soils, and by that associated formation of cracks could lead to stronger seepage water pulses which were shown to be an important driver in cell transport and microbial community assembly (Yan et al., 2020).

A temperature related rise in sea-level, while mostly unrecognized, will lead to increasing groundwater levels in coastal regions with devastating effects (Colombani et al., 2016). The impact of groundwater salinization can be two-fold. First, with a rise in sea-level, saline groundwater will ingress into

subterranean freshwater aquifers, and secondly due to an extended demand and a resulting increase in groundwater withdrawal, a decreasing groundwater table is pulling saline groundwater inland (Treidel et al., 2011). This is of particular concern when it comes to small islands, where fresh groundwater habitats are especially vulnerable due to their confinement by the sea. The groundwater ecosystems therein especially in proximity to shorelines could thus be threatened, and anchialine habitats will probably shift inland or disappear completely (Rotzoll and Fletcher, 2012; Moritsch et al., 2014; Rogers et al., 2019).

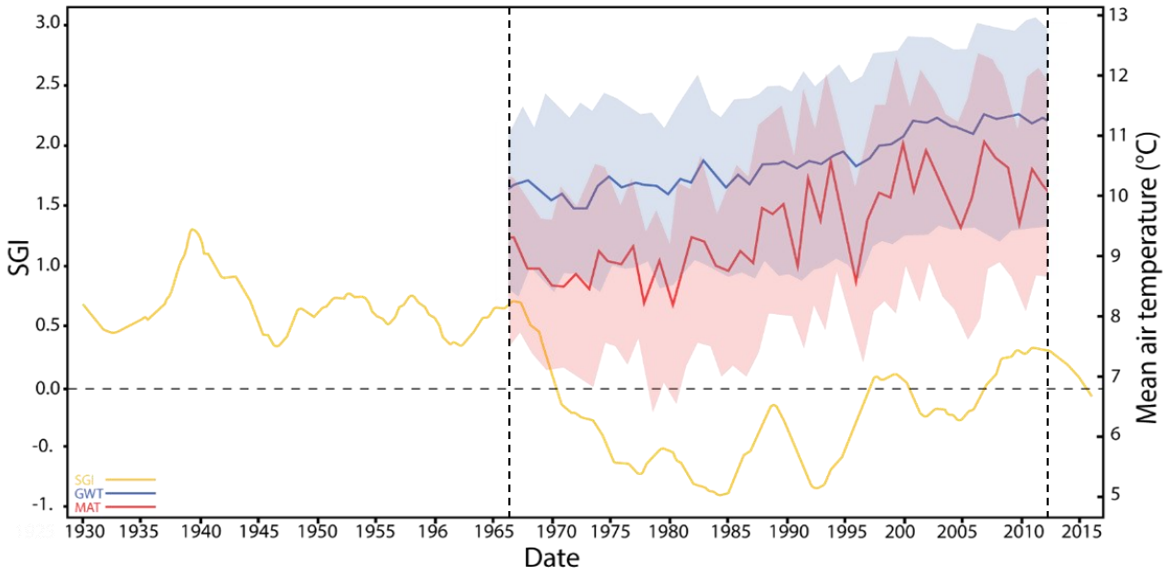

Figure 4 An exemplary time series from Austrian depicting trends in mean surface air temperature (MAT), and mean groundwater temperatures (GWT) (modified from Benz et al., 2018), as well as the trend of the standardized groundwater index (SGI) for Austrian groundwater (modified from Haas and Birk, 2019), showing the deviation from long-term average water levels, where negative values (<0.0) indicate declining groundwater levels; so called groundwater droughts (Uddameri et al., 2019). The general deviation between MAT and GWT relates to temperature measurements mainly in very shallow groundwater (see Benz et al., 2018).

Warming of permafrost and associated ground ice melting, as well as englacial temperatures have increased within the last decades (Ding et al., 2019). The melting of arctic permafrost leads to elevated emissions of carbon dioxide and other greenhouse gases that until now have been stored within the arctic soil. Conversely, a study of Voigt et al. (2019) showed that dry permafrost peatlands store methane rather than emitting it under warming scenarios. Without doubt, discontinuous permafrost and melting of glaciers alter the hydrology, chemistry and biology of the underlying groundwater bodies, which may experience a change from confined to unconfined aquifers. The latter will introduce the risk of groundwater pollution in case of existing above ground contaminant sources. Groundwater below glaciers and surface water ecosystems fed by them are influenced by several factors, such as thermal regimes (Ravier and Buoncristiani, 2017), the production of meltwater, and groundwater recharge, which changes when glacier size decreases (Haldorsen et al., 2010). The overall extent of climate change on aquatic habitats in polar regions can so far only be roughly estimated. They seem to harbor a distinct

microbiome (Wurzbacher et al., 2017), but further research on biodiversity as well as climate change induced dynamics of carbon- and nutrient in these remote regions is urgently needed.

The groundwater food web

Groundwater ecosystems provide habitats with constant darkness and stable temperatures, narrow space, as well as low carbon, nutrient, and energy levels. In fact, the subterranean biosphere is inhabited by a diverse selection of well adapted organisms, including microbes and metazoan. Because groundwater ecosystems are among the least-studied habitats on earth (Devitt et al., 2019), the majority of the members in subterranean communities, estimated at 50,000 to 100,000 obligate subterranean metazoan species and millions of microbial taxa, still await formal description (Culver and Holsinger, 1992; Griebler and Lueders, 2009). In light of the ongoing trend of global species extinction, there is a great risk of losing species even before their discovery.

Most dominant in groundwater food webs are microbes such as *Bacteria*, *Archaea*, *Fungi* and *Protozoa* - most of them sharing a heterotrophic lifestyle. However, as already stated, groundwater also harbours micro-, meio-, and macrofauna, including a multitude of crustaceans (i.e. copepods, isopods, amphipods, ostracods), worms (oligochaetes, polychaetes, nematodes, platyhelminths), gastropods and mites. In cave waters even salamander or fish are home. Metazoa depend on microbes as food source, although opportunistic predation within their own trophic levels occurs as well (Saccò et al., 2019b). Groundwater fauna often displays highest diversity and abundance at the upper boundaries of aquatic subterranean habitats where they take advantage of increased food availability (Fišer et al., 2014). Groundwater fauna in general is highly adapted to a narrow temperature range. There are exceptions with some taxa that endure a relatively broad temperature tolerance, however, always with a much better survival under temperature conditions resembling those of their natural habitats (Eme et al., 2014).

With the absence of major primary producers (i.e. photoautotrophs) groundwater food webs are typically truncated (Gibert and Deharveng, 2002). Trophic interactions appear to be bottom-up regulated, driven by the import of dissolved organic carbon, being important electron donor for microbial redox processes in groundwater (Hofbauer and Griebler, 2018; Saccò et al., 2019b). In situ microbial OM production in the light-deprived subsurface by chemolithoautotrophs is currently not considered a major contribution to the global carbon cycle. However, chemolithoautotrophic Bacteria have been shown to account for a large proportion of expressed functional genes in shallow alluvial groundwater and may share functionally redundant metabolic pathways (Jewell et al., 2016). Several studies (Lau et al., 2016, Hutchins et al., 2016; Sarbu et al., 1996; Galassi et al., 2017) underlined that locally, i.e. in caves and deep groundwater systems, chemolithoautotrophic production is playing a substantial role in driving trophic complexity and species abundances over long timescales. In any case, due to its huge dimensions,

groundwater ecosystems represent a significant storage of global organic carbon, for example, in form of microbial biomass (Whitman et al., 1998; Magnabosco et al., 2018).

Climate change effects to the groundwater microbiome

Much has been said about the 'abiotic' effects of climate change and global warming to groundwater in terms of quantity and physical-chemical quality. But what do these expected and already ongoing changes in salinity, temperature, as well as carbon import to the subsurface mean to groundwater microbial communities? For some of the effects that work on groundwater ecosystems or will gain importance in the future we have strong evidence, while in other cases we currently can only speculate.

It is well documented that a change in temperature alters microbial metabolic rates and affects microbial community composition. Elevated water temperatures, especially in temperate- and subpolar zones can be expected to influence the generation times and abundance of microorganisms in subsurface- and groundwater ecosystems (Rodó et al., 2013; Karthe, 2015; Shocket et al., 2018). Higher temperatures will speed up microbial mediated processes including the turnover of organic matter and respiration (redox processes). However, key to the expected effects is the magnitude of temperature change and the time scale. A temperature increase by only 1-2°C may not exhibit short-term (acute) changes in metabolic rates at the significance level taking the natural heterogeneity in groundwater systems into account. On a long-term perspective even slightly elevated rates of carbon turnover may exhaust carbon and nutrient pools available to the microbial communities if not replenished in time. There are complex scenarios to be considered, including as examples (1) elevated microbial activities along with decreasing pools of energy or (2) slightly increasing activities along with higher loads of carbon (including contaminants) arriving in pulses. In a field study targeting the influence of groundwater warming on various microbial patterns, it was concluded that in very clean groundwater, i.e. groundwater that lacks labile organic carbon as well as toxic compounds, a rise in temperature does not significantly affect the abundance and growth of prokaryotic cells (Brielmann et al., 2009). When overriding the energy limitation, as has been evaluated in subsequent lab experiments, even moderate temperature changes lead to shifts in microbial growth, biomass and respiration (Brielmann et al., 2011; Griebler et al., 2016). The limitation by an essential nutrient (e.g. phosphorus) may have similar effects. Bacterial cells that have enough bioavailable organic carbon but lack an element to build new biomass continue respiring OM without growth (Hofmann and Griebler, 2018). In consequence, OM is turned over without the production of prokaryotic biomass needed by other members of the (microbial) food web such as protozoa or meiofauna. Indeed, predator - prey interactions have shown to be temperature dependent, and the degree of dependency changes according to taxonomic affiliation, as well as species-, and food web traits (Hutchins et al., 2016; Archer et al., 2019). Not to forget, the solubility of gases is temperature dependent. Even small temperature

changes may turn groundwater with already low concentrations of dissolved oxygen into hypoxic or even anoxic habitats. There is ample evidence from lab experiments and small scale field studies that the switch from oxic to anoxic conditions in groundwater triggers a cascade of effects including (1) the onset of anaerobic processes, (2) the accumulation of unwanted solutes in the water (ferrous iron, hydrogen sulfide, methane), and (3) a dramatic shift in community composition. In summary, already small changes in groundwater thermal regimes may lead to a decoupling of balanced food web interactions with consequences to the cycling of elements and the composition and structure of communities. In addition to the direct effects of a changing climate, human activities intended to counteract climate change, such as the sequestration of CO_2 in deep geological layers and the use of geothermal energy as sustainable alternative, pose threats to groundwater communities and ecosystems (Bonte et al., 2013; Griebler et al., 2016; Lawter et al., 2017; Westphal et al., 2017; Oelkers et al., 2019).

As already mentioned briefly, changes in the availability of organic matter will modulate microbial activities and processes. Groundwater microorganisms drive carbon-, and nutrient cycling, catalyze the breakdown of contaminants, and modulate soil fertility and consequently global food webs (Cavicchioli et al., 2019). According to the scenario of irregular and more frequent extreme hydrological events, we may assume organic matter import into the subsurface on one hand to pause during extended periods of droughts and, on the other hand, to rapidly increase during times of heavy rain and floods. In the latter case, DOM may enter the subsurface in short-term pulses that cannot be processes spontaneously but are subject to a delayed microbial degradation (Foulquier et al., 2011; Reiss and Schmid-Araya, 2011; Saccò et al., 2019b). New degradation products will cause a reorganization of microbial communities in a compositional and functional manner. For instance, microbial communities in alkaline groundwaters were found to strongly relate to environmental factors such as pH, carbon monoxide, and methane concentrations (Twing et al., 2017). This will also impact the groundwater carbon cycle feedback to global warming (Allison and Martiny, 2009; He et al., 2010). Still, it is largely unclear to what extent groundwater habitats will experience a shift in the carbon sink-to-source ratio, and where to position groundwater within the global carbon cycle (Chapelle et al., 2016). Without doubt, changes in subsurface organic matter turnover will translate into changes in greenhouse gas emissions from the aquifers, a subject that so far received hardly any attention.

Changes in environmental conditions, i.e. temperature, salinity, carbon availability, all have in common that they directly or indirectly influence the composition of microbial communities. Microbial community composition in response to altered environmental conditions is linked to changes in ecosystem functioning. *Vice versa*, increased or decreased process rates affect microbial diversity (Jesußek et al., 2013, Stegen et al., 2016). It is a paradigm that ecosystems rich in microbial species diversity exhibit a

higher stability and resilience when facing disturbances due to a high functional redundancy. Species richness and biodiversity, on the other hand, are somehow connected to the energy available. There is conclusive evidence from several studies conducted in different aquatic environments that changes in organic matter supply in terms of quantity and quality steers shifts in microbial community composition (Shi et al., 1999; Baker et al., 2000; Findlay et al., 2003; Carlson et al., 2004; Judd et al., 2006; Kritzberg et al., 2006; Li et al., 2012; Wu et al., 2018). In particular, if the altered DOM supply lasts for longer than just a couple of hours or days (Herzyk et al., 2014; 2017; Grösbacher et al., 2016). However, we are only beginning to understand whether and how energy–diversity relationships known from macroecology apply to complex natural microbial communities. Diversity–productivity patterns from dozens of natural systems were found either negative (35%), positive (28%); or humped (23%) (Smith, 2007). From experimental studies that supplied sediment microbial communities with DOM, a change in community composition was reported, i.e. an increase in relative abundance of *Betaproteobacteria* with higher biodegradable dissolved organic carbon (BDOC) concentrations (Li et al., 2012). A lower BDOC was accompanied by a higher relative abundance of *Firmicutes*, *Planctomycetes*, and *Actinobacteria*, a pattern that mirrored our own findings. Overall, a decrease of microbial diversity in terms of richness and Shannon-Wiener diversity with higher feed concentrations was observed (Li et al., 2012, Li et al., 2013). In contrary, a positive relationship between bacterial richness and bioavailable organic matter was observed in Arctic deep-sea sediments, hinting at a positive energy–diversity relationship in oligotrophic environments (Bienhold et al., 2012). In fact, we can be sure that altered dynamics of organic matter input to groundwater systems will affect microbial community composition and mediated processes. The same holds true for nutrients (e.g. from fertilization) and in particular for contaminants. Undisturbed groundwater ecosystems are typically energy poor, they have a comparably low microbial biomass, activity and diversity (Griebler and Lueders, 2009) and, in consequence, are assumed to be less resilient to impacts. In other words, groundwater ecosystems and their communities are highly vulnerable.

Climate change will, without doubt, force us to adapt land use and agricultural practices. Several studies indicate that vegetation, land use and irrigation are important driver of the community composition and activity in shallow groundwater (Stein et al., 2010; Korbel et al., 2013; Iepure et al., 2016). The causality behind these effects is often not fully clear but the stressor 'land use' may be dissected into (1) fertilization and the application of pesticides that lead to an altered import of carbon, nutrients and contaminants, (2) deforestation and clearance of vegetation that alter conditions for groundwater recharge in terms of quantity and quality, (3) irrigation that changes the structure, moisture and water holding capacity of top soil layers and may lead to the accumulation of salts in case groundwater from deep aquifers is used as water source. Moreover, the application of manure to arable land constitutes a

source of pathogenic microbes and viruses which with seepage water may enter shallow aquifers. With the serious changes in hydrological conditions and a higher frequency of extreme weather events such as heavy rainfall, there is an increasing risk of a hygienic contamination (Krauss and Griebler, 2011).

There are significant differences in the composition of microbial communities in saltwater and freshwater habitats. While there are individual studies targeting wetland biodiversity in regard to sea-level rise, little research has been directed to groundwater systems so far. In general, freshwater microbial communities and communities in low-salinity habitats are prone to a loss in diversity in case of saltwater intrusion. As shown for wetlands, microbial community shift went along with changes in the carbon biogeochemistry (Franklin et al., 2017; Dang et al., 2019). In tidal wetlands, for example, the altered carbon cycling directly affected nitrogen turnover. A loss in the ability to remove excess nitrogen leads to an excess of nutrients and increased greenhouse gas emissions in these habitats. Even under reversed climate scenarios, it may take years to decades for these ecosystems to rebalance (Franklin et al., 2017; Dang et al., 2019). A shift in soil, and sediment salt content was shown to lead to an altered microbial community functioning, which in turn is depending on the duration and frequency of the salinity exposure (Liu et al., 2017; Rath et al., 2019). A study of Edmonds et al. (2009) underline the different time scales associated with changes in microbial community pattern. While a fast change in the functional component of the community with increased salinity was observed, taxonomic composition and metabolic potential on DNA level remain unchanged in response to salt stress. In summary, saltwater intrusion to fresh groundwater reservoirs poses complex changes that can be long lasting in zones with increasing salinity. Although only little is known for groundwater environments, similar effects as observed in other aquatic habitats can be expected to the groundwater biome.

Conclusions and Outlook

Climate change affects groundwater in many ways. On the one hand, it seriously alters the interplay of all important components of the hydrological cycle. Groundwater, at least the shallow portion, is changing in quantity and quality. On the other hand, climate change triggers a more intensive use of groundwater for the production of potable water, irrigation and cooling purposes. Both aspects have direct and indirect impacts to the groundwater communities in terms of composition and functions. This will have several consequences. Ecosystem functions and services mediated by microbes will change. In particular, this will apply to the subterranean carbon cycle. We may further expect that increasing temperatures impair groundwater food webs and effects to microbes will also translate into higher trophic levels.

While there is a multitude of models that try to predict climate change scenarios and related changes in carbon cycling or the loss of local to global biodiversity, groundwater ecosystems have so far been hardly

considered. Although a huge amount of carbon is stored in the terrestrial subsurface, groundwater ecosystems are not yet implemented in global scale carbon models. As a first step we suggest to analyze climate variability and change in relation to hydrological, meteorological and ecological patterns on the long term (50-100 years or more) including groundwater systems to identify drivers within the oceanic-terrestrial-atmospheric coupling to understand and to be able to predict spatiotemporal changes in precipitation, evapotranspiration, recharge, discharge and groundwater storage, biogeochemical processes, and the fate of contaminants, be it abiotic or biotic (Treidel et al., 2011). For sites than expected, long-term data series are available that await targeted analysis. We also need to have an eye on the climate change induced dynamics of the vadose zone that highly determines what ends up in the aquifer in terms of energy and matter. We further need to quantify groundwater recharge and discharge and the individual pools of carbon and other elements on different spatial scales. Moreover, information on carbon turnover rates including the production and emission of greenhouse gases from groundwater systems is urgently needed. Later, these details can be implemented into scale dependent groundwater models and coupled to already existing models on carbon cycling and climate change.

Currently, our knowledge about the effects of climate change on groundwater microbial communities and interlinked food webs and cycling of matter, as well as their response to global warming is still very limited. As mentioned above, many groundwater taxa, including microorganisms such as *Bacteria*, *Archaea*, *Fungi*, and *Protozoa* are still lacking a detailed taxonomic description (Nawaz et al., 2018; Mulec and Engel, 2019; Savio et al., 2019). Only a minute portion of microbes could yet be isolated and physiologically studied. Almost all information on the microbes' physiology and biochemistry comes from genome, transcriptome, proteome, and metabolome data. This situation can be illustrated with some examples. A study by Hug and coworkers (2016) discovered novel, unrelated lineages, and phyla of *Bacteria* and *Archaea* active in carbon fixation, as well as ammonia, and sulfur oxidation in sediment cores from the deep subsurface. Another study reported a novel lineage of nitrogen fixing *Bacteria*, sister to *Cyanobacteria*, that is living in anoxic zones in the subsurface (Di Rienzi et al., 2013). And last but not least, a study by Anantharaman et al. (2016) found a large number of undescribed bacterial phyla in groundwater which were affiliated to *Proteobacteria*, and Candidate Phyla Radiation (CPR), taking on many already known biogeochemical processes closely linked to carbon, hydrogen, and sulfur cycling, but also partly unknown metabolic pathways. Compared to other aboveground freshwater ecosystems, the knowledge about fungi in groundwater habitats, even though we have evidence that the groundwater in fact hosts numerous fungal taxa, is limited regarding species interactions, taxonomic composition and abundance, and community ecology (Grossart et al., 2019). Consequently, many known functions of the biogeochemical cycles in groundwater cannot be unambiguously linked to a certain species or group of

organisms and process rates are entirely missing. As such, we are far from integrating taxonomic and functional knowledge of the groundwater microbiome into climate change models. However, this is essential to estimate the contribution of microorganisms to carbon, and nutrient cycling in time and space (Antwis et al., 2017; Amend et al., 2019; Cavicchioli et al., 2019).

In the future, new bioinformatics approaches will help to refine ecological traits of microorganisms and their interplay with climate change (Simonsen et al., 2019). A comprehensive and standardized study and sampling design followed by the evaluation of genomic and metadata is asked for to allow meta-analysis (Field et al., 2008). These methods need to deal with the environmental complexity and interspecies interactions that often interfere with climate-associated patterns which become apparent on the large scale (Simonsen et al., 2019).

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Chapter II: From the Mountain to the Valley: Drivers of Groundwater Prokaryotic Communities along an Alpine River Corridor

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Retter, A., Haas, J.C., Birk, S., Stumpp, C., Hausmann, B., Griebler, C., Karwautz, C., 2023. From the Mountain to the Valley: Drivers of Groundwater Prokaryotic Communities along an Alpine River Corridor. Microorganisms 11, 779.

Abstract

Rivers are the “tip of the iceberg”, with the underlying groundwater being the unseen freshwater majority. Microbial community composition and the dynamics of shallow groundwater ecosystems are thus crucial, due to their potential impact on ecosystem processes and functioning. In early summer and late autumn, samples of river water from 14 stations and groundwater from 45 wells were analyzed along a 300 km transect of the Mur river valley, from the Austrian alps to the flats at the Slovenian border. The active and total prokaryotic communities were characterized using high-throughput gene amplicon sequencing. Key physico-chemical parameters and stress indicators were recorded. The dataset was used to challenge ecological concepts and assembly processes in shallow aquifers. The groundwater

microbiome is analyzed regarding its composition, change with land use, and difference to the river. Community composition and species turnover differed significantly. At high altitudes, dispersal limitation was the main driver of groundwater community assembly, whereas in the lowland, homogeneous selection explained the larger share. Land use was a key determinant of the groundwater microbiome composition. The alpine region was more diverse and richer in prokaryotic taxa, with some early diverging archaeal lineages being highly abundant. This dataset shows a longitudinal change in prokaryotic communities that is dependent on regional differences affected by geomorphology and land use.

Keywords: aquifer; prokaryotic diversity; community assembly; aquatic ecology

Introduction

Aquifers are of key importance for surface aquatic and terrestrial ecosystems, as well as for humans, in the form of drinking water, water for industrial uses, and water for irrigation. Invisible to the human eye, groundwater ecosystems are populated by diverse communities composed of microorganisms and metazoans well adapted to the darkness and energy scarcity that prevails in the subsurface (Gibert and Culver, 2009; Griebler and Lueders, 2009; Karwautz and Griebler, 2022; Malard, 2022). While the minute interstices of unconsolidated sediments and the often narrow channels and cracks in karst and fissured aquifers may represent a spatial limitation for metazoans, microorganisms are not restricted by this and are distributed ubiquitously throughout the subsurface (Herrmann et al., 2020; Inkinen et al., 2021; Karwautz and Griebler, 2022). Because of their diverse and metabolically flexible lifestyles, they are closely linked to most element cycles, with the carbon, nitrogen, and phosphorus cycles being relevant examples (Hofmann et al., 2020; Korbel et al., 2022; Starke et al., 2017). Be-sides being involved in processes such as organic matter (OM) sequestration, degradation, and mineralization, microorganisms serve as a source of food for other members of the groundwater food web (Karwautz et al., 2022; Marks, 2019).

Groundwater ecosystems in alpine regions are of particular interest. On one hand, mountainous areas are considered an important freshwater resource and biodiversity hotspot (Perrigo et al., 2020; Savio et al., 2018). On the other hand, alpine regions, as well as groundwater ecosystems are believed to be especially sensitive to external disturbances such as increased input of carbon, nutrients, pollutants, and heat (Auer et al., 2007; Beniston, 2003; Gobiet et al., 2014; Richts and Vrba, 2016). Shallow aquifers are at the transition between highly dynamic surface environments and deep, stable groundwater systems. While already depleted in organic carbon and most nutrients, they may receive irregular pulses of energy along with hydrological events such as heavy precipitation and floods, as well as intensive land use activities, such as the ap-plication of mineral fertilizers and manure. Agricultural practices play an important role in

the determination of the microbial community composition, as they often mobilize OM that is stably stored in the soil or alter hydrologic conditions in groundwater through irrigation (Korbel et al., 2013; Zhang et al., 2016). The composition and functioning of microbial communities in groundwater are generally closely linked to dis-solved organic matter (DOM) turnover, hydrochemical conditions, as well as aquifer lithology (Amalfitano et al., 2014; Guo et al., 2022; Langenheder and Lindström, 2019), but also by physical limitations, such as geographic distance (Barreto et al., 2014; Langenheder and Lindström, 2019). Microbial community assembly, on the other hand, which determines the identity and abundance of taxa, is the result of stochastic and deterministic processes (Stegen et al., 2012). The contribution of deterministic environmental factors and stochastic, or random, processes such as dispersal or disturbance (for example, precipitation, drought, anthropogenic impacts) can be determined (Ning et al., 2020) and used for predictive models and management purposes (Graham et al., 2017; Merino et al., 2022).

Besides general influence from nearby surface waters such as rivers and streams, precipitation, and agricultural activities, urban areas constitute another prominent threat to groundwater ecosystems (Becher et al., 2022). Groundwater below urban areas is highly impacted by surface sealing and reduced recharge, accumulation of heat, and the import of various kinds of contaminations including aromatic hydrocarbons, heavy metals, salts, and nanoparticles (Campos and Pestana, 2020; Cooper et al., 2014; McDonough et al., 2020).

There is a suite of ecological concepts proposed to be valid in macroecology and microbial ecology, while validation in microbial settings lag behind (Grilli, 2020; Konopka, 2006; Prosser et al., 2007). Hardly any of these concepts have been tested systematically for their validity in groundwater ecosystems (Griebler et al., 2022; Griebler and Lueders, 2009). Three ecological concepts are tested here. The species–area relationship, which describes the relationship between species richness as a function of habitat size (Horner-Devine et al., 2004), is still pending evaluation for groundwater systems. However, one would assume that here too, species richness in-creases with aquifer volume and groundwater catchment size. The second ecological concept, i.e., the intermediate disturbance hypothesis, assumes that microbial phylogenetic and functional diversity peak at intermediate intensities or frequencies of disturbances (Fierer et al., 2013; Ibekwe et al., 2002). Disturbances in aquifers can be the result of multiple concurrent stressors, including hydrological events and pollution (Bugnot et al., 2019). The third concept relates to the input of energy into the groundwater. Here, we will test whether, according to the existing productivity–diversity concept (Smith, 2007a), species diversity is highest at sites of moderate energy input, for example, in groundwater that is close to the surface or those that are hydrodynamically well connected to surface waters.

We examined physico-chemical and microbial characteristics, as well as drivers of microbial community composition and assembly in the groundwater, with respect to differences between groundwater and river water. To this end, shallow groundwaters were studied: along a gradient from mountainous to lowland areas; and from areas with natural vegetation to land impacted by agriculture and urban development. In addition, the ecological concepts mentioned above were tested with this dataset. More than 45 sites distributed along an alpine and pre-alpine transect of the River Mur (Austria) were sampled in early summer and autumn 2020.

We investigated the following questions:

(1) Do near-surface aquifers resemble adjacent surface waters, or are the environmental conditions (low energy, low temperatures, and darkness) and proximity to certain influencing factors such as land use or hydrological conditions determining community composition and assembly?

(2) What community assembly processes shape the prokaryotic community in groundwater, and do they differ from those in surface waters?

(3) Do patterns of groundwater microbial communities correspond to ecological concepts known from surface ecosystems in terms of biodiversity, energy, productivity, and disturbance?

The DNA-derived community is considered a species pool including dead or dormant cells that are negligible for the structure, functioning or response of the groundwater microbial communities at the time of sampling (Carini et al., 2016). To avoid this bias, we answered the aforementioned questions in the context of the metabolically active (i.e., RNA derived) fraction of the prokaryotic community (Wilhelm et al., 2014), where a comparison between the total (i.e., derived from the DNA) and the active prokaryotic community was made beforehand.

Materials and Methods

Field Work

The sampling of groundwater and surface water took place in June to July 2020 (early summer) and October to November 2020 (late autumn). Overall, 118 samples were collected in total, consisting of 90 groundwater samples (45 in early summer and late autumn each), and 28 surface water samples, i.e., 24 Mur river samples, and 4 samples from 2 tributaries (rivers Pöls and Lassnitz) (geographic coordinates see Table S1).

Study Area

The sampled area is located along the Mur Valley in the provinces Salzburg and Styria, Austria (Figure 1), and it covers a longitudinal transect of 325 km and an elevation gradient of approx. 1600 m. The Mur river emerges from a karst spring at an elevation of 1898 m a.s.l. in the Hohe Tauern National Park, at the main

chain of the alps, and flows downgradient through the alpine foreland to the lowlands at the border with Slovenia, where it leaves Austria at an elevation of about 260 m a.s.l. The transect combines 8 distinct groundwater bodies of different sizes, belonging to one large system of aquifers (Figure 1) that are, with the exception of the River Mur source, com-posed of alluvial and fluvio-glacial sediments, consisting of a mix of local and up-stream lithologies. In the upper section from about 2000 m a.s.l. down to the city of Graz at 350 m a.s.l., the bedrock lithology embedding the shallow porous aquifer of the Mur Valley consists mostly of metamorphic units of the Austroalpine Nappe, covering a wide range of lithologies from metamorphic, silica-rich basement to Palaeozoic limestones. Downstream of the city of Graz the underlying lithology is replaced by Neo-gene sediments which are also found in some alpine basins upstream such as the Aichfeld (Flügel and Neubauer, 1984).

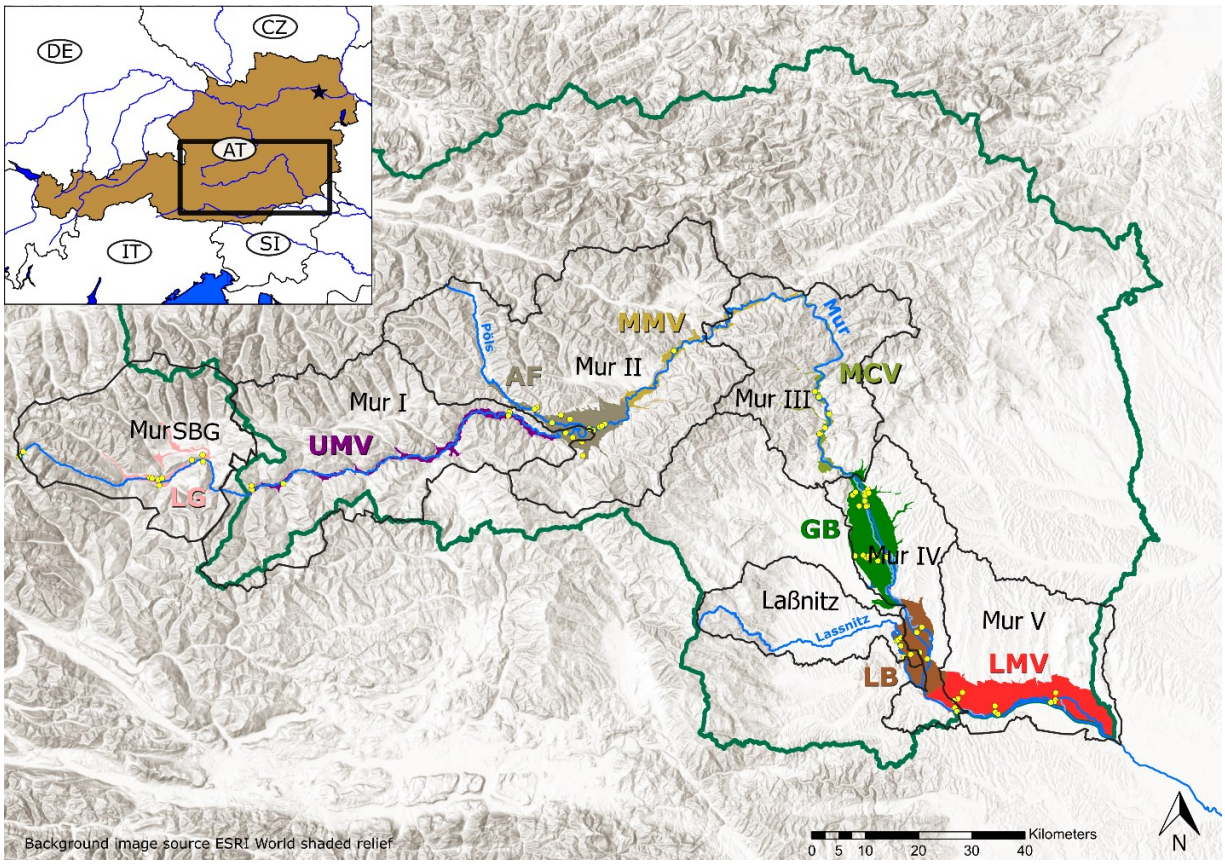

Figure 1. Groundwater, and surface water sampling sites (yellow points) along the Mur Valley in Styria (green boundaries) and Salzburg located along 8 distinct groundwater bodies that are named after their associated Mur Valley sub regions (color code, name (abbreviation) as follows: pink, Lungau (LG); violet, Upper Mur Valley (UMV); grey, Aichfeld (AF); ochre, Middle Mur Valley (MMV); olive, Mur Cross Valley (MCV); green, Graz Basin (GB); brown, Leibnitz Basin (LB); red, Lower Mur Valley (LMV)), as well as Mur river catchments sections (Mur SBG, Mur I, Mur II, Mur III, Mur IV, Laßnitz, Mur V). (Map redrawn from Haas & Birk (Haas and Birk, 2017).)

Samples Collection

Groundwater was withdrawn by a submersible pump (Grundfos MP1, Eijkelkamp Soil & Water, Giesbeek, Netherlands) from approx. 2 m below the water table in fully screened wells or otherwise from 2 m below

the screened zone. Before sample collection, stagnant well water was replaced by pre-pumping twice the well water volume until reaching stability in key physico-chemical parameters (EC, pH, Temp, Dissolved Oxygen) (Chapter A4. Collection of water samples, 2006). Surface water samples were collected from the banks of the rivers Mur, Laßnitz, and Pöls, lowering all bottles and vials at least 10–20 cm below the water level.

Analyses of Physico-Chemical and Microbial Parameters

Water samples were analyzed for major ions and for nutrient concentrations, prokaryotic cell counts, microbial ATP, stable water isotopes signature (δ18O, δ2H), dissolved organic carbon (DOC), and dissolved inorganic carbon (DIC), as previously described in Retter et al. (Retter et al., 2021). Groundwater and surface water samples for the measurement of microbial ATP, concentration of major ions, and stable water isotope ratios were filled into autoclaved glass bottles. For prokaryotic cell counts, samples were collected in sterile 15 mL plastic tubes and fixed with glutardialdehyde (0.5% v/v, final concentration) on site. Samples for DOC and DIC measurement were filtered through a 0.45 µm pre-rinsed PVDF syringe filter (STARLAB International, Hamburg, Germany) on site into acid-washed (5% HCl) glass vials. DOC samples were subsequently acidified with HCl to a pH ≤ 2. All samples were kept in the dark at 4–8 °C and analyzed within 48 h.

Water temperature, pH, EC, and concentration of dissolved oxygen (DO) of groundwater and river were measured on site using field sensors (WTW, Weilheim, Germany).

Total Prokaryotic Cell Count

The total number of prokaryotic cells in water samples was quantified by means of flow cytometry (Amnis CellStream, Luminex, Austin, TX, USA) equipped with a 488 nm blue light laser. Settings of the different detector channels were as follows: forward scatter, side scatter, trigger channel laser (488 nm) were all at 100%; speed was 'high' (14.64 µL min−1). To distinguish intact prokaryotic cells from damaged cells or inor-ganic particles, prokaryotes were stained with nucleic acid dye SYBR Green I (Invitro-gen, Darmstadt, Germany) at a volume ratio of 1:10,000 and incubated for 13 min at 37 °C. Cell counts were conducted in technical duplicates. Total cell counts (cells m L−1) were calculated using the Amnis CellStream Acquisition and Analysis software (v. 1.3.389).

Determination of Microbial Intracellular ATP

Microbial intracellular ATP concentrations were determined using the Bac-Titer-Glo Microbial Cell Viability Assay (Promega, Madison, WI, USA) based on the protocol by Hammes et al. (Hammes et al., 2010) with modifications as follows: The assay reagent (prepared following the manufacturer's instructions) and samples were pre-warmed separately to 37 °C for 3 min before mixing 180 µL of sample

with 20 µL of reagent, and subsequent incubation for 20 s at 37 °C while shaking at 600 rpm on a Thermomixer (Eppendorf). The luminescence signal was measured in a GloMax Navi-gator plate reader (Promega) with an integration time of 0.3 s. Concentrations were determined against ATP standards dissolved in ATP-free water. To correct for the contribution of extracellular ATP in the samples, each sample was measured twice, once untreated, and once after the sample was centrifuged for 30 min at 21,000× g and 4 °C resulting in a supernatant containing only extracellular ATP. The concentration of intracellular ATP was calculated by subtracting the concentration of extracellular from total ATP. Each sample was analyzed in technical triplicates.

Determination of DOC, and DIC

Concentration of dissolved organic carbon (DOC) and dissolved inorganic carbon (DIC) in surface water and groundwater samples were measured in a TOC-L Analyzer (Shimadzu, Kyoto, Japan). DOC was quantified as non-purgeable carbon in acidified samples (DIN, 2019). DIC was quantified from purged CO2 after acidification. Calculation of DOC and DIC concentration made use of pre-measured standard curves.

Water Stable Isotope Signature

Water samples were analyzed for its ratios of $^{18}O/^{16}O$ and $^{2}H/^{1}H$ using laser spectroscopy (Picarro, L2140-i). All samples were referenced to the VSMOW-SLAP (Vienna Standard Mean Ocean Water-Standard Light Antarctic Precipitation) standard, and results are reported as the ratios of isotopes in the delta notation as δ-value (‰). Precision of the instrument (1σ) was better than 0.15‰ and 0.6‰ for $\delta^{18}O$ and $\delta^{2}H$, respectively.

Major Ions and Nutrients

Major ion concentrations (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, SO_4^{2-}) were measured by ion chromatography (Dionex ICS-1100 RFIC; Thermo Scientific, Idstein, Germany) fol-lowing standard protocols (OENORM DIN EN ISO 14911, DIN EN ISO 10304-1). Nutrient concentrations (Table S2) were determined by photometric measurements.

