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„Impact of groundwater temperature on the composition of groundwater microbial communities in an urban aquifer"

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A special thank-you goes to my father for always believing in me.

Abstract

With recent public projects using groundwater as a heating and/or cooling agent, the impact of temperature manipulation on groundwater ecosystems becomes a popular subject, with new studies oftentimes connecting the groundwater temperature increase with a microbial community shift. Studies have so far shown that the urban areas provide a good opportunity to analyze microbial community composition in relation to groundwater temperature because of urban heat islands and urbanization's impact on temperature increase. Analyzing groundwater from aquifers in Vienna, Austria, shows that temperature does not have a direct impact on the microbial community composition, but could have an effect on the microbial diversity. An increase in temperature did not show a general connection to other environmental variables, including oxygen and pH levels, which are usually closely related to temperature in low energy groundwater systems, but aquifer-specific trends were implied. Implications that additional data on hydrogeology and wastewater leakage would help give better insight were found. Although the temperature did not show itself to be a significant driver in microbial community composition at this moment in time, that does not exclude that prolonged temperature stress would have an impact.

Keywords: urban microbial communities, temperature stress, urban heat islands (UHIs), urban groundwater

Zusammenfassung

Infolge der Nutzung von Grundwasser als Wärmequelle und Kühlmittel werden die thermischen Auswirkungen auf Grundwasserökosysteme messbar. Aktuelle Studien zeigen eine Veränderung der mikrobiellen Gemeinschaft mit steigenden Grundwassertemperaturen. Der urbane Raum eignet sich, aufgrund des hohen Grads an Verbauung und der Entstehung sogenannter Wärmeinseln, für die Untersuchung von mikrobiellen Gemeinschaften in Bezug auf Temperaturveränderungen. Die Analyse von Wasserproben aus den Grundwasserleitern (Aquifere) Wiens, Österreich, zeigt keinen direkten Zusammenhang zwischen Temperatur und der Zusammensetzung der mikrobiellen Gemeinschaft. In dieser Arbeit wurde die Auswirkung auf die mikrobielle Diversität untersucht. Obwohl höhere Grundwassertemperaturen keinen unmittelbaren Einfluss auf andere Umweltparameter z.B.: gelöster Sauerstoff oder pH hatten, konnten Aquifer-spezifische Trends aufgezeigt werden. Ergänzende Daten über die hydrogeologischen Bedingungen und das Aussickern von Abwasser würden genauere Vorhersagen ermöglichen. Obwohl zu diesem Zeitpunkt die Grundwassertemperatur kein signifikanter Einflussfaktor für die Zusammensetzung der mikrobiellen Gemeinschaft darstellt, müssen die langfristigen Auswirkungen auch in Zukunft berücksichtigt werden.

Schlüsselwörter: städtische mikrobielle Gemeinschaften, Temperaturstress, städtische Hitzeinseln, städtisches Grundwasser

Contents

1 Introduction

As the population becomes more and more concentrated in urbanized areas (UN Department of Economic and Social Affairs, 2018), the supporting infrastructure becomes more complex and interconnected, with a large portion finding itself underneath the surface. The distribution of water, wastewater, heating, cooling, as well as the traffic, all have their network beneath the cities' surface. The transport of energy and matter is accompanied by the heat it generates. This anthropogenic source of heat in the subsurface, combined with the warming effect which the surface urban design causes, creates a concerning trend in urban groundwater temperature distributions (Benz et al., 2018), and results with a disproportionately high impact of global warming on cities in comparison to rural areas.

Considering the ever-increasing urbanization and population-growth, we propose putting a bigger focus on the impact of temperature fluctuations on the groundwater ecosystem functionality and on the biotic communities inhabiting these ecosystems.

1.1 Effect of urbanization on temperature

1.1.1 Surface urban design and its temperature-affecting characteristics

The microclimate of an urban area differs from the climate patterns of the rural areas which surround it, due to a number of very thoroughly studied and well known factors: the main elements of urban design which impact the temperature are the **(1)** higher density of buildings, **(2)** lower percentage of green and water surfaces, and their replacement with paved and sealed surfaces, and **(3)** higher density of the population, along with all the activities which accompany today's living (transport exhaust fumes, industrial processing, etc.) (Mirza et al., 2022; Stewart & Oke, 2012).

More precisely, the height and density of the buildings in an urbanized area is a temperature-affecting factor **(1)**, since it creates an environment where solar radiation cannot freely reflect back from the Earth's surface, leading to an increased absorption of heat. The dense building structure is furthermore problematic by reducing the wind speed and changing the wind patterns, thereby affecting the amount of coolness brought to the area (Gago et al., 2013).

The urban layout, with fewer green surfaces **(2)**, caused by the removal of vegetation from urban areas, lowers evapotranspiration rates (Qiu et al., 2013), and affects the color scheme. The darker colors of buildings cause lower albedo, in comparison to the greenery of a forest or parks (Akbari et al., 2009). Both consequences of vegetation removal increase the temperature, since the paved surfaces take up more heat than the moist soils and plants, both because of their colour and texture (Heisler & Brazel, 2010). Another shift to sealed and paved surfaces is done by a decreasing the surface water area of the city by creating channels and removing the surrounding vegetation. While the countryside temperatures remain almost constant, the increase of temperature was tied to the percentage of sealed surfaces in the urban environment (Fokaides et al., 2016).

The impact of urban design on the microclimate gets additionally exacerbated by the human activities in urban areas **(3)**. The heat produced by industrial facilities, traffic and air-conditioning units cause an additional release of heat (Stewart & Oke, 2012). The temperature increase occurs through a positive loop, with higher temperatures leading to an increase in demand for air-conditioning. As the cooling industry becomes more energy-intensive, it leads to an even higher industrial pollution, which in turn increases the temperature again (Li et al., 2015).

All of the above leads to a phenomenon named "Urban heat islands" (UHIs), whose concept that the urban environments are continuously warmer than the surrounding rural areas (Alcoforado & Andrade, 2008) continues getting scientists' attention since it was first defined already in 1833, by Howard.

1.1.2 Subsurface urban heat islands (SUHIs)

Groundwater is known to be a relatively stable environment, with very little seasonal variation in its physicochemical parameters. Moreover, the limited availability of light, organic matter and oxygen, in comparison to the surface water, selects for organisms highly adapted to such specific environments (Gibert et al., 1994; Marmonier et al., 2023). While more stable than surface water ecosystems, groundwater is not isolated from the effect of urban microclimate patterns, especially in shallow aquifers. It has been shown that the groundwater temperature is correlated with the surface temperature and the density of building coverage above the surface (Previati & Crosta, 2021), and that certain subsurface areas have higher temperatures in comparison to those surrounding them. These areas have been referred to as subsurface urban heat islands (SUHIs) (Ferguson & Woodbury, 2007). The most pronounced urban heat sources that cause increase in subsurface temperature are the basements, sewage networks, the subway infrastructure, high-voltage cables (Menberg et al., 2013; Tissen et al., 2019) and underground tunnels (Previati et al., 2022), as depicted in Fig. 1.1. In Vienna, an additional source of heat are the heat district distribution systems, a well-developed network despatching cold and warm water to households, with the water going up to 160 °C temperature (Wien Energie

GmbH, 2023).