Microbial Community Analysis

For molecular analysis of *Bacteria* and *Archaea*, groundwater and river water samples were filled into sterilized (2% sodium hypochlorite solution) 10 L plastic containers. Within 48 h of sampling, in which containers were stored in a dark at 4–8 °C, a total of 10 L of groundwater and 4 L of surface water were filtered onto 0.22 µm Sterivex filters (Merck Millipore, Darmstadt, Germany) using a peristaltic pump. All tubing were pre-washed with sodium hypochlorite, rinsed with MilliQ and sample water before filtration. Filters were stored right away at −80 °C until further analysis.

Nucleic Acid Extraction and Illumina Sequencing

Genomic DNA and total RNA were extracted in two batches (early summer and late autumn samples), using the Norgen RNA/DNA purification kit (Norgen Biotek, ON, Canada), where nucleic acids are extracted and sequentially purified over two spin columns. Briefly, filters were first removed under sterile conditions and then cut to small pieces, and 800 µL of the kit's lysis buffer was added to the filter strips in a sterile 1.5 mL screw cap micro tube containing 0.2 mL of a mix of 1:1 0.1 mm and 0.7 mm Zirconia/Silica beads (Biospec, Bartlesville, USA). Cell lysis was enhanced by incubation for 10 min at 55 °C, followed by bead-beating. The entire liquid was transferred to a new micro-centrifuge tube and centrifuged at 14,000× g for 2 min. The supernatant was then purified according to the manufacturer's protocol and DNA and RNA was quantified using a Qubit 4 HS RNA, and HS dsDNA assay (Invitrogen). To digest undesired DNA within the RNA fraction, samples were treated with TURBO Dnase (2 Units/µL) (Thermo Fisher Scientific, Waltham, MA, USA), additionally adding RNaseOUT (Thermo Fisher Scientific, USA) to avoid RNA degradation during DNAse treatment, and cleaning up with the Norgen CleanAll DNA/RNA Clean-Up and Concentration Micro Kit to remove any downstream enzymatic reactions inhibi-tors (Norgen Biotek, Thorold, ON, Canada). The absence of DNA was checked by at-tempting the amplification of the 16S rRNA gene (35 amplification cycles), and TUR-BO Dnase treatment of individual samples was repeated if amplification occurred. For further downstream processing of the RNA samples, cDNA was synthesized with the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, USA) following the manufacturer's protocol. A primer combination of 515F (5'- GTGYCAGCMGCCGCGGTAA-3´, (Parada et al., 2016)) and 806R (5`- GGACTAC-NVGGGTWTCTAAT-3´, (Apprill et al., 2015)), annealing to the V4 region of the 16S rRNA was used for gene amplification, following the PCR protocol as described by Pjevac et al. (Pjevac et al., 2021). Sequencing was performed on a MiSeq platform (Il-lumina, San Diego, CA, USA), using a v3, 600 cycle chemistry (2 × 300 b paired-end sequences), in two separate runs. Negative controls (n = 3) were extracted from a blank sterile 0.22 µm Sterivex filter (Merck Millipore, Darmstadt, Germany), following the same extraction protocol as the biological samples.

Sequence Data Processing

Raw reads were processed in R (version 4.1.1, (R Foundation for Statistical Computing, 2023)) with DADA2 (v. 1.20.0; (Callahan et al., 2016)) as outlined in Callahan et al. (Callahan et al., 2016). Amplicon Sequence Variants (ASVs; (Callahan et al., 2017)) were inferred across all samples in pooled mode (details on sequence trimming, and quality filtering is outlined in Pjevac et al. (Pjevac et al., 2021)).

Sequence Classification and Decontamination

ASV sequences were subsequently aligned and classified using SINA version 1.7.2 (Pruesse et al., 2012) and the SILVA database SSU Ref NR 99 release 138.1 (Quast et al., 2013) using default parameters. ASVs

classified as eukaryotes, mitochondria, or chloroplasts, as well as ASVs not classified at domain level, were removed from the dataset prior to downstream analysis. One sample did not deliver any reads (groundwater sample 86). The dataset was bioinformatically decontaminated in R (v. 4.1.2, (R Foundation for Statistical Computing, 2023)) with decontam (v. 1.14.0, (Davis et al., 2018)), with a prevalence-based identification of contaminated sequences, using a strict threshold of 0.5, identifying all sequences as contaminants that are more prevalent in the negative controls (n = 3) than in the samples. Summer and autumn sequencing batches were analyzed separately against the respective negative control samples.

A species tree was estimated from bacterial and archaeal 16S rRNA-derived ASVs, adjusting read direction with MAFFT (v. 7.427; (Katoh and Standley, 2013)), subsequent multiple sequence alignment with Clustal Omega (v. 1.2.4; (Sievers et al., 2011)), and estimating the maximum likelihood tree with a GTR + F + ASC + R10 evolutionary model performing an SH-like approximate likelihood ratio test (SH-aLRT) (Guindon et al., 2010) as well as ultrafast bootstrap (Minh et al., 2013) with 1000 replicates using iqtree (v. 2.0.3; (Minh et al., 2020)).

Classification of Hydrology, Land Use and Disturbances

The groundwater wells were grouped according to their probability of being influenced by surface water intrusion based on the minimum surface water infiltration distance (with categories: none, weak, moderate, high). This distance was derived from a map of groundwater level contour lines, drawn from the water table data of public wells, as well as from expert knowledge about the local hydrogeological conditions.

In order to link precipitation events and dynamics in river levels, we used the Standardized Precipitation Index (SPI, (McKee et al., 1993)), the Standardized Groundwater level Index (SGI, (Bloomfield and Marchant, 2013)), and the Standardized River Stages Index (SRSI, (Haas and Birk, 2017)). These so-called drought indices allowed different time series to be compared by standardizing them into a range of ± 3 standard deviations. Additionally, the SPI allowed for the use of averaging periods (9–12 months in our case), taking into account that surface water and groundwater typically react to precipitation with some delay and attenuation such that short-term fluctuations are smoothed out. SPI and SRSI were correlated to SGI via a Pearson correlation, which were then categorized based on the resulting correlation coefficient in-to none (<0.3), weak (0.3–0.49), moderate (0.5–0.8), and strongly (>0.8) correlating wells.

Groundwater level fluctuations between the absolute long-term minimum and maximum measured for the time period 2000 to 2019 were used to categorize groundwater sites into low dynamic (average groundwater level fluctuation between 0 and 1.5 m), medium dynamic (average groundwater level fluctuation between 1.5 and 3 m), and highly dynamic (average groundwater level fluctuation between 3 and 7 m).

Moreover, information on land cover was taken from the Corine database (2012–2018; https://www.data.gv.at/, accessed on 1 February 2021). Surface water samples were manually classified as 'water ways'.

Factors chosen to test the intermediate disturbance hypothesis are (1) levels of nitrate in groundwater, a proxy for intensive agricultural land use, (2) absolute difference in groundwater level between the long-term minimum and maximum, as well as (3) the proximity to the nearest river water infiltration point. To test the productivity–diversity concept, total prokaryotic cell counts, and intracellular ATP concentrations were used as proxies for the prokaryotic secondary production in the groundwater ecosystem.

Statistical Analysis

For ordination of samples based on environmental parameters, a principal com-ponent analysis (PCA) was conducted, where parameters were standardized to z-scores beforehand. The correlation between individual environmental parameters was evaluated via a Spearman correlation matrix. To test if environmental variables change between groups (e.g., groundwater and the river, or early summer and late autumn), a multiple Student's t test was conducted, where p-values were false discovery rate (FDR) adjusted, and significance was given based on a cut-off of <0.05.

First, the analysis of the groundwater prokaryotic communities was based on DNA (total community) and RNA (active community) to compare both fractions in terms of their diversity and taxonomic composition. The subsequent analysis comparing groundwater to the river, as well as diversity within groundwater and its link to spatial, and temporal changes was solely based on the active community. For community diversity analysis, we used the R package breakaway (v. 4.7.9, (Willis and Bunge, 2015)) as well as divnet-rs (v. 0.2.1, (Willis and Martin, 2020)) to compute total rich-ness estimates (S) as well as Shannon diversity. The advantage over conventional sample-wise calculation methods is that in addition, it computes covariate-wise estimations, as well as variance estimates for each sample. To test diversity estimates between fixed combinations of the covariate-wise differences, we used the function 'betta', and 'betta_random' of the R package breakaway incorporating the sequencing batch affiliation as a random effect, as well as computing a global p-value with an F-test using a bootstrap analysis with 10,000 iterations implemented in the function 'test_submodel' in the R package breakaway. Shannon diversity estimates were computed from respective model matrices of co-variates of interest, with 4 replicates, and perturbation set to 0.01, otherwise with default tuning settings. A medium abundant base taxon (groundwater: *Rhodoferax ferrireducens/saidenbachensis*, river: *Limnohabitans* sp.) was used for log ratio computation. Shannon entropy (H′) was computed with the 'shannon_true' function implemented in DivNet (Hubálek, 2000). Due to the non-linear nature of H′ and inability to compare compositional

similarities of a set of species assemblages (Jost et al., 2011), we additionally calculated the Effective Number of Species (ENS) based on the Shannon entropy as:

$ENS(H') = \exp(H')$ (1)

with the standard error propagated as:

$\sigma_{ENS} = \sigma_{H'} \times e_{H'}$ (2)

where σ_{ENS} is the propagated error of the ENS. The effective number of species based on H′ is from here on referred to as Shannon diversity and is defined as the number of equally common species required to produce the same value of H′, or heterogeneity, in a given sample (Peet, 1974). Pielou's evenness (J) was calculated as a measure for evenness.

Rare taxa are from here on defined by making up less than 0.01% of the community, occurring in less than 20% of the samples. The core community is characterized by being present in at least 80% of the samples and making up more than 0.01% of the community.

Due to the compositional nature of our data (Gloor et al., 2017), we used the centered-log ratio (clr) transformation on ASV abundances, computed in the microbiome package (v. 1.16.0, (Lahti and Shetty, n.d.)), and a non-parametric multivariate analysis of variance (PERMANOVA) using the function 'adonis2' implemented in the pack-age 'vegan' (v. 2.5-7, (Oksanen et al., 2016)) to test for differences in community com-position between regions, land use, seasons, correlation of groundwater levels with precipitation (measured as SPI), correlation of groundwater levels with Mur river water levels as SRSI, as well as the probability that the groundwater is influenced by the River Mur. For analysis of species turnover, we excluded samples with fewer than 500 reads. Environmental differences between groups (e.g., land use and region) were calculated as standardized Euclidean distances between samples, whereas differences between groups in community composition were calculated as Aitchison distances. The Aitchison distance is the Euclidean distance between clr-transformed abundances (Aitchison, 1983), and it is an equivalent to Bray–Curtis, but better suited for compositional data (Gloor et al., 2017; Silverman et al., 2017). To identify differences in dispersion between groups, we used a permutational analysis of multivariate dispersion (PERMDISP, (Anderson, 2006)) using the functions 'betadisper', and 'permutest' implemented in the vegan package. The vegan function 'varpart' was then used to partition the response matrices of Aitchison distances with respect to measured variables (Borcard et al., 1992). The relevance of parameters was beforehand identified via a distance-based redundancy analysis (db-RDA).

To investigate the relationship between community composition, and environmental parameters we used a Constrained Analysis of Principal Coordinates (CAP) based on the 'capscale' function in the R package 'vegan' (Anderson and Willis, 2003). Prior to CAP, the collinearity of environmental parameters

was tested. The scaled environmental parameters were selected by stepwise regression in both directions of variables previously selected via the calculation of the variance inflation factors (VIF) (<10).

To estimate the contribution of various community assembly processes in groundwater and the Mur river, we used the iCAMP package, calculating the beta net relatedness index (βNRI) of the RNA-derived data (Stegen et al., 2012; Webb et al., 2008). The contribution of assembly processes is estimated for each bin based on a null model analysis of the taxonomic β-diversities using a modified Raup–Crick metric and the phylogenetic diversity. Bins are here defined by the phylogenetic threshold (ds = 0.2) and a minimum bin size limit (=12). For each bin, the fraction of pairwise comparisons with βNRI < −1.96 is considered as the percentage of homogeneous selection (HoS), those with βNRI > +1.96 as the percentage of heterogeneous selection (HeS), based on the threshold applied previously (Stegen et al., 2015; Zhou and Ning, 2017). Next, the taxonomic Raup–Crick diversity metric is used to partition the remaining pairwise comparisons (i.e., |βNRI| ≤ 1.96). The fraction of pairwise comparisons with RC < −0.95 is treated as the percentage of homogenizing dispersal (HD), while those with RC > +0.95 as dispersal limitation (DL). The remaining with |βNRI| ≤ 1.96 and |RC| ≤ 0.95 represent the percentages of drift, diversification, weak selection, and/or weak dispersal designated as 'drift' (Ning et al., 2020). If selection occurs, we may further differentiate between homogeneous (e.g., caused by similar environmental conditions) and heterogeneous selection (i.e., species performance). In addition, dispersal rates are divided into dispersal limitation (i.e., high compositional turnover caused by a low dispersal rate) and homogenizing dispersal (i.e., low turnover caused by high dispersal rates), leaving all other assembly effects to drift. To test the correlation of the most dominant processes in groundwater and the river to environmental parameters, we applied a Mantel test and a mixed regression model (MRM) as described in Ning et al. (Ning et al., 2020). Spatial distances were transformed via a principal coordinates of neighborhood matrix (PCNM) prior to regression analysis, capturing the most variation in latitude and longitude between sites (Oksanen et al., 2016). The relative im-portance of each process, as well as the environmental variables were either log-transformed or not to explore the best model for each factor. For each measurement, both the mean and variation between sample pairs were used to investigate the correlation with the relative importance of the individual process (Ning et al., 2020).

To test for differentially relative abundant taxa across different parameters, we used the 'differentialTest' function using a Wald test with a false discovery rate (FDR) cut-off of 5%, implemented in the corncob package (v. 0.2.0, (Martin et al., 2020)). The resulting abundance coefficient estimate is the estimate from the beta-binomial model that models the ASV against a parameter. The Venn diagram of shared taxa across categories of nucleic acid fractions and water types was created with the function 'ps_venn' in the MicEco package (v. 0.9.17) that was weighted by relative abundance, computing counts of ASVs as well as

percentage of sequences per total sequence number. There has been filtering of the sequences applied beforehand, such as decontamination, filtering of only archaeal and bacterial taxa, and removal of samples with less than 100 reads. We did not apply any further filtering since the tools we use for diversity estimation are dependent on the rare fraction, which is large in groundwater (89% of the ASVs that make up 31% of the reads).

Results

Physico-Chemical Characteristics of Groundwater in the Mur river Valley

The physico-chemical characteristics, the concentrations of nutrients, as well as the microbial productivity in terms of prokaryotic cell numbers clearly distinguished groundwater from surface water (Figure 2A). In general, the river has significantly less nitrate, a lower electrical conductivity, and a lower water temperature (p adj. < 0.05). On the other hand, the river water contained higher DOC and DO concentrations, and was characterized by a higher pH, and a higher microbial productivity (in terms of cellular ATP and cell count) (Table 1) (p adj. < 0.05). Individual features of groundwater bodies are the highest productivity in the Lower Mur Valley (LMV), with high DO concentrations in the Graz Basin (GB) and the Aichfeld (AF). The sub region Aichfeld (AF) delivered groundwater with slightly alkaline pH values (Figure 2B, Table 1). In groundwater, differences between samples from summer and autumn are especially pronounced for temperature, and pH (in autumn, the groundwater has higher water temperatures and a lower pH) (p adj. < 0.05), whereas with the river water samples, temperature, pH, and DO are significantly affected by the season (in autumn, the river has lower water temperatures, a higher DO content, and a lower pH) (p adj. < 0.05) (Table 1).

Table 1. Key physicochemical features of groundwater (GW) and river water samples collected in the River Mur Valley in summer and autumn 2020. Data are means ± SD.

	Regions	N	Altitude (m a.s.l.)	T (°C)	pH	DO (mg/L)	EC (µS/cm)	NO_3 (mg/L)	DOC (mg/L)
GW	LG	6	1032 ± 13	9.8 ± 1.2	6.8 ± 0.4	5.8 ± 2.2	785 ± 124	13.9 ± 7.9	0.94 ± 0.27
	UMV	6	834 ± 89	9.2 ± 1.5	7.2 ± 0.2	4.6 ± 0.9	377 ± 155	5.9 ± 3.0	0.39 ± 0.15
	AF	20	653 ± 65	10.3 ± 0.7	7.1 ± 0.4	6.2 ± 2.3	515 ± 267	11.6 ± 9.6	0.54 ± 0.22
	MMV	2	584 ± 0	9.3 ± 0.8	7.7 ± 0.1	1.8 ± 1.2	413 ± 14	4.0 ± 0.0	0.46 ± 0.05
	MCV	8	402 ± 19	11.9 ± 0.6	7.3 ± 0.2	6.8 ± 1.3	599 ± 102	9.1 ± 2.9	0.54 ± 0.16
	GB	22	350 ± 21	13.7 ± 1.0	7.2 ± 0.3	6.1 ± 2.2	700 ± 154	17.8 ± 9.1	0.60 ± 0.12
	LB	8	280 ± 6	13.3 ± 1.3	7.2 ± 0.2	4.7 ± 3.8	660 ± 112	23.8 ± 15.2	0.84 ± 0.32
	LMV	18	256 ± 26	13.0 ± 1.5	6.8 ± 0.4	3.7 ± 2.4	546 ± 189	41.1 ± 21.7	1.01 ± 0.39
	Autumn	45	-	12.5 ± 2.1	7 ± 0.4	5.2 ± 2.5	602 ± 219	20.6 ± 16.9	0.66 ± 0.35
	Summer	45	-	11.4 ± 1.6	7.2 ± 0.4	5.5 ± 2.6	585 ± 201	18.4 ± 17.5	0.72 ± 0.29
	Agricultural areas	52	516 ± 247	11.6 ± 2	7.1 ± 0.3	5.8 ± 2.3	562 ± 161	20.9 ± 19.9	0.65 ± 0.29
	Artificial areas	28	454 ± 208	12.6 ± 1.7	7.1 ± 0.4	6.1 ± 2.1	680 ± 271	17.5 ± 11.5	0.63 ± 0.29

Forest and semi natural areas	10	364 ± 217	12 ± 2	6.9 ± 0.5	1.3 ± 1.1	515 ± 162	18 ± 15.6	1.08 ± 0.31
River								
Autumn	14	-	7 ± 2.5	7.8 ± 0.1	11.4 ± 0.6	223 ± 69	4.4 ± 2.6	1.5 ± 0.7
Summer	14	-	12.7 ± 3.3	8.1 ± 0.2	10.2 ± 1	196 ± 76	3.3 ± 2.2	1.8 ± 1.3

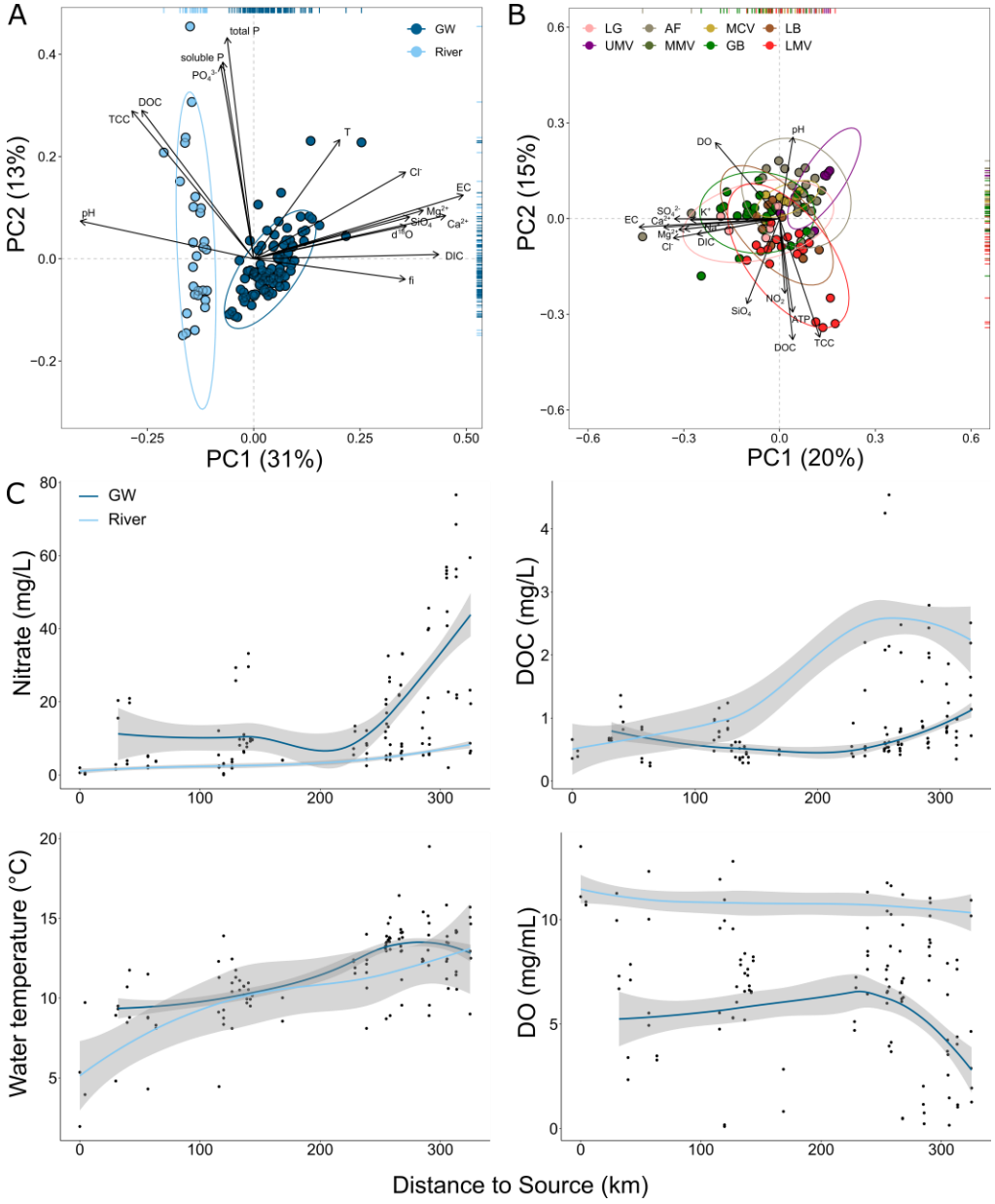

Figure 2. (A) Principal component analysis (PCA) based on selected environmental parameters clearly separates groundwater and river water samples. (B) Groundwater samples colored ac-cording to their origin in terms of subregions (groundwater bodies). The ellipses depict the 95% confidence interval ellipses (A, B). (C) Nitrate, DOC, and DO concentrations, as well as water temperature (each sampling site represented by a summer and autumn value), are plotted against the distance to the Mur river source. Grey shading indicates the 95% confidence intervals. (TCC: total cell count; ATP: cellular adenosine triphosphate; DOC: dissolved organic carbon; T: water temperature; DO: dissolved oxygen; EC: electrical conductivity; $\delta^{18}O$: stable ^{18}O isotope signature in water; NO_2: nitrite; PO_4^{3-}: orthophosphate; Na^+: sodium; K^+: potassium; Ca^{2+}: calcium; Mg^{2+}: magnesium; Cl^-: chloride; SO_4^{2-}: sulfate; soluble P: soluble phosphorus; total P: total phosphorus; SiO_4: silicate; DIC: dissolved inorganic carbon.)

In groundwater, the following physico-chemical parameters are changing significantly down the river valley with distance from the Mur river source: There is an in-crease in groundwater temperature ($p < 0.05$), nitrate concentration ($p < 0.05$), DOC concentration ($p < 0.05$), SiO_4 concentration ($p < 0.05$), and an increase in the water δ^2H signature ($p < 0.05$) (Figure 2C). In groundwater, DO is negatively correlated with DOC (r_s −0.49, $p < 0.05$) and prokaryotic cell numbers (r_s −0.66, $p < 0.05$). Prokaryotic cell numbers are positively correlated with cellular ATP (r_s 0.67, $p < 0.05$) and DOC (r_s 0.76, $p < 0.05$). On the abiotic side, EC correlates strongly with sodium (r_s 0.75, $p < 0.05$), magnesium (r_s 0.69, $p < 0.05$), and DIC (r_s 0.72, $p < 0.05$) (Figure S2).

In our dataset, 'agricultural areas' make up 58% of the land cover, followed by 'artificial surfaces' (31%) and 'forest and semi natural areas' (11%). Land cover could be connected to some of the environmental parameters, i.e., 'agricultural areas' were characterized by high pH, and nitrate concentrations (from fertilization), and 'artificial surfaces' (including urban land) showed high EC values (Figure S3). In more detail, nitrate was highest in the Corine land cover categories 'land principally occupied by agriculture with significant areas of natural vegetation', 'broad-leaved forests', and 'non-irrigated arable land', and it was lowest in 'mixed forests' and 'pastures'. DOC was highest in 'mixed forests' and 'broad-leaved forests' and lowest at groundwater sites located below 'pastures' (Table S3).

The influence of surface water to groundwater can be described as follows: groundwater that is likely to receive inflow from the Mur river, as based on ground-water level triangulation, also showed on average a shorter minimum distance to the river than groundwater that is not, weakly, or moderately influenced (Figure S4) (Kruskal–Wallis $p < 0.05$).

Microbial Productivity in Groundwater and River Water

The microbial productivity is estimated from the prokaryotic cell density, and the cellular ATP concentration (cell activity). For both seasons, surface water was found more productive (ATP: median = 175.1 pM (Interquartile range (IQR) = 222.7), cell counts: median = 4.4×10^8 cells L^{-1} (IQR = 6.6×10^8)) than groundwater (ATP: median = 2.5 pM (IQR = 6.6), Cell count: median = 1.05×10^7 cells L^{-1} (IQR = 2×10^7)). The samples from the source of the Mur river (MS) were more similar to surface water productivity in summer and more similar to groundwater in autumn (Figure 3). Overall, the microbial productivity of river water in the upper mountainous reaches, i.e., Lungau (LG) and Upper Mur Valley (UMV), were similar in values to groundwater of the lowermost region of the Mur river transect, the Lower Mur Valley (LMV) (Figure 3). In ground-water, prokaryotic cell counts did not differ between seasons, but cellular ATP concentrations were higher in summer than in autumn ($p < 0.05$). In river water, cell counts were higher in summer ($p < 0.05$), but ATP concentrations are statistically similar in summer and autumn. In autumn, there is a

stronger linear relationship (adj. $R^2 = 0.75$) ($p < 0.05$) between ATP and prokaryotic cell density compared to summer (adj. $R^2 = 0.64$) ($p < 0.05$) (Figure 3).

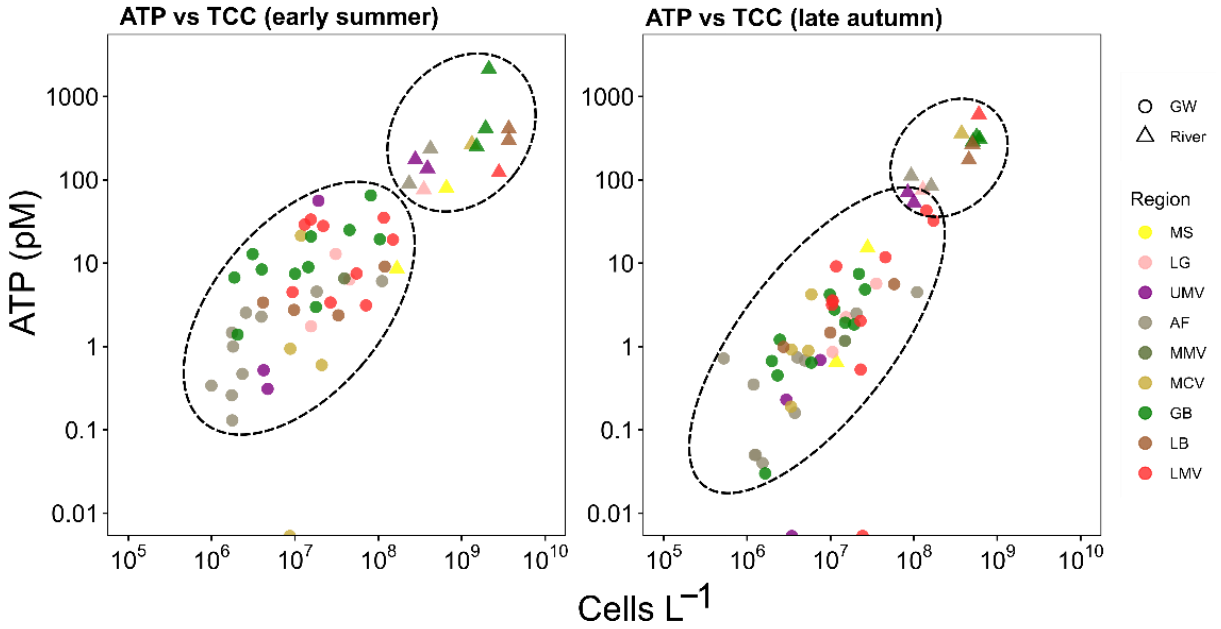

Figure 3. Bivariate plot of the prokaryotic cell counts, and the cellular ATP concentrations of groundwater and river water samples collected in summer (left) and autumn (right) 2020.

Prokaryotic Diversity and Community Composition in Groundwater and Comparison of the Total and Active Fractions

Prevalence-based contaminant identification was performed independently for each sequencing batch and found 11,326 ASVs and 728 contaminants overall in the dataset. Furthermore, this dataset, composed only of *Bacteria* and *Archaea*, consists of ASVs that could be classified to 94% at phylum level, 90% at class level, and 80% at order level. Summary statistics can be found in Tables S4 and S5.

The taxonomic composition of the prokaryotic communities based on DNA (total community) and RNA (active community) extracted from groundwater in summer and autumn 2020 is visualized in Figure 4A. The majority of sequences are from *Bacteria*. However, almost half of the bulk community in groundwater is made up of *Archaea* (42.2% ASVs, 43% of the reads), which become less abundant in the RNA-derived fraction (18% ASVs, 19% of the reads). In both nucleic acid fractions, *Archaea* became significantly less abundant with increasing distance from the River Mur source (Figure S5).

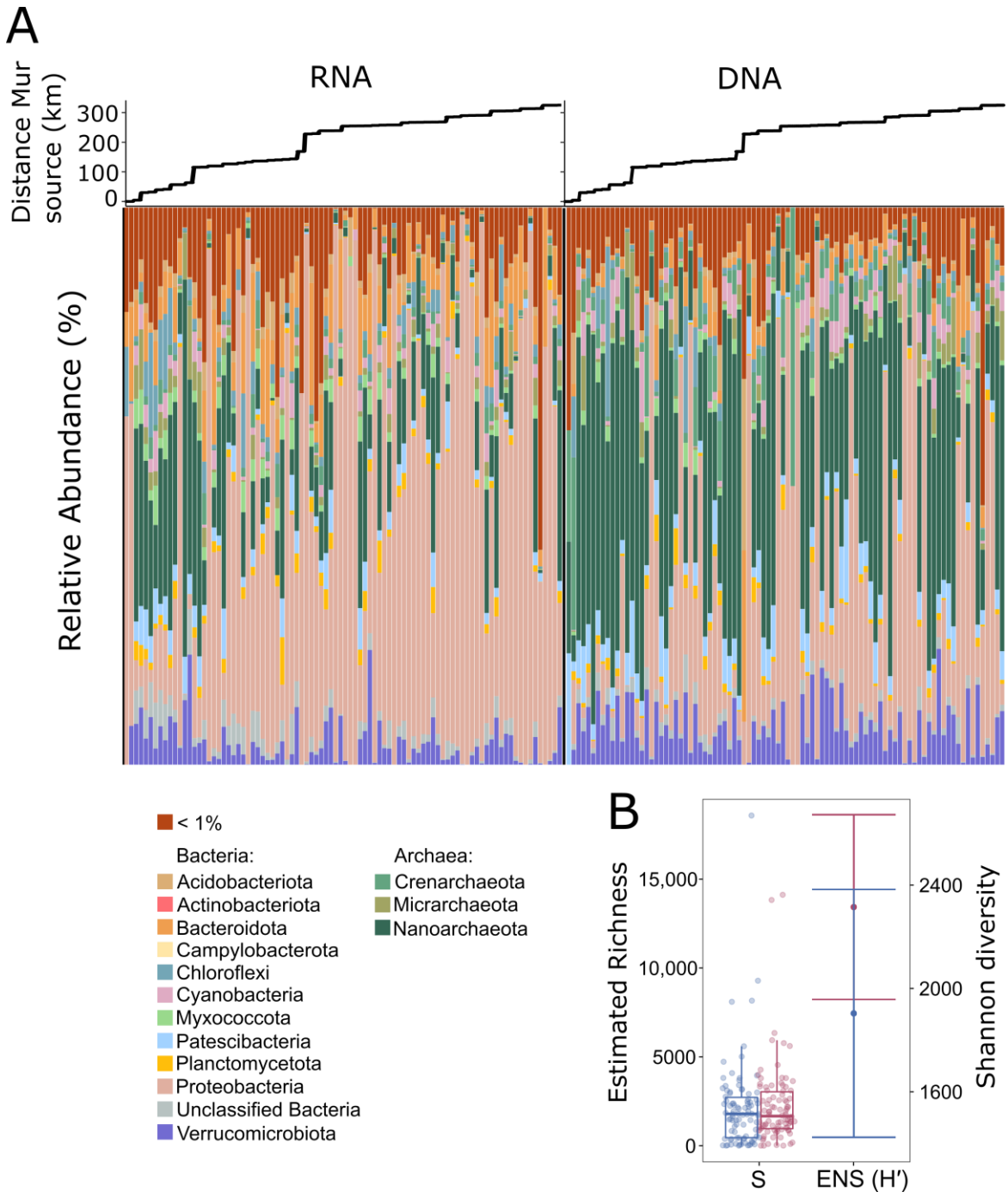

Figure 4. (A) Relative abundance plot of total, and active fractions of groundwater prokaryotic communities. Samples were ordered according to their distance to the source of the Mur river, where the source itself is on the far-left side of the plot. (B) Total richness estimates (S) are plotted in a boxplot showing differences in RNA (blue)- and DNA (red)-derived communities, as well as covariate-wise mean estimates of DNA- and RNA-derived communities of the Shannon diversity (ENS (H')) showing error bars as error of the mean.

In groundwater, the most abundant phyla are *Nanoarchaeota* (DNA: 34.3%, RNA: 14.2%), *Proteobacteria* (DNA: 30.4%, RNA: 46.4%), *Verrucomicrobiota* (DNA: 6.2%), and *Bacteroidota* (RNA: 7.2%), respectively. Of those, *Nanoarchaeota* and *Verrucomicrobiota* significantly decrease in the RNA-derived community. *Proteobacteria* significantly increase with increasing distance from the Mur source in both DNA- and RNA-

derived communities (Figure S6). *Proteobacteria* in groundwater are mainly composed of *Gammaproteobacteria* (DNA: 83%, RNA: 86% of all *Proteobacteria* ASVs), which represent 28.8% of the total reads in the DNA fraction and 42.5% in the RNA fraction. Archaeal lineages belonging to unclassified Woesearchaeales, part of the class *Nanoarchaeia*, are most abundant in the groundwater (DNA: 52%, RNA: 57% of all archaeal ASVs), which represent 24% of the total reads in the DNA fraction and 11% of the total reads in the RNA fraction. Archaeal lineages significantly decreasing over the course of the alpine river transect in the active fraction are unclassified Archaea and representatives of the Deep Sea Euryarchaeotic Group (DSEG), being part of *Aenigmarchaeota*. Additionally, there is a non-negligible number of unclassified *Bacteria* found throughout the groundwater communities (DNA: 1.4%, RNA: 2.3% of ASVs), also being significantly more abundant at higher elevations in both the DNA and RNA fraction ($p < 0.005$).

Overall, total estimated prokaryotic richness does not differ significantly between the active and total fraction of the community (RNA, S: 1100 ± 63, DNA, S: 1196 ± 89). The differences in Shannon diversity, however, are significant over all samples, being 18% higher within the DNA fraction of the groundwater ($p < 0.05$) (Figure 4B, Table 2). In short, groundwater species diversity is higher in the DNA fraction, declining in low abundant taxa, with common species starting to play a larger role in the active fraction of the community, as richness does not seem to diminish. In groundwater, 55% of the ASVs and 85.5% of the reads are shared between the active and total fraction (Figure S7).

Table 2. Groundwater alpha diversity metrics of estimated total richness (S), Shannon index (H'), effective number of species based on the Shannon index ENS (H'), and Pielou's evenness (J). Significant differences are highlighted in bold. Values are means ± SE.

	Total Estimated Richness (S)	Shannon Index (H')	ENS (H')	Evenness (J)
RNA	1261 ± 81	**7.55 ± 0.009**	**1903 ± 30**	**0.98 ± 0.002**
DNA	1379 ± 114	**7.75 ± 0.012**	**2314 ± 38**	**1.00 ± 0.004**

When testing for overall differences in species turnover between nucleic acid fractions with PERMANOVA (999 permutations), the difference between nucleic acids (DNA and RNA) alone explains around 4.5% of the variation in community composition based on the clr metric.

ASVs showing the largest differences in abundance between nucleic acid fractions over the whole dataset are visualized in Figure S8. All communities show homogeneous dispersion among nucleic acid groups.

Active Prokaryotic Community Diversity between Groundwater and the River

Groundwater and river samples share 22.7% of the ASVs, and 66.7% of the reads (Figure S9), which indicates a high degree of microbial exchange between the two habitats, but also a certain degree of niche specialization. The river only harbors a small fraction of unique ASVs (7.7% ASVs and 4.1% reads),

while ASVs unique to groundwater make up 69.6% of the groundwater ASVs, and 29.3% of the reads (Figure S9). The active groundwater core community comprises six taxa making up 16% of the total reads (*Ferribacterium limneticum*, 0.68%; *Actimicrobium* sp., 3.0%; *Rhodoferax ferrireducens*, 2.6% as well as three taxa belonging to *Pseudomonas sp*. (5.8%, 2.5%, 2.2%)), but they are significantly varying in abundance between wells (Kruskal–Wallis; $p < 0.05$). Rare species make up around 89% of the ASVs and 31% of the reads in the groundwater active community. The rare groundwater community comprises taxa belonging to unclassified Bacteria, *Acinetobacter lwoffii*, *Gallionella sp*., unclassified SAR202 clade, unclassified Xanthomonadales, and unclassified Magnetospirillaceae, namely, the five most abundant ones. The river core community comprises thirteen taxa (the five most abundant taxa with percentage of relative abundance in parenthesis are: *Limnohabitans* sp. with 7.9% abundance, followed by *Malikia granosa* (7.8%), *Pseudarcobacter suis* (7.6%), *Rhodoferax ferrireducens* (2.8%), and *Acinetobacter bohemicus* (2.0%)) that represent 28% of the total reads, which vary significantly in abundance between samples (Kruskal–Wallis; $p = 0.000497$). The river ASVs consist of 70.7% rare taxa, which make up 9% of the total sequences.