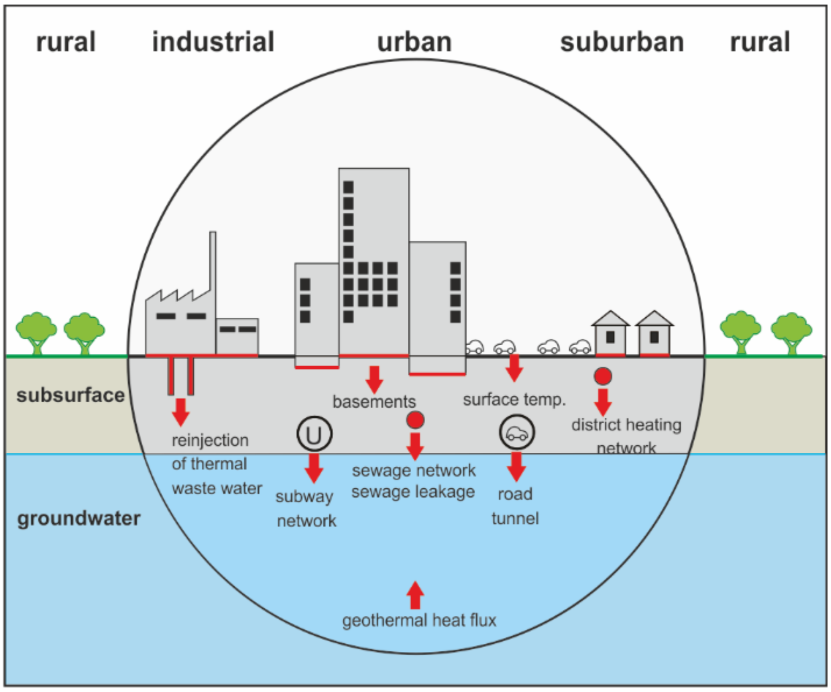

Figure 1.1: Schematic depiction of subsurface heat sources in urban areas which affect groundwater temperatures, source: Menberg et al. (2013)

With the motivation of localizing SUHIs, groundwater temperature profiles have already been measured in several cities, including Cologne, Paris, Peking, Winnipeg. Some studies are focused on making a profile of a few cities (Menberg et al., 2013), and some aimed to design a thorough complex model of just one city (Zhu et al., 2015), going into hydrological patterns and taking into consideration the recharge patterns. Previati et al. (2022) showed the importance of vertical temperature flow from the surface and analyzed in detail what processes have lead to higher temperatures in Milan. However, there are not many studies which aimed to connect the urban temperature variation to microbial communities and their biodiversity, community composition, or an overall ecosystem functionality.

1.2 Heat Below the City project - an ecological overview of Viennese groundwater

Many of the groundwater ecological studies focusing on temperature as the main environmental variable try to recreate conditions in a laboratory environment (Bonte, Röling, et al., 2013; Brielmann et al., 2009; Griebler et al., 2016; Qu et al., 2022), with very few focusing on the microbiome in its natural habitat.

Vienna, populated by 1.8 million people, i.e. a highly urbanized area, as a city with a long culture of groundwater research and many installed groundwater wells, should provide a good insight on urbanization's impact on temperature, and consequently, on microbial communities. We analyzed the groundwater microbiome together with physicochemical measurements of $\tilde{3}20$ groundwater samples taken directly from wells within Vienna and its surrounding. The objective of this thesis is to provide a comprehensive overview of the composition of urban groundwater communities and their relation to the environmental conditions of a natural microbial habitat.

The distribution of surface temperatures in Vienna follows a spatial trend of temperature increasing from the outskirts to the city center. The surface waters (the Danube river, and its tributaries and channels), parks and the Lobau national park, have lower temperatures in comparison to the inner city and the Viennese transdanubian area (Fig. 1.2). The warmer parts of Vienna are both densely populated, have high percentage of sealed surfaces, and can be designated as urban heat islands.

The Viennese Environmental Protection Department is not only aware of the problem of urban heat islands (Fig. 1.2), but has also made a strategy to try to buffer its influence (Brandenburg et al., 2018). Still, the study focuses on how the UHIs affect us, and not how we affect them - their diversity and/or stability. The study left out groundwater from its calculations completely and focused on air temperatures, implying that the main focus of official urban temperature-related developmental strategies does not lie in groundwater systems, despite their importance. Whether anthropological manipulations of temperature with various subsurface structures can be done without causing instability of groundwater microbial communities remains yet to be explored. The Heat Below the City project focuses on the subsurface temperatures of urban areas and aims to shed light on their influence on both the Microbiocenosis and Zoocenosis. Using water sampled directly from Viennese groundwater wells, and measuring the temperature of the water at the time of sampling, could point to the consequences of temperature increase on ecosystem functions of city water, which would be less abstract to the general public than the currently widely published thermal facilities' temperature-related microbial shifts.

Within the Vienna area, aquifers originate from different geological ages, and are characterized by a different set of physico-chemical properties (Grupe et al., 2021). The most complex set of environmental conditions characterizes the Schichtwasser, while the most homogenous one is confined by the Danube gravel, and therefore referred to as Donauschotter (Fig. 1.3). Aquifers are not isolated from the surface influences, and they exchange nutrients and organisms with streams, lakes, and wetlands, resulting in changes in the groundwater chemical composition and its biological characteristics (Winter et al., 1998). The biggest portion of groundwater recharge occurs through rainfall and snowfall (Aquilina et al., 2023). In Vienna, the main sources of surface water are the Danube river,

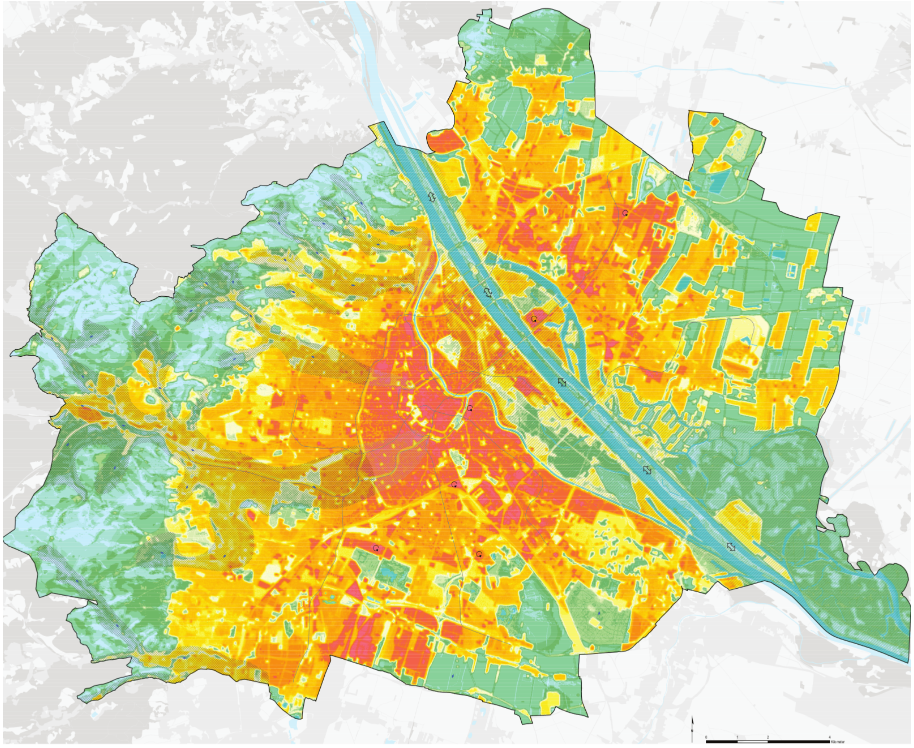

Figure 1.2: Thermal imaging of Vienna, lower temperatures shown with hues of green, corresponding to green surfaces, parks, forests and surface water bodies, the yellow colored areas exhibit moderate surplus in expected temperature, while the strongly overheated areas are shown in red, mostly located in the inner city and areas with high building density and high percentage of paved surfaces, source: https://www.weatherpark.com/stadtklimaanalyse-wien/.

its tributaries, and channels. The connectivity and aquifer type were taken into account while analyzing the microbial communities of Vienna.

1.3 Effect of temperature on microbial communities

1.3.1 Interconnectedness of temperature and other environmental variables

As a result of the complex nature of groundwater ecosystems, environmental variables are in a constant interaction and inseparable one from another (for example, gas solubility, nutrient availability and temperature), with one parameter triggering multiple other changes. More specifically, the environmental cascade put into motion by temperature variation, is well-described (Bonte, Röling, et al., 2013; Griebler et al., 2016; Riedel, 2019). A temperature increase in groundwater often leads to an anoxic environment with changed physicochemical parameters (pH, O_2, DOC, pCO_2) and changed ecosystem functionality

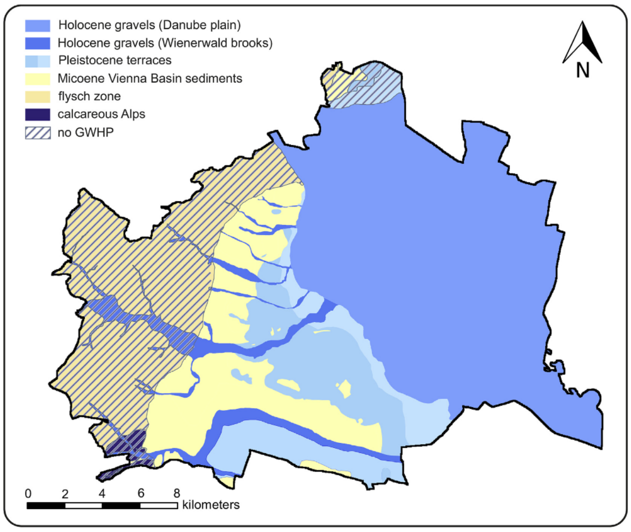

Figure 1.3: Imaging of aquifer terraces in Vienna. Donauschotter (Danube plain), and Schichtwasser (Pleistocene terraces), source: Tissen et al. (2021).

(organic matter mobilization/cycling, gas solubility, heavy metal concentrations), and mobilizes harmful heavy metals (Bonte, Breukelen, & Stuyfzand, 2013; Griebler et al., 2016). With that in mind, it should be stressed that the temperature is not only an important direct factor for the microbial composition, but could impact the composition also indirectly, through its relationship with other environmental variables. However, it must be noted that these changes are not expected with high likelihood at the moderate temperatures that *in situ* conditions provide (Brielmann et al., 2009), but could be visible if other anthropological stresses add up to the temperature stress even at small temperature deviations such as ±5 °C (Brielmann et al., 2011), where a temperature increase is defined as a deviation from the known groundwater temperature mean for the area of study.

Possible anthropogenic impacts on communities are: wastewater from humans and livestock, agricultural/industrial land use and their contribution to nitrogen and pesticide levels in water, and irrigation and surface water manipulation (United States Environmental Protection Agency, 2023b). Besides land use, aquifer categorization based on the aquifer type and age was also considered as an important factor in temperature response, since a difference in response of individual aquifers to stressors was already reported (Jesußek et al., 2013). Hence, both land use and aquifer type were considered in our study, and interaction of the two with the temperature change was regarded as the more likely factor than just the temperature alone.

Taking into consideration the aquifer type of samples could provide more accurate models - Lima et al. (2020) report that putting the temperature increase within the context of the area-specific temperature trends is an important part of the community response modelling. Microbial communities of those reef areas which experience bigger temperature fluctuations (a 13-15 °C seasonal difference between summer and winter temperatures) cannot be significantly modelled with a constant based on the temperature mean. In comparison, the community response of the reef area that experienced lower fluctuations was successfully modelled with temperature as a constant, even though yearly temperature range was also not negligible (10 °C). Therefore, responses to temperature stress could be aquifer-specific, if their temperature ranges differ.

Additionally, temperature and its dynamics are affected by the groundwater depth - the susceptibility to temperature fluctuations is higher for shallow aquifers (Taylor & Stefan, 2009). Therefore, extracting groundwater at depths under 20 meters can help register changes in ecosystem at lower rates, possibly at moderate increases in surface temperature. However, the challenge that comes with it is isolating the trends from the background scatter when performing data analysis, as was the case in our study.

1.3.2 Temperature and diversity

A temperature increase was shown to affect the alpha diversity, sometimes causing an increase (Brielmann et al., 2009), sometimes a decrease (Metze et al., 2021) in the diversity of groundwater microbial communities. Studies showing no effect of temperature on communities emphasize that with longer exposure they could exhibit different results (Keller et al., 2021), and even studies that do show a significant effect in both the alpha and beta diversity, reveal that prolonged exposure would possibly give clearer and more specific outcomes (Metze et al., 2021). As opposed to laboratory conditions, the natural habitat provides better possibilities for long term exposure. While no significant effect of increased groundwater temperature on bacterial activity and cell counts has been found in an energy-poor shallow aquifer (Brielmann et al., 2009), longer exposure or more extreme heat stress could be a deciding factor with this variable, as well.