In the river, the following phyla are significantly more abundant when compared to groundwater: *Campylobacterota*, *Bacteroidota*, *Fusobacteriota*, *Armatimonadota*, and *Cyanobacteria* (Figure S10). The observed richness captures around 20% of the total estimated richness of the active fraction in groundwater, and about 35% of total estimated richness in the river samples, respectively (Table S6). Groundwater (S: 1272 ± 97 ASVs) harbors a significantly higher total estimated richness compared to the river (S: 780 ± 188 ASVs) ($p = 0.007$), as well as a higher Shannon diversity (Groundwater: 1442 ± 11 ENS, River: 339 ± 86 ENS) ($p < 0.05$). DNA-derived communities show a similar pattern. Prokaryotic diversity and complexity are therefore clearly higher in the active community of the groundwater ecosystem compared to the river water.

The active river prokaryotic communities are also significantly different from groundwater in their taxonomic community composition ($R^2 = 0.115$, $p < 0.05$), with river communities (57.4 ± 10.5) being on average more similar to each other than groundwater (73.7 ± 11.4) (Wilcoxon: $W = 8{,}406{,}047$, $p < 0.05$) (Figure S11). Using a distance-based redundancy analysis, we identified potential environmental drivers (out of physico-chemical variables, and nutrients) that cause the differences between surface water and groundwater communities. Taxonomic differences are largely driven by variation in DO, pH, SiO_4, water temperature, DIC, DOC, δ^2H, sodium, and potassium which explains around 17% (adj. R^2) of the variation in taxonomic community composition between groundwater and river water.

Community Assembly Processes Differ between the River and Groundwater

To determine which assembly processes shape the prokaryotic communities in groundwater and the Mur river, prokaryotic 16S RNA sequences were grouped into a total of 110 phylogenetic bins. Pairwise comparison of all river sampling sites (with-in-group, number of comparisons = 1265), as well as between groundwater sites (n = 13,505) were evaluated. Our results show large differences regarding the assembly processes of prokaryotic river water communities in comparison to groundwater. Deterministic homogeneous selection (HoS, similar habitats harbor similar communities), dispersal limitation (DL, high compositional turnover between sites, mainly caused by low dispersal rates) and ecological drift (DR, stochastic changes in relative abundance) are the most relevant processes for structuring the communities. In general, the impact of homogeneous selection (HoS) is significantly greater (Wilcoxon: $W = 539{,}030$, $p < 0.05$) in the river than in groundwater. In contrast, dispersal limitation (DL) plays a greater role for the groundwater prokaryotes than for the lotic communities (Table 3).

Table 3. Proportions as percentages of community assembly processes in groundwater and the river.

	Heterogeneous Selection (HeS)	Homogeneous Selection (HoS)	Dispersal Limitation (DL)	Homogenizing Dispersal (HD)	Drift (DR)
River	0.41%	55.80%	17.54%	1.75%	24.98%
GW	3.09%	34.16%	37.86%	3.17%	22.36%

Environmental Factors Influence Community Assembly Processes in Groundwater

Environmental factors that correlate with the most important assembly processes in groundwater were tested via a Mantel test and a mixed regression model (MRM) under both seasons (Ning et al., 2020). These two processes were dispersal limitation (DL) and homogeneous selection (HoS) (Table 3). Both processes could be linked to environmental factors related to nutrient concentrations, as well as geographical distance, which could explain around 44% of the variation of both HoS and DL (Figure 5). In contrast, environmental factors were able to explain much more of the variation ($R^2 > 0.8$) in community assembly processes in each HoS and DL in the river (Figure S12). In groundwater, HoS correlated most strongly with nitrate concentrations and elevation ($R^2 > 0.098$) and more weakly with longitude, the first two principal coordinates of neighborhood (PCNM) as well as water temperature and dissolved oxygen concentrations ($R^2 > 0.06$). DL on the other hand correlated most strongly with phosphate concentrations, as well as elevation ($R^2 > 0.09$), and more weakly with longitude, dissolved oxygen, as well as nitrate concentrations and between-sample differences in phosphate concentrations and water temperature ($R^2 > 0.068$). Even though these correlations are significant, they are quite weak ($R^2 < 0.1$).

The most important contributions of different variables were determined with an MRM. Regarding processes of HoS, again, nitrate concentrations, as well as elevation, had the largest effects (Figure 5C). DL had an opposite relationship to various environmental factors (e.g., nitrate, phosphate, longitude,

elevation, and dissolved oxygen), out of which phosphate and longitude had the largest effects on DL (Figure 5D). In summary, DL is positively related to elevation (coinciding with the geographic location of the groundwater sites along the Mur Valley). This means that within alpine groundwater (in this dataset at high elevation, high latitude, and low longitude), DL plays a more important role in community assembly, whereas in the alpine foreland (here at lower elevations, lower latitude, and higher longitude), HoS is the prevalent process for community assembly that is mostly related to changes in nutrient concentrations.

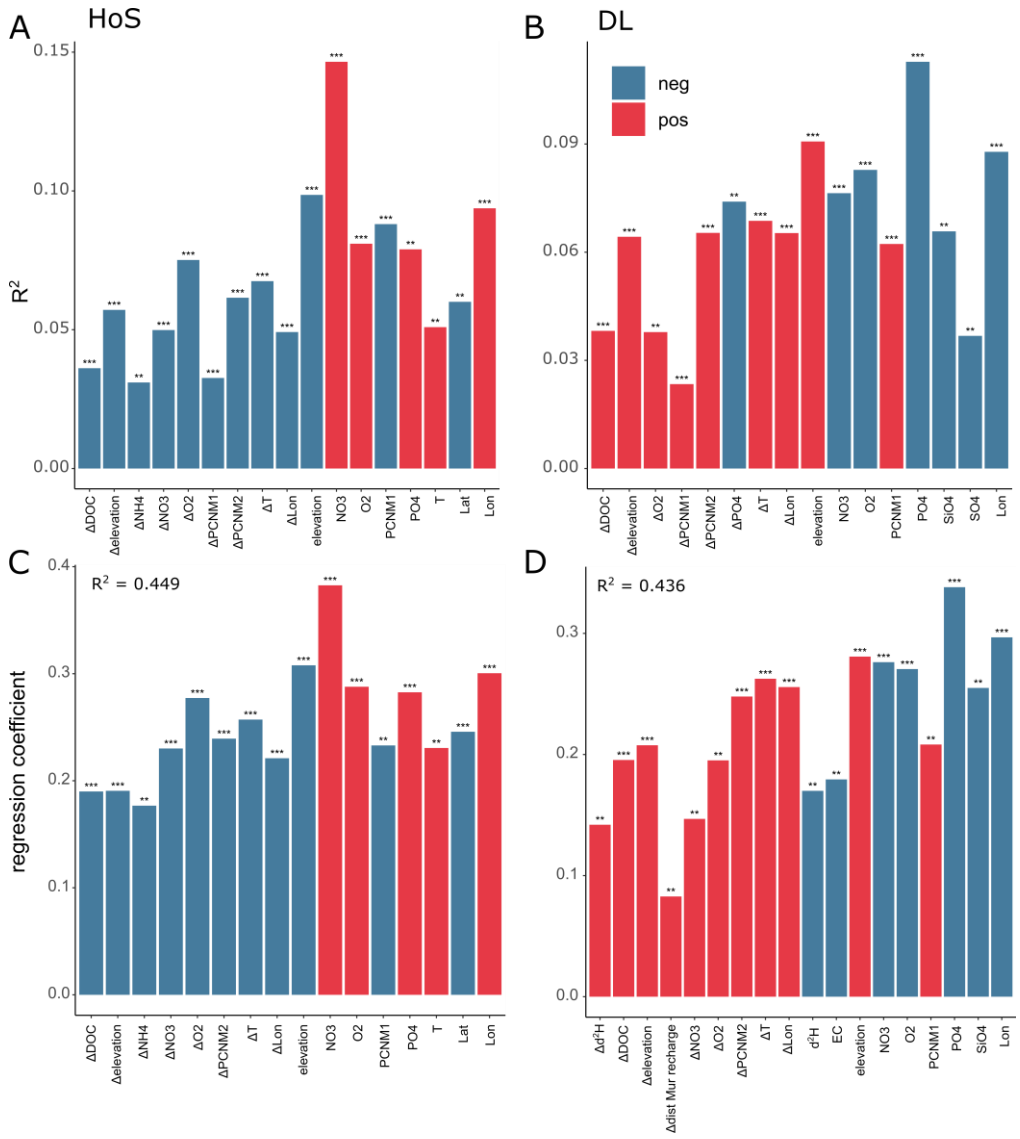

*Figure 5. Correlations of environmental variables showing factors with significant correlations for HoS (A) and DL (B) based on a Mantel analysis. Multiple regression on distance matrix (MRM) for HoS (C) and DL (D) with regression coefficient on the y-axis and R^2 shown based on the model from the Mantel test, which was based on log-transformed data. Negative correlations are colored in blue, and positive are colored in red. Correlation was based on the difference (Δ) or the mean of the variables between each pair of samples (*** $p < 0.01$; ** $p < 0.05$).*

Ecological Concepts

The species area relationship (SAR) applied for groundwater and river water prokaryotes follows the common assumption that the number of species is a positive function of the area sampled (Figure 6). The way groundwater diversity (observed richness and Shannon diversity) is linked to external disturbances and impacts from the surface is visualized in the context of the 'intermediate disturbance hypothesis' (Figure 6). Out of the relationships tested, observed richness does not show a statistically significant relation to any of these disturbances. However, the Shannon diversity revealed a negative linear relationship with nitrate concentrations (adj. $R^2 = 0.04$) (Figure 6B).

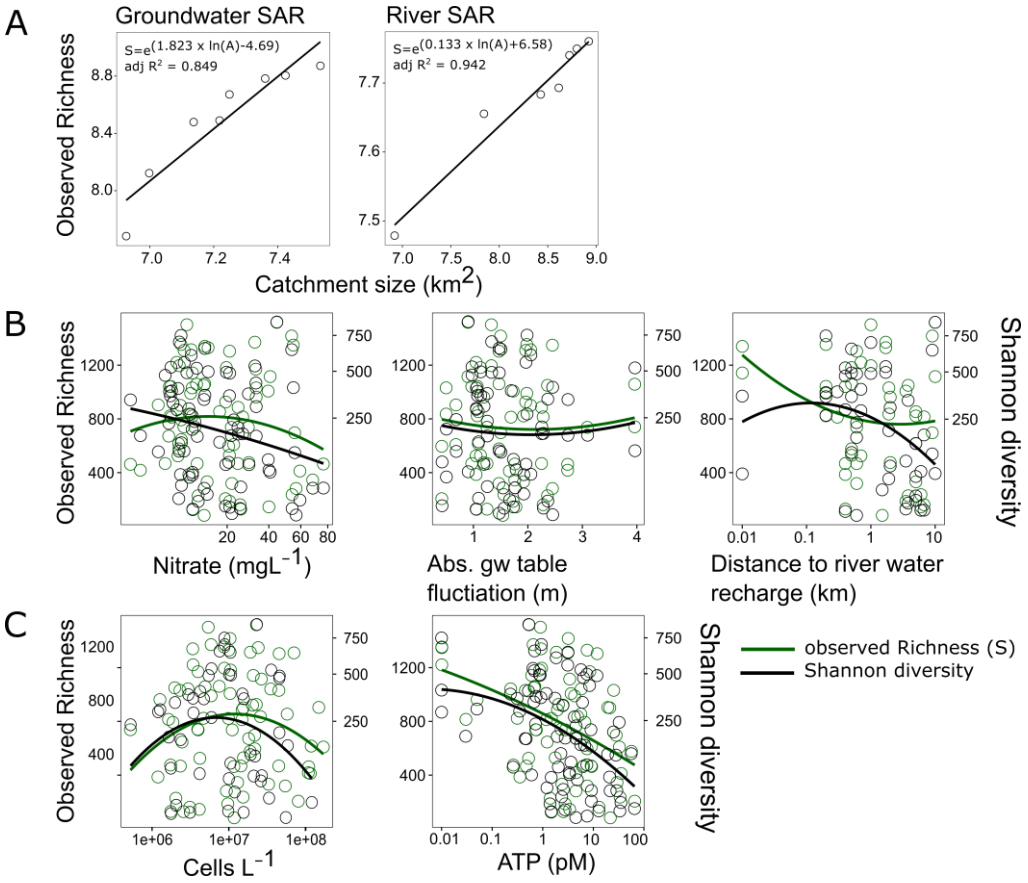

Figure 6. Bivariate SAR-linearized log–log power function plot of cumulatively added groundwater and Mur river ASVs (RNA fraction) with increasing catchment area. Groundwater catchment area and observed richness were cumulatively summarized from the groundwater regions closest to the source of the River Mur (LG) to the groundwater regions furthest away (LMV), only adding taxa that were not found in the previous groundwater region. The Mur river catchment sections (from top to bottom: MUR SBG, MUR I, MUR II, MUR III, MUR VI, Laßnitz, MUR V) were also summarized from the source of the Mur river in the Hohe Tauern to the area furthest downstream (MUR V) (A). Intermediate disturbance hypothesis (B) and productivity–diversity concept (C): Biplot of square root-transformed Shannon diversity (black), and observed richness (S; green), as well as square root transformed nitrate (mg L−1), log10 transformed Mur inflow distance (km), and un-transformed groundwater level fluctuation between absolute max and min since the year 2000 (m), fitting a 2nd-degree quadratic regression line of the groundwater data, as well as log10 transformed prokaryotic cell counts (cells L−1) and cellular ATP (pM) to meet criteria of a normal distribution.

The relationship between observed richness and cell numbers is significantly explained by a quadratic model (adj. $R^2 = 0.022$) and is linearly negatively related to cellular ATP (adj. $R^2 = 0.15$). The Shannon

diversity does not vary with cell numbers but shares a significant linear negative relationship with cellular ATP (adj. R^2 = 0.17). This means that in our groundwater dataset, diversity is principally highest at lower levels of disturbance. Investigating the productivity–diversity concept regarding our data, the Shannon diversity is not changing with cell numbers, while observed richness is highest at intermediate levels of cell density. Observed richness and the Shannon diversity are changing with the concentration of intracellular ATP, both being lowest when ATP is highest.

Temporal Shifts in Diversity and Community Dynamics in the Active Groundwater Fraction

Comparing total estimated richness between summer and autumn, groundwater has significantly higher richness in autumn (S: 1626 ± 121) compared to summer (S: 1062 ± 164) ($p < 0.05$). Moreover, the Shannon diversity in groundwater is higher in autumn as well (summer: 622 ± 96 ENS, autumn: 1847 ± 67 ENS) ($p < 0.05$), which hints at a more evenly distributed community towards autumn, which is richer in species. In the DNA fraction, the picture is similar, with no difference in the richness between seasons but higher diversities in autumn (Figure S13).

Regionality and Land Use Influence Diversity in the Active Groundwater Community

Specifically, in groundwater, there are overall regional differences in total estimated richness ($p = 0.04$), where richness is highest in the region Mur Cross Valley (MCV) (S: 2175 ± 461) and lowest in the Leibnitz Basin (LB) (S: 500 ± 398). There are also overall differences in the Shannon diversity between regions ($p < 0.05$), which be-comes gradually less towards lower elevations, being highest in the region Aichfeld (AF) (3072 ± 85 ENS), and lowest in the region Graz Basin (GB) (259 ± 46 ENS) (Figure 7A). In principle, groundwater located in the alpine regions shows a higher species diversity and complexity, with exception of the Upper Mur Valley (UMV) with only a few samples collected, than regions in the alpine foothills, where there are a few abundant species dominating the community. UMV is probably less diverse, as the communities are not as evenly distributed compared to the region Lungau (LG) ($p < 0.05$) and is more similar in evenness to wells located in the downstream part of the Mur Valley transect of Graz Basin (GB), Leibnitz Basin (LB), and the Lower Mur Valley (LMV). A similar trend can be observed in the DNA fraction (Figure S14).

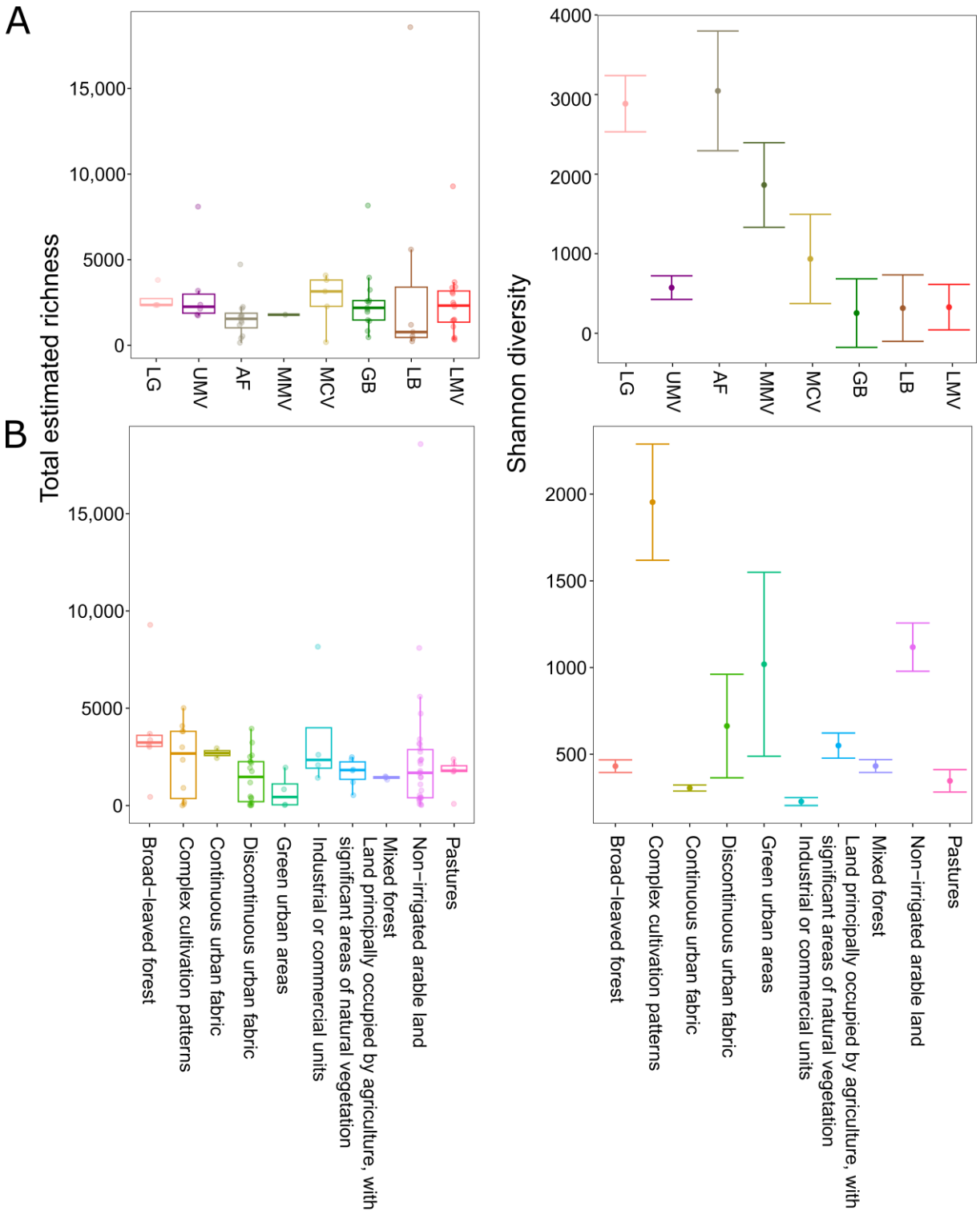

Figure 7. (A) Diversity metrics of groundwater prokaryotic communities between regions and (B) related to different land use types. Total estimated richness is depicted in form of boxplots showing median values as midline, first, and third quantiles as edges, and whiskers representing the max/min values. Shannon diversities are shown as effective species numbers with error bars showing the mean estimates from covariate-wise estimations ± SE.

In groundwater, total estimated richness is comparable over all land use types, but the Shannon diversity is significantly different between categories of land use ($p < 0.05$). The groundwater Shannon diversity is highest in wells located beneath 'Complex cultivation patterns' (2503 ± 163 ENS), and lowest in wells

beneath 'Industrial or commercial units' (164 ± 35 ENS) (Figure 7B). Results are similar for the richness and Shannon diversity in the DNA-derived community (Figure S15).

Land Use Affiliation, and Regionality as a Major Factor for Species Turnover

In groundwater, the categories land use and regionality explain most of the variation in the taxonomic community composition of the prokaryotic communities. The PERMDISP analysis, designed to test if some groups are more variable than others, reveals significant differences for season, land use, and groundwater level correlation with 9–12-month SPI (Table 4). Notably, the heterogeneity of overall environmental parameters between seasons does not seem to cause the observed patterns of beta diversity. Differences in environmental conditions, however, could offer an explanation for the multivariate homogeneity of group dispersions between land use categories ($p < 0.05$), and strengths of well level correlation with 9–12-month average precipitation ($p < 0.05$).

Table 4. PERMANOVA and PERMDISP analysis of the effect of sampling region, season, land use, correlation of groundwater levels with precipitation, and Mur river water levels on beta diversity of prokaryotic communities measured by clr (999 permutations) (SRSI: Standardized River Stages Index, SPI: Standardized Precipitation Index).

PERMANOVA				PERMDISP	
Taxonomic (CLR)	R^2	F	p-Value	F	p-Value
Land use	0.178	1.998871	0.001	2.0506	0.044
Region	0.139	2.006402	0.001		
Season	0.073	7.343749	0.001	8.5672	0.007
SRSI	0.047	1.594799	0.001		
SPI 9–12 months	0.044	1.469684	0.001	5.4924	0.001
Recharge probability Mur	0.038	1.294373	0.012		

Next, we plotted key physico-chemical parameters and nutrients using a con-strained analysis of principal coordinates (CAP) of the Aitchison distances. The taxonomic composition is significantly explained by differences in elevation, cellular ATP, pH, SiO_4, groundwater level, DO, Mg^{2+}, and DOC (adj. R^2 = 0.108), where elevation (adj. R^2 = 0.037) and pH (adj. R^2 = 0.036) alone explain the most variation in taxonomic composition of groundwater prokaryotic communities (Figure 8).

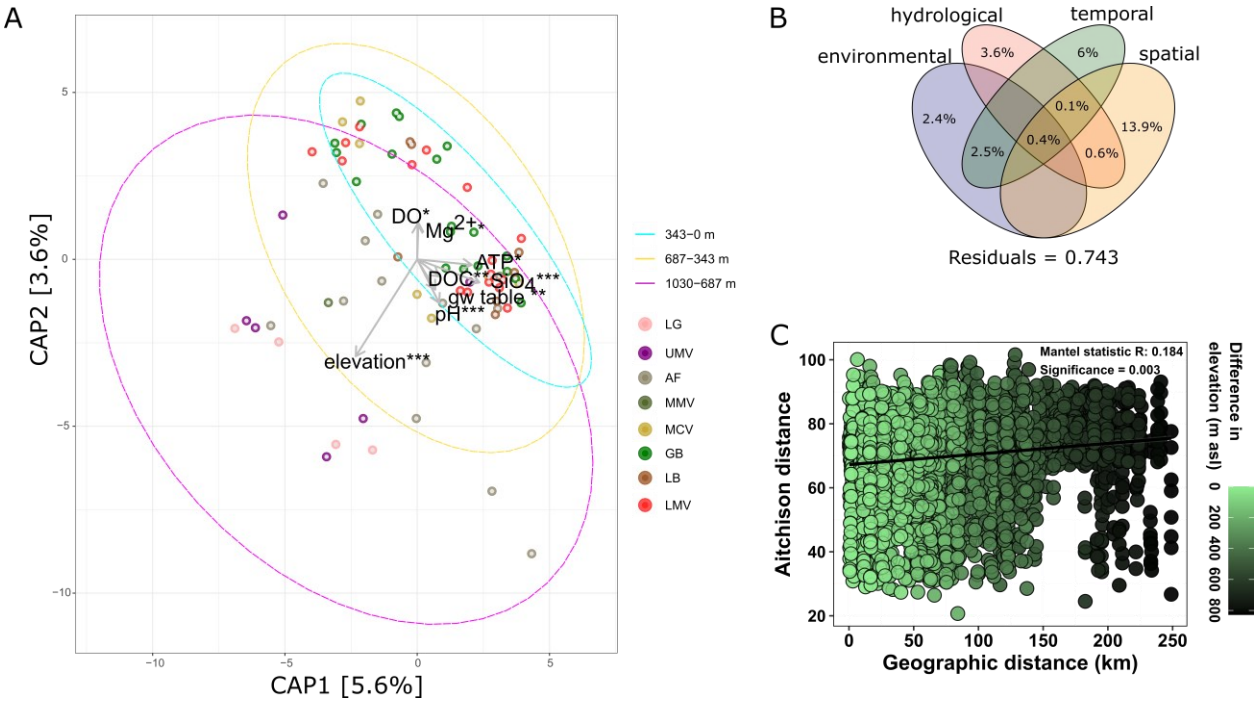

*Figure 8. (A) Ordination of species turnover with a constrained analysis of principal coordinates (CAP) based on clr-transformed abundances between groundwater wells in association with respective environmental parameters (*** p < 0.001, ** p < 0.01, * p < 0.05). (B) Venn diagram showing the proportions of variation explained by four explanatory factors: environmental factors, hydrological factors, temporal, and spatial factors. (C) Biplot of the Aitchison distance and geographic distance depicting the distance decay relationship with circles colored according to the difference in elevation between sample pairs, showing Spearman correlation coefficient (R) as well as p value.*

Based on a variation partitioning analysis, excluding variables that are correlating beforehand, differences in significant environmental factors (pH, K^+, DO) explain 2.4%, while the significant hydrological fraction (SRSI, and probability that groundwater is influenced by the river water) explains 3.6%, the temporal factor season explains 6%, and spatial factors (region, land use) explain 13.9% of the variation in prokaryotic community composition. Environmental, hydrological, temporal, and spatial factors are still significant when controlling for the respective others. The dataset covers a distance gradient of almost 300 km. The distance–decay relationship (Figure 8) depicts an overall positive correlation that indicates a higher species turnover between samples as the distance increases between them (Figure 8). These results suggest that spatial components such as region, land use, geographic distance, and elevation account for most of the variability in community turnover. Furthermore, communities originating from the low altitude section (0–343 m a.s.l.) seem to be most similar to each other, compared to communities derived from the alpine region (Figure 8). This indicates a higher structural heterogeneity in alpine aquifers and some separation of high-er altitude groundwater bodies (> 400 m a.s.l.), where members of the archaeal phyla *Nanoarchaeota*, *Aenigmarchaeota*, *Micrarchaeota*, as well as unclassified Archaea, together with bacterial phyla WOR-1, *Patescibacteria*, *Chloroflexi*, *Verrucomicrobiota* and unclassified

Bacteria are significantly more abundant, from groundwater bodies in the lowland (200–400 m a.s.l.) *Proteobacteria* gain significantly in abundance ($p < 0.05$).

Discussion

In our study, we collected and characterized shallow groundwater and corresponding river water from eight regions along a 300 km river valley stretch with a focus on the active microbial diversity and community. The dataset was further explored in relation to hydrochemical composition and physicochemical conditions, microbial productivity, as well as community composition with respect to aspects of altitude, season, and land use, as well as local and regional hydrology influenced by precipitation and interaction with the river. We investigated how the groundwater prokaryotic communities differed in their active (RNA derived) and total (DNA derived) composition in terms of structure, richness, and diversity. The active batch of the prokaryotic communities was then analyzed regarding different community assembly processes. Moreover, ecological concepts established in macroecology and partly in microbial ecology of soil ecosystems and surface waters were tested for the Mur Valley groundwater ecosystem.

Groundwater is often perceived as an environment stable in hydrological and physico-chemical conditions carrying a rather low productivity and biodiversity (Griebler and Lueders, 2009). Due to protective overlying soil layers and extended water residence times, groundwater systems are usually more self-contained and less affected by external seasonal disturbances (e.g., extreme weather events), land use, and accidental pollution when compared to surface ecosystems such as rivers and lakes. In contrast, several recent studies provide solid evidence that aquifers in karst and shallow unconsolidated alluvial and glacio-fluvial sediments exhibit quite some dynamics in environmental conditions translating into dynamics in microbial community characteristics, including community composition and productivity (Fillinger et al., 2019; Griebler et al., 2022; Savio et al., 2018; Zhou et al., 2012). Evidence is also growing that although depleted in readily biodegradable organic carbon and poor in energy, groundwater ecosystems may harbor highly diverse microbial communities absolutely comparable to surface waters in terms of richness and diversity (Fillinger et al., 2023; Ji et al., 2022; Karwautz and Griebler, 2022).

A river corridor originating from the mountainous region extending into lowlands offers the possibility to systematically study microbial community composition along various gradients, i.e., a change in elevation and temperature regime, variation in the concentration and quality of natural organic matter, an increase in land use types and intensity translating into an increase in nutrients and pollutants, and a change in microbial diversity and productivity in surface waters and groundwater (Besemer et al., 2013;

Harjung et al., 2023; Kamjunke et al., 2019; Weitere et al., 2021). Indeed, groundwater downgradient of the source of the River Mur at about 2000 m a.s.l. continuously increased in temperature, DOC, and nitrate concentrations (Figure 2). In addition, silicate is enriched in groundwater and, to a lesser degree, in the Mur river and tributaries over the course of the valley, with the Laßnitz River especially being a contributor draining a silicate-rich area. The strong relationship between DOC and DO concentrations suggest a high bioavailability of DOC (Chapelle et al., 2012) in the Mur Valley groundwater. Harjung et al. (2023) (Harjung et al., 2023) pointed at a correlation between groundwater DOC quality and concentration with prokaryotic biomass in the large river valleys of Austria, including the Mur river valley.

As a proxy for microbial productivity, we used prokaryotic cell density and cellular ATP. In general, groundwater and surface water differed significantly in productivity, with groundwater being less productive (Figure 3). Within groundwater, productivity increased from the alpine region to the lowlands, and was lower in great-er depth (e.g., in the Aichfeld and Graz Basin region) compared to shallow groundwater (e.g., in the Lower Mur Valley). Prokaryotic cell densities observed in groundwater exhibited values typical for groundwater ranging from 10^6 to 10^8 cells L−1 (Figure 3) and only in exceptional cases hint at serious pollution (Griebler and Lueders, 2009; Retter et al., 2021). Temporal differences in productivity were evident between groundwater collected in summer and autumn. This variation can be related to sea-sonal inputs of energy from the surface (i.e., winter and spring are the periods of main groundwater recharge), a change in microbial community composition, and/or a change in physiological state (Hammes et al., 2010). Evidence for the latter comes from elevated cellular ATP values in summer when compared to autumn, with prokaryotic cell densities showing no significant differences between seasons (Figure 3). In case of an increased availability of organic matter in parallel to a lack of an essential nutrient such as phosphorus, cells may increase physiological activity (and thus ATP content) without cell reproduction (Hofmann and Griebler, 2018).

Based on molecular analysis, differences between the active (based on total RNA) and the total fraction (based on total DNA) of the prokaryotic communities in groundwater are especially pronounced in the different relative abundance of *Bacteria* and *Archaea* (Figure S8). Since the total and active fraction are equally rich in species (ASVs), but the Shannon diversity is higher in the total fraction, the difference in abundance of some prokaryotic groups may explain this outcome. This hints at both communities, the total and the active, being differently structured. In groundwater, *Archaea* make up > 40% of the species in the total, DNA-derived fraction, whereas in the active community, *Archaea* account for < 20%. This indicates that a large proportion of *Archaea* in the groundwater obviously is inactive, dead or in a dormant state. Overall, the most abundant archaeal lineages in groundwater are members of unclassified Woesearchaeales. Members of *Woesearchaeales* (Candidatus Woesearchaeota) have been discovered

from a diverse variety of aquatic habitats (Castelle et al., 2015; Liu et al., 2018; Somervuori et al., 2021) and are hypothesized to have a symbiont and/or fermentation-based lifestyle. Their presence in the active fraction of the groundwater microbiome suggests that they likely take part in organic carbon cycling; however, a detailed process understanding is lacking. In the RNA-derived fraction, there is an increasing presence of *Proteobacteria*, mainly composed of *Gammaproteobacteria*, comprising almost half of the reads of the active groundwater community. No matter the nucleic acid fraction, *Proteobacteria* comprise the most abundant phylum present, as has also been observed in other groundwater studies (Ma et al., 2022; Morrissy et al., 2022; Yan et al., 2020). A more extensive picture into the groundwater microbiome could emerge if microbial communities attached to the aquifer matrices as well as microeukaryotes were also included in a future analysis, since they will con-tribute significantly to compositional aspects as well as functions (Groult et al., 2022).

Groundwaters and surface waters analyzed in this study were fundamentally different in their species diversity in both the active and total fraction, with groundwater being on average 76% more diverse and 38% richer in prokaryotic taxa. A comparable or even higher microbial diversity in groundwater and surface waters, respectively, have been found repeatedly in modern studies (Ji et al., 2022), mainly attributed to the improved sensitivity of molecular tools for rare taxa. The active river prokaryotic biome shares 67% of the reads and 23% of the ASVs with the active groundwater prokaryotic community, with groundwater harboring a higher fraction of uniquely occurring taxa. The active groundwater prokaryotic community comprises six core taxa, predominantly consisting of *Pseudomonas* species. The larger proportion of the active groundwater community is composed of species with a relative low abundance and composed of many unclassified Bacteria, suggesting the presence of yet undescribed bacterial lineages in the groundwater. These rare microbial taxa in groundwater shall be addressed in more detail in future studies. There is evidence that taxonomic minority members in microbial communities may be responsible for major activities (Munson-mcgee et al., 2022; Wilhelm et al., 2014). The active core community in the river consists of 13 species and shows a relatively lower proportion of rare taxa compared to the total fraction. This confirms the assumption that species that are abundant within one site tend to occupy many sites, while those that are locally rare will not be detected in many sites (Shade et al., 2018). Physico-chemical drivers of the variation in groundwater and river prokaryotic community composition are DO, pH, SiO_4, and ($R^2 > 0.2$), followed by water temperature, concentrations of DIC and DOC, δ^2H (water origin), and Na^{2+} and K^+ concentrations (Figure S11), leaving around 83% of the variation in community composition unexplained. Other studies investigating the influence of environmental parameters on microbial community composition also found low degrees of explanation (Langenheder and Lindström, 2019). This unexplained proportion of variation can be related to other, yet unmeasured

environmental factors, the huge spatial heterogeneity with a low spatio-temporal resolution in sampling, dispersal dynamics (e.g., community assembly linked to stochastic processes), and/or methodological shortfalls (Zhou and Ning, 2017).

We show that the assembly of prokaryotic communities was shaped by a combination of environmental selection of similar local conditions, dispersal limitation (DL), and ecological drift. Considerable differences in community assembly processes dominating in groundwater and river water habitats were found (Table 4). Previous studies investigating prokaryotic communities in the subsurface (Danczak et al., 2018a; Fillinger et al., 2019) provide further evidence for homogeneous selection (HoS) and DL to be dominating community assembly processes.

DL was most important in groundwater, meaning that in the absence of selection, prokaryotic communities have fewer taxa than expected by chance. A low rate of dispersal of taxa can happen when habitats or sites are spatially not connected, for instance, in cases of limited groundwater flow between aquifers (Maamar et al., 2015). DL does play a role for the river prokaryotic assembly as well, for instance, by limiting the turnover of species along the flow path of the river in the downstream direction. However, species assembly processes in the river are to a larger extent influenced by HoS, likely caused by homogeneous biotic and abiotic environmental conditions, leading to a more similar community structure than expected by chance. In accordance with Danczak et al. (Danczak et al., 2018b), we found that the groundwater, which experiences higher turnover of taxa than the river, is more strongly affected by stochastic processes, such as DL. On the other hand, little variation in community compositional turnover between river sites can dominate, if communities occur in the same selective environment and if selection is strong.

The longitudinal change in assembly processes (HoS and DL) reflects the hydro-geomorphic conditions along the Mur river valley. This could partly be related to the land management, as reoccurring and predictable environmental conditions have been shown to cause homogeneous selection (Aguilar and Sommaruga, 2020). It seems that homogeneous selection (HoS) is a more important community assembly process in the alpine lowland, where it is mostly driven by changes in nutrient concentrations (phosphate, nitrate) and oxygen availability (Figure 5). In the alpine region, however, prokaryotic communities are more strongly influenced by dispersal limitation (DL), eventually caused by existing physical barriers (e.g., mountains, narrow valleys) and/or possibly altered hydrological conditions affecting the groundwater flow rates.

Making use of our unique dataset, we tested a selection of ecological concepts that are well established in macroecology but controversially debated and hardly validated in microbial ecology of soil ecosystems and surface waters (Griebler et al., 2022), i.e., the species–area concept (SAR), which describes the

relationship between species richness as a function of habitat size (Horner-Devine et al., 2004), the intermediate disturbance hypothesis, which assumes microbial phylogenetic and functional diversity peaking at intermediate intensities or frequencies of disturbances (Fierer et al., 2003; Ibekwe et al., 2002; Zhang et al., 2018), and finally, the productivity–diversity concept, which assumes an increase in species richness and diversity with increasing available energy, though similarly to disturbances, a decline in richness and diversity happen in systems which are over-stressed and eutrophicated (Smith, 2007b). None of the concepts have been challenged with a solid groundwater dataset (Griebler et al., 2022; Griebler and Lueders, 2009). First, the species–area relationship holds true for groundwater and river water. A cumulative increase in prokaryotic richness was observed with increasing catchment size (Figure 6). The intermediate disturbance hypothesis did not prove similarly valid for the conditions tested. As disturbances, we tested (i) nitrate as a proxy for agricultural land use and the input of fertilizers and pesticides, (ii) the absolute groundwater level fluctuations as proxy for the hydrological dynamics aquifer vulnerability, and (iii) the distance to the river as a proxy for surface water impact to groundwater. These disturbances (in the range observed) did not reveal a significant quadratic relationship with prokaryotic richness in groundwater. Only the Shannon diversity was negatively related to the intensity of agricultural land use, as classified by nitrate concentration. The intermediate disturbance hypothesis has been frequently questioned and refuted in other studies and might not hold true for every type of ecosystem and all kinds of communities (Fox, 2013). On the other hand, the influence of available energy and resulting productivity as assessed by total prokaryotic cell counts showed a quadratic relationship with prokaryotic richness, being highest at an intermediate prokaryotic cells density (~10^7 cells L^{-1}). Moreover, we observed linear relationships with both the prokaryotic richness and Shannon diversity decreasing proportionally with increasing ATP concentrations (Figure 6). This could be related in part to the fact that pristine aquifers in the alpine region have high habitat heterogeneity and low productivity, which together lead to higher species diversity. In conclusion, there is proof for the SAR, there is support for a productivity–diversity relationship, with highest richness and diversity at lowest energy levels, and intense agricultural activities represented by high nitrate concentrations (but putatively also by elevated pesticide concentrations) had a negative effect to prokaryotic diversity (Figure 6).

Similarly to a few other studies (Zhou et al., 2012), our work revealed temporal dynamics in groundwater prokaryotic diversity, being higher in autumn where com-munities were found more evenly distributed and richer in taxa.