Composition of the groundwater microbiome was also temperature-dependent in some laboratory-based studies, with the most dominant phyla differing at moderate temperatures (15 °C) from increased temperatures (+25 °C) (Metze et al., 2021; Qu et al., 2022), i.e. temperature-related shifts from *Proteobacteria* to *Actinobacteria* were marked, as well as an increase in *Firmicutes.*

1.3.3 Temperature and functionality

Lastly, even though the microbial community composition cannot give a thorough insight into ecosystem functionality directly, it can still point towards a possible further line of study by finding the relative abundance of specific function-performing taxa. Microbes drive important biogeochemical processes such as carbon and nutrient cycling, as well as provide regulating services such as water purification, pathogen elimination and pollutant biodegradation (Griebler & Avramov, 2015), so any temperature-dependent compositional shift could imply a temperature-dependent functional shift. This would have far more reaching consequences, and should be kept in mind when attempting to influence temperature through technology.

1.4 Research question and hypothesis

The temperature maximums of urban areas, and the unwanted warming of the groundwater caused by the specificity of the urban design, were taken as a point of interest and communities of these urban groundwater wells were studied.

The main question of our study was how the temperature stress in urban areas of Vienna impacts the groundwater microbial communities. We hypothesize that the microbiome will exhibit a response to temperature stress even when exposed to moderate temperature increases, particularly when multiple stressors are present and the duration of exposure is sufficient. This response can manifest as alterations in diversity and/or composition.

By exploring this scientific question and testing our hypothesis, the aim is to deepen the understanding of the impact of rising groundwater temperatures on microorganisms. Considering the role of microbes in various vital biogeochemical processes, it is crucial to foresee the changes in community dynamics that will accompany climate change, in order to be able to counteract them in time.

2 Materials and Methods

2.1 Sampling

In autumn and spring of 2021/2022, two sampling campaigns were performed throughout the urban area of Vienna, Austria, within two PhD projects (C. English, University of Vienna and E. Kaminsky, BOKU). Around 150 groundwater wells have been sampled during each of the two campaigns, located throughout the city, as shown in Fig. 2.1. Autumn samples were taken during the period from September to December 2021, while the spring samples ranged from February to April 2022. Groundwater was collected from wells maintained by the official provincial water authority (source: MA45), from the Vienna city area. The wells were categorized by the aquifer type (hydrological unit) and land use they belong to. The wells' depth ranges from 5.66 to 35.3 meters. Groundwater wells were pumped for two volumes, with a submersible pump (Grundfos MP1, Eijkelkamp Soil Water, Giesbeek, Netherlands) in order to remove the stagnant well water and obtain the fresh groundwater. After the pumping time has passed, temperature, oxygen and pH were measured on site with field sensors (WTW Multi 3620, Weilheim, Germany), and water samples were collected in different vials, in order to perform further analyses in the laboratory. Groundwater for hydrochemical analysis was filled into 50 mL plastic tubes without further treatment. Samples taken for the DOC measuring were filtered with a 0.45 µm syringe filter. For molecular analysis, additional samples were taken by filtering 10 liters of water through a 0.22 µm Sterivex filter. For the samples where the filter had clogged, an additional one liter was filtered in a new sampling vial. Such filters were further referred to as the "replicates" of the corresponding wells. All samples were stored at -20 °C until they could be further processed.

2.2 Physicochemical and cell count measurements

All the hydrochemical measurements, heavy metal measurements and categorizations based on heat sources and wastewater impact were performed by Eva Kaminsky within her PhD project - electrical conductivity, nitrate, nitrite, ammonium, sulfate, and ions such as Cl^-, Na^+, K^+, Ca^{2+}, Mg^{2+}, Fe^{2+}) were measured in the laboratory with ion chromatography (DIONEX ICS-1100; Thermo Scientific, Idstein, Germany) after being filtered through the

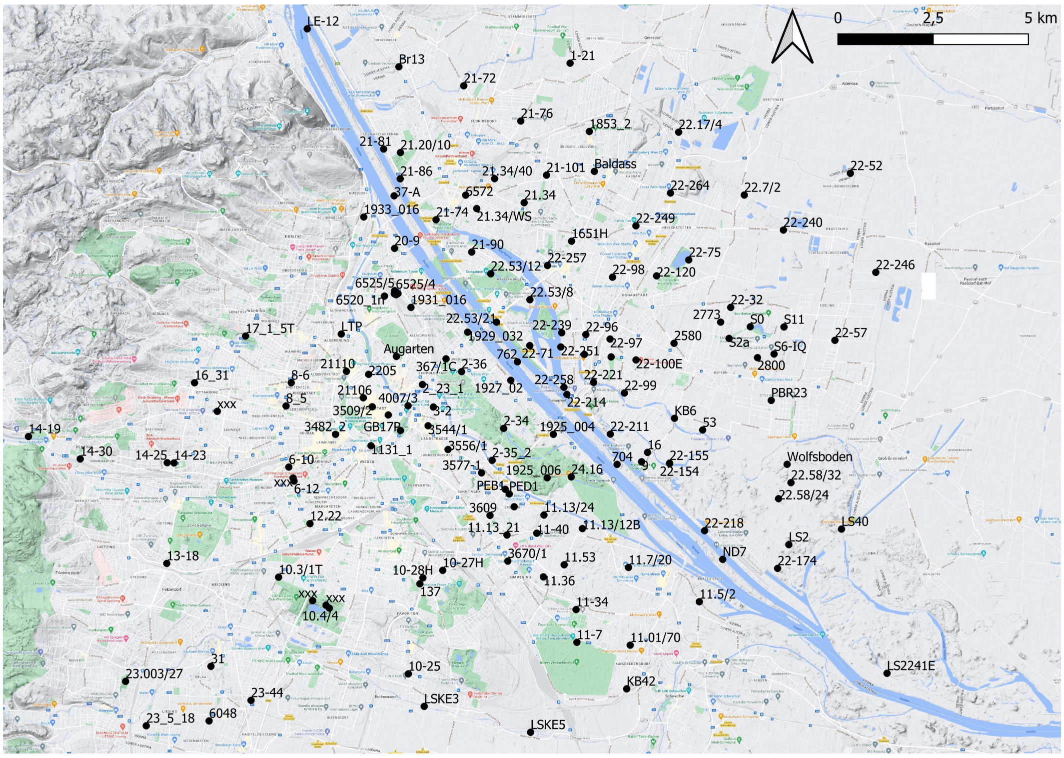

Figure 2.1: Map of Vienna. Locations of groundwater wells are black dots with names of wells shown, courtesy of Constanze Englisch.

0.45 µm membrane. The detection limit was 1 mg/L. The phosphate was also first filtered (0.45 µm membrane filtration). Sequentially, photometric measurements (Hitachi U-2000 Spectrophotometer) were taken, with the reduced phosphomolybdic acid method ($\lambda =$ 890). Dissolved organic and inorganic carbon (DOC and DIC, respectively) were measured with catalytic oxidation method with NDIR (Shimadzu TOC-L). Prior to measuring, a filtration with 0.45 µm filter was performed for dissolved inorganic carbon (DIC) and additional acidification with HCl (pH = 2) for dissolved organic carbon (DOC). The microbial activity (internal and external ATP) and prokaryotic cell counts were measured by Constanze Englisch, as a part of a PhD project. The total microbial activity was measured with a luciferin-oxidation based method with luminator plate reader (Promega, Madison, WI, USA), in triplicates, after which the microbial activity outside the cells was measured, in order to obtain the internal cell activity. Total cell counts of prokaryotic cells were measured with a Flow Cytometer (Amnis CellStream, Luminex, Austin, TX, USA).

2.3 Molecular analysis

DNA was extracted for all samples based on a protocol published by Pilloni et al. (2019), in order to prepare the samples for 16S rRNA gene amplicon sequencing. Since the phenol chloroform extraction continues to prove itself to be the most effective method (Busi et al., 2020), this was the chosen approach. All vials were autoclaved prior to use. The 0.22 µm Sterivex filters were cut into the 2ml vials which contained 0.2 mL of zirconia-silica beads (Biospec, Bartlesville, USA) in a 1:1 ratio of 0.1-mm and 0.7-mm size. Then 750 µL of PTN buffer (phosphate-Tris-NaCl) was added, after which the addition of lysozyme (50 mg mL^{-1}) and proteinase (10 mg mL^{-1}) followed. With this, the lysis process started. The incubation period of 15 minutes at 37 °C in a Thermomixer was combined with a sporadic manual mixing of vials, after which 100 µL of 20% SDS was added to denaturate the proteins. The second incubation also required a Thermomixer, with shaking at 500 rpm at 65 °C for 15 minutes, and then 100 µL of PCI (phenol-chloroform isoamyl alcohol in 25:24:1 ratio) was added. The next step consisted of a physical lysis through bead beating (MP Biomedicals, Santa Ana, CA, USA), for 45 sec at 6.0 m sec^{-1}, which was then followed by centrifugation at 4 °C for 5 min at 6000×g. Subsequently, 600 µL of the supernatant was transferred to an autoclaved 2 mL vial. The remaining of cell fragments was subjected to one more bead beating, this time for 20 sec at 6.5 m sec^{-1}, after which it was once again centrifuged at 4 °C for 5 min at 6000×g, to extract residual DNA. Both supernatants were pooled together in the fresh vial, and 900 µL (1 volume) of PCI was added. After strong manual shaking, centrifugation for 4 min at 21 000xg and 4 °C followed. The supernatant (800 µL) and 24:1 chloroform:isoamyl alcohol (800 µL) were mixed in a fresh Phase Lock Gel vial, manually shaken, and centrifuged as before. After 650 µL of supernatant was mixed with 2 volumes of the PEG buffer (30% polyethylene glycol) in a fresh vial, the samples were left to precipitate at 4 °C for 2–12 hours. After the precipitation period, another centrifugation was performed to pellet the DNA, for 30 min at 21 000×g and 21 °C. With supernatant removed, the pellet was washed from the vial walls with 150 µL of the 70% ethanol, cooled at -20 °C. Following the 5-minute centrifugation at 21 000×g and 4 °C, the ethanol was removed, and the pellet was left to dry for >5 min. Finally, it was resuspended in 30 µL EB buffer (10mM Tris-HCl, pH 8.5) and stored at -80 °C until it was sent for gene amplification and sequencing of the V4 region of the 16S rRNA, with the 515F/806R primers, per Pjevac et al. (2021) protocol.

2.4 Data analysis

The bioinformatic processing of the raw sequence data was done using DADA2 (Callahan et al., 2016) to select the viable amplicon sequence variants (ASV). It was performed by the Joint Microbiome Facility (JMF) of the Medical University of Vienna and the University of Vienna under the project ID JMF-2207-07, where they did quality filtering, merged forward and reverse reads, and further processed the ASV abundance table by removing the ASVs classified as organelles (mitochondria or chloroplasts), and those ASVs that could not be classified as neither Bacteria nor Archaea at the domain level. Classification of sequences was done with SINA (version 1.6.1) (Pruesse, Peplies, & Glöckner, 2012) based on the SILVA database (release 138.1) (Quast et al., 2012).

All data was analyzed in R (version 4.3.0.) (R Core Team, 2022) and visualized with the R package "ggplot2" (Wickham, 2016). The sampling site was mapped with "sf" (Pebesma, 2018) and "mapview" (Appelhans et al., 2023) packages. After data selection of only the wells which had complete measurements of physicochemical parameters, sampling information, total cell counts, ATP levels and were sequenced in both campaigns, the total number of data points was 321 samples and a total of 26 729 ASVs. From this, replicate abundances were averaged for the wells which had more than one filter per sampling well. The average number of sequence reads was 7390 $\pm$ 4112, with the highest number in one sample being 16 693.

The "decontam" (Davis et al., 2017) was used for identifying possible contaminants occurring during the DNA extraction process, by subtracting from the samples those ASVs which had an increased prevalence in negative controls (blanks), based on a strict threshold of 0.1. Since the extraction was done in batches, each of the 45 blanks was connected to the $\sim$10 samples of the same extraction method, and analyzed for contaminants separately. Overall, there were 5763 ASVs categorized as contaminant ASVs, where the majority belonged to class *Nanoarchaeia*. After removing them from the count table, ASV abundances were then rarified with "vegan" (Oksanen et al., 2022), to account for the variation in the total number of sequence reads between samples. It resulted in an equal number of 2000 reads per sample, and the final ASV table contained a total of 228 samples and 23 730 taxa.

The package "vegan" (Oksanen et al., 2022) was used to perform both alpha and beta diversity analyses, Shannon diversity with the command "*diversity*" and richness with "*specnumber*". Effective number of species was calculated as an exponential of Shannon diversity, *"exp(H')"*. The indices were compared across the groups with an ANOVA, if parametric, or a Kruskal-Wallis test, when non-parametric in nature. They were further compared with the Dunn test from the package FSA (Ogle et al., 2023), with the Hochberg correction. The relationship between diversity and other environmental variables was modelled with multiple linear regression, again removing any variable with variance inflation factor (VIF) over 10, and the significant variables were chosen with a *"stepAIC"* command.

The "vegan" package was further used to obtain a Principal component analysis (PCA) and the non-metric multidimensional scaling (nMDS), on a genus level. Because of the compositional nature of microbial data, nMDS was performed on the hellinger-transformed abundances (Legendre & Gallagher, 2001). A PERMANOVA for the compositions across the groups was done with "*adonis2*", while their dispersions were tested with the "*betadisper*", both "vegan" package commands. The "*pairwise.adonis2*" from the "pairwiseAdonis" package (Arbizu, 2020) provided the closer inspection of the nMDS ordination.