In addition to temporal dynamics, there are significant spatial changes in prokaryotic community composition. Along the alpine to lowland river corridor, prokaryotic diversity is altered, being overall higher at higher elevations (Figure 7). In alpine aquifers, environmental connectivity increases

downgradient, and groundwater bodies at lower altitudes experience unification and anthropogenic degradation with multiple pressures acting in a concerted way, obviously negatively affecting microbial diversity. The alpine region harbored a more diverse and complex prokaryotic community in its groundwater, hinting at the possibility that mountainous areas are not only important biodiversity hotspots for other domains of life (Mráz and Ronikier, 2016; Perrigo et al., 2020), but also for prokaryotes living in the aquatic realm below ground. In alpine groundwater, Archaean lineages especially seem to play a yet unexplored role with unclassified Archaea, as well as members of the *Aenigmarchaeota* (DSEG) being highly abundant. *Aenigmarchaeota* are one of the earliest diverging lineages within the DPANN superphylum meanwhile discovered in a wide range of habitats, and often found to have a symbiotic lifestyle (Li et al., 2021).

We looked at land use information as another important spatial and temporal driver that not only affects the surface environments and underlying soils but may significantly shape groundwater conditions and communities (Banks et al., 2011; Korbel et al., 2022; Schmidt et al., 2007). Groundwater located beneath areas classified as 'complex cultivation patterns' and 'pastures' exhibited the highest species diversity, being described as a mosaic of small plots of land consisting of different types of cultivation with occasional houses and gardens, and pastures combining all those areas where grassland and pasture farming is practiced. Lowest diversity on the other hand was discovered from groundwater located beneath 'Industrial or commercial units' (e.g., factories, warehouses, and storage facilities typically located in urban or suburban areas). Besides that, 'forest and semi natural areas' as part of the broader Corine land cover category, harbored significantly higher abundances of some archaeal line-ages, as well as the bacterial phylum Dependentiae, which contains as yet mostly uncultured Bacteria found from a wide variety of environments, mainly discovered from metagenomic studies (Brown et al., 2015). Differences in the prokaryotic species turnover (beta diversity) between groundwater habitats is mainly driven by the different land use types, but also by the characteristics of the different groundwater bodies, i.e., the regions. Considering the elevation gradient that is represented by the various regions, groundwater bodies at lower elevations (200–343 m a.s.l.) are more similar to each other than groundwater bodies found at higher elevations (Figure 8), where dispersal of prokaryotic taxa is more limited (Figure 5). Land use and region (as a summary of hydrogeological and climatic regional characteristics) explain by far most of the variability in taxonomic prokaryotic community composition in groundwater, while all physicochemical and microbial variables tested in this study explain only a much smaller fraction of variation.

Conclusions

Microbial diversity and controlling assembly mechanisms in aquatic systems are an active area of research. However, the groundwater ecosystem has often been overlooked, although its understanding is crucial due to its potential impact on human health and ecosystem functioning, as well as its important role in the overall water cycle. Yet, high-throughput gene amplicon sequencing of both the metabolic potentially active (RNA-derived) and total (DNA-derived) groundwater microbial communities, their comparison, and diversity analyses based on the metabolically active fraction have been few.

In this study, the active groundwater community was found to be largely composed of *Proteobacteria* (mainly *Gammaproteobacteria*), whereas the archaeal fraction was dominated by unclassified Woesearchaeales. This also indicates that members of *Archaea* take an active part in the energy turnover of groundwater environments. Moreover, the groundwater harbors relatively more rare taxa than the river, which is composed of many unclassified Bacteria, suggesting the presence of bacterial lineages with unknown functions that are yet to be described. Several studies have highlighted the importance of unclassified Prokaryotes including the bacterial CPR group and the archaeal DPANN taxa. These microorganisms seem to be abundant in the subsurface and are characterized by a minute cell size and reduced genomic repertoire. Metagenomic and metatranscriptomic analysis have shown a coupling of processes (i.e., metabolic hand-off) and the importance of autotrophic lifeforms in the subsurface. Still, metabarcoding remains the method of choice for rapid and representative microbial community analysis. The complexity of groundwater habitats in terms of physico-chemical, hydrological, and geological heterogeneities in combination with the limitations of groundwater sampling requires careful analysis and interpretation. In general, aggregate parameters such as land-use and geomorphic regions were more reliable in explaining variation of the community composition, than variation of individual parameters. Current community assembly models gave further evidence that dispersal limitation and homogeneous selection are the driving processes in groundwater. Here, dispersal limitation seems to play a larger role at higher altitudes, whereas homogeneous selection explains a larger share in the alpine foothills, where it is related to the availability of nutrients. The alpine region is also more diverse and richer in prokaryotic taxa, with some unclassified Archaea and early diverging archaeal lineages being highly abundant, again underlining the great need to study and appropriately protect this unique habitat, including its groundwater reservoirs.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microorganisms11030779/s1.

Author Contributions: conceptualization, S.B. and C.G.; formal analysis, A.R., J.H., C.S., B.H. and C.K.; funding acquisition, S.B. and C.G.; methodology, A.R., J.H. and C.K.; project administration, S.B. and C.G.; resources, C.G.; supervision, S.B. and C.G.; visualization, A.R., J.H. and C.K.; writing—original draft, A.R., C.G. and C.K.; writing—review and editing, S.B., J.H., B.H., C.G. and C.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the ÖAW (Österreichische Akademie der Wissenschaften), grant number: ESS-Project 2018: 'Integrative Groundwater Assessment'. Open Access Funding by the University of Vienna.

Data Availability Statement: Gene amplicon sequencing data, generated by the JMF under Project IDs JMF-2009-1 and JMF-2106-09, were deposited under the BioProject accession number PRJNA926931.

Acknowledgments: We thank Peter Rauch, Barbara Stromberger (Steiermärkische Landesregierung), Harald Huemer, and Margot Geiger-Kaiser (Salzburger Landesregierung) for their valuable information and help in selecting appropriate groundwater sampling wells. We also thank Lucas Fillinger, Constanze Englisch, Filipa Sousa, Grit Rasch, and Hubert Kraill for their help and support during sampling, lab work, and data analysis.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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Chapter III: The groundwater mycobiome: Fungal diversity in terrestrial aquifers

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Retter, A., Nawaz, A., 2022. The Groundwater Mycobiome: Fungal Diversity in Terrestrial Aquifers, 2nd ed, Encyclopedia of Inland Waters. Elsevier Inc.

Abstract

This chapter aims to present the accumulated knowledge on the groundwater mycobiome and characterize the fungal fraction present in groundwater based on available case studies. An attempt is made to answer questions regarding groundwater species diversity, relationships with emphasis on their geochemical role, systematics, and possible ecological functions. In addition, this chapter takes a closer look at the role of unexplored fungal diversity in groundwater and places it in context with other aquatic habitats. We also attempt to highlight possible ways to address potential challenges in assessing fungal diversity in terrestrial aquifers, outlining mainly cultivation-independent, molecular methods used in the framework of environmental studies of other aquatic systems. One of them is the frequently employed high-throughput sequencing of parts or the entire Internal Transcribed Spacer (ITS) region of the nuclear ribosomal DNA. This approach may come with various constraints and advantages, such as the choice of sequencing platform, the selection of primers and barcodes, or the decision of which taxonomic reference database to use. As it now stands, we are only aware about a fraction of groundwater fungal diversity, as well as their lifestyles and functions. It is clearly a race against time to characterize the groundwater mycobiome in detail and understand it in its entirety before many fungal communities undergo alteration or disappear from groundwater altogether.

Keywords: groundwater fungi, freshwater ecology, fungal diversity, mycobiome, terrestrial aquifers, biodiversity, molecular taxonomy

Glossary of main terms

- *Fungi:* Taxonomically, fungi constitute their own kingdom. They are eukaryotic, heterotrophic, mostly mesophilic organisms that produce spores for reproduction and dispersal and branching

hyphae (that make up the so-called mycelium) for propagation, organic matter decomposition, and uptake.

- *Groundwater:* Water that is located beneath the earth's surface in pore spaces and cracks of the solid, rocky crust of the earth.
- *Diversity:* The number and relative abundance of taxa, which are often species or functional groups of organisms, as well as the genetic variation within a taxon.
- *Next-Generation Sequencing (NGS):* Inexpensive, routine, high throughput method for sequencing short genetic barcodes, often from environmental samples.
- *Barcode:* Conserved to highly variable gene segments, such as the ribosomal gene cluster, proteins, or enzymes, that are universal within the target group of organisms. They are, for instance, utilized in metabarcoding efforts for rapid species identification of environmental samples.
- *Dark Matter Fungi:* Fungal taxa that are uncultured and poorly known, mostly belonging to lineages diverging early in the fungal tree.
- *Oogamy:* Fertilization by the fusion of a small, usually motile male gamete and a large, much less motile female gamete.
- *Convergent evolution:* Organisms not closely related that develop traits with similar form or function, also called homoplasies.
- *Cryptic:* Morphologically indistinguishable.
- *Anamorph:* Asexual, mold-like reproductive stage of members of Dikarya.
- *Teleomorph*: Sexual lifecycle stage of members of Dikarya, often producing macroscopic fruiting bodies.

Introduction

Fungi are among the dominating lineages within the ecosphere, ubiquitously present in both terrestrial and aquatic habitats. Evidence from fossil records even suggests that fungi / fungal-like organisms may have existed in the deep biosphere as early as about 2.4 billion years ago (Bengtson et al., 2017; Loron et al., 2019). Owing to their diverse life strategies as decomposers, symbionts, parasites, and pathogens, fungi play vital ecological roles in the organization of different biological communities and contributed to shaping the world as we know it in its present form. Fungi can survive and thrive under a wide range of oxygen, pH, temperature, water potential, and nutrient level conditions (Schlosser, 2012). Thus, these fantastic creatures have managed to establish themselves in various types of ecological niches and habitats such as the snow and ice as found naturally in glaciers, the tropics, deserts, the oceans, and any

kind of freshwater habitat – which simply means that fungi are everywhere. Along with this omnipresence, global fungal diversity has for some time been estimated to lie between 3.5 and 5.1 million species (Blackwell, 2011). In contrast, a more recent study showed that fungal diversity probably ranges between 2.2 and 3.8 million worldwide (Hawksworth and Lücking, 2017). However, with only ~150,000 formally described fungal species so far (Kirk, 2021), the fungal inventory is far from being fully explored.

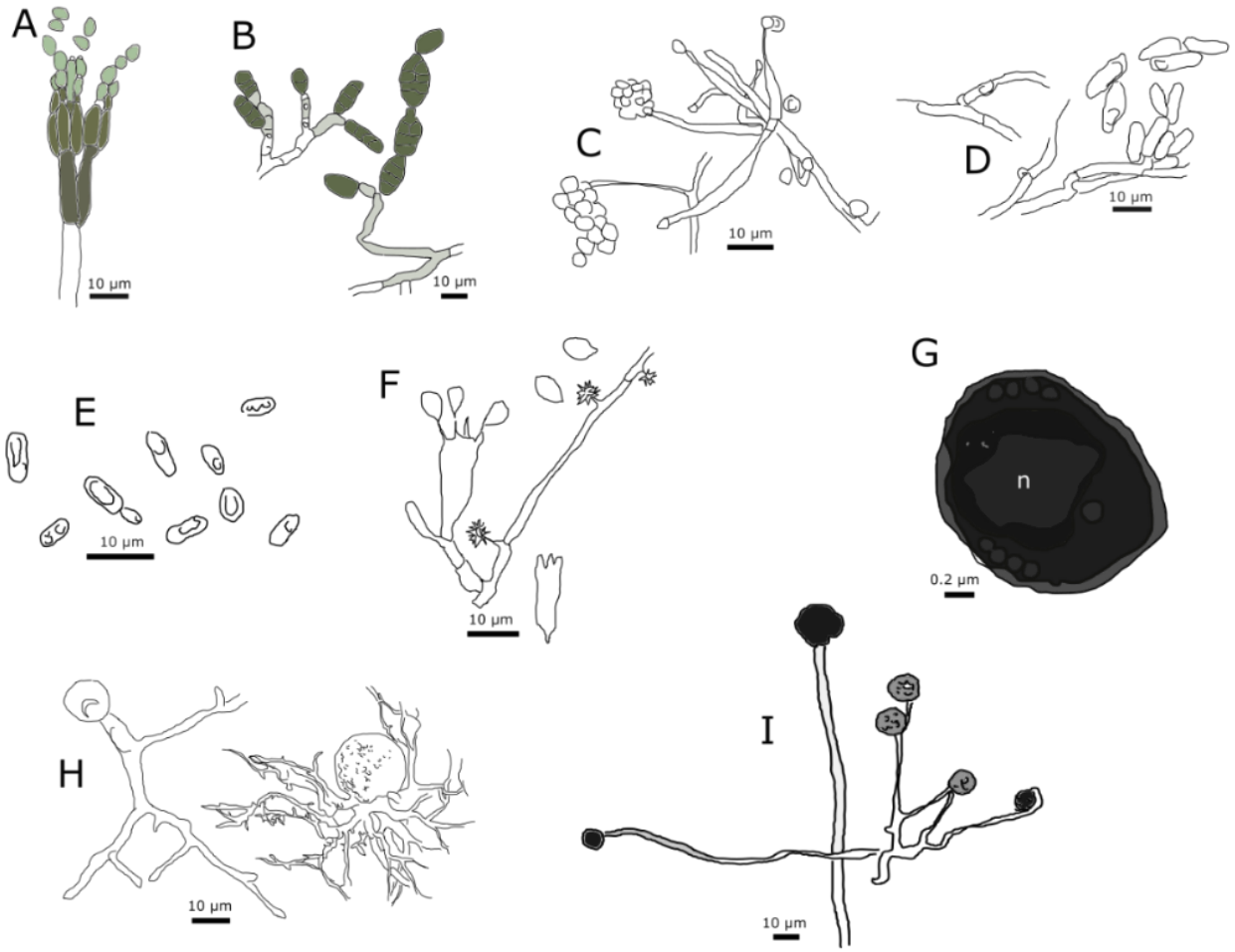

Figure 5 Some examples of the variety of fungal lifestyles found within groundwater. ***A*** *Ascomycetous saprotroph and plant pathogen/parasite (Penicillium oxalicum).* ***B*** *Ascomycetous plant- and animal pathogen, wood saprotroph (Alternaria sp.).* ***C*** *Animal pathogen (Pochonia sp.).* ***D*** *Saprotrophic, basidiomycetous yeast with filaments (Dioszegia sp.). E Low temperature tolerant, basidiomycetous budding yeast (Vishniacozyma sp.).* ***F*** *Saprotrophic basidiomycete (Resinicium sp.). G Microsporidian hyperparasite spore (n = nucleus).* ***H*** *Freshwater chytrid (Rhizophydiales)).* ***I*** *Endophyte / saprotroph (Mortierella sp.).*

Fungi are talented decomposers of dead organic matter (saprotrophs) and recyclers of inorganic materials (e.g., N and P), and their bioavailability constitutes a critical factor for fungal activity. In aquatic environments, fungi display various trophic modes, often occurring as parasites or pathogens, harming the host's cells, which can be animals, plants, or sometimes even other fungi. Apart from being saprobes or pathotrophs, fungi can form mutualistic associations (symbiotroph) with animals, plants, and various

microorganisms that bring forth a multitude of phenotypically distinct lifestyles, some examples being mycorrhizae or lichens (Figure 1).

About 90 % of existing plants form such mycorrhizal associations, which, among other things, promote plant growth, increase plant resistance to biotic and abiotic stressors, and help plants acquire absorbed soil nutrients through fungal hyphae. Interestingly enough, some early diverging lineages of fungi, for instance, neocallimastigomycetes, even co-evolved within the digestive tracts of ruminant mammals, insects, and other invertebrates (Moore, Robson and Trinci, 2011; Stefani et al., 2016; Picard, 2017). Consequently, fungi can take on a broad range of metabolic roles, naming just a few examples in table 1.

Fungi reproduce and disperse by means of spores, which are formed during meiotic- or mitotic reproduction. Different taxa can bring forth a great variety of spore types, all with an individual morphological and functional identity. Many aquatic fungal species, such as members of *Chytridiomycota*, and *Blastocladiomycota* can produce motile, flagellate spores (zoospores) (Figure 2), or non-motile spores with a variety of unique shapes, optimal for dispersal in aquatic environments.

Table 1 Metabolic roles of fungi, summed up from Watkinson et al. (2015).

Decomposing of OM (organic matter)
Organic and inorganic nutrient cycling (e.g., C, H, O, N, P, S, metals)
Translocation of water and nutrients
Change in local geochemistry (e.g., redox potential, pH, O_2)
Production of metabolites and exopolymers
Degradation of xenobiotics (e.g., pesticides, drugs, radionucleotides), complex compounds (polysaccharides, lipids, and proteins, and other complex structure polymers), and contaminants (e.g., fuel, oil, gas, explosives)
Mineral formation (e.g., carbonate, limestone) and dissolution (e.g., weathering and leaching)

It is well established that fungi sometimes have complex and obscure life cycles that may involve stages of different growth forms (e.g., filamentous and/or unicellular, such as yeasts) (Figure 1), hosts, and sexual states (e.g., anamorphs and teleomorphs in Dikarya) (Naranjo-Ortiz and Gabaldón, 2020). Fungi are largely cryptic, driven for instance, by convergent evolution, which is widespread throughout the fungal tree of life and can be caused by mechanisms such as interspecific hybridization (Torruella et al., 2015; Shang et al., 2016; Sun et al., 2019; Steensels, Gallone and Verstrepen, 2021). Traditional morphological taxonomic species identification is, in reality, often not possible, as stages of the life cycles in which fungi form characteristic fruiting bodies are unknown or not inducible during cultivation, and their somatic structures only show limited cell differentiation (Watkinson, Boddy and Money, 2015). Thus, purely

phenotypic recognition of fungi is difficult, and it is no wonder that the taxonomic history of fungi resembles a roller coaster ride (Naranjo-Ortiz and Gabaldón, 2020).

The lack of knowledge about the sexual states of fungi and the fact that many species cannot be cultured in the laboratory consistently results in formal species descriptions being ambiguous or missing altogether. The large amount of molecular data derived from environmental sequencing creates an additional layer of complexity to this problem. Environmental sequencing, such as Next-generation sequencing (NGS) or Third-generation sequencing is based on classifying a molecular barcode into a hypothetical species. These techniques enable rapid species identification in unprecedented numbers, utilizing subregions located in the nuclear ribosomal gene cluster, such as the formally accepted internal transcribed spacer (ITS) barcodes ITS1, or ITS2, or other, sometimes longer or more conserved parts of the rDNA (ribosomal DNA) (Heeger et al., 2018, 2019; Nilsson et al., 2019; Wurzbacher et al., 2019). The exactness of the classification depends a lot on the comprehensiveness and up-to-dateness of the various classification databases of choice. Additionally, these sequencing techniques are subject to several factors that may lead to biased species classifications, such as choice of primers, barcode, or sequencing technology (Tedersoo et al., 2015).

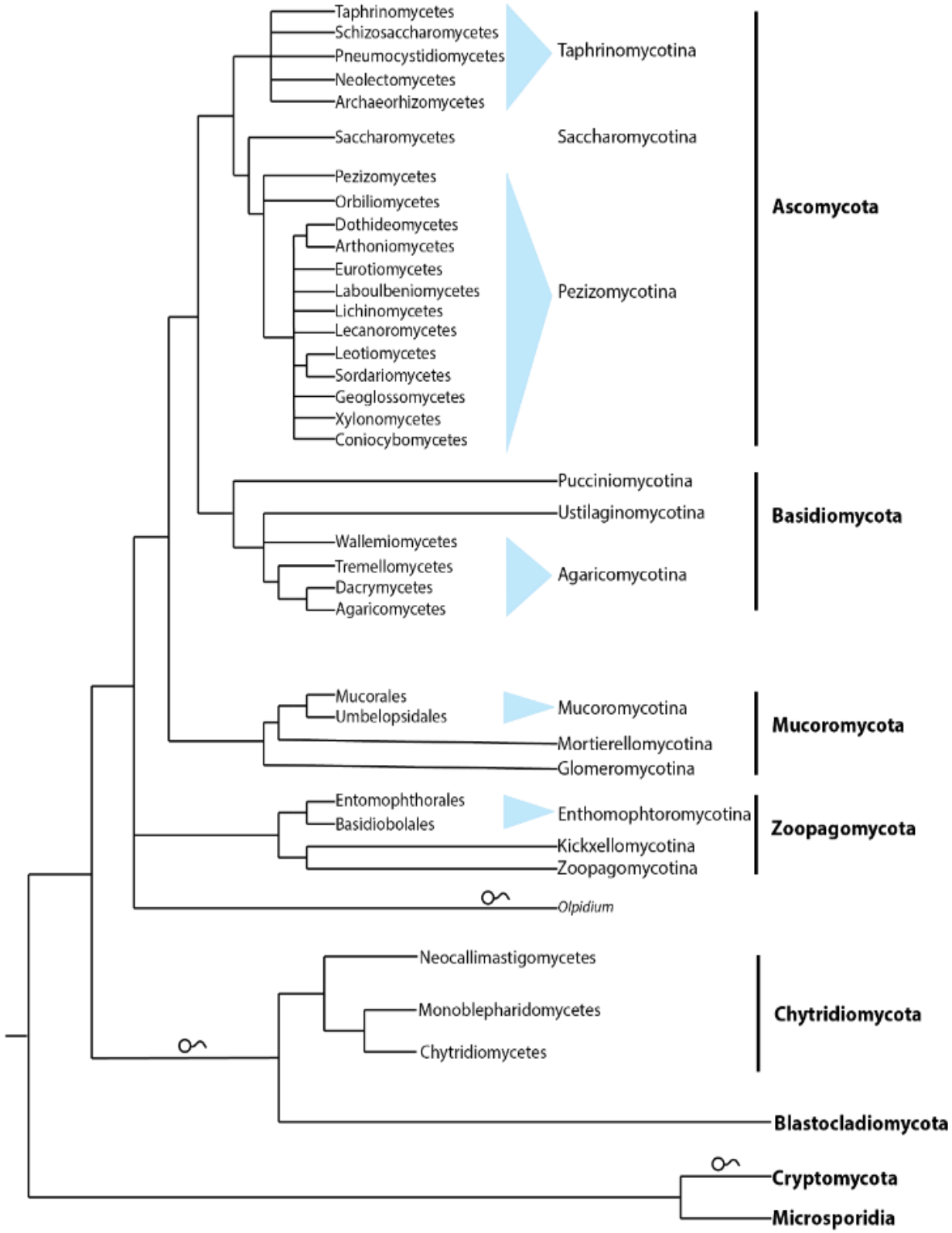

Figure 5 Current understanding of the phylogenetic backbone of the fungal kingdom, compiled and negotiated from several phylogenetic studies (James et al., 2006, 2013; Hibbett et al., 2007; Schoch et al., 2009; Rosling et al., 2011; Sekimoto et al., 2011; Gazis et al., 2012; Zhuang and Liu, 2012; Nguyen, 2015; Spatafora et al., 2016; Tedersoo et al., 2018). Polytomies are representing yet unresolved relationships between lineages. Zoosporic fungi are indicated by a schematic drawing of a zoospore (James et al., 2006).

Unknown fungal richness in groundwater

Compared to the earth's terrestrial ecosystems, where many fungal global datasets are available, the aquatic environment has been historically understudied regarding its fungal diversity. It is important to mention that despite the huge estimates of ~2.2 – 3.8 million extant fungal species, currently, only a little over 4,000 have been classified as aquatic (Jones, Hyde and Pang, 2014). Such a small proportion of aquatic fungi within the global fungal diversity estimates may not hold when acknowledging that around 70% of the earth is covered with water and aquatic environments are potentially ideal habitats for many fungal species (Wurzbacher et al. 2011). This highlights the need to revise the extent of aquatic fungal diversity in the light of recent advances in molecular methods and their broader applications in biodiversity estimates of various ecosystems.

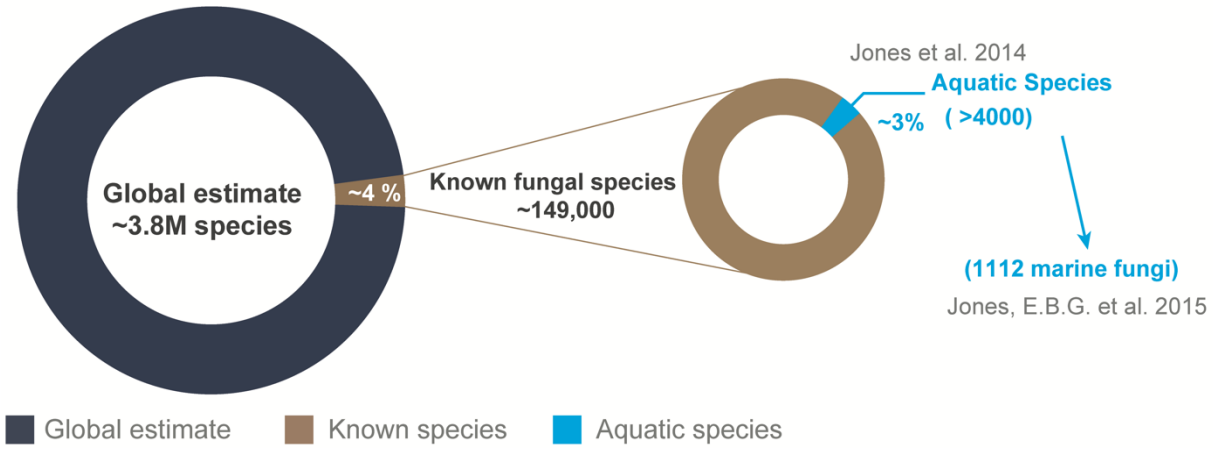

Figure 3 The actual representation of known/described species of aquatic fungi (Jones, Hyde and Pang, 2014; Jones et al., 2015) in the overall fungal diversity estimates.

Similar to the deep oceans, the world's groundwater habitats are seriously underexplored for fungi (Grossart et al., 2019). Especially fungal members belonging to early diverging lineages (Figure 2) are mostly underrepresented in fungal studies of aquatic systems (Picard, 2017; Retter, Nilsson and Bourlat, 2019). There are several reasons for such underrepresentation of aquatic fungi: first, many aquatic ecosystems/landscapes of the world are still not very well explored for their fungal inventory; second, reproduction life cycles in which fungi produce distinct fruiting bodies are often entirely missing (or yet undiscovered) for many fungal taxa; and third, due to molecular bias caused, for example, by choice of primers or the DNA barcode marker. The ITS - region, for instance, is not sufficient to target and resolve the taxonomy of fungi at a deeper level (Tedersoo et al., 2015; Berbee, James and Strullu-Derrien, 2017; Picard, 2017; Grossart et al., 2019; Nilsson et al., 2019).

Groundwater food sources and replenishment

The groundwater hydrosphere is generally regarded as an oligotrophic environment regarding the abundance and bioavailability of organic material. Due to the complete absence of light, an important energy source for primary production is missing. Even though chemoautotrophic organisms, for instance, as part of microbial biofilms, can sometimes contribute substantially to biomass production in groundwater, most of the energy is derived from the land surface (Herrmann et al., 2020). This includes organic material mainly in the form of allochthonous DOM (dissolved organic matter), which is supplied with seepage water. The availability and composition of DOM and essential nutrients are crucial for the survival and flourishing of the fungal communities in groundwater. In groundwater, the input of organic material will usually change dynamically during periods of precipitation and subsequent recharge or drought. Likewise, the quality and quantity of DOM that ultimately reaches the saturated zone is influenced by various biotic and abiotic factors, such as the surface land cover, surface water to catchment connectivity, the porosity, and the geology of the unsaturated zone, as well as the distance of the land cover to the aquifer (groundwater table depth). It is also dependent on the chemical and physical properties (e.g., polarity) of the dissolved organic compounds, if they have undergone photodegradation on the surface, and the degree of biodegradation by resident microbial communities in the soil layers through which the DOM passes (Shen et al., 2015). As a result, typical groundwater DOM is largely deprived of lignin-derived phenols, which are derivatives of secondary metabolites of vascular plants, and higher molecular weight (HMW) compounds (Shen et al., 2015). These fractions of DOM are highly reactive and have the ability to readily adsorb to the surface of solids (Singh et al., 2016; Rutlidge et al., 2021). Therefore, in terms of DOM composition, groundwater is similar to the deep ocean (Shen et al., 2015).

In contrast, in rivers, streams, and lakes, POM (particulate organic matter), e.g., animal and plant detritus, is an essential energy source. However, POM is not present in significant quantities in groundwater, as it is attenuated or degraded already within the pedosphere. From this point of view, groundwater represents a rather hostile environment for fungal organisms. On the other hand, some limiting factors such as UV-irradiation, competition for food or host species, or extreme temperature fluctuations do not exist or are less pronounced, which in turn may prove advantageous for the survival of various fungal species and their spores.

Not surprising, groundwater is different to surface water systems in several other aspects, such as high-water residence times (sometimes decades or millennia) and limited space, mainly in the form of (interconnected) pore cavities in unconsolidated sediments or fractures in the rock matrix. As a result, the groundwater hydrosphere may only serve to a limited extent as a vector for (long-distance) dispersal of

fungal taxa and their spores. In summary, groundwater, represented by water in various types of aquifers, underground streams, and cave waters, forms a unique ecosystem with some potential limitations and advantages for its fungal and overall microbial life, on which the stability of this ecosystem is founded. Since groundwater is usually recharged by rain and snowmelt, as well as surface water passing through soil and sediment zones, this ecosystem provides a rare opportunity to study both transient and resident fungi in their natural settings.

The diverse ecological roles of fungi

Despite the challenging conditions prevailing in groundwater, there have been several studies conducted within the last decades that were able to detect both total and active proportions of fungal communities in various groundwater environments (Ekendahl et al., 2003; Livermore and Mattes, 2013; Jasrotia et al., 2014; Sohlberg et al., 2015; Schwab et al., 2017; Nawaz et al., 2018, 2019; Perkins et al., 2019; Wurzbacher et al., 2020). A study that has been published as early as 1989 already points at the highly diverse and distinct microbial communities, including fungi that could be recovered from a deep coastal plain aquifer (Fliermans, 1989), and fossilized fungi have also been reported from the subsurface (Reitner, Schumann and Pedersen, 2006).

Concrete examples highlight the potentially important and yet unexplored roles that fungi play in the oligotrophic environment of the water-saturated subsurface. The first is that they can potentially restructure the groundwater matrix through their mineral weathering and mineralization properties, and thus also provide essential minerals for their (and other microorganisms) growth and metabolism (Ivarsson et al., 2018).

Second, they are excellent degraders of complex organic compounds, which they may mineralize to CO_2 and water in the presence of oxygen. Macropolymers often have a poor biological degradability, making them persist in the groundwater environment over a long period of time. Poorly biodegradable leftovers (refractory OM) of organic matter can be decomposed and transformed by fungi via particular enzymatic pathways, and they are also believed to have the capability to produce highly aromatic organic compounds themselves (Collado et al., 2018; Masigol et al., 2019). Some lignin-derived compounds, such as humic substances, make up a significant part of the refractory DOM available in groundwater (Hofmann and Griebler, 2018; Perkins et al., 2019). The effective degradation of such humic compounds by fungi seems to be dependent on, e.g., its concentration, oxygen availability, and temperature (Collado et al. 2018), possibly leading to slower degradation rates (i.a. supporting long-term growth) of complex organic substances by fungi in groundwater.

If pulses of DOM passing through the aquifer are long-lasting, at least bacterial communities were found to adapt and utilize the labile fraction of DOM. However, it appears that the more labile carbon there is available, the less it is converted into bacterial biomass for reasons that are still ambiguous (Hofmann et al., 2020). On the other hand, it seems that a more diverse composition of DOM promotes a higher bacterial diversity (Hofmann et al., 2020). Bacterial and fungal communities do not seem to be intimately connected, but as the structural setup of DOM is a primary driver for both, these scenarios could prove to be true for fungi as well (Fabian et al., 2017).

In other words, this could mean that if pulses of DOM are short, labile DOM is not converted into biomass to the same extent, as the microbial communities are highly adapted to the energy-poor conditions prevailing in groundwater. Thus, labile DOM may be retained in the gravel matrix or transported further down into the aquifer depending on hydrology and sorption capacities (Hofmann and Griebler, 2018). Fungi, especially saprotrophic taxa such as yeasts, are known to degrade substantial amounts of labile carbon (de Vries and Caruso, 2016), therefore potentially playing an important role in the degradation of this "unused" DOM fraction. Taking all of this into account, one can still only speculate about the dimension of the groundwater fungal biomass.

In situ sources of OM include microbial biofilms that form on the gravel matrix and rock surface of aquifers, byproducts of mineral-fluid interactions, chemolithoautotrophic primary production, production of secondary metabolites, fungal spores, as well as dead groundwater biota (Reitner, Schumann and Pedersen, 2006; Kagami, Miki and Takimoto, 2014; Drake and Ivarsson, 2018; Ivarsson et al., 2018). Although a certain percentage of groundwater fungal communities consist of saprotrophic species, Sohlberg and colleagues (2015) did not find a correlation between fungal diversity and activity and the input of dead organic material in their study of a deep groundwater mycobiome. This suggests that fungi potentially take on different ecological roles within the groundwater than those of pure decomposers, again emphasizing that fungi have complex life cycles and lifestyles within the groundwater habitat. This would be in line with the discovery that significant parts of fungi recovered from groundwater are parasites and pathogens (Figure 4). For example, fungal members of *Microsporidia* have been found to parasitize groundwater amphipods - an ecological role that is still awaiting a more thorough exploration (Grabner, Weber, and Weigand 2020). Other studies hint at the important role of fungi sustaining anoxic (deep) subsurface environments, for example, by recycling fossil DOM stocks (Perkins et al., 2019). Here they act as producers of substrates, such as small organic compounds or hydrogen gas that are readily available for autotrophic processes of other members of the microbial loop (Pomeroy et al., 2007; Drake and Ivarsson, 2018; Purkamo et al., 2018). Another study hints at the role fungi play in providing and transporting dissolved iron in anoxic to suboxic karstified groundwater

environments (Schwab et al., 2017). These specific metabolic abilities indicate that fungi could play a role in sustaining bacterial communities locally, where *Bacteria* are known to play an essential role in OM decomposition and biomass generation and effectively contributing to groundwater biomass themselves (Watkinson, Boddy and Money, 2015).

Where do groundwater fungi originate from?

The question of what the source of fungi recovered from groundwater forms is not so easy to answer. Nevertheless, aquatic fungi in general and groundwater fungi, in particular, can be divided into two broader groups. These are "resident fungi" and "transient fungi". Resident fungi are those specimens that can complete their life cycle in the aquatic realm and are not encountered in terrestrial habitats. In contrast, transient fungi are those that enter the aquatic environment from terrestrial sources and can adapt well to local conditions or remain inactive in the form of spores (Shearer et al., 2007). For instance, a robust adaptive capacity is exhibited by groundwater fungi found in karst aquifers, which make up the majority of the fungal OTUs found in these landscapes. These are presumably transported from the surface into the groundwater system, facilitated by fractures, underground drainage systems, as well as the large voids of karst during recharge events (Nawaz et al., 2016). The presence of terrestrial fungi in aquatic systems (transient fungi) raises a relevant question about the ability of different fungal taxa to adapt to the aquatic environment.

A recently published study on subterranean aquifers has shown the presence of various fungal OTUs of the terrestrial origin or known to be parasitic fungi of various crops in the active fraction of groundwater fungal communities recovered (Nawaz et al., 2018). This raises the question of whether and how the fungi have undergone specification in groundwater and whether obligate groundwater species exist at all. With the current state of information available on groundwater fungi, these questions are difficult to answer unless there are hypothesis-driven studies on these topics either in natural settings or in well-designated lab experiments.

Taxonomic composition and systematic overview of groundwater fungi

Fungal taxonomic and functional diversity in groundwater habitats is manifold, although there is currently no systematic overview of groundwater species. Keeping the fungal tree of life (Figure 2) in mind, fungal taxa recovered from groundwater habitats so far are mostly affiliated with pathogenic and/or saprotrophic lineages, and a major part of them belong to Dikarya (*Ascomycota* + *Basidiomycota*) (Figure 4). Wurzbacher and colleagues (2020) found that around 8% of the fungal diversity recovered from volcanic springs was made up of early diverging lineages of fungi, and a significant part belonged to yet unexplored yeast lineages. Yeasts, exclusively occurring within Dikarya, are especially remarkable, as they

could be recovered from basically every habitat of the world (McLaughlin and Spatafora, 2015; Rojas-Jimenez et al., 2020).

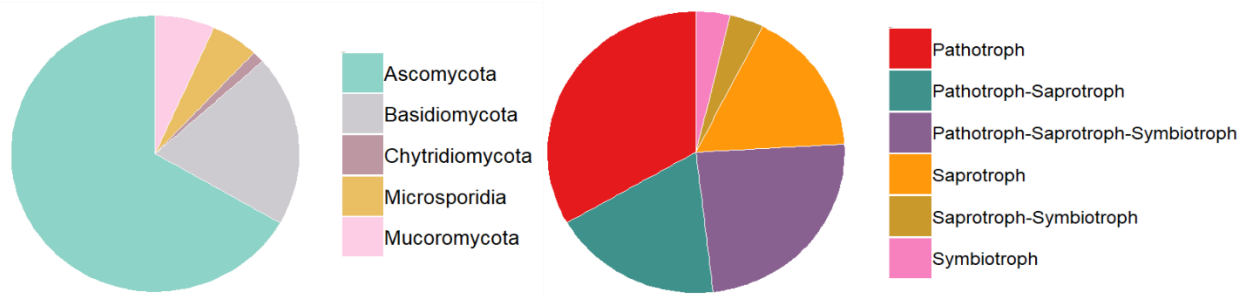

Figure 4 Taxonomic affiliation on phylum level (left) and their trophic state (right) of 78 fungal taxa that represent the most commonly recovered fungi from groundwater habitats so far, summarized from several environmental sequencing studies (Ekendahl et al., 2003; Euringer and Lueders, 2008; Fester, 2013; Livermore and Mattes, 2013; Jasrotia et al., 2014; Sohlberg et al., 2015; Bohu et al., 2016; Hoque and Fritscher, 2016; Nawaz et al., 2018; Purkamo et al., 2018; Wurzbacher et al., 2020; Grabner, Weber and Weigand, 2020). Trophic states of taxa were annotated, where possible, with FUNguild (Nguyen et al., 2016).

Early diverging lineages

The following paragraphs attempt to address the ecology and systematics of the individual fungal phyla against the background of groundwater ecosystems. According to most recent phylogenetic studies, the branch diverging earliest in the fungal tree is the clade uniting members of *Cryptomycota*, and *Microsporidia* which are, according to most recent phylogenetic studies, sister to the rest of the fungi. This clade is found to be almost as old as the entire fungal tree of life, and emerges mostly from environmental sequencing efforts, most often in marine ecosystems, but also from groundwater (Richards et al., 2012; Letcher et al., 2018; Grabner, Weber and Weigand, 2020; Wurzbacher et al., 2020). Many cryptomycetes and microsporidians are endoparasites, for instance, of other fungi or fungal-like organisms (Gleason et al., 2012). Cryptomycetes have likely secondarily lost their chitin cell wall – a feature most members of the fungal kingdom share – in certain stages of their life cycle, such as during host infection (Jones et al., 2011; Richards et al., 2012; James et al., 2013). They also seem to have a degenerate mitochondrion, emphasizing the parasitic signature this group of early diverging fungi shares. Members of *Microsporidia* are also intracellular parasites that have evolved to infect a wide range of organisms, from insects to mammals, including aquatic species (McLaughlin and Spatafora, 2014). Microsporidians have to harvest their energy from a host as they have lost their mitochondria in the course of evolution, being left only with a reduced version called a mitosome (James et al., 2013).

Members of another ancient branch of fungi comprised of zoosporic chytrids and blastocladiomycetes have been found within groundwater ecosystems (Jasrotia et al., 2014; Sohlberg et al., 2015; Nawaz et al., 2018). The emergence of zoospores within this aquatic sister group to the “terrestrial fungi” once again underlines their adaptation to the aquatic realm. They are ubiquitous in various habitats, the majority of

them being found in water. They have also been recovered from highly challenging environments, such as hydrothermal vents in the deep ocean (Le Calvez et al., 2009). Nawaz and colleagues (2018) discovered that the chytrid fraction made up the third-largest phylum detected in their groundwater study site. In the groundwater, chytrids are mainly parasites of other fungi or invertebrates. In surface water ecosystems, they also infect amphibians, and plants, including algae and phytoplankton. Some orders within Chytridiomycotina are also known to saprotrophically feed on various structural polymers (e.g., keratin and chitin). Monoblepharidomycetes, on the other hand, are exclusively saprotrophic and form a distinct clade, which is attributable to their oogamous sexual reproduction (McLaughlin and Spatafora, 2014). Neocallimastigomycetes are obligate anaerobic species, which can be found in terrestrial and aquatic anaerobic environments but are best known for their occurrence in the guts of ruminant herbivores. In the course of evolution, they came forth with a degenerate version of a mitochondrion that allows them to break down complex compounds, such as cellulose, by fermentation. Thus, together with facultative anaerobic fungal species, which include some members of *Blastocladiomycota*, they have the potential to sustain oxygen-deprived groundwater environments (Moore, Robson and Trinci, 2011; Risse-Buhl et al., 2013).

Fungi belonging to the *Zoopagomycota* and *Mucoromycota* are thought to only occur sparsely within aquatic habitats. On the other hand, these claims are potentially obsolete as it is now well established that this group of fungi is underrepresented in environmental sequencing efforts, primarily due to molecular biases. They are non-flagellate fungi and known to comprise the earliest terrestrial lineages that are important to understand the transition of fungi from water to land (Heitman et al., 2017). Members of *Zoopagomycota* are mostly predators (for instance, of nematodes and their eggs, or amoebae), parasites (e.g., of other fungi), but also obligate symbionts, for instance, in the gut of arthropods (Heitman et al., 2017; Spatafora et al., 2017). Mucoromycetes are predominantly characterized by their associations with plants, for example saprotrophic, or mycorrhizal, and their most recent ancestors probably gave rise to the modern fungus-plant relationships. Evidence in favour of the theory of a molecular bias towards these lineages of fungi is given by Grossart and colleagues (2019), who name this phylum as one of the dominant lineages recovered from groundwater aquifers.

Dikarya

Most sequences recovered from groundwater metabarcoding efforts are affiliated with Dikarya, many of them yeast or yeast-like taxa (Figure 4). In other words, they are probably dominating the subsurface hydrosphere from what we know so far. The largest fungal phyla are *Ascomycota*, containing around 65,000 described species, and *Basidiomycota*, which encompasses approximately 31,000 described species. All known yeast species are placed within Dikarya but do not form a monophyletic group. Yeasts

consist of those taxa that are known to grow vegetatively by budding or fission, and they are usually single-celled, although there exist exceptions that can produce hyphae. They can be free-living, attached to the substratum, or living within a host. Besides that, the majority of members of Dikarya grow in filaments on all kinds of organic substrates, such as carbohydrates, some being able to function well even under oxygen-deprived conditions (facultative anaerobes). Many members cannot be unambiguously placed within existing phylogenetic clades (Jones, Hyde and Pang, 2014), and a significant number of described species within this group have their binomial name only linked to their asexual state, and the description of sexual morphs is often missing.

Moving from unknowns to knowns of aquatic fungal diversity

As in the previous sections, we have established that aquatic fungi are highly diverse in their taxonomic composition and functional abilities. Unlike the conventional microbiology tools such as microscopic or direct observations of different fungal spores, the molecular-based environmental surveys employing metabarcoding techniques (e.g., NGS) helped to explore unusual aquatic fungal niches, for instance, deep-sea sediments, subseafloor, hydrothermal vents, and terrestrial groundwater. However, apart from the environmental sequences that could be taxonomically assigned, a significant proportion of the sequences remained unclassified, a common observation in most aquatic fungal surveys. Likewise, from DNA and RNA-based analysis of groundwater samples from terrestrial aquifers, Nawaz and colleagues reported that a significant proportion of fungal sequences remained unclassified (Nawaz et al. 2018 and 2019). Such high proportions of unclassified fungi detected in the sequence-based datasets from the aquatic habitats are primarily due to the lack of their representation in the reference databases (Grossart et al. 2019, Heeger et al. 2019, Hibbett et al., 2011) and Grossart et al. (2016) has termed these unknown/unclassified aquatic fungi as “fungal dark matter - FDM” – the composition and ecology of which is largely unknown.

At present, the fungal databases are dominated by sequences from two major lineages, namely *Ascomycota*, and *Basidiomycota* with little representation of early diverging lineages, such as *Cryptomycota*, *Chytridiomycota*, and *Zoopagomycota*. This explains the possible reason for a considerable proportion of unclassified aquatic fungal query sequences against the available databases. One way forward to resolve the problems associated with fungal dark matter is the unambiguous communication of such unclassifiable fungal species (Kõljalg et al., 2020), or the application of single-cell omics, which involves the lysis and subsequent single-cell whole-genome sequencing of uncultured fungi. In 2018, a team of scientists from the Joint Genome Institute (JGI) and the University of Michigan has successfully amplified the whole genomes of a group of uncultivated fungi (Ahrendt et al., 2018). Application of such

methods in aquatic fungal diversity studies will help to expand the current scope of information of aquatic fungi from mere pieces of barcoding regions of fungal DNA to whole genomes of the representatives of different fungal lineages, which consequently not only help to catalog the early diverging fungal species but also to understand their evolution and unique metabolic potentials.

Outlook on fungal groundwater diversity and their protection

In aquatic ecosystems, both organic and inorganic matter sources are altered by influences such as human activities, changed groundwater characteristics, or extreme meteoro-hydrological events (climate change) (Retter, Karwautz and Griebler, 2020), which ultimately affects the resident fungal communities. These events can also potentially influence important drivers for fungal diversity, such as pH (e.g., leading to possible heavy metal leaching), temperature, or salinity. A change in local salt concentration could have very specific consequences, as it is relatively more difficult for land and freshwater species to conquer marine habitats, and salinity, therefore, plays a fundamental role in shaping fungal diversity (Jones and Pang, 2012). To better protect fungal communities in groundwater and the aquatic realm, there is an urgent need to improve the understanding of their diversity, as well as functions they provide, to protect their habitats, to act against climate change, to reduce anthropogenic influences on water bodies, such as the unrestricted use of fertilizers and fungicides in agriculture, and to accelerate the description and accurate classification of yet undescribed fungal species.

Conclusion

In this chapter, we underlined the diversity and capabilities of groundwater fungi as well the bottlenecks and way-outs in studying groundwater fungi using next-generation molecular platforms. There is a general agreement that fungi in terrestrial habitats are well studied and researched compared to aquatic ecosystems and that terrestrial fungi can be described as both richer and more diverse. However, the advent and application of new molecular biological tools in the field of mycology has revolutionized the approach and understanding of mycologists towards aquatic fungi. Despite this technological progress, groundwater mycologists are still faced with fragmented, or distorted information and literature on certain fungal groups and taxa, while research on others is entirely lacking.

It is worth noting that although groundwater represents only a small fraction of the water available on the planet, nearly 1.6 billion people worldwide depend on this underground reservoir for domestic, agricultural, and industrial purposes. From this context emerges a lack of consensus on the occurrence of diverse fungal taxa in groundwater, as the focus lies mostly on disease-causing taxa or few others that provide potentially interesting functions for biotechnology. All of this gives rise to an incomplete puzzle of groundwater mycology.

Conversely, it can be concluded from the available information on groundwater fungi that, in terms of species diversity and occurrences, we are currently only scratching the tip of the iceberg. A coherent message from recently published literature on groundwater fungi using the most-recent molecular methods (based on sequencing) is that largely, fractions of unknown fungi represent the mycobiome of groundwater. This indicates a significant knowledge gap regarding the contribution of groundwater fungi to the sustainability of such a unique ecosystem, along with their evolution, ecology, metabolic properties, and interaction potential with other organisms in the aquatic groundwater food web.

Knowledge gaps

- Accounting for the proportion of fungal respiration in groundwater.
- Estimating the proportion of fungal biomass in comparison to other microorganisms, such as *Bacteria*.
- Exploring ecological roles that fungi cover within groundwater and defining their contributions to the groundwater food web, carbon, and nutrient cycling dynamics, as well as quantifying their turnover rates.
- Determining fungal diversity and formally describing novel fungal species living in groundwaters to reduce the number of "dark taxa" and accurately assess the full taxonomic and functional potential of fungi in groundwater.
- Investigating the fungal spore hydrodynamics and their dispersal in groundwater.
- Determining the number of obligate and facultative groundwater species.

Important case studies

Name of site	Country	Coordinates	Main topic/concept covered	Key reference
Marching spring	Germany	48.820217, 11.715458	biodiversity survey; mercury-removal function analyses	(Hoque and Fritscher, 2016)
Former gasworks site	Germany	51.222720, 6.817301	biodiversity survey; microeukaryote- targeted PCR/T-RFLP fingerprinting	(Euringer and Lueders, 2008)
Former uranium mining district	Germany	50.854577, 12.147187	Mn(II)-oxidizing microbial communities structures	(Bohu et al., 2016)
Äspö Hard Rock Laboratory	Sweden	57.433191, 16.660323	Cultivation, phenotypic, and phylogenetic analysis of yeasts	(Ekendahl et al., 2003)
Constructed wetland	Germany	51.325078, 12.027310	Arbuscular mycorrhizal bioremediation; phylogenetic analysis	(Fester, 2013)
	Germany,		Microsporidian diversity survey;	(Grabner, Weber and Weigand,

	Belgium, Luxembourg, France, Poland, Great Britain, Ireland, The Netherlands, Czech Republic		barcoding	2020)
OakRidge Integrated Field Research Challenge	USA	35.979929, -84.290411	biodiversity survey; ITS sequencing	(Jasrotia et al., 2014)
Deep Archaean bedrock fracture aquifer	Finland	64.218147, 29.944645	biodiversity survey; metabolic functionality	(Purkamo et al., 2018)
Collaborative Research Centre AquaDiva	Germany	51.112186, 10.436933	rDNA, rRNA ITS sequencing	(Nawaz et al., 2018)
Deep saline groundwater	Finland	61.236213, 21.478502	rDNA, rRNA ITS sequencing	(Sohlberg et al., 2015)
Groundwater springs along volcanic zone	Iceland		biodiversity survey; ITS sequencing	(Wurzbacher et al., 2020)

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Relevant internet resources

- 'The Unite database' https://unite.ut.ee/
- 'Mycocosm – A fungal genomics resource' https://mycocosm.jgi.doe.gov/mycocosm/home
- 'The FUNguild database' http://www.funguild.org/
- 'Global Biodiversity Information Facility' https://www.gbif.org/
- 'The fungal database of the Species 2000 project' http://www.speciesfungorum.org/
- 'FungiDB – an integrated Bioinformatic Resource for Fungi and Oomycetes' https://fungidb.org/fungidb/

'The International Commission on the Taxonomy of Fungi' https://www.fungaltaxonomy.org/

Chapter IV: Metabarcoding reveals ecologically distinct fungal assemblages in river and groundwater along an Austrian alpine to lowland gradient

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Abstract

Biodiversity, the source of origin, and ecological roles of fungi in groundwater are to this day a largely neglected field in fungal and freshwater ecology. We used DNA-based Illumina high-throughput sequence analysis of both fungal gene markers 5.8S and internal transcribed spacers region 2 (ITS2), improving taxonomic classification. This study focused on the groundwater and river mycobiome along an altitudinal and longitudinal transect of a pre-alpine valley in Austria in two seasons. Using Bayesian network modeling approaches, we identified patterns in fungal community assemblages that were mostly shaped by differences in landscape (climatic, topological, geological), and environmental conditions. While river fungi were comparatively more diverse, unique fungal assemblages could be recovered from groundwater, including typical aquatic lineages such as *Rozellomycota* and *Olpidiomycota*. The most specious assemblages in groundwater were not linked to the input of organic material from the surface, and as such, seems to be sustained by characteristic groundwater conditions. Based on what is known from closely related fungi, our results suggest that the present fungal communities potentially contribute to mineral weathering, carbon cycling and denitrification in groundwater. Furthermore, we were able to observe the effects of varying land cover due to agricultural practices on fungal biodiversity in groundwater ecosystems. This study contributes to improving our understanding of fungi in the subsurface aquatic biogeosphere.

Keywords: fungi, fungal ecology, terrestrial aquifers, groundwater, hydrological cycle

Introduction

Fungi are a diverse group of heterotrophic eukaryotic organisms with a multitude of ecological roles. Recent estimates suggest that there may be up to 6 million extant species of fungi (Baldrian et al., 2021; Lücking et al., 2020). They include economically and ecologically relevant groups such as molds, mushrooms, and yeasts, as well as many less studied fungi that do not seem to produce tangible fruiting bodies. Fungal communities are ubiquitous (Debeljak and Baltar, 2023; Naranjo-Ortiz and Gabaldón, 2020) and have been reasonably well characterized in a number of habitats, such as soils, rivers, lakes, and oceans, or in association with plants or animals (James et al., 2020; Mikryukov et al., 2023). In spite of all scientific progress, fungal communities remain difficult to study due to the limited number of distinct morphological features, high physiological variation, and often cryptic ecology, especially in an ecosystem as inaccessible as groundwater (Grossart et al., 2019; Retter and Nawaz, 2022).

Terrestrial groundwater, Earth's largest reservoir of liquid freshwater, is found below the land surface, and is generally described as thermally stable. The lack of light makes groundwater oligotrophic by nature (Overholt et al., 2022), being characterized by a scarcity of organic matter. This allows groundwater organisms to maintain slow metabolic rates while exhibiting a comparatively lower productivity than surface water ecosystems (Griebler and Lueders, 2009). Nonetheless, groundwater is known to support a diverse variety of microbes, for instance *Bacteria* and *Archaea*, but also *Protozoa* (Fillinger et al., 2023; Herrmann et al., 2020; Karwautz and Griebler, 2022; Korbel et al., 2013). It provides a relatively stable environment and furthermore shelters its inhabitants from desiccation. In terms of energy, underground layers of water-bearing material (aquifers) may exhibit various redox gradients which microorganisms can exploit for their metabolic processes (Fillinger et al., 2023; Maamar et al., 2015).

To date, our knowledge of the ecological importance of fungi in groundwater, including the suitability of groundwater as a fungal habitat, their diversity and distribution, as well as the pathways of their entry, or the overall existence of resident communities is limited. Recent studies have shown that fungi play fundamental roles in the subsurface ecosystem through their widespread anaerobic metabolism (Drake and Ivarsson, 2018; Hoque and Fritscher, 2023; Müller et al., 2012), trophic relationships (e.g., symbiosis, parasitism, predation) (Bengtson et al., 2014; Galindo et al., 2018; Grabner et al., 2020), and evolutionary traits such as flagellated unicellular forms and hyphae formation (Rojas-Jimenez et al., 2017; Voigt et al., 2021). To this end, we sampled 45 groundwater wells and 14 river sites at two times of the year along a 300 km altitudinal gradient in the Austrian alps stretching from the alpine region to the lowland (i.e., the foothills of the alps). Hydrological exchange between groundwater and river systems, and subsequent

groundwater recharge is largely determined by climatic conditions, soil properties, the depth of the groundwater, and the properties of the surface of the Earth (e.g., altered by anthropogenic degradation such as increased surface sealing, or surface erosion due to intensive agriculture) (Uhl et al., 2022). Thus, fungal communities along the groundwater and river network should potentially be influenced by various landscape (present climatic, topologic, and geologic) and land use characteristics, and we are expecting corresponding patterns in their biodiversity. Understanding the ecological dynamics that shape microeukaryotes across freshwater habitats enables us to relate groundwater to an ecosystem (i.e., the river) that is both hydrologically interdependent and much better understood in terms of fungal and freshwater ecology (Barros and Seena, 2022; Grossart et al., 2019; Swan et al., 2021). Additionally, the majority of fungi have been shown to be saprotrophic, feeding on detritus in groundwater (Foulquier et al., 2011; Inkinen et al., 2019; Shamshedenova et al., 2019; Somervuori et al., 2021). We therefore propose that fungi play a vital role in the element and nutrient cycle of groundwater through their versatile metabolic and physiological characteristics. We hypothesize that the effects of local landscape characteristics and environmental conditions, e.g. as a result of hydrologic fluctuations, are reflected in the presence of locally adapted fungi (De Cáceres and Legendre, 2009). Our study addresses the complexity of fungal communities in groundwater and the adjacent river in an alpine valley and intents to unravel drivers that determine their biodiversity, ecology and distribution in the context of landscape characteristics, hydrological and environmental conditions as well as local land cover.

Materials and Methods

Study area, field sampling and data acquisition

Sampling took place in the provinces Styria and Salzburg (Austria) from June to July (spring) and October to November (autumn) 2020. 118 samples were collected in total, consisting of 90 groundwater and 28 surface water samples, including the Source of the Mur river in the Hohen Tauern national park as well as the Lassnitz and Pöls tributaries (Figure 1). To roughly categorize the river catchment which follows an altitudinal, land use, and climatic gradient, we defined three different basins which we called the alpine region, the Graz basin, and the lowland (Figure 1). To determine land cover in the Mur valley catchment at each sampling site, we used the CORINE Land Cover (CLC) data (2012–2018; https://www.data.gv.at/, accessed on 1 February 2021). For categorizing lithological characteristics, we utilized the OneGeology survey (https://onegeology.org/), with the permission of OneGeology. Bioclimatic variables of mean annual air temperature (bio1, °C), annual precipitation amount (bio12, kg m-2), and seasonal precipitation as the coefficient of variation (bio15, kg m-2) from the years 1981 – 2010 were downloaded

from CHELSA Bioclim (Karger et al., 2017) to subsequently calculate zonal statistics of our sampling locations.

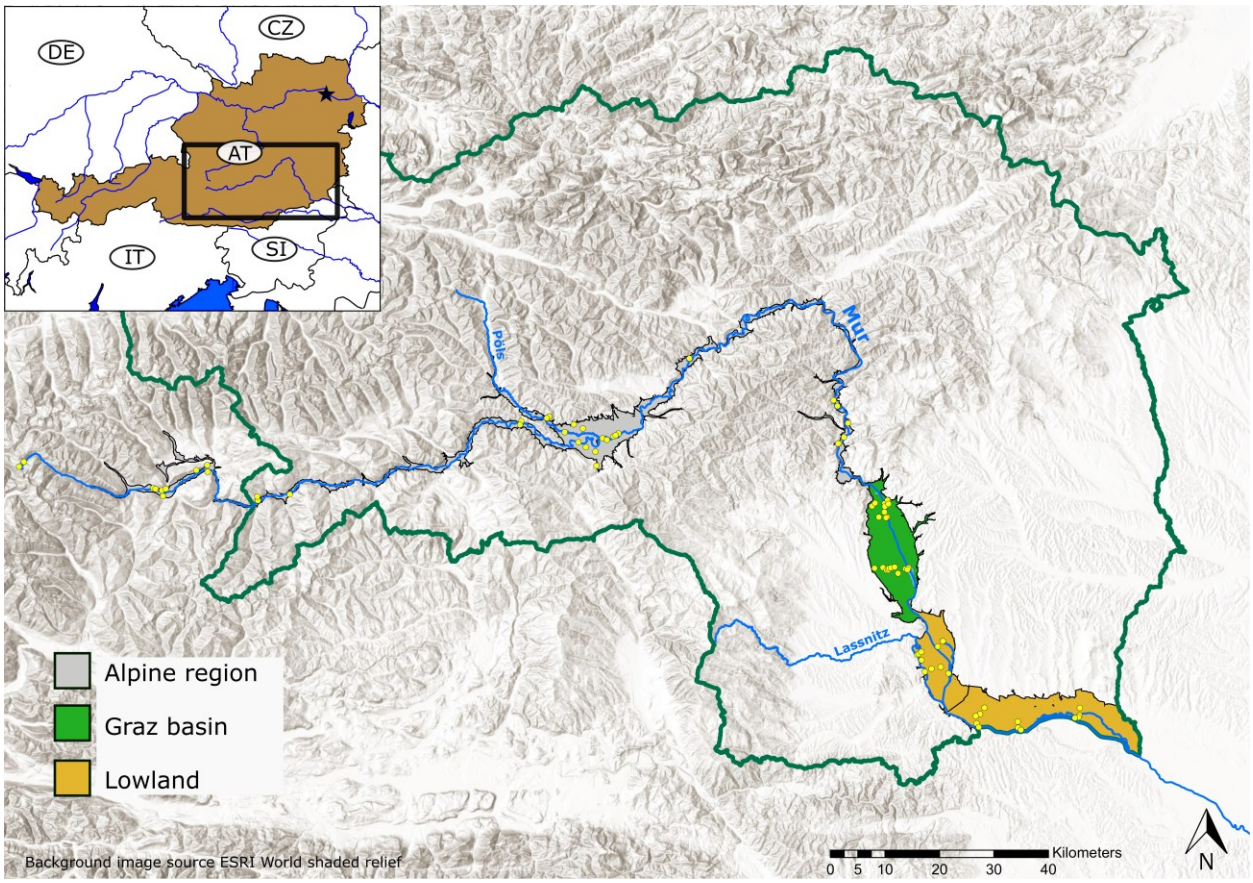

Figure 1 Map showing the sampling locations of groundwater and surface water as yellow points along the Mur valley in Styria (green boundaries) and Salzburg, as well as groundwater bodies along the valley depicted in their regional landscape types (alpine region, Graz basin with the city of Graz, and the lowland).

Sample collection for fungal communities, physicochemical parameters, - and basic microbial variables

Groundwater was withdrawn from observation wells established within the past few decades by the provinces of Styria and Salzburg to monitor groundwater quality and quantity (locations, groundwater table-, and well depths can be found in SI Table 1). Consequently, nearly all the wells from which groundwater was sampled have extensive long-term datasets on hydraulic head (groundwater table) and temperature, accessible via www.ehyd.gv.at. Groundwater was withdrawn using a Grundfos submersible MP1 pump (Eijkelkamp Soil & Water Corp., Netherlands), placed 2 meters below the groundwater table. The pumping rate maintained a maximum drawdown of 0.5 meters of water table in the well vicinity. Before sample collection, stagnant well water was purged by pre-pumping twice the well water volume and stability of key physicochemical parameters (electrical conductivity: EC, pH, water Temperature, concentration of dissolved oxygen: DO) was considered. During sample collection, the pumping rate was

reduced to avoid dislodgement of microbial biofilms and withdrawal of fine sediments. River water samples were collected from the riverbank, if possible, from the free-flowing section. Water temperature, pH, EC, and DO of groundwater and river water were measured on site using field sensors (WTW, Weilheim, Germany). All samples were filled into sterilized canisters washed with a 2% sodium hypochlorite solution and rinsed three times with MilliQ lab water, as well as with sample water. Within 48 h of sampling, in which canisters were stored in a dark cool place, a total of 10 L of groundwater and 4 L of surface water were filtered onto 0.22 µm Sterivex filters (Merck Millipore, Darmstadt, Germany) with a peristaltic pump. Filters were stored right away at -80°C until molecular analysis.

All water samples were analyzed for the concentration of major ions, nutrients (NO3-, NO2-, NH4+, PO43-), dissolved organic carbon (DOC), dissolved inorganic carbon (DIC), composition of dissolved organic matter (DOM), and water stable isotope (δ18O, δ2H) signatures as previously described in Retter et al. (2023, 2021). A detailed description of the environmental characteristics of sampling sites is also included therein. DOM was characterized in terms of different fluorescence indices, i.e., HIX (Humification Index), BIX (Freshness/Biological Index), and FI (Fluorescence Index), as well as the commonly referenced peaks obtained from aquatic DOM (i.e., Coble peaks b, t, a, m, c; Coble, 1996; Coble et al., 2014; Parlanti et al., 2000).

Nucleic acid extraction and Illumina sequencing

Genomic fungal nucleic acids were extracted in two batches using the Norgen RNA/DNA purification kit (Norgen Biotek, ON, Canada). In brief, filters were first removed under sterile conditions and cut up, and 800µL of the kit's lysis buffer was added to the filter strips in a sterile 1.5 mL screw cap micro tube containing 0.2 mL of a mix of 1:1 0.1 mm and 0.7 mm Zirconia/Silica beads (Biospec, Bartlesville, USA). Cell lysis was enhanced by incubation for 10 min at 55° C followed by bead-beating. The entire liquid was transferred to a new microcentrifuge tube and centrifuged at 14,000 x g for 2 min. The supernatant was then purified according to the manufacturer's protocol. DNA was quantified using a Qubit 4 dsDNA assay. Three negative control samples were co-extracted from sterile 0.22 µm Sterivex filters. A primer combination of forward primer ITS3-Mix2 (Tedersoo et al., 2015) and reverse primers ITS4-cwmix1 and ITS4-cwmix2 (Wurzbacher et al., 2017), annealing to the 5.8S gene and the ITS2 region respectively, were used for amplification following the PCR protocol described by Pjevac et al. (2021), with PCR conditions as described in Retter et al. (2019). Sequencing was done at the Joint Microbiome Facility (JMF) of the Medical University of Vienna and the University of Vienna on a MiSeq platform (Illumina, San Diego, CA, United States), producing 2 x 300 b paired-end sequences. Sequencing data, generated by the JMF under

Project IDs JMF-2009-1 and JMF-2106-09, were deposited under the BioProject accession number PRJNA926931.

Sequence data processing and classification

We applied a two-marker metabarcoding approach making use of a conserved region which help to anchor the ITS (Heeger et al., 2018; Lücking et al., 2020). Raw reads were processed and classified using the metabarcoding pipeline of Heeger et al. (2019) utilizing the two marker regions 5.8S and ITS2 with default settings (pipeline configurations: conflictBehavior = "mark", primerError = 0.2, maxAmplLen = 550, minAmplLen = 150). Here, operational taxonomic units (OTUs) were classified by the lowest common ancestor classification using the UNITE reference database release for eukaryotes (ITS2) (v. 9.0, 29.11.2022, Abarenkov et al., 2022) and RFAM database (5.8S) (v. 14.2, Kalvari et al., 2021). OTUs that were classified by either 5.8S or ITS2 as fungi were retained. Taxonomic annotations of the 600 largest (i.e., most abundant) OTUs were manually examined and verified as suggested by Nilsson et al. (2012) using BLAST (Altschul et al., 1997). Clustering sequences into OTUs has previously been shown to outperform amplicon sequence variants (ASVs) in regards to recovering species diversity when it comes to fungal metabarcoding datasets (Tedersoo et al., 2022).

The dataset was decontaminated in R (v. 4.3.1, R Foundation for Statistical Computing, 2023) with 'decontam' (v. 1.18.0, Davis et al., 2018) with a prevalence-based identification of contaminated sequences using a strict threshold of 0.3. Spring and autumn sequencing batches were analyzed separately against their respective negative control samples. Samples with less than 100 reads were discarded.

Statistical analysis

In this study, we aim to conduct a comprehensive biodiversity assessment of fungal communities in groundwater and adjacent river ecosystems based on high-throughput amplicon sequencing. To test for differences between groundwater and the river in terms of total estimated richness and Shannon diversity based on OTU abundances, we used the R packages breakaway (v. 4.8.4, Willis and Bunge, 2015) and divnet-rs (v. 0.2.1, Willis and Martin, 2020). Shannon diversity was calculated as effective number of species (Jost, 2006) as Hill-numbers (q = 1) and was calculated from model matrices of tested co-variates with 6 replicates, perturbation set to 0.01, and a medium abundant base taxon, otherwise using default tuning settings. To test diversity estimates between fixed combinations of the covariate-wise differences, we used the functions 'betta' and 'betta_random' incorporating the sequencing batch affiliation as a random effect, as well as computing a global p-value with an F-test using a bootstrap analysis with 10,000 iterations implemented in the function 'test_submodel' in the R package breakaway. To test for

differentially relative abundant phyla across groundwater, and the river, we used the 'differentialTest' function using a Wald test with a false discovery rate (FDR) cut-off of 5%, implemented in the corncob package (v. 0.2.0, Martin, 2017).

The interpretation of complex multidimensional sequencing datasets require the implementation of efficient dimensionality reduction techniques to effectively retrieve relevant fungal communities rather than single species/OTUs (Bálint et al., 2016; Leite and Kuramae, 2020). To that end, we employed a Latent Dirichlet Allocation (LDA) analysis, which employs a probabilistic model to identify groups of OTUs that frequently appear together i.e., communities of fungi which we term "assemblages". Here, each sample represents a mixture of assemblages computed as Dirichlet-distributed proportions and thereby reduced to a probability distribution on a fixed set of assemblages (Blei et al., 2003). This method of deconstructing samples into overlapping assemblages of co-occurring fungal taxa has been shown to be a powerful and computationally efficient model-based approach (Sommeria-Klein et al., 2021, 2020). It also enables the linkage of community composition of local communities with environmental data, a task that is frequently challenging due to the size and complexity of metabarcoding datasets. LDA is suited for this task due to its adaptability to accommodate uneven sample sizes (i.e., no prior rarefaction required) and large, sparse datasets commonly encountered in these environments (Sommeria-Klein et al., 2020; Weiss et al., 2016).

We used the R version of the pipeline for LDA analysis of metabarcoding data provided by Sommeria-Klein et al. (2021). We determined the optimal range for the number of assemblages, denoted as K, spanning from 5 to 40. This evaluation of K involved the utilization of two distinct methodologies: a) a comparison based on Akaike's information criterion (AIC) between different algorithm realizations and b) perplexity-based cross-validation. The perplexity defines the level of coherence within the dataset. For cross-validation we randomly split the dataset into 10-sample validation sets. Median perplexity across all validation sets was used to compare K values. Given the relatively unstructured nature of the groundwater dataset, we employed Gibbs sampling for LDA with 100 Markov Chain Monte Carlo (MCMC) chains, each running for 5,000 iterations. The process began with random assemblages, and concentration parameters for the distribution of assemblages over sites and for the taxonomic composition over assemblages were set to α = 0.067, and δ = 0.067 respectively. Following an initial 2,000 iterations (burn-in), we captured samples every 25 iterations during the final 3,000 iterations. We followed the approach by Sommeria-Klein et al. (2021) and used a heuristic approach to select a K value near the plateau's onset of mean perplexity (K = 35). We began by fitting the model to the entire dataset with the K value that yielded the lowest mean perplexity. We then refined this K value based on repeating the model computation with a range of K values (5 to 15, where 15 was the number of assemblages that had a

cumulative prevalence above one sample i.e., the ideal number of assemblages) and choosing the number of K (K=10) that lead to the least spatial autocorrelation, as well as retrieving similar assemblages across K values based on correlations with environmental parameters. Correlations of environmental data and assemblage distributions were done by computing the Pearson correlations between the best realization and environmental variables. Spatial autocorrelation of assemblage probabilities was assessed by computing Moran's I ('Moran.I' in the R package ape; Paradis and Schliep, 2019) on each assemblage distribution based on hydrological distances between sites. Hydrological distances were computed as network distances in meters between all sample pairs based on their sub catchment IDs created from the Mur catchment stream network with the function 'get_distance_graph' in the hydrographr package in R (Schürz et al., 2023). The stream network graph was calculated based on the Hydrography90m dataset taking flow accumulation, flow direction, drainage basins, outlets, stream distance metrics along the network into account (Amatulli et al., 2022). Zonal statistics of bioclimatic variables derived from CHELSA Bioclim were also computed in hydrographr in R with the function 'extract_zonal_stats'.

To determine how much of the changes in fungal assemblage composition across ecosystems could be explained by broader categories such as landscape characteristics (climatic variables, water temperature, lithological bedrock characteristics, topology), hydrological (hydrological distances), and environmental (DO, EC, pH, nutrients, major ions, DOM coble peaks, DOM fluorescence indices) conditions as well as local land cover, we applied a variation partitioning analysis based on distance-based redundancy analysis (dbRDA) on response matrices of Euclidean distances of assemblage probabilities between samples. Environmental variables were represented by the first 10 axes of principal components (PCs) derived from a principal component analysis (PCA) on z-score transformed environmental data using the function 'prcomp'. Climatic variables were specified according to the CHELSA Bioclim codes bio1, bio12, and bio15, as well as water temperature. Hydrological conditions were given as hydrological distances between sites based on distance-based Moran's eigenvector maps (db-MEMs) (Declerck et al., 2011). The db-MEM matrix was created with the 'dbmem' function in the R package adespatial (Guénard and Legendre, 2022). All variables were selected by forward selection using the adjusted R2 of the full db-RDA model (landscape characteristics, environmental variables, hydrology, land use) as stopping criterion with the function 'ordiR2step' (10 000 permutations). The variance inflation factor (VIFs) for each full model was checked before and collinear variables (VIF > 10) were excluded (Kleinbaum, 1978). The marginal significances of each group of variables tested were determined using the 'anova.cca' function with 10 000 permutations both implemented in the R package vegan (Oksanen et al., 2022).

Results

Different fungal diversity in river and groundwater

Based on relative OTU abundances, the river water contained a higher total estimated fungal richness ($p < 0.001$, F-stat= 237.456) as well as Shannon diversity given as effective number of species (Table 1) ($p < 0.001$, F-stat= 728.2094). It furthermore harbored a larger proportion of unique fungal OTUs (51%). Groundwater and river water shared 16% of the OTUs, and these shared fungi (i.e., generalist or transient taxa) were classified as, e.g., *Cladosporium*, *Fusarium*, *Epicoccum*, *Vishniacozyma*, *Filobasidium*, *Pyrenochaetopsis*, *Paranamyces*, and *Mrakia*. Especially *Cladosporium* and *Epicoccum* showed large log2-fold increases in relative abundance in groundwater (2.1, and 1.5, $p < 0.05$), and significant increases for *Betamyces* and *Mrakia* (-1.3, -0.95, $p < 0.05$) were observed in the river water. More generally, some fungi were significantly differently abundant in surface- and groundwater. Members of *Chytridiomycota* and *Basidiomycota* were more abundant in the river, whereas *Mortierellomycota*, *Ascomycota*, and *Rozellomycota* were relatively more abundant in groundwater ($p < 0.05$, SI Table 1). *Olpidiomycota* only occurred in groundwater (Table 1).

Table 1 The summary statistics of taxon richness and relative abundances in the different types of habitats (groundwater and river) of the most abundant fungal phyla. Total estimated richness and Shannon diversity based on OTU abundances are shown as mean values and standard errors. Read numbers of each phylum are also shown.

	Ground-water	rel. Richness %	rel. abun. %	River	rel. Richness %	rel. abun. %
tot # reads	64740			26434		
tot # OTUs	1406			1942		
Total estimated Richness	46.7 ± 9.8			240.2 ± 15.1		
Shannon diversity	142.8 ± 0.3			219.3 ± 2.8		
Ascomycota	26970	48.1	41.7	7979	41.8	30.2
Basidiomycota	9276	30.6	14.3	6738	25.4	25.5
Mortierellomycota	12562	3.3	19.4	22	0.2	0.1
Chytridiomycota	2137	2.8	3.3	8480	12.9	32.1
Rozellomycota	3314	3.4	5.1	354	3.0	1.3
Olpidiomycota	1505	0.4	2.3	1	0.0	0.0
Unclassified Fungi	8749	9.9	13.5	2808	16.2	10.6
Mucoromycota	101	0.7	0.2	9	0.0	0.0

Biodiversity of fungal assemblages across ecosystems

In this dataset, 10 distinct fungal assemblages could be recovered by LDA modeling and average assemblage probabilities across samples were the highest in assemblage 1 (~ 0.20) and decreasing towards assemblage 10 (~ 0.03) (SI Figure 2). Some assemblage probabilities differed significantly along the river valley basins (Figure 1, alpine region, the Graz basin, and lowland) as well as between

ecosystems (groundwater and river) and seasons (spring and autumn) (SI Table 2). Assemblage 10 showed significant spatial autocorrelation and was not well resolved by the model in terms of fungal taxa (i.e., all fungal OTUs present in the data had a non-zero probability to occur in this assemblage) and was therefore excluded from further discussion (SI Table 2).

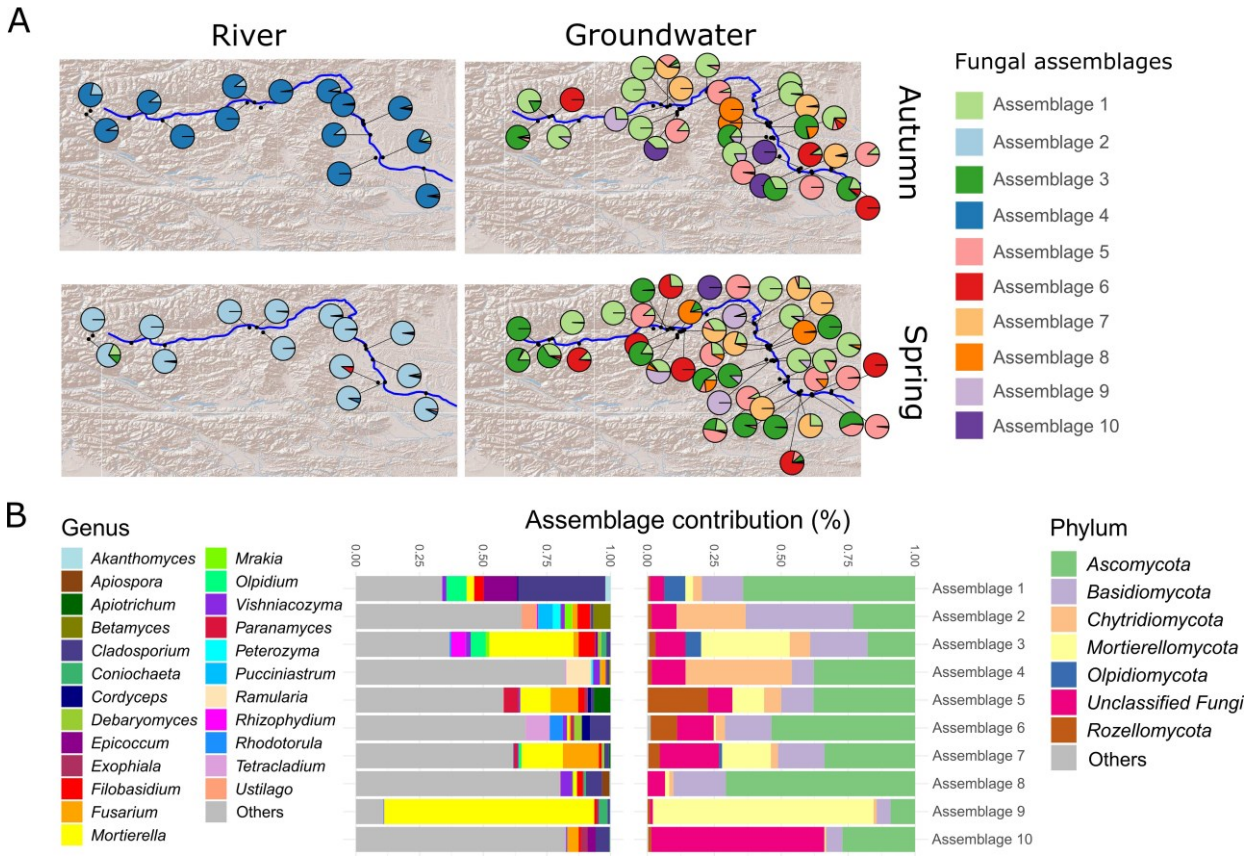

Figure 6 Groundwater and river assemblages of co-occurring OTUs along the alpine gradient. (A) Base map (ESRI World Shaded Relief) with sites depicting the relative assemblage proportion of the 10 recovered assemblages as pie charts with assemblages 1 to 10 shown in their respective colors. (B) Relative contributions of fungal taxa to assemblages on genus (left) and phylum (right) level, respectively.