The "phyloseq" (McMurdie & Holmes, 2013) and "microViz" (Barnett, Arts, & Penders, 2021) packages were also utilized for a taxonomic categorization and identification of abundant classes and phyla. Abundances were analyzed at the class level (*"tax_glom"*), and transformed to relative abundances (*"transform_sample_counts"*). The data was then summarized with "dplyr" (Wickham et al., 2023) to find the values for mean ± standard deviation. Rare and core taxa were identified with "microbiome" (Lahti & Shetty, 2017) and its commands "*rare_members*" and "*core_members*" were used on relative abundances.

Vegan's "*capscale*" on environmental variables with variance inflation factor <10, and the corresponding *"anova"* of this, was used to detect significant environmental variables and to plot them on an ordination plot.

2.4.1 Categorization of samples

For a more comprehensive analysis, samples were categorized based on their temperature values (Table 2.1.). Samples colder than 10 °C were considered colder than the average groundwater temperature of a rural undisturbed aquifer, and further on categorized as *"<10"*. Samples within the undisturbed groundwater temperature range were assigned to *"10-12"*, and those close to the Viennese temperature average, as defined from the results, were categorized as *"12-14"*. Samples with temperatures higher than 20 °C, which was regarded as extremely high for groundwater, were grouped together as *">20"*. The remaining samples (14-20 °C) were broken into three groups with a two degree range. This resulted with three additional categories, *"14-16"*, *"16-18"* and *"18-20"*.

Category	<10	10-12	12-14	14-16	16-18	18-20	>20	reference
Sample size	5	8	68	89	39	9	13	6

Table 2.1: Sample size of each of the designated temperature groups.

Reference samples

Samples with selected parameters (DOC, oxygen) and microbial measurements (total cell counts) below defined thresholds were considered as a reference - meaning that samples with elevated DOC (> 1.4 mg/L), low oxygen (> 2 mg/L), and temperatures higher than 14 °C were left out, as well as those with big fluctuations in cell counts between the sampling campaigns. The groundwater wells located close to densely populated areas were also left out when identifying reference samples. The remaining seven samples were located in the Marchfeld area, i.e. outside the densely urbanized Viennese area (Fig. 3.2.). They were chosen as an approximation of a non-urbanized groundwater habitat, and a starting point for further analyses.

3 Results

All analyses were done with the aim of understanding the impact of the groundwater temperature on the composition of microbial communities inhabiting the urban groundwater in Vienna. Therefore, the main focus was put on the temperature as the important environmental variable for the microbial community composition and community dissimilarity. Responses in diversity and taxonomic composition of communities were evaluated for the different temperature categories (Tab. 2.1.). Additional environmental variables (water chemistry, concentrations of greenhouse gases, depth of the groundwater table, microbial activity), were taken into consideration in order to have a comparative overview in regard to temperature influence. The combined effect of temperature, land use and aquifer type was explored, to account for the possibility of an additive impact on microbial communities.

3.1 Temperature distribution in Vienna

3.1.1 General overview of temperature measurements

Groundwater temperature differed between spring and fall (Fig. 3.1, right), with the fall groundwater temperature averaging at 15.28 ± 2.32 °C, and the spring average reaching 14.22 ± 2.63 °C ($W = 8251$, $p < 0.01$). Most of the groundwater sampling sites were within the 13-16 °C temperature range (52% of samples). The maximum temperature measured in fall was 25.7 °C at location "10-27H", while the spring maximum was 23.3 °C at "10-28H", both of these sampling wells were located within the densely built city area.

3.1.2 Seasonal distribution of temperature

The groundwater temperatures varied substantially between the two sampling surveys, even at the same sampling location (Fig. 3.1, left). Most of the samples with temperature maxima were grouped together (almost exclusively located in the inner city), and were repeatedly, i.e. in both samplings, warmer than the average groundwater temperatures of rural areas. While most wells remained within a narrow temperature range (the average difference in water temperature within the same well was 1.064 °C), some wells exhibited an extreme temperature variation from autumn to spring. The maximum difference was measured in well "KB6" (10.5 °C - varying from 19.2 °C in the fall to 8.7 °C in the spring) and well "762" (10.4 °C - varying from 16.6 °C in the fall to 6.2 °C in the spring). Both of the wells with these extreme variations in temperatures are located close to the Danube River and its channels, and showed substantial oscillations in oxygen and dissolved organic carbon levels, as well.

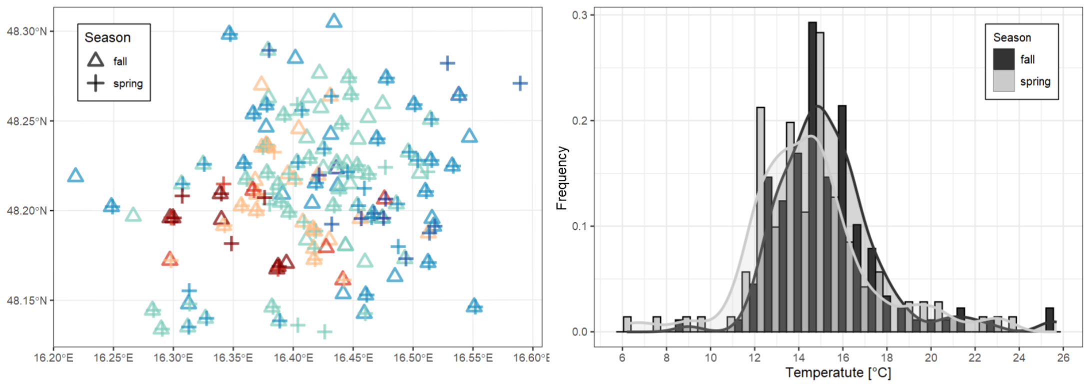

Figure 3.1: Left. Geographic locations of sampling wells. Temperature scale depicted with a color scheme on a blue-red continuum, where red is the highest temperature. Autumn temperature depicted by a triangle symbol, spring by a plus symbol. Right. Distribution of groundwater temperatures. Frequency signifies the number of well within the same temperature bin. Fall temperatures in dark gray, spring in light gray, with density curves in the same color.

There were 25 wells with temperatures within the temperature range of undisturbed groundwater habitats (as prior mentioned, between 10 and 12.5 °C), which makes 14.8% of analyzed wells. Out of those, 13 wells did not have severely depleted oxygen levels ($>$ 2 mg/L, as defined by United States Environmental Protection Agency (2023a)) nor elevated DOC levels ($<$ 1.5 mg/L), which makes up only 5.5% of urban wells roughly resembling the groundwater habitats not affected by urbanization (Chapelle (2022) dictates $\leq$ 1 mg/L DOC is a characteristic of pristine groundwater).