Linking LDA derived assemblages to environmental drivers

Assemblage 2 was strongly associated (i.e., high probabilities) with the river during spring season (Figure 2 A, SI Table 3) and correlated positively with microbial productivity (prokaryotic cell counts and cellular ATP concentrations), pH, annual precipitation, DOC, DO, DOM peaks for protein-like coble fluorophores b (Tyrosine-like DOM) and t (Tryptophan-like DOM), and negatively with annual mean air temperature °C), major ions and conductivity, and HIX (humified DOM) (Figure 3). Autumn river fungal communities were characterized by a shift towards assemblage 4 (Figure 2), when the river system became less productive and generally lower in water temperature. Assemblage 4, in contrast to the river spring assemblage, was correlating to a lesser extent with microbial productivity and protein-like DOM, but additionally with DOM

derived coble peaks a, c (both terrestrial humic substances), and m (i.e., marine and terrestrial humic material).

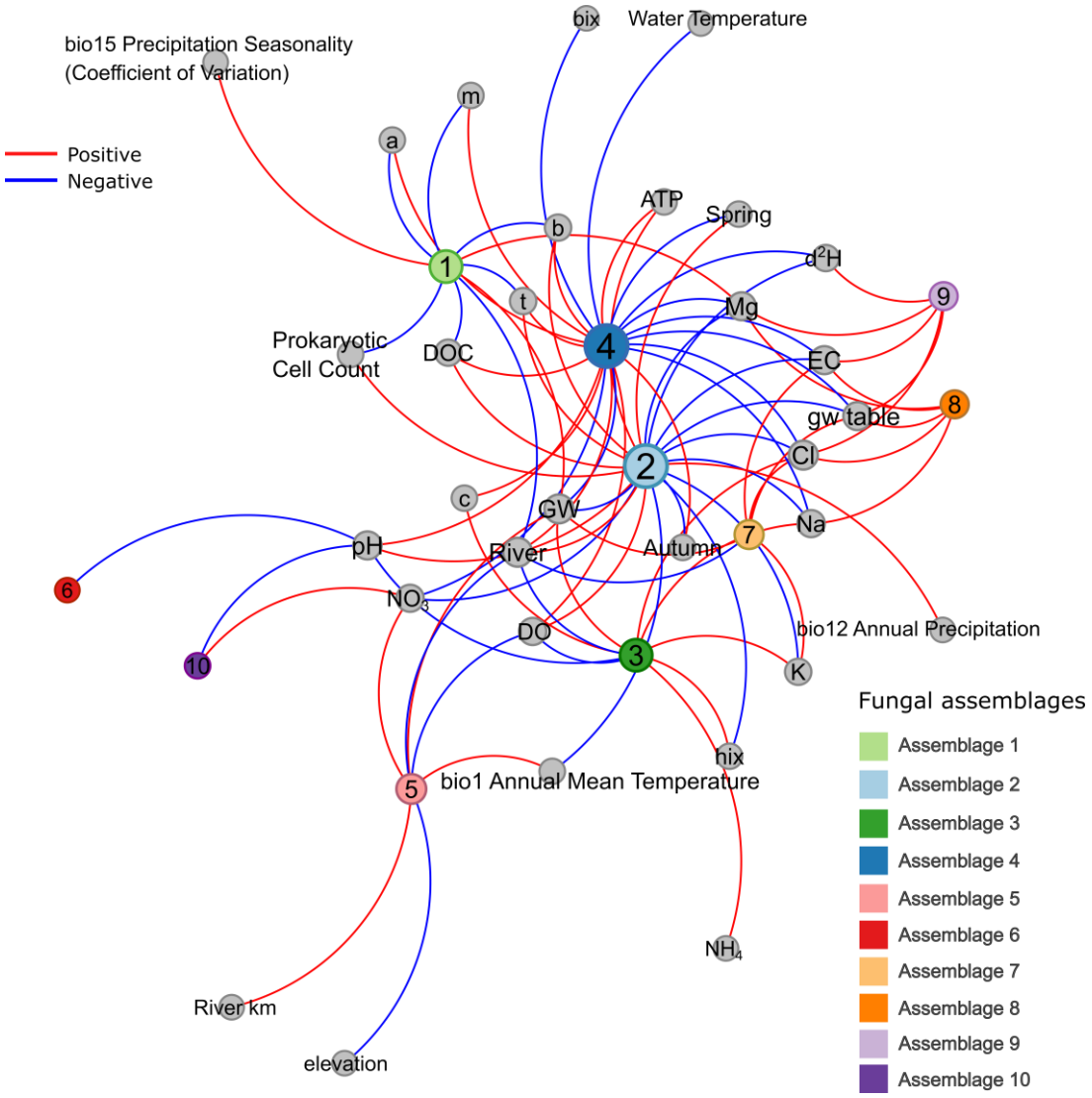

Figure 7 Network plot of assemblages correlating with environmental variables (Pearson correlation; $p < 0.05$) visualized using the Force Atlas layout in Gephi (https://gephi.org/) with blue edges depicting negative correlations and red edges depicting positive correlations. The size of nodes represents the node degree, which depicts the total number of edges (environmental variables) connected to that node. (c= coble peak c, b= coble peak b, t= coble peak t, a= coble peak a, m= coble peak m, hix= humification index, bix=biological/freshness index, δ^2H= ratio of stable hydrogen isotopes, DO= dissolved oxygen, ATP = intracellular ATP)

The assemblages 1, 3, 5, and 8 were predominantly associated with groundwater and are further characterized in the following (SI Table 3). High assemblage1 probabilities were associated with groundwater low in organic material (DOC and DOM) and located in areas with high seasonal variation in precipitation. The combined effect of season, ecosystem type, and regionality (SI Table 3) hints at this assemblage being more prevalent in autumn throughout the alpine mountain range. Assemblage 3 on the other hand seemed to predominate groundwater high in ions (Na, K, Cl), ammonium, and humified organic material of terrestrial origin (HIX, coble peak c) coinciding with a lower pH (on average 6.8) and

lower DO. This groundwater assemblage weakly coincided with the spring season (Dunn's test, with Bonferroni adjusted p-value = 0.025). In contrast, assemblage 5 was characteristic for the lowland and correlated positively with nitrate concentrations and annual mean air temperature, and negatively with DO. Assemblage 8 showed high probabilities in groundwater recovered from the Graz basin (SI Table 3), and the assembly correlated strongly with ions (Mg, Na, Cl), EC, and was predominantly recovered from deeper groundwater (i.e., low groundwater levels).

Fungal taxa linked to LDA depicted assemblages

River assemblages 2, and 4 are the most similar in terms of the presence of fungal taxa (Figure 4 B). Assemblage 2 (River spring) contains the highest diversity in terms of OTU richness (1305 OTUs), and most of the fungal taxa belong to *Ascomycota* (34.2%), *Basidiomycota* (25.7%), unclassified fungi (24.4%), and *Chytridiomycota* (11%). Common genera (i.e., high proportions) in this assemblage are *Ustilago*, *Pucciniastrum*, *Filobasidium*, *Peterozyma*, *Betamyces*, *Mrakia*, and *Aureobasidium*, where members of the genus *Pucciniastrum* are unique to this assemblage (Figure 4 A). Some of the most OTU-rich genera that occur uniquely in this assemblage belong to *Mrakia*, *Betamyces*, and *Rhizophydium*. In assemblage 4 (River autumn), fungal richness is 884, of which 48.8% are *Ascomycota*, 21.2% *Basidiomycota*, 17.1% unclassified fungi, and 8.9% *Chytridiomycota*. Genera predominantly linked to this assemblage are *Ramularia*, *Fusarium*, and *Flagellospora*. Here, members of the genus *Ramularia* present only in this assemblage are also comparatively rich in OTUs. OTU diversity further decreases towards the groundwater assemblages. Assemblage 1 richness is 411, which is the highest among groundwater assemblages. It is mainly composed of *Ascomycota* (43.1%), *Basidiomycota* (34.5%), unclassified fungi (13.4%), and *Chytridiomycota* (4.9%). Genera prevalent in this assemblage are *Cladosporium*, *Epicoccum*, and *Olpidium*, where some members of *Olpidium* are unique to this assembly, but only containing two species. The second richest assemblage in groundwater is 3 (379), which is mainly characterized by *Ascomycota* (45.1%), *Basidiomycota* (36.4%), and unclassified Fungi (10.6%). Common genera include *Mortierella*, *Olpidium*, and *Rhizophydium*, with some species of *Mortierella* and *Rhizophydium* being unique to this assemblage. In both assemblage 1 and 3 *Vishniacozyma*, *Dioszegia*, *Mortierella*, and *Filobasidium* are particularly rich in OTUs. Assemblage 5 has a fungal richness of 329 and most fungi belong to *Ascomycota* (49.8%), *Basidiomycota* (31.3%), and unclassified fungi (10.3%). Common genera are *Fusarium*, *Mortierella*, *Apiotrichum*, and *Paranamyces* with some species belonging to *Mortierella*, *Cutaneotrichosporon*, and *Exophiala* being unique to this assemblage. Here, *Exophiala*, *Mortierella*, and *Fusarium* are especially rich in OTUs. The groundwater associated assemblage with the lowest diversity (130) is assemblage 8 and comprises mainly of *Ascomycota* (45.5%), *Basidiomycota* (38.5%) and unclassified fungi (9.2%). Genera tightly linked to this assembly are *Cladosporium*, *Vishniacozyma*, and

Apiospora where *Apiospora*, *Rhizophlyctis*, and *Psathyrella* are uniquely occurring. The genus richest in OTUs is *Filobasidium*.

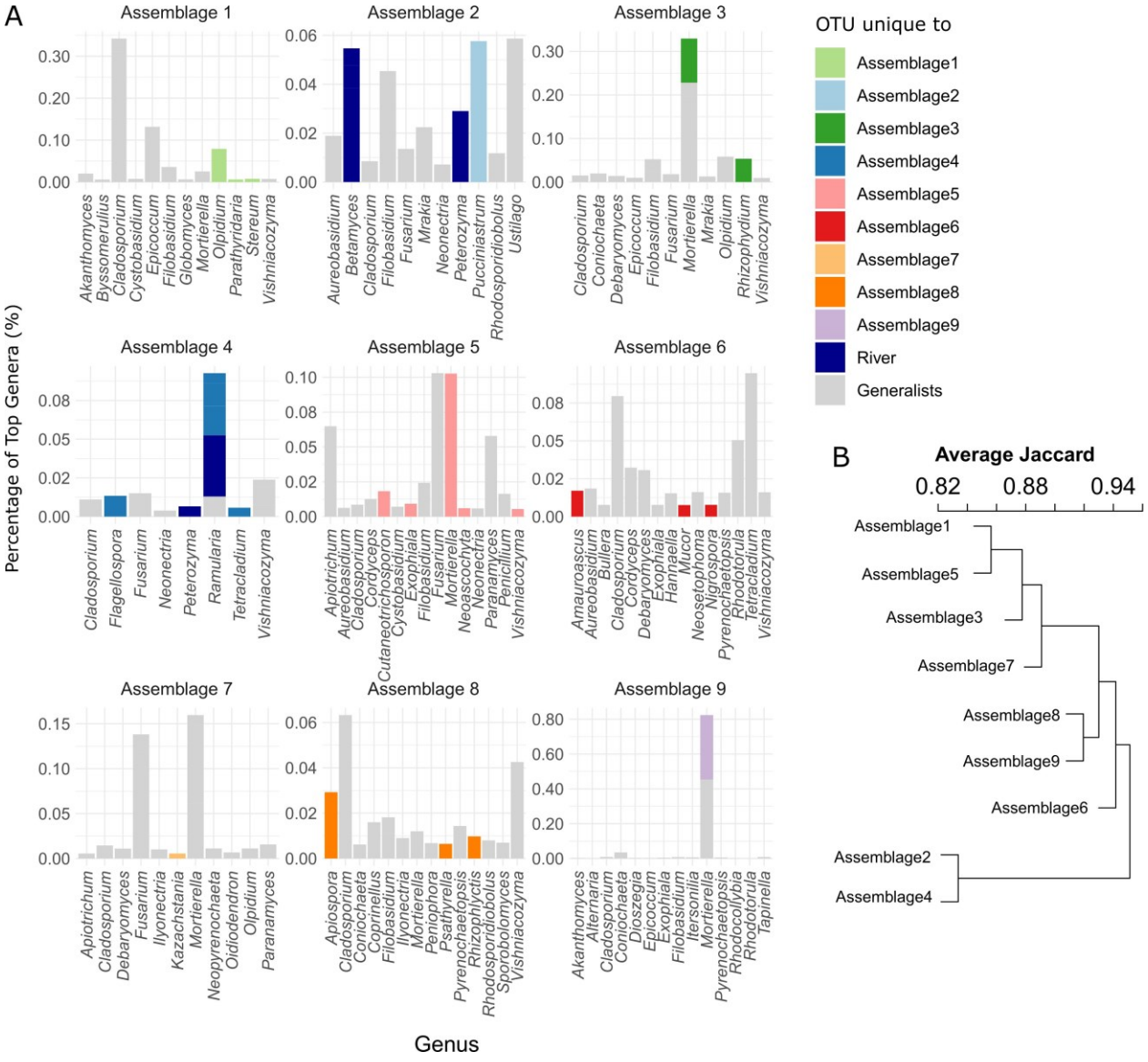

Figure 4 (A) Taxonomic composition of individual assemblages showing the most prevalent OTUs on genus level and proportions shown in respective assembly colors when OTUs are unique to an assembly, colored in grey when they occur in all assemblies (Generalists) or are colored in blue when they are unique to the river assemblies (River). (B) Dendrogram based on the Jaccard distance between assemblies computed from presence or absence of fungal OTUs in each assemblage.

Trophic modes of fungal assemblages

In this dataset, OTUs present in each assemblage were matched to growth form and primary lifestyle at genus level utilizing the database of Põlme et al. (2020). Fungal river assemblages (2, and 4) contained a larger share of OTUs not classifiable to the genus level (mean proportion = 64.4%) compared to groundwater (mean proportion = 51.6%) (SI Figure 3 A). Fungi forming filamentous mycelia as well as

dimorphic yeasts were more prevalent in groundwater assemblages compared to the river communities (Dunn's multiple comparisons, Bonferroni adjusted $p < 0.05$). Dung saprotrophs, pollen saprotrophs, and algal parasites were significantly more prevalent in river assemblages, whereas groundwater assemblages contained a relatively larger share of soil saprotrophic, animal-, and mycoparasitic fungal OTUs (Dunn's multiple comparisons, Bonferroni adjusted $p < 0.05$) (SI Figure 3 B).

Landscape characteristics shape fungal assemblages

To estimate the contribution of landscape characteristics, environmental and hydrological conditions, and local land cover to the observed distribution patterns of fungal assemblages along the river valley a variation partitioning analysis was done. When assessing the total variation explained by broader categories, landscape characteristics explained the largest portion of total variation in fungal probabilities between sites (10.8%) and was followed by environmental differences (9.5 %), and land use patterns (4.6%) (Table 2). Hydrological connectivity between sites played only a minor role.

Table 2 Partition of variation in assemblage probabilities over sites between environmental, biogeographical, and hydrological conditions, and local land cover. Group|Group+Group represents the marginal fraction of variation explained by each group after controlling for the respective others. Significance of each individual group was assessed by 10 000 permutations.

Group	Significant variables	R^2 adj	DF
environment	PC1 + PC6 + PC3 + PC4	0.176	4
landscape	Water temp + lithological char + bio15	0.105	11
land use	Land use 2nd level	0.053	12
hydrology	dbMEM1	0.009	1
environment | landscape +land use+ hydrology		0.095	4
landscape | environment +land use+ hydrology		0.108	9
land use| environment + landscape + hydrology		0.046	10
hydrology| landscape +land use+ environment		0.010	1
Residuals		0.701	

Discussion

In the present study we explored an Austrian alpine-river gradient for fungal diversity and distribution across groundwater and river water. Diversity was considered both in terms of taxonomy and ecological roles. A number of significant patterns was recovered for both ecosystem types and are discussed in detail below.

The ecology of fungal river assemblages

It is intriguing that in this study, some fungal taxa have been found exclusively in certain assemblages, potentially serving as indicators for local environmental conditions. The river community in spring (assemblage 2) was the richest in fungal taxa and exhibited a strong association with key environmental

parameters as well as with concentrations of DOC and protein-like DOM. Elevated protein-like DOM and DOC during spring have shown to be associated with the breakdown products of lignin, or leaching of exudates from leaf emergence (Singh et al., 2014). It could also correspond to recent in-stream production (i.e., phytoplankton growth) and processing of OM, or could indicate influence by human activities (i.e., a shift from more plant/soil derived DOM to more microbial/algal DOM) (Jørgensen et al., 2011; Lambert et al., 2017; Ren et al., 2021). This assemblage contained the only *Pucciniastrum* species present in this data set, *P. areolatum*, which is the causal agent of spruce rust and produces basidiospores during spring. These basidiospores are propagated by wind and are probably introduced into and spread by the river system (Zhang et al., 2021). Many species of *Mrakia* associated with this assemblage are psychrophilic and produce both yeast and hyphal states (dimorphic). They have the ability to produce cold-active pectinases and different hydrolytic enzymes, which indicates their potential to decompose organic matter (OM) in cold environments (Bezus et al., 2022). A number of *Mrakia* species have previously been reported from freshwater ecosystems, including polar regions, glacial meltwater, snow, rivers, and wetlands (Fell, 2011; Grossart et al., 2019; Jones et al., 2014). The chytrid genus *Rhizophydium* contains many zoosporic taxa known to be parasitic on Algae and Cyanobacteria (e.g., *Planktothrix*) or saprotrophic (e.g., cellulosic and keratinophilic) in soil or aquatic habitats or on pollen. This chytrid potentially coincides with phytoplankton river spring blooms (SI Figure 4) (Letcher et al., 2004; Longcore and Simmons, 2012; Maier and Peterson, 2017; Wagner et al., 2023). The zoosporic chytrid *Betamyces* was also characteristic of and specious in this assemblage, including the only known species *Betamyces americae-meridionalis*. *Betamyces* spp. have previously been reported from pollen and soil, and they have been found to be highly abundant in association with microplastics in Arctic freshwater lakes (Gonçalves et al., 2022; González-Pleiter et al., 2021; Letcher et al., 2012).

The fungal river assemblage coinciding with autumn (assemblage 4) was characterized by significantly cooler water temperatures and higher terrestrial and humic, but fewer freshly produced DOM compared to spring river conditions. It only contained one exclusive fungal genus, namely the plant pathogen *Ramularia*, above all its species *R. miae* and *R. unterseheri*. *R. miae* was first observed causing black leaf spots in a bloodwort species native to South Africa, but has since been isolated from multiple hosts (Videira et al., 2015a). *R. unterseheri* is also plurivorous, often recovered from dead or alive leaves of *Fagus* (Videira et al., 2015b). Most plants undergo changes in growth and physiology during autumn and are therefore more vulnerable and susceptible to Ramularia infections, probably leading to higher prevalence of infections and completion of the pathogen's life cycle and the dispersal of spores during that time. An increase in terrestrial organic and humic substances can be linked to an increased import of usually fungi-rich substrates (e.g., leaf litter - a primary source of allochthonous OM in rivers and streams) during

autumn and winter (Chrismas et al., 2023). Indeed, we found that the remaining extant unique genera were well-known leaf-litter decomposers and/or leaf inhabitants (i.e., "hyphomycetes", *Flagellospora*, *Tetracladium*, *Tetrachaetum*, *Moellerodiscus*), some of them being sensitive to water pollution (Baschien et al., 2013; Charcosset and Gardes, 1999; Duarte et al., 2012; Ghate and Sridhar, 2015; Pietryczuk et al., 2018). The most specious genera in this assemblage, *Dioszegia*, and *Vishniacozyma*, belong to psychrophilic/psychrotolerant, halotolerant and/or osmotolerant yeasts often recovered from glacial, cold, and/or arid regions from e.g., meltwater, groundwater and volcanic springs (Buzzini et al., 2018; Jones et al., 2014; Nuppunen-Puputti et al., 2021; Wurzbacher et al., 2020). They are known for instance to inhabit soil rich in organic matter, and potentially contribute to nutrient cycling and the decomposition of diverse types of organic compounds (Abu-Mejdad et al., 2019; Maeng et al., 2022).

The prevalence of dung and pollen saprotrophs as well as algal parasites in the river is compatible with the available OM sources and hosts. River systems are heavily impacted by their surrounding environments (through land cover), and they are typically characterized by high primary production. Dung and pollen saprotrophs benefit from the influx of organic matter from terrestrial sources, while algal parasites thrive in environments with abundant host biomass. Taxonomic classification to genus level was only possible for around 36% of the OTUs from the river assemblages, probably due to the high abundance of early diverging fungi (i.e., Chytridiomycota) in the river known to fail during ITS classification, as well as their underrepresentation in the UNITE database (Heeger et al., 2019).

The ecology of fungal groundwater assemblages

The specific environmental conditions shaping the specious groundwater assemblage 1 follows the regional distribution pattern of the number of rainy days. A higher coefficient of variation in seasonal precipitation indicates a larger variability in precipitation throughout the year due to convective precipitation (i.e., increased rain and storm events) along the mountain range compared to the lowland. This is also corresponding to the existing weather gradient between the rainy north and the less rain-rich south of the alpine region, and in the south-eastern foothills of the Alps to a decrease in rainy days with distance from the mountain range (Wakonigg et al., 2008). Higher variation in precipitation can potentially lead to increased hydrological mixing in groundwater, promoting the dispersal and introduction of fungal propagules (e.g., spores or filaments), and subsequently a higher fungal diversity. The assemblage was also typical for groundwater largely deprived of protein-like, terrestrial, and aquatic humic OM (e.g. stream, and algal DOM), possibly due to dilution effects (Coble et al., 2014; Foulquier et al., 2010; Søndergaard et al., 2003). Unique fungal taxa were identified as *Olpidium brassicae*, *Stereum hirsutum*, *Parathyridaria clematidis*, and *Pseudotaeniolina globosa*. O. brassicae is a unicellular, zoosporic plant endoparasite, often in the roots of vascular plants and known to be transmitted when water is

recirculated and exchanged (Runia, 1994). *S. hirsutum* is a lignicolous white-rot fungus which is known to produce a wide range of metabolites (terpenes, acetylenic aromatic, alkyl and benzoate derivates) (Liu et al., 2023; Pu et al., 2021). Not much is known about *P. clematidis* on the other hand, even though a few other members of the genus have previously been isolated from the marine environment (Dayarathne et al., 2020; Poli et al., 2020). *P. globosa* is a known rock inhabiting fungi that is meristematic and melanized and associated with mineral weathering (Necropolis et al., 2021).

The second richest assemblage in groundwater (assemblage 3), was characterized by elevated ammonium concentrations under more reducing conditions and lower pH, possibly due to denitrification of infiltrated nitrate as a result of the application of fertilizers, and irrigation (Huang et al., 2016; Korbel et al., 2022). Additionally, potassium and recalcitrant, aromatic, fulvic-like DOM (coble peak c) was comparatively higher in groundwater coinciding with assemblage 3. This DOM component seems to be the major form of DOM found in groundwater throughout the year and could be elevated in spring due to the input of terrestrial derived OM following snow melt (Harjung et al., 2023). Its degradation in a reducing environment could additionally lead to increased ammonium levels in groundwater (Huang et al., 2021). Unique fungal taxa in this assemblage belonged to the genera *Mortierella*, and *Rhizophydium*, the latter being more variable than what was previously understood (James et al., 2006; Longcore, 2004; Sigee, 2005). Species rich genera present in both groundwater assemblages 1 and 3 were members of the yeast genera *Dioszegia*, and *Vishniacozyma*, as well as *Mortierella*, including *M. alpina*. The latter is an oleaginous species that accumulates a diverse range of fatty acids, comprising of up to 50% of its dry weight (Wang et al., 2011). It has previously been found in association with soil, seaweed, and peatlands (Asemaninejad et al., 2017; Gonçalves et al., 2023; Poli et al., 2022). More generally, members of *Mortierella* are saprotrophic, known for decomposing labile carbon. They are commonly isolated from soil and have previously been identified as one of the dominant genera in groundwater (Asemaninejad et al., 2017; Nawaz et al., 2018; Nuppunen-Puputti et al., 2021).

Assemblage 5 was indicative of reduced groundwater influenced by agricultural land use, as suggested by high nitrate concentrations at these sites and the fact that these land use practices intensify in the lowland, spatially coinciding with increased assemblage probabilities (Graeber et al., 2012) (Figure 4 A). Here, unclassified members of *Peniophora* were particularly rich in taxa. *Peniophora* encompasses corticioid fungi with lignolytic and antiprotozoal activities, some of them causing white-rot, but also found to be associated with beetles and sea grass (Esser, 2018; Heitman et al., 2017; Valmaseda et al., 1990; Xu et al., 2023). Moreover, dominant taxa, such as members of *Fusarium*, *Apiotrichum*, and *Mortierella*, are known for their nitrate utilization capabilities, and fungal denitrification is indeed a major pathway in natural systems, sometimes exceeding bacterial denitrification capacities (Shoun et al., 2012;

Takaya, 2002; Zhong et al., 2022; Zuo et al., 2023). The oleaginous yeast *Cutaneotrichosporon*, unique to this assemblage, has been shown to utilize a broad spectrum of especially aromatic compounds for fatty acid biosynthesis (Yaguchi et al., 2020). These are compounds that most *Bacteria* are not capable of degrading. Some species of *Cutaneotrichosporon*, as well as the other exclusively associated fungal genus with assemblage 5, *Exophiala*, may furthermore be parasitic on animals, including humans (Aliyu et al., 2020; Najafzadeh et al., 2018).

The last groundwater assemblage (8), which was also lowest in species diversity, coincided with higher concentrations of ions and lower groundwater levels in the Graz basin, suggesting a community locally adapted to infiltration of, e.g., road salt, industrial discharges, or effluents from local waste-water treatment systems (Becher et al., 2022; Howard and Gerber, 2018). Fungi unique to this assemblage were for example *Apiospora marii*, and *Rhizophlyctis*, specifically *R. rosea*. *A. marii* could previously be isolated from stems and roots of plants causing plant dieback and wilt (Agustí-brisach et al., 2023). *R. rosea* is one of the most ubiquitous cellulose degrading zoosporic soil chytrids, and their presence might suggest a dynamic groundwater-surface exchange in urban areas (Jones and Bennett, 2014; Longcore and Simmons, 2012). Since it produces motile zoospores, we propose that the vadose zone could serve as a potential habitat for this chytrid, as long as oxygen is abundant (Gleason et al., 2019; Willoughby, 2001).

The overall high prevalence of soil saprotrophic fungi in groundwater could putatively be explained by ongoing exchange processes of groundwater with the surface via the soil. We think it is likely that soil fungi may find a suitable habitat in groundwater, or the vadose zone, contributing to ongoing ecosystem processes with their unique, versatile metabolic capabilities. This would have to be further investigated by targeting not the total but the active fungal communities in groundwater via e.g., RNA sequencing or omics approaches. Our results agree with previous findings that filamentous fungi, as well as fungi that form yeast stages are more often found in groundwater compared to the river system (Afonso et al., 2021; Wurzbacher et al., 2020). The fact that animal pathogens (some of which are also known to infect humans) are primarily present in groundwater affected by intensive agriculture should raise concerns regarding their increased transport facilitated by habitat fragmentation and human use. These pathogenic fungi are possibly diluted in streams after their introduction via fertilization with manure, whereas they can reside in groundwater for a prolonged period of time.

Landscape characteristics drive differences in fungal assemblages

Our analysis showed that local landscape characteristics, including climatic conditions that translate into river-, and groundwater temperatures, bedrock lithology, as well as precipitation patterns were detrimental factors in shaping fungal communities across groundwater and river ecosystems. Differences in local environmental conditions, as well as variations in land use were additional drivers. Even though

lithological conditions do not seem to be a major driver for bacterial communities (Maamar et al., 2015), we show that they are significantly shaping fungal assemblages in groundwater. For instance, some fungal species are known for their granite weathering capabilities and different rock surfaces have been found to harbour a diverse selection of fungal communities (Knudsen and Vesterholt, 2008; Li et al., 2022; B. Liu et al., 2022; Nuppunen-Puputti et al., 2021).

In conclusion, our study revealed significant differences in fungal diversity and relative abundances of fungal groups between groundwater and the river. Unlike bacterial communities, where biodiversity in groundwater exceeds that of surface waters, fungi are less likely to establish diverse communities in groundwater (Ji et al., 2022; Retter et al., 2023). We attribute this difference both to the restricted exchange of groundwater systems with the land surface and surface waters, and to the reduced input of organic material compared to riverine environments. While it is apparent that fungal communities in groundwater contain numerous degraders of organic matter and that fungal diversity was relatively high in groundwater receiving terrestrial input in the form of humic and fulvic OM, the most specious assemblage was existing at sites containing lower OM. This, in turn, is likely attributable to the highly variable precipitation and storm events at these sites, possibly diluting organic material and at the same time introducing transient fungi into groundwater. The presence of transient fungal taxa found in both groundwater and the river supports the notion of ecological connectivity between these two contrasting ecosystems. Even though spanning both ecosystems, OTUs belonging to *Cladosporium*, and *Epicoccum* seem to be abundant in - and characteristic of - groundwater while *Dioszegia*, and *Vishniacozyma* are found across ecosystems in comparable proportions (Afonso et al., 2021; S. Liu et al., 2022; Nawaz et al., 2018; Perkins et al., 2019). Moreover, parasitic fungi regularly recovered from aquatic ecosystems are likely to require a certain amount of hosts - mostly phototrophs - and are therefore absent in the groundwater environment (Frenken et al., 2017; Klawonn et al., 2023; Van den Wyngaert et al., 2022). Our study emphasizes that a consistent and diverse group of fungi perceives the vast, energy limited, and light deprived groundwater as a suitable habitat and that these fungi could be linked to specific environmental conditions, corresponding ecological functions, and niches.

Funding

This work was supported by the ÖAW (Österreichische Akademie der Wissenschaften), grant number ESS-Project 2018: 'Integrative Groundwater Assessment'.

Conflict of interest. None declared.

Acknowledgements

We thank Peter Rauch, Barbara Stromberger (Steiermärkische Landesregierung), Harald Huemer, and Margot Geiger-Kaiser (Salzburger Landesregierung) for their valuable information and help in selecting appropriate groundwater sampling wells. We also thank Lucas Fillinger, Constanze Englisch, Filipa Sousa, Grit Rasch Hubert Kraill, Santiago Gaviria for their help and support during sampling, lab work, and data analysis.

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Chapter V: Application of the D-A-(C) index as a simple tool for microbial-ecological characterization and assessment of groundwater ecosystems – a case study of the Mur river Valley, Austria

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Retter, A., Griebler, C., Haas, J., Birk, S., Stumpp, C., Brielmann, H., Fillinger, L., 2021. Application of the D-A-(C) index as a simple tool for microbial-ecological characterization and assessment of groundwater ecosystems—a case study of the Mur river Valley, Austria. Österreichische Wasser- und Abfallwirtschaft 73, 455–467.

Abstract

The assessment and monitoring of the ecological quality and status of groundwater is a timely issue. Currently, various assessment tools have been developed that now await application and validation. One of these, the D-A-C index, evaluates the microbiological-ecological quality of groundwater by means of prokaryotic cell counts, microbial activity measurements, and the qualitative characterization of dissolved organic carbon (DOM). The purpose of this paper is to introduce the various ways of application of the D-A-(C) index making use of a recently collected data set (n=60) originating from the river Mur

valley, Austria. First, we introduce an extension of the D-A-(C) index by including measurements of dissolved organic matter quality (DOM) derived from fluorescence spectroscopy as additional variables to supplement the analysis of microbial cell density and activity levels. Second, we illustrate how the definition of a reference status for a 'good' microbiological-ecological state can improve the analysis and allow for a more sensitive and accurate detection of impacts on groundwater ecosystems. Based on our results, we advocate that the analysis be performed by making use of expert knowledge for the definition of reference sites to which target sites are to be compared.

Keywords: aquifer; surface water; ecological monitoring; ecosystem health; microbial total cell counts; microbial intracellular ATP; dissolved organic matter fluorescence spectroscopy.

Introduction

Besides being a vital resource, groundwater is the largest, yet still mostly unexplored freshwater ecosystem on our planet, harboring a rich diversity of microbial and metazoan life (Gibert and Culver, 2009; Griebler and Lueders, 2009). These organisms are key to ecosystem services provided by groundwater, including, among many others, the attenuation of anthropogenic contaminants and pathogens (Griebler and Avramov, 2015; Herman et al., 2001). Such ecosystem services are directly linked to human welfare and the health of all groundwater-dependent ecosystems (Griebler et al., 2014a). At about 20-30% of the global land surface, shallow groundwater is feeding surface waters, wetlands and the roots of plants (Ribeiro et al., 2013). These shallow groundwater bodies are particularly vulnerable to impacts from the surface such as hydrometeorological changes, rising sea water levels, as well as anthropogenic pressures like intensive agriculture (Khan et al., 2018; Lee et al., 2019; Menchen et al., 2017), industrial activities (Brielmann et al., 2009; Rehman and Cheema, 2016; Singh et al., 2016), and urbanization (Hassane et al., 2016; Howard and Gerber, 2018; Minnig et al., 2018). In addition, surface waters that receive wastewater and which are subject to hydrological extremes such as flood events may carry pollutants from the surface into aquifers, thereby seriously compromising groundwater quality (Corada-Fernández et al., 2017).

It is these shallow groundwater systems that are also of greatest ecological interest. Shallow groundwater bodies are more likely to receive sufficient energy from the surface (mostly in the form of dissolved organic matter (DOM)) to sustain active microbial and metazoan communities, and are therefore home to most of the biodiversity found in groundwater habitats (Hubalek et al., 2016; Ward et al., 2017). However, despite early reports on groundwater biodiversity (Spandl, 1926), until recently groundwater was primarily viewed as a commodity in the eyes of policymakers, with little consideration for groundwater

biodiversity and ecosystem health (Griebler et al., 2010). Yet, the conservation and protection of groundwater as a unique habitat forms the basis for maintaining its diverse ecological functions on which mankind is ultimately highly dependent (e.g. water provided for drinking, irrigation, industrial processes) (Griebler and Avramov, 2015).

Legal guidelines for groundwater monitoring have almost exclusively focused on the assessment of physico-chemical and hygienic parameters, whereas frameworks demanding assessments of ecological parameters, comparable to those defined for surface water ecosystems including the EU Water Framework Directive (EU WFD, 2000/60EC, European Commission, 2000), have long been lacking. Yet, policymakers in different parts of the world have started to become increasingly aware of the inextricable link between groundwater quality and ecosystem health, which has led to new environmental directives incentivizing the incorporation of ecological parameters in routine groundwater monitoring practices. Prominent examples of such directives come from Switzerland and Australia, as well as the EU (SWPO, 1998; NSW-SGDEP, 2002; EPA, 2003; EU-GWD, 2006). Despite these developments, ecological assessments have not been widely implemented in routine groundwater monitoring so far, because reliable and validated monitoring criteria and tools have long been unavailable.

Recent research efforts have started to fill this gap by developing practically applicable toolboxes and assessment schemes that target the different biological facets of groundwater ecosystems ranging from microorganisms to metazoan groundwater fauna (Hahn, 2006; Steube et al., 2009; Korbel and Hose, 2011, 2017; Griebler et al., 2014b; Di Lorenzo et al., 2020). Prokaryotic microorganisms are particularly suited as monitoring targets in groundwater ecosystems, as they are ubiquitous and occur under environmental conditions that exclude metazoan fauna, such as naturally anoxic groundwater. We have recently developed the D-A-(C) index (in German termed B-A-(E) Index) as an approach to detect biological disturbances of groundwater ecosystems based on the microbial parameters cell density (D), activity (A) measured as intracellular ATP levels, and, the quantity and/or quality of organic carbon (C) as an optional parameter (Griebler et al., 2018; Fillinger et al., 2019). The D-A-(C) index combines all parameters in a multivariate outlier analysis yielding a single index value that flags individual outlier samples in a dataset as potentially ecologically disturbed. One of the main advantages of this approach is that its parameters are fast and easy to measure and its universal applicability is independent from environmental conditions. Furthermore, the multivariate nature of the approach allows for fully exploiting the data of the measured parameters, and we showed that it enables a more robust and sensitive detection of outliers indicative of ecological disturbances in groundwater compared to univariate outlier analyses applied to each microbiological parameter separately (Fillinger et al., 2019). As an additional advantage, the D-A-(C) index can be easily extended by additional variables, without compromising the simplicity of the analysis,

as the signals of multiple parameters are integrated into a single index value that is easy to interpret. For instance, we previously showed how concentrations of microbially assimilable organic carbon (AOC) derived from batch incubation assays (Hammes and Egli, 2005) can increase the sensitivity for detecting impacts from agricultural land use on groundwater ecosystems (Fillinger et al., 2019).

The detection of an ecological disturbance, as well as the assessment of the groundwater ecosystem condition into 'good' or 'impaired', depends critically on the definition of a regional or even local reference. Yet, due to the heterogeneous nature of groundwater ecosystems, defining a 'good' ecological reference status can be challenging. To date, the definition of such references has been severely hampered by the lack of robust and long-term datasets on key ecological variables. For this study, we analyzed a dataset that covers key parameters that are easy to come by, i.e., excluding molecular methods. These consisted of microbiological, physico-chemical, and hydrological information of 46 groundwater samples, 13 river water samples, and 2 samples from springs, collected all along the Austrian part of the river Mur, from its highly alpine origin (~2000 m asl (above sea level)) to its exit to Slovenia (~200 m asl). Criteria considered in these analyses include the individual water types, sub-regions (combining information on groundwater bodies and geology), and possible land use impacts, among others. This study strives to assess how the definition of a natural reference groundwater status affects the outcome and sensitivity of the D-A (C) analysis. In other words, the reliable assessment of the microbiological-ecological quality of groundwater and the extent to which groundwater systems are 'impaired' largely depends on what is defined as an intra-regional pristine reference status. The aim is to determine the natural state of groundwaters within each sub-region, to detect intra-regional outliers, and to explore possible reasons for disturbances (e.g., influence of groundwater-surface water interactions) based on this extensive data set.

Furthermore, we further explored the usefulness of DOM quality indices derived from fluorescence spectroscopy as promising additional parameters to be included in the D-A-(C) index. Fluorescence spectroscopy of DOM has previously been used mainly to examine shifts in DOM composition in surface water systems with season, changing environmental conditions, or in response to anthropogenic impacts, (Benk et al., 2019; Coble et al., 2014; Harjung et al., 2020). Moreover, it has been used to study risks of fecal contamination in groundwater and drinking water systems (Frank et al., 2018; Nowicki et al., 2019; Sorensen et al., 2015, 2020). Fluorescence spectroscopy of DOM is simple and inexpensive, and thus DOM indices are highly suitable for the incorporation into routine monitoring practices. Ohno (2002) and Huguet et al. (2009) have introduced two indices for the characterization of fluorescent DOM, i.e. the humification index (HIX) and the biological index (BIX). HIX provides information on the degree of humification of DOM, while BIX represents the degree of freshly produced, autochthonous, protein-like

DOM. Due to the typically low biological productivity of oligotrophic groundwater environments, the bulk of the DOM is usually composed of recalcitrant, humic compounds derived from the surface with a comparatively small contribution of fresh microbially produced DOM (Gooddy and Hinsby, 2009; Hofmann et al., 2020; Shen et al., 2015). Furthermore, we hypothesize that in general the increased inputs of organic matter from the surface can cause changes in groundwater DOM quality either directly, or indirectly via the stimulation of biological productivity. This can be caused, for example, by intensive agriculture (e.g., application of manure) or heavy rain events that cause pulses of DOM passing to shallow aquifers through seepage water. Therefore, combining information on DOM quality with information on prokaryotic cell densities and microbial activity can be a promising avenue for further increasing the sensitivity of the D-A-(C) index approach.

Finally, to demonstrate the versatility and sensitivity, as well as the limitations of the D-A-(C) index, the dataset at hand comprising microbiological, physico-chemical, and hydrological data was analyzed in three different ways: (1) using an unguided approach where we did not make any assumptions about the ecological condition of the groundwater, but simply calculated the D-A-(C) index based on the full dataset regardless of location, or land use categories; (2) the data were analyzed at the resolution of sub-region and land use type to take into account regional differences within the data; and finally (3) we illustrate a case where prior expert knowledge of sites (information on reference is available) is used to guide the analysis.

Materials and methods

Site description and sample collection

The sampling was conducted in the federal provinces of Styria and Salzburg, Austria, during a five-week sampling campaign from June to July 2020. Samples (N = 61) were collected from groundwater wells, springs, and from the river Mur, as well as two tributaries, covering a section of around 300 km and an altitude gradient of around 1800 m (Fig. 1). The river Mur passes through the southeastern part of the province of Salzburg, with its spring located at approximately 2000 m asl, and through most of Styria, until it passes the Slovenian Border at around 200 m asl. Over this course, it passes through differing geological and morphological settings as well as different aquifers distinguished into eight groundwater bodies, in the following termed sub-regions.

From the border of the Tauern-window at its origin, the river Mur passes through various – mostly metamorphic – units of the Austroalpine nappes until it reaches Neogene sediments south of the city of Graz as well as in some of the inner alpine basins, most notably in the Aichfeld. The aquifers following the river Mur are (glacio) fluvial valley fillings, mostly comprised of gravel and sand, locally with bodies of

alluvial fan or rockslide sediments. The transversal and vertical dimensions of the Mur valley aquifers range from narrow valleys (e.g., the Murdurchbruchstal, where the valley is sometimes only a few hundred meters wide), to deep and wider, inner-alpine basins, and finally broad but shallow foreland basins (Fig. 1). The alpine glaciations played a considerable role for the formation of the aquifers, either directly due to the glaciation of the approximately upper third of the Mur valley upstream of the Aichfeld or indirectly by the formation, erosion, and re-sedimentation of terraced sediments in most of the downstream aquifers. The investigated region of the Mur valley can therefore be divided into eight sub-regions (Fig. 1), roughly correspond to the delineated groundwater bodies characterized by different hydrological and geological settings (Haas and Birk, 2017).

The study area covers different land use types, which include areas of intense agriculture in the Alpine foreland in the south of Styria, the industrial and commercial areas south of and around the city of Graz, urban areas in Graz, as well as sites used mainly for forestry and grassland farming in the peripheral mountainous and alpine regions of Styria and Salzburg. The land use types were classified into three levels using the Digital Atlas of the Province of Styria, which makes use of the CORINE Land Cover Database from 2018 (www.landesentwicklung.steiermark.at/cms/beitrag/12723062/142970647/). The lowest level of characterization in the CORINE data base was used for further evaluation of the data (i.e., agricultural area, urban area, and forest and semi-natural area). Most of the groundwater wells sampled in this study fall into the category 'Agricultural use' (30 sites), and each of the sub-regions contain some groundwater sites of this type of classification. 'Forests and semi-natural areas' (6 sites) were only covered by wells belonging to the lower Mur valley, Lungau (including the Mur source in the Hohe Tauern mountains), and one well in the Murdurchbruchstal. The category 'Urban areas' (12 sites) included wells in the lower Mur valley, Grazer Feld, and Aichfeld.

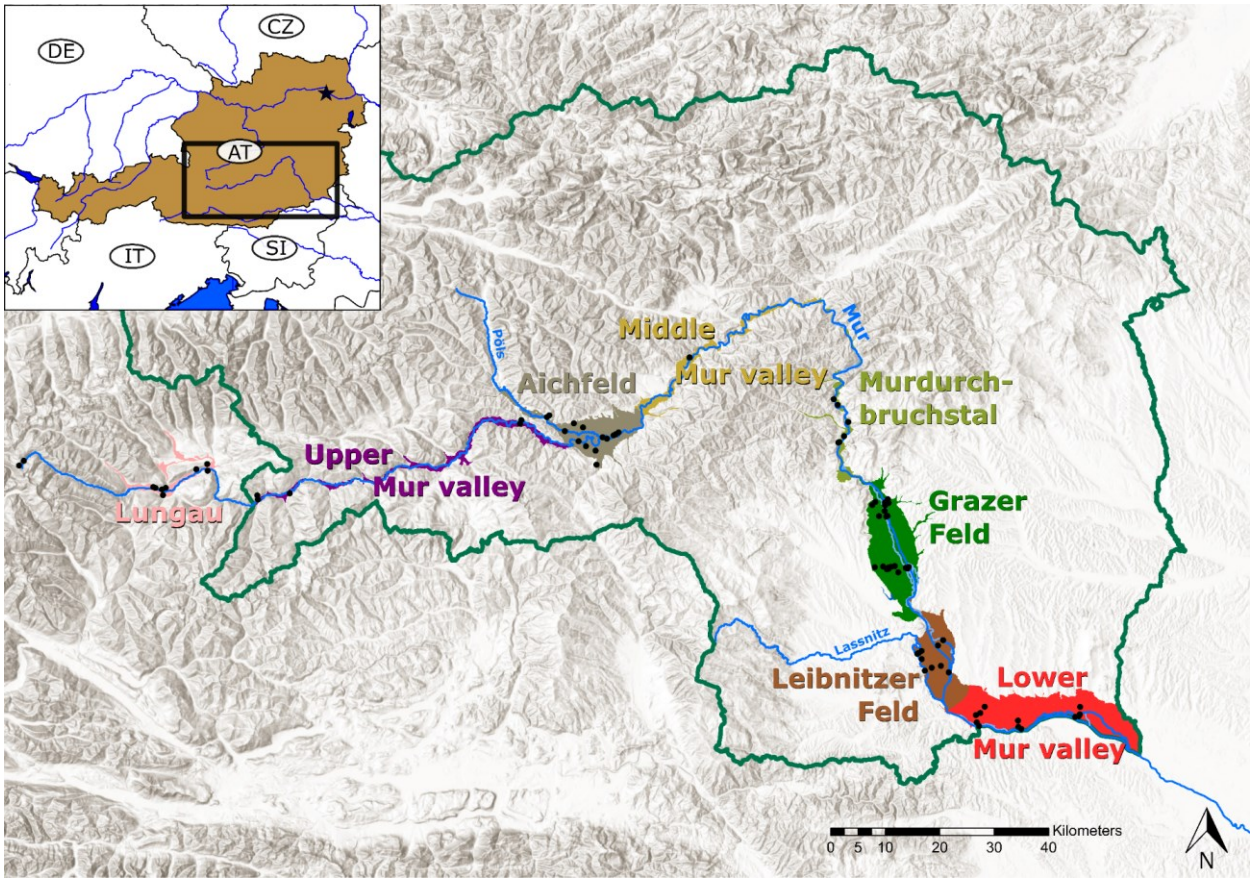

Figure 1: Location of the study area within Styria (green boundaries), and Salzburg, as well as the sub-regions within the Mur valley (The Upper Mur valley, Lungau, Aichfeld, Middle Mur valley, Murdurchbruchstal, Grazer Feld, Leibnitzer Feld, and Lower Mur valley). The position of sampling sites of groundwater and surface water along the river Mur and its tributaries is indicated by black dots.

Groundwater sampling

Groundwater was sampled from already existing observation wells, which were installed within the last decades by the provinces of Styria and Salzburg for the purpose of groundwater quality and quantity monitoring. Therefore, for almost all the sampled groundwater wells long-term data series on hydraulic head (groundwater table) and temperature are available (www.ehyd.gv.at). Prior to the investigations, these long-term monitoring data were used to select appropriate spots and wells to be sampled in the different sub-regions. Groundwater was withdrawn by a Grundfos submersible MP1 pump (Eijkelkamp Soil & Water, Giesbeek, Netherlands) which was placed 2 meters below the groundwater table. The pumping rate was set to allow a maximum drawdown of the water level of 0.5 m. Before sample collection, stagnant well water was purged by pre-pumping twice the well water volume until reaching stability in key physico-chemical parameters (EC (Electric Conductivity), pH, Temperature, DO (Dissolved Oxygen)). Water temperature, pH, electrical conductivity (EC), and concentration of dissolved oxygen (DO) were measured on-site using field sensors (WTW, Weilheim, Germany). During subsequent sample collection, the pumping rate was reduced to avoid dislodgement of microbial biofilms and withdrawal of fine sediments.

Groundwater, and surface water samples for the determination of microbial ATP, concentration of major ions, and stable water isotope ratios were filled into autoclaved glass bottles. For total prokaryotic cell counts, samples were collected in sterile 15 mL Falcon tubes and fixed with 0.5% (v/v, final concentration) glutardialdehyde on-site. Samples for DOC measurement were filtered through a 0.45 µm PVDF syringe filter (STARLAB International, Hamburg, Germany) into acid-washed (5% HCl) glass vials and were immediately acidified to a pH ≤ 2 with HCl. Samples for fluorescence spectroscopy of DOM were passed through a baked 0.7 µm glass fiber filter (Whatman GF/F; GE Healthcare Life Sciences, Little Chalfont, UK) into pre-combusted glass vials (450°C, 4 h). All samples were kept in the dark at below 4-8°C until further analysis.

Laboratory analyses

Determination of total prokaryotic cell counts (cell density, D)

The total number of prokaryotic cells in the water samples was quantified in an Amnis CellStream Flow Cytometer (Luminex, Austin, TX, USA) equipped with a 488 nm blue light laser. Settings of the different detector channels were as follows: forward scatter, side scatter, trigger channel laser (488 nm) all at 100%, speed 'high' (14.64 µL min^{-1}), recording everything, and counting the gated area depending on the nature of the water sample (e.g., groundwater has a low cell density with usually only a few hundred events sec^{-1}, whereas surface water can have up to a few thousand events sec^{-1}), but measuring for a minimum of 10 sec. If necessary, samples with more than 2000 events sec^{-1} were diluted. To distinguish intact prokaryotic cells from damaged cells or inorganic particles, prokaryotes were stained with SYBR Green I nucleic acid stain (Invitrogen, Darmstadt, Germany) at a volume ratio of 1:10000 and incubated for 13 min at 37°C. Cell counts were conducted in technical duplicates. Each replicate was measured at three different settings for events sec^{-1} (e.g., 100, 500 and 1000 for groundwater samples). Total cell counts (Cells mL^{-1}) were calculated using the Amnis CellStream Acquisition and Analysis software.

Determination of microbial intracellular ATP (activity, A)

Microbial intracellular ATP concentrations were determined using the BacTiter-Glo Microbial Cell Viability Assay (Promega, Madison, WI, USA) based on the protocol by Hammes et al. (2010) with modifications as follows. The assay reagent (prepared following the manufacturer's instructions) and samples were pre-warmed separately to 37°C for 3 min before mixing 180 µL of sample with 20 µL of reagent in a 96 well plate. The plate was incubated for 20 sec at 37°C while shaking at 600 rpm in on Thermomixer (Eppendorf). The luminescence signal was measured in a GloMax Navigator plate reader (Promega) with an integration time of 0.3 sec. Concentrations were determined against external ATP standards dissolved in ATP-free water. To correct for the contribution of extracellular ATP in the samples, each sample was

measured in two separate fractions, one fraction of the sample was centrifuged for 30 min at 21000×*g* and 4°C to spin down cells such that the resulting supernatant contained only extracellular ATP. A second fraction was not centrifuged to obtain the total ATP concentration in the sample. The concentration of intracellular ATP was calculated by subtracting the concentration of extracellular from total ATP. Each sample fraction was analyzed in technical triplicates.

Determination of DOC concentrations and DOM fluorescence spectroscopy (carbon, C)

DOC concentrations were measured with a TOC-L Analyzer (Shimadzu, Kyoto, Japan), using a 25 ppm TOC calibration curve standard, and additionally measuring MilliQ water, as well as control samples with a defined DOC concentration for quality control.

DOM fluorescence spectra from water samples were captured in an Aqualog spectrofluorometer (Horiba Scientific, Kyoto, Japan) using a quartz cuvette with a 1 cm optical path length and the Aqualog software version 3.6. Emission spectra were recorded from 212.53 to 621.80 nm at 1.64 nm increments at excitation wavelengths from 230 to 480 nm (4 nm increments) with an integration time set to 3 sec. Samples were measured against MilliQ water as a blank to subtract water Raman fluorescence peaks and convert fluorescence intensities to Raman units normalized relative to Raman scattering band intensity. The two DOM indices HIX and BIX were calculated from the EEM matrices according to Ohno, (2002) and Huguet et al. (2009) based on Equations 1 and 2, respectively:

$$HIX = \frac{\sum\left(I_{Ex:254nm;Em:435nm\rightarrow480nm}\right)}{\sum\left(I_{Ex:254nm;Em:300nm\rightarrow345nm}\right)} \quad \text{(Eq. 1)}$$

where *I* is the fluorescence intensity summed over the indicated range of emission wavelengths measured at excitation wavelength of 254 nm.

$$BIX = \frac{I_{Ex:310nm;Em:380nm}}{I_{Ex:310nm;Em:430nm}} \quad \text{(Eq. 2)}$$

where *I* is the fluorescence intensity at the indicated excitation-emission wavelength. Normalization of fluorescence spectra and computation of HIX and BIX were done in R (v 4.0.3; R Core Team, 2020) using the 'staRdom' package (v 1.1.14; Pucher et al., 2019).

Stable water isotope measurements and determination of hydrochemical parameters

Water samples were analyzed for ratios of $^{18}O/^{16}O$ and $^{2}H/^{1}H$ using laser spectroscopy (Picarro, L2140-i). All samples were referenced to the VSMOW-SLAP (Vienna Standard Mean Ocean Water-Standard Light Antarctic Precipitation) scale, and results are reported as the ratios of isotopes in the delta notation as δ-value (‰). Precision of the instrument (1σ) was better than 0.15‰ and 0.6‰ for $\delta^{18}O$ and $\delta^{2}H$.

Major ion concentrations (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, SO_4^{2-}) were measured by ion chromatography (Dionex ICS-1100 RFIC; Thermo Scientific, Idstein, Germany) following standard norms (OENORM DIN EN ISO 14911, DIN EN ISO 10304-1). NO_3^- and NH_4^+ concentrations were determined by photometric measurements (UV-method in the filtrate (λ = 220 nm); APHA 4500-NO3/B, and Indophenol blue method in filtrate (λ = 655 nm): DIN 38406-5, OENORM ISO 7150-1).

Determination of the MWWI

The indicator test for municipal wastewater (MWWI) is based on the presence/absence of selected typical municipal wastewater derived compounds, i.e. acesulfame, tolyltriazoles, carbamazepine, benzotriazole, sotalol, metoprolol, 10,11-dihydro-10,11-dihydroxycarbamazepine (CBZ-DiOH) and diclofenac. All compounds were measured by direct injection in a LC-MS/MS system (Waters Xevo TQS) using a Waters Acquity UPLC BEH C18 1,7µm 2,1x 50mm column and MilliQ water with 0,1% HFA as eluent. Acesulfame was analyzed in negative mode, while benzotriazole, carbamazepine, 10,11-dihydro-10,11-dihydroxycarbamazepine (CBZ-DiOH), diclofenac, metoprolol, sotalol and tolyltriazole were analyzed in positive mode. Based on the comparison of measured concentrations with mean concentrations of these compounds in wastewater effluents and literature values, the municipal wastewater index (MWWI) classified samples into 4 categories (0 – no influence, 1- influence unlikely, 2 – influence likely, 3 – influenced by municipal waste water).

Data analysis

Essentially, the approach to assessing our data using the D-A-(C) index is a multivariate outlier analysis. Under normal undisturbed conditions, samples are expected to display a certain distribution in a multivariate space along the variables included in the analysis. 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, and the covariance matrix determining its shape and slope. A disturbance that causes severe changes in one or more of these variables increases the distance of the affected sample to the center of the ellipse beyond the range of distance expected due to random variation. In other words, samples affected by a disturbance can be considered outliers. The distance of a single sample *i* to the center of the multivariate distribution is calculated as the Mahalanobis distance (*MD*) according to Equation 3:

$$MD_i = \sqrt{(X_i - \mu)' \times S^{-1} \times (X_i - \mu)} \quad \text{(Eq. 3)}$$

where X_i is a vector with the values of the individual D-A-(C) variables for a single sample, and μ and S^{-1} are the vector with variable means and the inverse of the covariance matrix, respectively, calculated from the full dataset.

Because *MD* takes values of the square root of chi-squared distribution with as many degrees of freedom as variables included in the analysis (e.g. df=2 if only microbial cell density and activity are included), statistical thresholds for *MD* can be defined above which a sample is considered a significant outlier at a given confidence level. For instance, 97.5% of the values of a chi-squared distribution with df=2 are <7.38. Hence a sample with *MD* larger than the critical value of in a two-parameter analysis would be declared an outlier at a 0.975 confidence level.

However, calculating *MD* from a full set of raw data directly is problematic, because outliers present in the data can severely distort the estimates of mean values (μ) as well as variance and covariance (S) of the data. To overcome this problem, the D-A-(C) index is based on robust estimates of μ and S that are calculated using the algorithm for fast estimation of the minimum covariance determinant (Fast-MCD) by Rousseeuw and Van Driessen (1999), which can provide reliable robust estimates of μ values and S in datasets contaminated with potential outliers (for details see: Hubert and Debruyne, 2010; Rousseeuw and Van Driessen, 1999). The D-A-(C) index is then simply the *MD* calculated for a given sample based on these robust estimates. Robust estimates of μ and S for the calculation of the D-A-(C) index were computed using the implementation of Fast-MCD in the 'covMcd' function of the 'robustbase' R package (v 0.93-6; Maechler et al., 2020). All variables were $\log_{10}$-transformed prior to the calculation to yield a normal data distribution.

Testing the sensitivity of the D-A-(C) Index with three approaches

The data set at hand was analyzed in three different ways:

(1) First, we took an unguided approach to identify differences within this large data set of different water body types. We did not make any assumptions about the ecological condition of the groundwater and simply applied the Fast-MCD algorithm to the full dataset regardless of location, or land use categories. The D-A-(C) was subsequently calculated for each sample based on the resulting robust estimates of μ and S.

(2) As a second approach, we analyzed the data for each sub-region, and land use type separately to take into account regional differences within the data, as we have previously shown that multivariate D-A-(C) fingerprints in groundwater can vary between different geographic regions (Fillinger et al., 2019).

(3) Finally, we illustrated a case where prior expert knowledge of sites is used to guide the analysis. For this approach, we defined a reference group of samples which we assumed to be representative of

groundwater in the Mur valley under natural or close-to-natural conditions in absence of major anthropogenic impacts. This reference group consisted of samples from the Aichfeld sub-region, which were additionally curated by removing samples which showed signs of anthropogenic impacts based on the municipal wastewater index (samples where MWWI≠0, Table 1), and surface water intrusion based on the stable water isotope data (e.g., evaporation; Fig. 6). Assuming, that this group did not contain outliers, μ and S were calculated for this group of reference samples directly without using robust estimates from Fast-MCD and subsequently used to calculate the D-A-(C) index as Mahalanobis distances for all remaining groundwater samples relative to this reference group.

Results

Applying the D-A Index we analyzed our dataset to assess the microbiological-ecological status and a possible ecological disturbance within the individual groundwater samples collected. We further implemented two DOM indices (HIX and BIX) into a D-A-C index. Both the D-A Index and the D-A-C index were performed (1) in an unguided, simple but insensitive manner, (2) with an increased spatial resolution taking sub-regions and land use into account, an (3) in the high-sensitivity mode with application of a reference data set.

Approach one: Detecting differences in the full data set without any prior knowledge

Multivariate plots of the individual samples, as well as the D-A-(C) signatures are here calculated for all samples with respect to the groundwater data set and no prior knowledge (Fig. 2). The plots show the distribution of groundwater and surface water samples in terms of prokaryotic cell numbers and microbial activities. The black, dashed ellipse depicts the 0.975 confidence level of the groundwater data set and shows that river samples clearly cluster separately from groundwater samples. Additionally, if we label the samples according to their affiliation to sub-regions or according to their origin from areas of different land use 'post-analysis', we see that sub-clusters are visible, which indicate that groundwater samples collected from the same sub-regions are more similar to each other than to those of other sub-regions. However, at the same time there is a considerable overlap between the samples of different sub-regions, and land use types. Samples that deviate significantly from the distribution of the groundwater data are considered outliers, and consequently are regarded as 'disturbed' with respect to their overall microbiological-ecological status. Approach one revealed that several river water samples, were classified as outliers, while all but one groundwater sample, two spring water samples and the rest of the river water samples were classified 'undisturbed' by the D-A Index. When having a closer look at the origin of the river sample outliers, they all stem from the lower river Mur sub-regions (i.e., Lower Mur valley, Leibnitzer Feld, Grazer Feld, and Murdurchbruchstal) that experience strong influences from land use and

urbanization. The portion of river samples clustering within the confidence ellipse originate from the upper stretch of the Mur river located in the alpine region (i.e., Upper Mur valley, Lungau, Aichfeld), where the morphology and water quality of the river is natural or only moderately anthropogenical impacted. The groundwater samples that group close to these uncontaminated surface water samples can be considered as most contaminated within the groundwater data set, ignoring possible regional or local differences in natural groundwater quality. Besides the D-A Index (Fig. 2B; left), we also calculated the Index further incorporating the DOM humification (HIX) and biological index (BIX) referred to as D-A-C Index (Fig. 2B; right). The D-A-C Index reveals that all surface waters are outliers (rivers and springs), and also some of the further downstream groundwater monitoring wells, as well as wells that have a MWWI≠0, or are otherwise conspicuous (e.g., samples 86, and 9 have a strikingly high concentration of ammonium (400-500 µg L^{-1}), samples 5 and 7 show the highest measured nitrate concentrations (13-15 mg L^{-1}) as is summarized in Fig. 3).

Because no reference data set on the 'good ecological status' was available, the approach introduced here displayed low sensitivity in separating undisturbed from disturbed samples in the group of samples that are more alike. As demonstrated, the D-A Index could not even separate all surface water samples from the groundwater data set. Some river samples were classified as 'undisturbed', although microbiological characteristics of surface waters and natural groundwater typically differ significantly (see discussion below). The D-A-C index performed better, and separated, all surface waters from groundwaters with physico-chemical features that were clearly at the upper end of the variance in measures from the rest of the groundwater samples. In our next approach, we excluded the surface water samples from the analysis and further consider regional differences that may influence groundwater quality and ecological status, such as hydrogeology and land use.

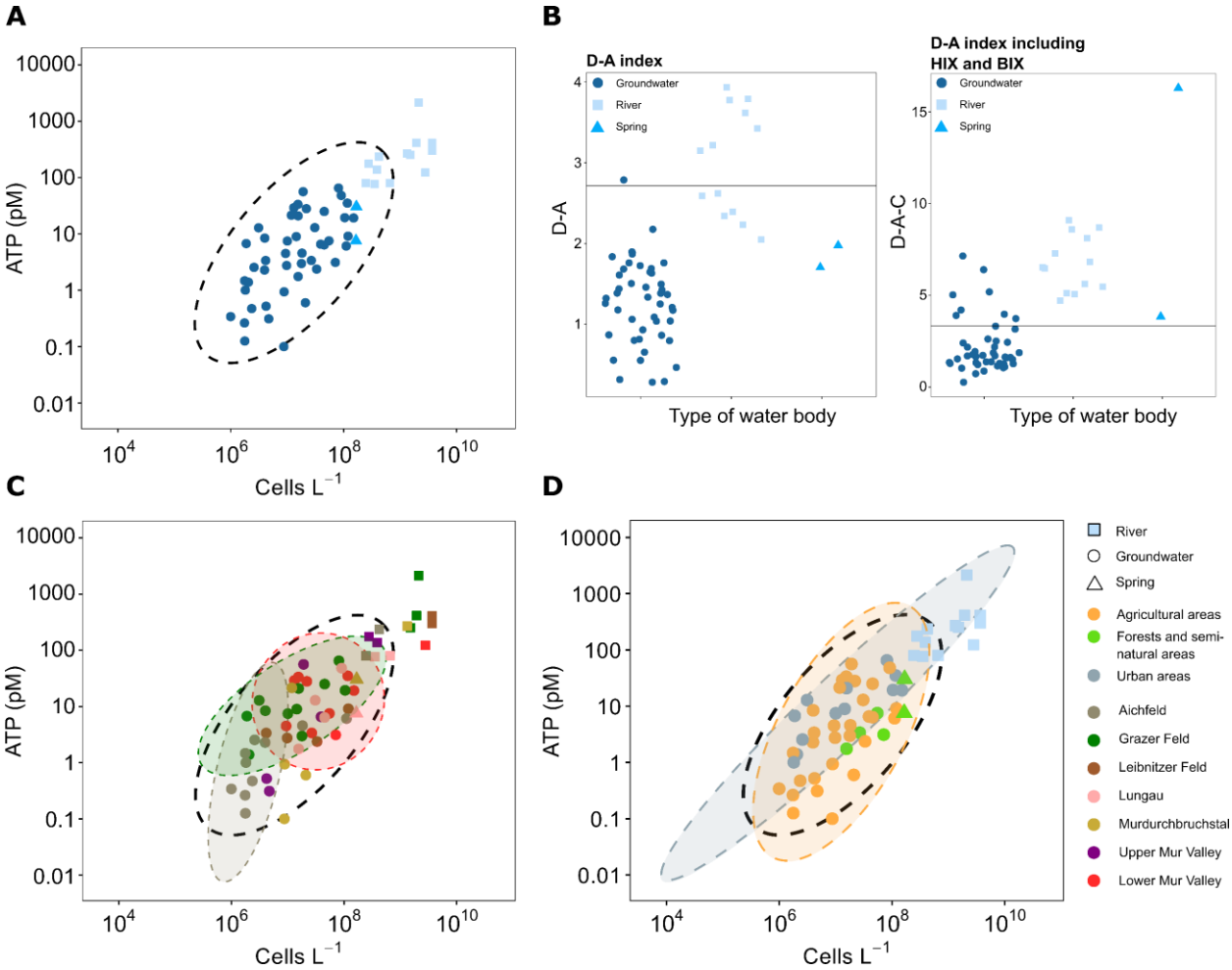

Figure 2: Bivariate plot of internal ATP, and microbial cell counts shown in A, C, and D, depicting the mean values of the random variation within the groundwater dataset as a black, dashed 0.975 confidence ellipse, as well as showing the respective groups (type of waterbody (A), sub-regions (C), and land use (D)); 0.975 confidence ellipses in the groups corresponding color. (B) shows the D-A Index (left), and D-A-C Index (right) that are computed according to approach 1 without prior knowledge, mean and covariance based on the groundwater dataset, but calculated for all samples. The solid black vertical line depicts the critical value of the chi-squared distribution with a confidence level of 0.975 and two degrees of freedom (D-A) and four degrees of freedom (D-A-C), and the x-axis shows the data points fanned out into the various water types.

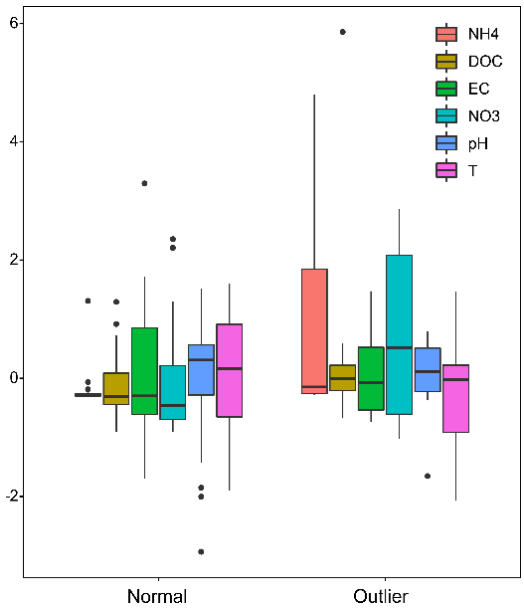

Figure 3: Boxplot for the z-values of the groundwater data set of most commonly measured hydrochemical parameters (NH4, DOC, EC, NO3, pH, and T) for the D-A-C Index calculated according to approach one.

Approach two: Detection of disturbed groundwater taking sub-regions and land use into account

In our second approach, we based the calculation of the D-A-(C) index on our groundwater dataset only, as well as factoring different categories, i.e., the Mur valley's different sub-regions and different land use types into its computation. In approach two, the D-A Index classified five groundwater samples as outliers within the individual sub-regions (Fig. 4A) and one sample within the land use types (Fig. 4B). Further consideration of the HIX and BIX again improved the sensitivity of the assessment, with an increase in the number of outliers (Fig. 4C, D). Still the assessment of disturbed samples from a large set of groundwater samples analyzed was biased. Since approach two again lacked the information on a reference status of undisturbed groundwater, the outliers in our calculations may depict the disturbed samples only in case most other samples are undisturbed. Vice versa, in case most samples analyzed were disturbed, the natural and undisturbed groundwater samples were flagged as outliers. One example is shown in Figure 4D, where outliers are comprised of those groundwater samples that can be regarded as most undisturbed, as is indicated by the differences in physico-chemical parameters. For each plot, the differences between outliers and the remaining data points in relation to the five most commonly measured environmental parameters, namely DOC, electric conductivity, nitrate, pH, and temperature (the entire set of measured parameters can be found in Table 1) are shown. Most of the outlier samples not only differed in D-A-C but also in their physico-chemical pattern. Worth mentioning, since the group of samples that can be analyzed in each category (sub-region and land use type) must at least contain one data point more than there are variables in the index calculation, the D-A-C index could be calculated for

only three sub-regions (Fig. 4C) and two land use types (Fig. 4D). For a maximum sensitivity of the D-A-(C) Index, we introduce one well defined reference data set in the next step of analysis, i.e. approach three.

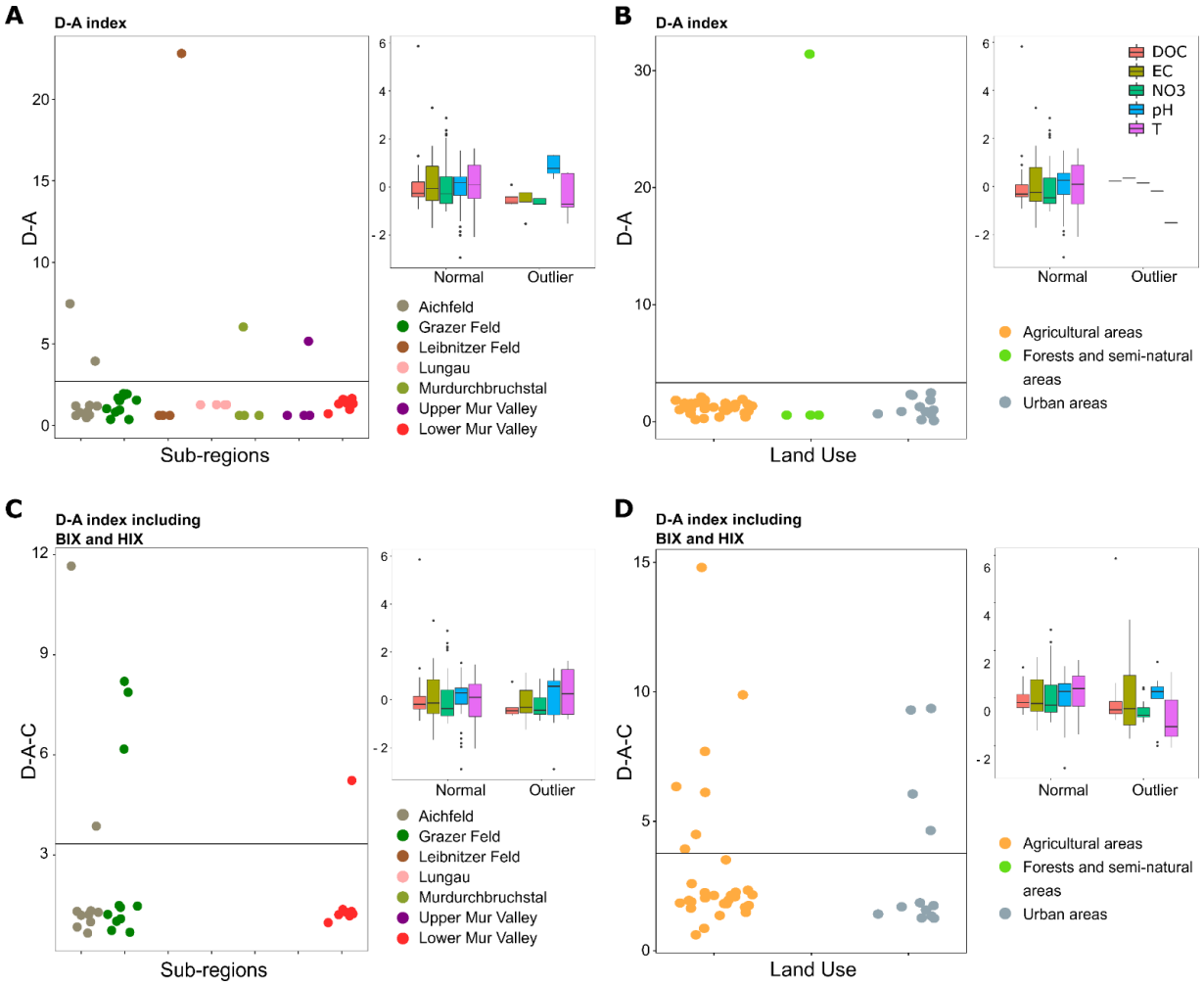

Figure 4: D-A index (A, B), and D-A-C index (C, D) computed for groundwater samples in reference to their different sub-regions (A, C), as well as land use classifications (B, D). Outliers shown here thus deviate from the regional average of internal ATP, and microbial cell counts, as outlined in the second approach. In every plot the deviation from the mean value of the most commonly measured hydrochemical parameters (DOC, EC, NO3, pH, and T) are illustrated as their z-scores in form of a boxplot. The calculation of the D-A index including HIX and BIX was only possible for 3 subregions (Aichfeld, Grazer Feld, and Lower Mur Valley), and two land use types respectively (Agricultural areas, and Urban areas) due to the insufficient number of samples. The solid black line depicts the critical value of the chi-squared distribution with a confidence level of 0.975 and two degrees of freedom in plot A and B, and four degrees of freedom in plot C, and D. In all plots, the x-axis shows the data points fanned out into the various sub-categories the index computation was based upon.

Approach three: Detection of disturbed groundwater samples integrating pre-existing expert knowledge

As in approach two, the inclusion of HIX and BIX increased the number of outliers (Fig. 5) for approach three. Similarly, the physico-chemical parameters of the outliers appeared to be slightly different from the other 'undisturbed' samples, i.e. they tended to be higher in temperature, electrical conductivity, nitrate and DOC, but lower in pH, which permits the assumption that the groundwaters identified as outliers here were in fact influenced or disturbed in some way. In other words, in contrast to approach two, the outliers

here formed the group of disturbed groundwater samples. Moreover, when comparing the D-A-C index results from approaches two and three, the number of disturbed groundwater samples in this comprehensive data set now outnumbered the number of 'good', uncontaminated groundwater samples. It can thus be said, that with the availability of a reliable reference data set and the inclusion of HIX and BIX can in fact noticeably increase the sensitivity of analysis.