3.1.3 Spatial distribution of temperature

All samples within the "*16-18*", "*18-20*", and "*20<*" categories, meaning warmer than 16 °C, were located in the densely built inner city of Vienna (Fig. 3.2.). The urbanized areas in the proximity of surface waters (the Danube's branches and its channel) did not measure temperatures higher than 16 °C, and neither did the green outskirts of the city.

All samples with extreme temperatures ("*20<*"), were shallower than 10 meters. The average depth of the 13 wells with 20+ °C (Table 2.1.) was only 3.9 meters. The difference

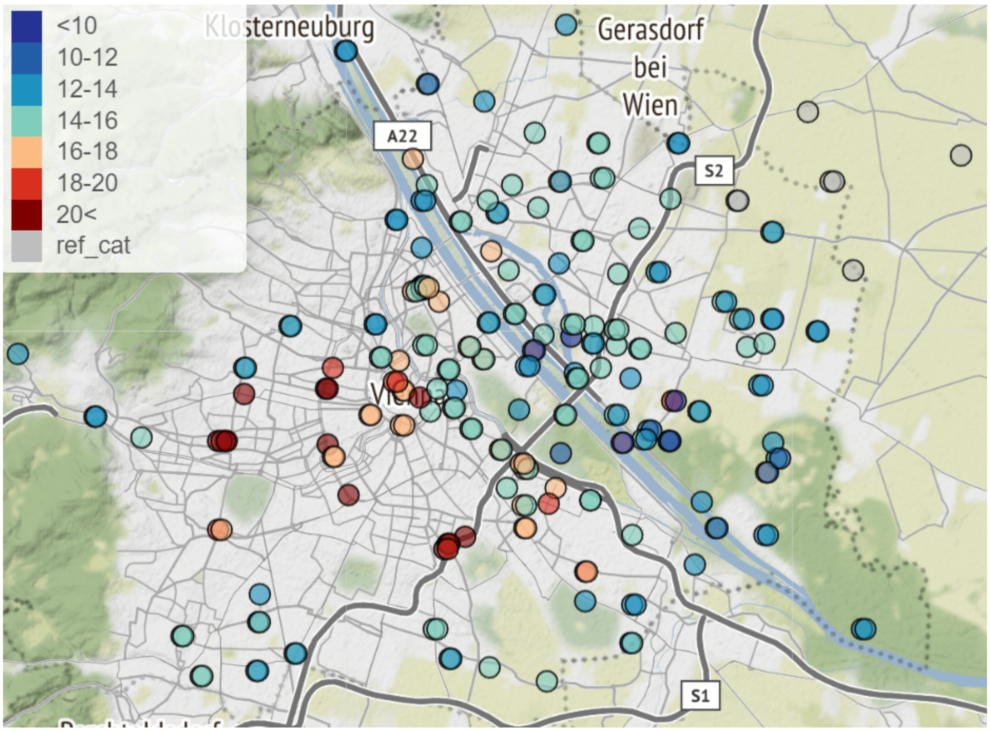

Figure 3.2: Map of Vienna with sampling wells. Circles represent a well colored by a temperature scale corresponding to the temperature group categorization, "ref_cat" representing the reference samples category. Spring wells in foreground, with autumn dots shifted slightly to the left.

in temperature distribution between wells deeper than 10 meters, and those less than 10 meters deep ($W = 4432.5$, $p < 0.01$) showed in average temperatures, with shallow wells averaging at 15.16 °C, and deep wells at 13.7 °C.

3.2 Impact of temperature on the community composition

3.2.1 Overview of groundwater microbial communities at class level

The taxonomic composition of all the samples, divided by the temperature category they belong to, is visualized in Fig. 3.3, with individual samples ordered by similarity. The figure depicts the sample sizes of the groups, and the composition variability within the group. Only the most abundant 15 classes are shown, while the rest is grouped as *"Other"*. Most samples have similar composition, consisting of *Nanoarchaeia*, *Omnitrophia*, *Gammaproteobacteria* and *Nitrososphaeria*, except for a small cluster of ten samples.

The ten clustered samples which were dominated by *Cyanobacteriia* (with relative abundance within the cluster amounting to 23.1% ± 16.1%), *Actinomycetia* (14.2% ± 9.11%) and *Bacteroidia* (26.9% ± 14.5%) are seen to be distributed across most temperature categories (Fig. 3.3), and hence not connected to temperature. As opposed to the rest of the samples, where a class from the Archaea domain was the most abundant, it made up less than 0.5% of relative sample abundance for each sample in these 10 samples. The phylum *Proteobacteria* was represented within this cluster with *Gamma-* and *Alphaproteobacteria* classes in higher percentage than in the rest of the samples (13.6% ± 6.93%, and 7.29% ± 4.76%, respectively).

For the majority of samples, the most prevailing class was *Nanoarchaeia*, from the Archaea domain (37.9% ± 15.4% across all samples). The most abundant class from the Bacteria domain was *Omnitrophia* (8.10% ± 4.55%), and the fourth most abundant class overall also belonged to the Archaea domain - *Nitrososphaeria* (5.08% ± 4.67%). Relative abundances averaged by temperatures are shown in Fig. 3.4. From the phylum *Proteobacteria*, the most abundant class was *Gammaproteobacteria* (7.96% ± 6.22%), followed by *Alphaproteobacteria* (2.73% ± 3.14%).

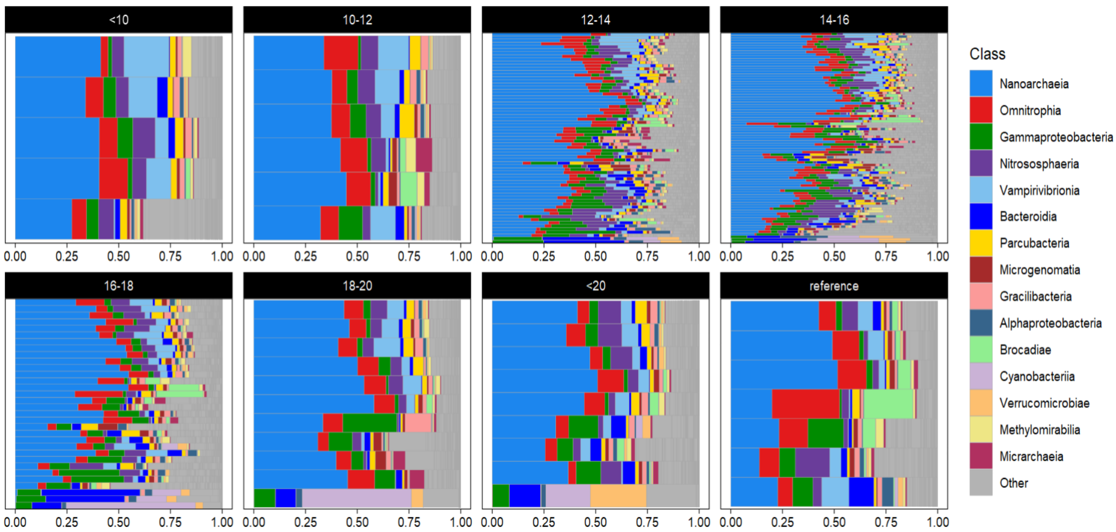

Figure 3.3: Relative abundance of the 15 most abundant classes. The remaining classes are classified as "*Others*". Communities were analyzed for each temperature category. Samples were ordered by their dissimilarity.