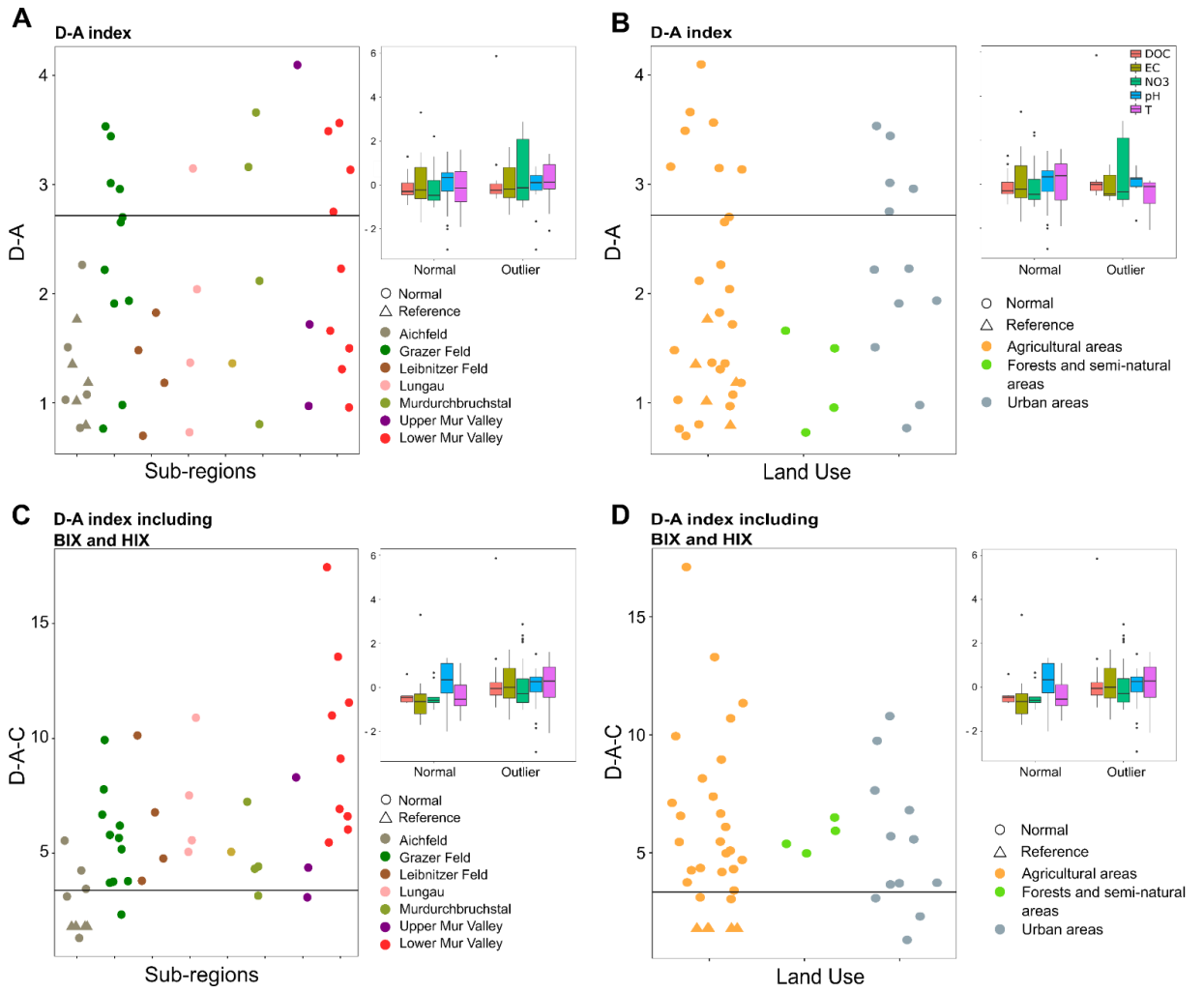

Figure 5: D-A index (A, B), and D-A-C index (C, D) computed for groundwater samples in reference to their different sub-regions (A, C), as well as land use classifications (B, D). Outliers shown here thus deviate from a unique pristine reference group in terms of internal ATP, and microbial cell counts, as outlined in the third approach. In every plot the deviation from the mean value of the most commonly measured hydrochemical parameters (DOC, EC, NO3, pH, and T) are illustrated as their z-scores in form of a boxplot. The solid black line depicts the critical value of the chi-squared distribution with a confidence level of 0.975 and two degrees of freedom in plot A and B, and four degrees of freedom in plot C, and D. In all plots, the x-axis shows the data points fanned out into the various sub-categories the index computation was based upon.

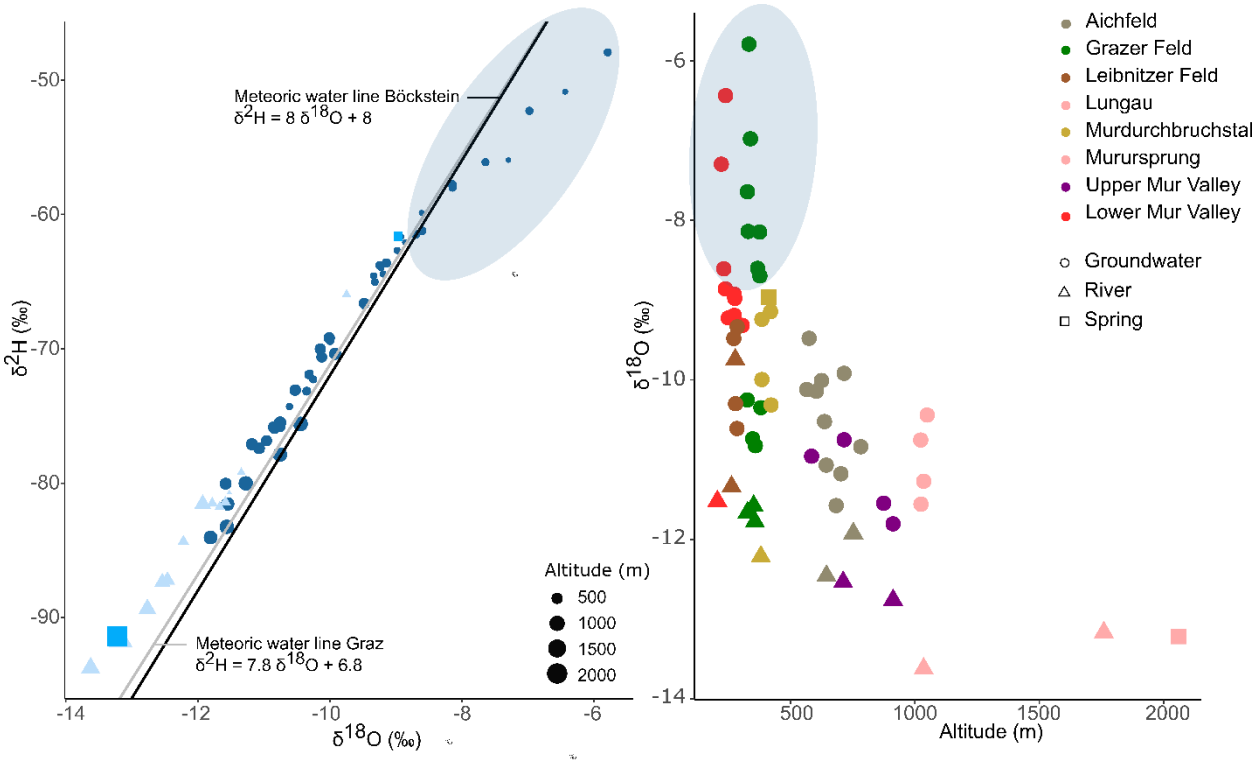

Figure 6: Bivariate plot of isotope data of the full dataset. The blue ellipse marks those samples which lie below the regional meteoric water lines of Graz (alpine foreland) and Böckstein (alpine region) and are therefore probably affected by evaporation. Local meteoric water lines for Austria were taken from Hager and Foelsche (2015).

Discussion

Aquifers harbor diverse organismic communities, microorganisms and metazoans, which are essential to providing pivotal ecosystem services (Griebler and Avramov, 2015). In the recent past, an increasing awareness of the 'ecological' characteristics of groundwater could be noticed, underlined by the numerous targeted international and national research projects that got funded (e.g. AQUALIFE 2020 GroundCare 2020), scientific reports and papers that got recently published (e.g. Griebler et al. 2014; Korbel and Hose, 2017; Griebler et al., 2018; Fillinger et al. 2019; di Lorenzo et al., 2020), and novel directives that got into action (Hahn et al., 2018). The D-A-(C) index is one of the various tools that have recently been developed for the easy, fast, and inexpensive assessment of the groundwater microbiological-ecological status (Fillinger et al. 2019). Here, we elaborate the applicability of the D-A-(C) Index considering different frame conditions.

The D-A-(C) Index can be easily applied to every kind of data set, with the restriction that the data group to be analyzed needs to be larger than the measures implemented in the index. However, as obvious from the results obtained by 'approach one' (uninformed and unguided evaluation), the size of the data set as well as the distribution of undisturbed and disturbed samples in the data group strongly influence the outcome of the analysis. Without consideration of spatial heterogeneities (e.g. appropriate local to

regional resolution) and temporal variabilities (e.g. seasonality), as well as without the availability of a well-defined reference data set, the index cannot discriminate between 'good' and 'bad', and between 'very good' and 'less good' in terms of the microbiological-ecological status. This approach is useful to evaluate differences in space and follow changes over time, but is seen as only a very first step. Already, consideration of local and regional particularities, such as hydrogeological conditions, increase the discrimination power of the index significantly. However, a reliable separation of groundwater that exhibits a natural or close-to-natural ecological status and groundwater that shows signs of an ecological disturbance, is only possible with a reference data set in hand.

The delineation of a perfect reference data set is challenging. Without a doubt, available knowledge on physico-chemical conditions, possible anthropogenic and natural influences, as well as expert knowledge are important ingredients. The question is, how specific a reference data set needs to be. Which resolution and sensitivity in the Groundwater quality assessment is approached. With different spatial scale, different drivers are active. While on the local scale within an individual groundwater body, key drivers are land use and the distance to the next surface water. On the regional scale, the type of aquifer (porous, fissured or karstic) and the hydraulic connectivity comes into play. At the supra-regional (national, continental, global) scale climate and earth history (e.g. ice ages) did and still do play a major role in shaping groundwater systems.

A first step in defining reference conditions at a spatial scale can be the classification of groundwater systems based on the aquifer type and specific hydrogeological units (e.g. Weitowitz et al., 2017). Subsequently, natural (pristine) and unpolluted representative sites are then selected within each unit (Korbel and Hose (2011, 2017). Importantly, few if any sites or regions of the planet remain unaffected by human activities, such that 'pristine' and 'natural' sites may be unavailable, and reference sites may be based on relatively undisturbed or 'best available' sites (Bailey et al., 2004). In practice, one may consider spots in protected areas (e.g. nature reserve, drinking water protection zones) or high alpine regions that experience less anthropogenic impacts. Robust classifications require adequate assessment of the natural variation within and between units, and may require intensive sampling of a large number of sites to adequately identify site 'types' and characterize the reference condition state (Hose et al., 2020). Complicating the definition of reference conditions is the natural heterogeneity in the subsurface. Among the hydrochemical conditions, oxygen is a key factor that shapes groundwater communities and influences biological processes. Also regional temperature anomalies must be considered. In lack of location- or region-specific knowledge, reference conditions and data sets can alternatively be delineated using national groundwater quality standards and guidelines. A clear advantage of such an individual

assembly of a reference data set is that there is always the possibility to upscale the already established reference group in order to improve or counter-adjust it as was done in this paper.

At the moment, the multivariate analysis is still based on very general factors, some of which may correlate with each other. As exemplified with the establishment of the D-A-C index in this study, it may be useful to include other parameters in the assessment to better evaluate the qualitative and ecological status of groundwater. However, it is also important to mention that there are clear limits to the sensitivity of our index with respect to groundwater microbiological-ecological conditions. Any disturbances that do not affect microbes in terms of biomass standing stock, growth and physiological activity cannot be detected. Such disturbances may include the presence of pollutants in trace concentrations (Meckenstock et al., 2015) or moderate temperature alterations (Griebler et al., 2016). Certain limitations in this regard may indeed be overcome by including fluorescence spectroscopy data of DOM in the analysis, which may detect traces of organic pollutants under certain conditions (Wasswa et al., 20019). Disturbances resulting from other types of contaminants such as traces of heavy metals, as well as moderate changes in temperature, however, will be more difficult to capture if they do not significantly affect microbial cell numbers or activity levels. Hence, it is important to note that the ecological assessment provided by the D-A-(C) index is not intended to replace existing physicochemical (Steinbacher et al., 2021) analyses in groundwater. Rather, the approach is to be used as an amendment to current monitoring approaches in order to provide a more holistic assessment of groundwater that captures its chemical as well as biological-ecological status.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

This work is part of the project ‘Integrative Groundwater Assessment’ funded by the Earth System Sciences (ESS) research program of the Austrian Academy of Sciences (ÖAW). We thank Peter Rauch, Barbara Stromberger (Steirische Landesregierung), Harald Huemer and Margot Geiger-Kaiser (Salzburger Landesregierung), for their valuable information and help in selecting appropriate groundwater sampling wells. We thank Sandra Kulscar und Michael Roll (Umweltbundesamt GmbH) for the analysis of the municipal waste water indicators. Finally, we deeply acknowledge the fire brigade of Mureck that have literally pulled us out of the mud in the Mur riparian forest.

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Table 1: Summary of environmental parameters showing the Minimum (Min), Maximum (Max), Mean values (Mean), and Standard deviation (SD) for Groundwater samples, Spring samples, and River samples (Altitude: Altitude (m asl), DOC: Dissolved Organic Carbon (mg/L), T: Temperature (°C), pH, DO: Dissolved Oxygen (mg/L), EC: Electric Conductivity (µS/cm), TCC: Total Cell Count (Cells/L), i.ATP: internal ATP (pM), $\delta^{18}O$: ratio of stable oxygen isotopes (‰), $\delta^{2}H$: ratio of stable hydrogen isotopes (‰), Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^- all in mg/L, NO_3^- and NH_4^+ both in µg/L, MWWI: Municipal Wastewater Index, BIX: biological index, HIX: humification index).

	Altitude	DOC	T	pH	DO	EC	TCC	i.ATP	$\delta^{18}O$	$\delta^{2}H$	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	NO_3^-	NH_4^+	MWWI	BIX	HIX
Groundwater																				
Min	221	0.24	7.81	6.2	0.11	241	9.94E+05	0.1	-11.8	-84	1.5	1	33.5	4.5	1.2	5	1	0	0.7	1.58
Max	1049	4.37	14.1	7.8	8.73	1243	1.50E+08	65.1	-5.79	-48	84.6	14	191	36.6	200	15467	502	3	1.2	33.2
Mean	491.8	0.8	11.4	7.2	5.29	582.5	3.02E+07	11.64	-9.68	-68.5	17.5	3.6	75.9	13.8	37.5	4058.6	30.5	1.04	1	8.67
SD	249.4	0.61	1.72	0.4	2.69	200.5	3.84E+07	15.42	1.4	8.87	13.6	2.6	30.3	6.33	34.8	3975.7	98.3	1.19	0.1	5.85
Springs																				
Min	412	0.36	5.35	7.8	10.6	11.9	1.60E+08	7.58	-13.2	-91.4	0.2	0.1	1.8	0.1	0.1	135	0	0	0.8	0.84
Max	2060	1.35	9.3	8.1	13.5	368	1.70E+08	30.45	-8.96	-61.7	4.8	1	55.8	6.3	10.8	1875	2	3	3.2	5.57
Mean	1236	0.85	7.33	8	12.1	190	1.70E+08	19.02	-11.1	-76.5	2.5	0.6	28.8	3.2	5.45	1005	1	1.5	2	3.2
SD	1165.3	0.7	2.79	0.2	2.03	251.8	2.00E+06	16.17	3.01	21.03	3.25	0.6	38.2	4.38	7.57	1230.4	1.41	2.12	1.7	3.35
River																				
Min	205	0.48	8.92	7.8	8.89	79.5	2.40E+08	76.61	-13.6	-93.7	0.3	0.2	13.7	1.2	0.2	45	0	0	0.6	2.18
Max	1760	4.54	19.5	8.3	10.7	305	3.70E+09	2137.8	-9.75	-66	9.6	3.1	35	7.5	16.1	1883	137	3	0.9	7.49
Mean	613.2	1.9	13.3	8.1	9.93	210.5	1.50E+09	360.88	-12	-83.5	4.95	1.4	26.8	5.49	6.27	785.15	30.4	2.38	0.8	4.47
SD	436.5	1.35	2.71	0.2	0.46	58.79	1.30E+09	546.38	0.97	6.98	3.52	0.8	5.6	1.71	4.76	502.08	42.6	1.12	0.1	1.65

General Discussion

Since all chapters in this thesis have their own discussion section, the general discussion intents to summarize and highlight the doctoral thesis's contribution to advances in the field of microbial and microeukaryotic freshwater ecology in a short and comprehensive manner. A particular emphasis is laid on providing a bigger picture of groundwater ecosystems in mountainous regions and its relation to fresh surface water under varying spatiotemporal conditions, such as land use, or climatic conditions. As such, this thesis provides valuable insights into the impact of environmental heterogeneity on groundwater microbial communities in an alpine setting. Chapter I emphasizes the need for further research to integrate groundwater ecosystems into climate change models and to improve our understanding of microbial ecology in shallow groundwater environments. Chapter II challenges the traditional perception of aquifers as stable ecosystems with low prokaryotic productivity and biodiversity. It provides evidence for the impact of landscape characteristics, including land use, and environmental conditions on groundwater communities ranging from their diversity to the drivers of community assembly processes over time and space. Chapter III summarizes known literature on fungal diversity in groundwater and highlights the need for comprehensive research efforts to enhance the understanding of fungal communities in groundwater ecosystems. Chapter IV examines the diversity and distribution of fungal communities in an Austrian alpine river valley in both groundwater and river water, considering taxonomy and potential ecological roles. It highlights the uniqueness of both ecosystems in terms of fungal communities, as well as their ecological connectivity. Chapter V introduces the D-A-(C) Index as a tool for assessing the microbiological-ecological status of groundwater. It underscores the importance of factors such as spatial and temporal variability, as well as the establishment of a representative reference dataset for effective groundwater status assessment.

The pivotal role of prokaryotic and fungal groundwater communities in biogeochemical cycles and ecosystem services was elucidated by Illumina amplicon sequencing. Genetic markers of interest were the V4 region of the 16S SSU (prokaryotes) and 5.8S and ITS2 region (fungi) of the ribosomal DNA and RNA, targeting the total and active communities, respectively. An improved primer pair amplifying the 16S region was chosen to characterize the prokaryotic fraction of groundwater and river water with an improved detection of archaeal diversity. A two-marker metabarcoding approach was chosen for fungi, including the hypervariable region ITS2, as well as its highly conserved upstream region 5.8S. The taxonomic annotation of sequences could thereby be improved, especially for early diverging lineages of fungi, such as members of *Chytridiomycota* (Heeger et al., 2019). Besides, an additional effort was made to measure and compute data such physico-chemical variables, major ions, nutrients, DOM quality and

quantity, a set of wastewater indicators based on the quantity of known trace pollutants (e.g., pharmaceuticals, anti-corrosives, artificial sweeteners), water stable isotopes, prokaryotic cell counts, as well as intracellular ATP as a proxy for microbial activity. Through an interdisciplinary effort, as well as integrating long-term monitoring data we also hydrologically characterized the groundwater flow patterns along the Mur river valley. This included the relationship of groundwater tables with river water levels, and the amount of precipitation. These were given as the strength and direction of the groundwater level long-term correlation with the Mur river water level, as well as with local precipitation frequency and quantities. Besides, we characterized the groundwater sites based on their probability to be hydrologically and quantitatively correlated to the Mur river given as the distance to the closest possible infiltration location inferred from groundwater flow charts. On top of that, the 20-year average groundwater level fluctuation was calculated, indicating how dynamic groundwater levels were around their mean. We also included GIS data, such as land use data derived from Corine Land Cover from the year 2018, as well as available bedrock lithologies. We also utilized bioclimatic variables derived from CHELSA Bioclim, namely the mean annual air temperature (bio1, °C), annual precipitation amount (bio12, kg m^{-2}), and seasonal precipitation as the coefficient of variation (bio15, kg m^{-2}) from the years 1981 – 2010. In contrast to many other studies, this highly interdisciplinary approach provided a deeper understanding of underlying drivers and mechanisms with respect to prevailing microbial community dynamics.

The Mur river valley is heterogeneous, and its freshwater resources are spatiotemporally dynamic

The sampled area covered an altitudinal range from 221 to 1 760 m.a.s.l. and a river stretch of around 300 km in the Austrian Central Eastern Alps. A key driver of microbial dynamics in groundwater was constant temperatures, which were on average 3°C cooler compared to the dynamic river water across time and space. Groundwater was around 1°C cooler in early summer compared to late autumn, with an increasing trend further down the river valley (Chapter II). Groundwater temperatures were different over the year depending on the regional gradient with different land use practices (e.g. alpine region, urban, or arable lowland). While groundwater temperatures were highest, but relatively stable in the urban catchment in the city of Graz, groundwater temperatures were noticeably more dynamic in the alpine region and the lowland of the Mur river valley. The temperature difference between seasons was much more pronounced in the river system, with an increase of around 5°C from late autumn to early summer. Altogether river water temperatures increased along the alpine region to the lowland, and this was less pronounced than in groundwater (Chapter II). The input of organic matter in terms of quantity and quality

was found to be an additional factor determining microbial community composition and assembly, especially in its dissolved form (Chapter II, IV). DOC concentrations were around 3 times higher in the river compared to groundwater. In the river, DOC concentrations were highest and most variable in the city of Graz but altogether increasing along the river corridor. In groundwater, DOC was also increasing towards the lowland, however, it was highest beneath forested areas, often found when overlying soils were rich in organic material (e.g. wetlands) (Chapter II). One relevant nutrient found to be driving microbial and fungal community composition and microbial assembly patterns was nitrate. It constitutes one of the most prominent groundwater contaminants originating from agricultural land use and wastewater discharge. Nitrate was found to be typically much higher in groundwater, and our data support this pattern, being on average 4 times higher in groundwater compared to the river (Chapter IV). Nitrate concentrations increased down the valley in the river, doubling its amount towards the lowland, whereas it quadrupled towards the lowland in groundwater compared to the alpine region. Not surprisingly, the river was significantly microbially more productive, with cellular ATP concentrations substantially higher (> 100 times) and prokaryotic cell numbers around 2.6 times higher in river water compared to groundwater (Chapter II, V).

The Alpine Region

Alpine groundwater consistently showed a more depleted stable isotopic signature and was therefore likely fed by a variety of natural sources such as glacial melt water in the Hohen Tauern, and melt from snow formed during the colder season (Gaillard et al., 2024). Seasonal variation in precipitation and subsequent groundwater table fluctuations were high in the alpine region and nutrient inputs were closely linked to surface water intrusion possibly driven by these dynamic precipitation patterns (Figure 1). This is supported by a study conducted on a subset of groundwater sites under scrutiny here, where high-resolution monitoring supporting a close link between short-term fluctuations in nitrate and groundwater level increases associated with precipitation (Haas et al., 2023). We found that groundwater in the alpine region was largely hydrologically connected to the Mur river, which was morphologically more natural and qualitatively more pristine than downstream river sections and was only moderately impacted by anthropogenic inputs. While some studies support that groundwater recharge in alpine regions is dominated by river discharge, the aquifers ability to attenuate floods and sustain downstream baseflow can however sometimes be marginal (Halloran et al., 2023; Müller et al., 2024). On the other hand, groundwater recharge dynamics in alpine regions seem to be spatially highly variable and non-linearly impacted by slight changes in precipitation and evaporation. As a consequence, already drier alpine regions might become more vulnerable to groundwater loss and susceptible to consequences of climate change (Berghuijs et al., 2024).

ALPINE REGION

Groundwater Tables: Highly dynamic, with a significant decline from early spring (about 4 m below the surface) to late June (around 12 m below the surface).
Around 70% of wells significantly correlate with Mur River water levels, while none correlate significantly with precipitation.
Drying Periods: Groundwater levels continue to decrease when precipitation declines from August onward, resulting in similarly low water tables by November, comparable to June levels.
Precipitation Patterns: Precipitation is highly variable, dominated by convective storm events and rainfalls, indirectly contributing to rapid changes in recharge.
Temperature: Groundwater temperatures lowest, but following river water intrusion according to water isotopic signatures.
Water Isotope Signatures: Groundwater isotopic signatures closely follow river water isotopic signatures throughout the year. This is driven by early summer rain and preceding snowmelt from higher elevations and colder periods, which recharge both river and groundwater.
DOC and Nitrate: Both exhibit low concentrations but show high temporal variability, primarily due to dilution through groundwater recharge.
Microbial Productivity: Lowest microbial productivity occurs in regions with minimal organic material input. Cell activity peaks with organic material influx, while cell numbers reflect a mix of dead, dormant, allochthonous, and autochthonous microbes.
Prokaryotic Diversity: Prokaryotic diversity is high, driven by habitat heterogeneity in alpine environments. Many lineages of *Archaea*, including undescribed taxa, contribute to this great diversity.
Fungal Diversity: Fungal diversity, intead of following a strict elevation gradient, is rather highest in groundwater systems with typical conditions, i.e. that are undisturbed by anthropogenic activities, such as road salt intrusion and intensive agriculture.

URBAN AREA

Groundwater Tables: On average, deeper but less dynamic.
Around 70% of wells do not or only weakly correlate with Mur River water levels, while over 90% correlate significantly with 9-12 month average precipitation.
Replenishment: Groundwater is primarily replenished by local rainfall, but surface sealing limits infiltration.
Drying Periods: Groundwater levels start to decline in early May and groundwater is not significantly recovered until November. This is likely due to increased surface runoff (impervious surfaces), higher water demand, and elevated evapotranspiration during summer.
Precipitation: Receiving, on average, more rainfall with a lower variation in precipitation compared to the alpine region.
Temperature: Groundwater shows the highest temperatures throughout the year, with significant warming.
DOC and Nitrate: Dissolved organic carbon (DOC) increase during the summer months, but remain relatively stable over time compared to the alpine region, or the lowland. In contrast, nitrate concentrations increase sharply in summer. Both could be explained by e.g. accumulated surface inputs, limited infiltration, or evapoconcentration during dry months.
Microbial Productivity: Intermediate between lowland and alpine regions, reflecting moderate levels of microbial activity.
Prokaryotic Diversity: Comparatively low in urban areas, especially beneath warehouse and storage facilities, likely due to limited nutrient input and road runoff (e.g., road salts, heavy metals, organic and inorganic pollutants).
Fungal Diversity: Low fungal diversity, impacted by factors such as road salt, wastewater infiltration, or industrial discharges. The fungal community tends to include soil saprotrophs and plant pathogens.

LOWLAND

Groundwater Tables: Shallow and relatively static.
Around 60% of wells correlate significantly with Mur River water levels, whereas 70-90% correlate significantly with both 6 and 9-12 month average precipitation.
Groundwater Warming: Groundwater disproportionately warms up during and after summer, with a time lag of about three months following river temperatures.
Evaporation: Groundwater is evaporated throughout the year, e.g., due to irrigation practices. There is hydrological connectivity to the river, with combined recharge from local rainfall.
Precipitation: While variation in precipitation is lowest among regions, this area experiences the highest average rainfall possibly driven by large-scale weather systems (e.g., weather fronts) and a consistent moisture supply from the Mediterranean, and Adriatic region.
Nutrient Load: Microbial productivity, DOC, and nitrate levels are the highest compared to other regions, likely driven by agricultural practices.
Prokaryotic Diversity: The lowest prokaryotic diversity is found in the agriculturally used lowland. Community structure is primarily influenced by environmental conditions such as nutrient loads and soil management practices.
Fungal Diversity: Fungal diversity is low, likely due to increased use of fertilizers and fungicides. The fungal community includes animal pathogens and fungi with known denitrification abilities, adapted to the nutrient-rich environment.

Figure 1 Table describing and summarizing groundwater characteristics along the Mur river valley depicting the alpine region on top, the urban area including the city of Graz in the middle and the lowland which is impacted by intense agriculture and industrial and commercial surfaces at the bottom.

Since extended drying periods during summer, accompanied by shorter and more-frequent wet spells has increased over the last century in the European Alps, this is affecting the overall water balance and

might deteriorate groundwater replenishment during the summer months (Menegoz et al., 2020). As shown here, this would in turn not only lead to an increased input of soil, and surface water organisms (e.g., fungi) into groundwater aquifers during that time, but also to a more productive microbial community, as groundwater productivity was directly related to the input of DOC (Chapter II). For instance, an increased input of soil OM, and nutrients could in turn lead to a loss of rare (i.e. microbial seedbank) or otherwise sensitive taxa well-adapted to the nutrient poor, oligotroph groundwater environment. Despite their low abundance, rare taxa often fulfill crucial ecological functions in aquatic ecosystems, or in the subsurface (Hug et al., 2016; Kritzberg et al., 2006; Wilhelm et al., 2014), however the relationship between diversity and functioning is not always straight forward (Pholchan et al., 2013).

This thesis found that prokaryotic diversity in groundwater, although lower in cell density, far exceeds that of surface water, which decreases when the system becomes more metabolically active. This is probably due to a few generalist species dominating the active microbial community when there is, for instance, an excess of nutrients in groundwater, favoring fast-growing taxa. Prokaryotic communities form in the face of complex structural and hydrological barriers in alpine groundwater, potentially leading to a limited or absent gene flow on an evolutionary timescale, and higher speciation rates and subsequent diversification (Suárez et al., 2022). This is in line with a peak in microbial diversity found in the alpine region, following a relationship with reduced productivity. A high diversity of understudied lineages of *Archaea*, including *Aenigmarchaeota,* are resident to these alpine groundwater habitats and as such could be lost before being fully characterized and described, as much about them is currently not well understood. Ultra-small prokaryotes, such as CPR *Bacteria* and DPANN *Archaea* have a reduced genome size and thus are likely to be dependent on other, either prokaryotic or eukaryotic organisms via symbiosis or parasitism. They exhibit high genomic flexibility and are involved in key ecosystem processes in groundwater via their hydrogen, sulfur and nitrogen metabolism (Amano et al., 2024; Li et al., 2021), where strain diversity might be shaped by specific physiological conditions (Gios et al., 2023). One hypothesis is that they could be dispersed throughout the aquifer by slow flow-rates and selected for by a groundwater matrix of low porosity (Calvo-martin et al., 2022; Gios et al., 2023).

Fungal diversity and biomass are, on the other hand, overall, significantly lower in groundwater compared to surface water. Rich fungal communities were recovered from groundwater under natural conditions and with organic matter typical in quantity and quality throughout the river valley (Harjung et al., 2023), however most of them situated in the alpine region. These fungal assemblages include abundant genera such as *Cladosporium*, *Epicoccum* and *Olpidium*. *Cladosporium* is known to degrade complex polymers and participate in Mn (II) sequestration (Somervuori et al., 2021). *Epicoccum* is a known drought-depleted fungus in soils and could for instance pose as a proxy for groundwater that is less

affected by warming and evaporation, but this would have to be further investigated (Yue et al., 2024). *Olpidium* is occurring in association with algae, diatoms, insect exuviae or plant pollen – their exact roles in groundwater not fully understood yet. One of the prevalent species in alpine groundwater is *O. brassicae*, an obligate root parasite that thrives at lower temperatures (Zelyüt and Ertunç, 2021).

Urban areas

The urban area, including the city of Graz, lies at the base of the mountain range and features, on average, deeper groundwater, meaning the water is located further below the surface (Kokimova et al., 2024). The climate in the urban area and downstream is altogether drier compared to the alpine region (Prettenthaler et al., 2020), caused by elevated solar radiation and increased evapotranspiration, counteracting the higher amount of precipitation this region receives annually. While there is on average quantitatively more rain fall in the city of Graz and even more so in the lowland – i.e. when it rains it pours, Prettenthaler et al. (2020) show that it rains less frequently compared to the alpine region, with the number of precipitation days decreased more strongly around the city of Graz compared to the rest of the Mur river valley over the last decades. In other words, precipitation events in the foreland tend to occur less frequently, with a disproportionally more infrequent trend for the future, but with a greater intensity and more uniformly distributed over the year, whereas alpine areas experience more frequent but smaller-scale rain events.

In terms of water temperature, urban groundwater is influenced by heat island effects caused by increased surface sealing and runoff, making it significantly more susceptible to warming (Griebler et al., 2016; Menberg et al., 2013; Taylor and Stefan, 2009). A drier climate, together with the formation of urban heat islands, translates into elevated Mur river water, and groundwater temperatures in the urban region to various extents. Additionally, groundwater aquifers are potentially more vulnerable to drought in the southern foreland of the alps for another reason. Compared to the alpine region, quaternary sand and gravel deposits are altogether decreased, leading to a decreased groundwater thickness (Birk et al., 2023; Haas et al., 2018; Haas and Birk, 2017). Besides, groundwater warming in urban areas is especially problematic in light of co-occurring groundwater pollution influencing ecosystem functioning and food web dynamics, with shallow groundwater being particularly vulnerable (Archer et al., 2019; Brielmann et al., 2011).

Our data show that during summer months groundwater is continuously lost in the city by e.g., increased water demand, evapotranspiration fuelled by a warming climate and intense solar radiation, shifts in precipitation patterns and increased surface runoff that is lost from groundwater recharge. It is only during colder seasons from around December to March that groundwater is replenished again, when air temperatures drop significantly and precipitation can reach the aquifer, with seasonality often being

critical for large-scale groundwater recharge processes (Gaillard et al., 2024). Here, we find that based on elevated groundwater temperatures, nutrient concentrations, water stable isotope depletion and inorganic carbon loads, different communities of prokaryotic taxa will establish in groundwater. The effect of nitrate and phosphate on microbial community composition and assembly is even more prominent in the lowland further downstream the river Mur, fuelled by intensified agricultural practices, however this will be discussed in detail later. Together with peaking salt loads, we show that road runoff, or wastewater discharge after e.g. flooding events are particularly problematic for fungal and prokaryotic biodiversity (Figure 1), including diffuse sources such as runoff from transportation and industrial facilities often entering groundwater in rapid pulses (Cooper et al., 2014; Reid et al., 2019). Fungal biodiversity in urban groundwater is lowest among our dataset and include mainly a few cosmopolitan, eurybiont and/or soil saprophytic taxa, such as *Rhizophlyctis* and *Psathyrella*. Prokaryotic diversity is also low, but slightly higher than in agriculturally impacted groundwater catchments located in the lowland. The metabolically active prokaryotic fraction in groundwater includes mainly ubiquitous taxa, such as members of *Proteobacteria*, but also not well characterized, ultra-small *Nanoarchaeota* harbouring metabolically diverse, as well as thermophilic taxa which are often enriched in sediment-rich groundwater (Castelle et al., 2015; Lauzon et al., 2024; Stephenson et al., 2023). Together, these data let us conclude that urban groundwater is disproportionally affected by warming, and that there is a dynamic exchange with soils driven by local precipitation via preferential (i.e., unsealed) flow paths through soil layers.

The lowland

After the river Mur leaves the urban region, it enters the agriculturally intensively used lowland, with some wetland forests shaping the riverbank landscape furthest downstream of the river corridor. Groundwater tables remain close to the surface over the year, being recharged by a combination of intense rain fall and river recharge, depending on local hydrology. Groundwater temperatures were highest around September, lagging peak river summer temperatures for about two months. Groundwater showed consistent signs of evaporation over the year, for instance caused by irrigation of agriculturally used fields when groundwater is brought back to the surface and quickly evaporates when it reaches the land cover (Gaillard et al., 2024). Here, fungal diversity was somewhat higher than in groundwater beneath the city, and contained some fungal taxa with known nitrogen assimilation, remineralization, but also dissimilatory cycling of nitrogen capabilities, sometimes exceeding those of microbes (Gulis et al., 2006; Peng et al., 2024; Takaya, 2002). Interestingly, an undescribed *Olpidium* sp. was found to be potentially parasitizing or competing for resources with isopods in the lowland, but not with other more sensitive stygofauna such as amphipods (Birk et al., 2023; Marmonier et al., 2023; Sime-Ngando, 2012; Young et al., 2014). At these sites, groundwater temperatures were higher, and oxygen was abundant. Prokaryotic

diversity was hitting its minimum in lowland aquifers with fewer, and less evenly distributed species dominating the community. As mentioned, prior, the excess input of inorganic nutrients into groundwater (i.e., concentrations of phosphate and nitrate exceeding denitrification capacities) have a significant effect on prokaryotic community composition and complexity (Chapter II), which could possibly lead to functional shifts and consequently reduce ecosystem resilience. The accumulation of eutrophicants, such as nitrate, in groundwater is suggested to be controlled by local redox-conditions, but also the presence of large networks of surface canals along agriculturally used fields (Young et al., 2010). This is problematic as discharge of contaminated groundwater into surface water ecosystems can lead to eutrophication and surface water deterioration. Toxic algal blooms or sedimentation of algal biomass on the river bed can then impact higher trophic level diversity (e.g. filter feeders, water plants), and can be attenuated by intact and active floodplain ecosystems (Brunke and Gonser, 1997; Köhler et al., 2024; Spiteri et al., 2007).

Conclusion and Outlook

This thesis underlines the urgent need for future studies to explore how microeukaryotic, and prokaryotic communities present in aquatic ecosystems, such as groundwater, respond to short-and long-term environmental changes, such as altered precipitation patterns and land use shifts. Additionally, when it comes to determining the health status of groundwater, there should be a strong focus and effort towards creating and evaluating reference data sets of pristine groundwater bodies from other geographic regions.

In a way, this work and other collaborative studies have laid a framework for future research by demonstrating the benefits of a highly interdisciplinary approach, combining aquatic ecology with microbial ecology, taxonomic, and hydrological expertise, the incorporation of GIS data, as well as the involvement of federal agencies to understand the complex underlying dynamics of aquatic ecosystems. Especially in urban and agricultural areas, further research is needed on a higher temporal and spatial resolution to understand the impacts of groundwater warming and nutrient pollution on a micro-scale. Ideally, following studies will focus on additionally integrating (meta)genomic, and (meta)transcriptomic data to understand species and population level impacts of a changing environment on community diversity and functional capacities in a broader context, i.e. without relying on PCR based approaches. In this context, the analysis of a subset of groundwater and river sites is still underway and will be investigated for potential sources and sinks for extant fungi in the groundwater-river-soil realm. Additionally, we will show how, for the metacommunity, gene expression and genetic potential varies among river and groundwater sites under varying environmental conditions (i.e. high and low nitrate input over the year), facilitated by omics approaches. Moreover, we will make use of the sequenced rRNA,

thereby characterizing the present community as is, which will serve as a more unbiased approach towards understanding community dynamics in a meta sense. The benefit of having available metagenomic data is that the rRNA can in theory be normalized by copy numbers of retrieved MAGs and the community should be relatively quantifiable. This will help us to understand in a more direct way food web dynamics and existing interactions.

While this study provides a comprehensive analysis of groundwater microbial diversity, several limitations must be acknowledged. First, the temporal resolution of sampling was limited to two seasons, which may not capture longer-term shifts in microbial communities. Besides, even though the sampling effort was high, it should be increased for such a heterogenous environment as is the alps, as statistical support for subsequent analysis was sometimes not as strong as we would have wished, or as we saw for the more homogeneous Mur river system.

Subtle changes seem to have a detrimental impact on groundwater ecosystems, and small and targeted improvements might attenuate consequences of climate change and maintain natural levels of productivity, biodiversity and altogether the sustainability of groundwater as a resource. Mitigation strategies, such as stronger regulations and incentives e.g., integration of biological parameters into long term monitoring, a controlled withdrawal of groundwater resources, closely evaluating effects of old and new agricultural and building practices, controlling the use of pesticides, fungicides, and antibiotics in agriculture, the use of more water-efficient crops, or the deconstruction of paved areas and renaturation of buffering zones such as wetlands should be prioritized to preserve groundwater biodiversity, particularly in the vulnerable lowland and urban areas of the Mur valley. This thesis shows that these ecosystems are already quite heavily impacted considering ongoing anthropogenic pressures and climatic changes, making it hard to find pristine reference aquifers in this region. We cannot continue to treat groundwater as a lifeless and endless resource and its alteration and diminishing being of no consequence.

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