Phyla with more than one class with > 1% relative abundance were *Cyanobacteriota* (with classes *Cyanobacteriia*, 1.13% ± 5.72%, and *Vampirovibrionia*, 4.27 % ± 4.63 %) and *Patescibacteria* (with classes *Gracilibacteria*, 1.58% ± 1.23%, *Paceibacteria*, 2.55% ± 1.93%, *Microgenomatia*, 1.79% ± 2.33%).

The remaining classes represented with more than 1% of relative abundance in each sample they are present in, were: *Bacteroidia* (4.20% ± 6.44%), *Brocadiia* (1.55% ± 3.34%), *Methylomirabilia* (1.06 % ± 1.06%), *Verrucomicrobiae* (1.27% ± 2.69%), and

Micrarchaeia (1.43 % ± 1.76%).

None of the most abundant classes are significantly different between the groups they appeared in, but classes *Verrucomicrobiae* and *Actinomycetia* seem to show an increase in abundance at higher temperatures (left, Fig. 3.4). They appeared from 20 °C and 16 °C onwards, respectively. The class *Cyanobacteriia* appeared in substantial abundance from 16 °C onward, and *Campylobacteriia* were present in the 14 to 20 °C range. On the other hand, class *Methylomirabilia* was only present in temperatures below 12 °C. Class *Brocadiia* was the most abundant in the reference samples, and did not appear in substantial amounts at temperatures higher than 18 °C, nor in samples with temperatures lower than average.

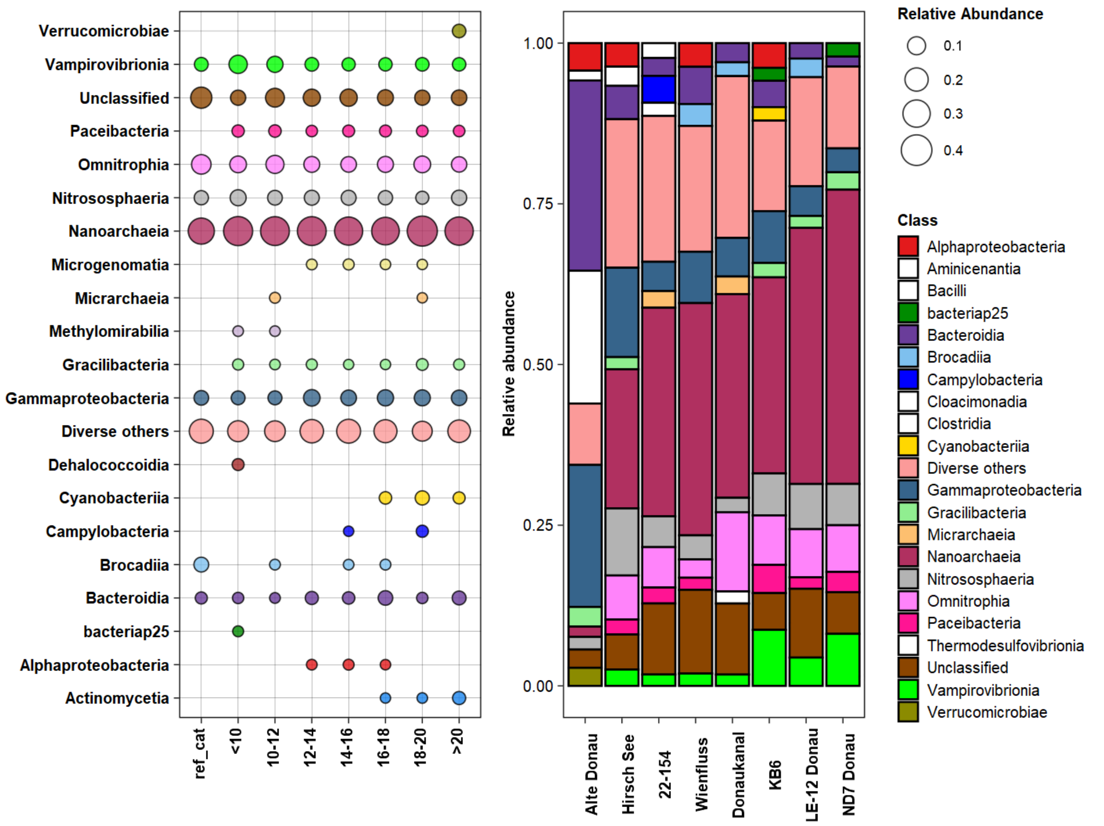

Figure 3.4: Left. Relative abundances of the most abundant classes. Classes with less than 1% are classified as "Diverse others". Communities were analyzed for each temperature category. Right. Overview of microbial composition of the 8 surface water sampling sites. The taxa not present in the groundwater was plotted in white color.

Classes which differed between the urban sampling sites and the reference sampling sites were *Gracilibacteria* and *Paceibacteria*. Both classes were absent from the sampling sites which were not exposed to the urban influences, and were consistently present in all the urban wells, regardless of the temperature values. Classes which were present only at sampling sites with groundwater temperatures below 10 °C, were bacteriap25 and *Dehalococcoidia*.

Microbial communities of samples collected from urban surface waters were dominated by *Nanoarchaeia*, just like the groundwater microbial communities (right, Fig. 3.4). Only the Alte Donau sampling site (the Old Danube) exhibited a different trend by being *Bacteroidia*- and *Gammaproteobacteria*-dominated. Bacteriap25 was present at two surface water sampling sites, while *Dehalococcoidia* was absent from the surface water sites we sampled.

3.2.2 Overview of groundwater microbial communities at order level

Considering the scarce values of abundance when analyzing individual orders, it was not sensible to proceed with the previously established temperature categories (Table 2.1), and only three categories for temperature were chosen (Fig. 3.5). Categories were limited at 13 °C, and 15 °C, which gives three groundwater temperature categories that had similar samples sizes. With this analysis, the most pronounced results were seen for the Candidatus Roizmanbacteria and *Desulfobacterales* abundances. These orders showed an increase in abundance counts with an increase in groundwater temperatures ($X^2_{(2)}$ = 5.62, p = 0.06; and $X^2_{(2)}$ = 5.26, p = 0.07, respectively).

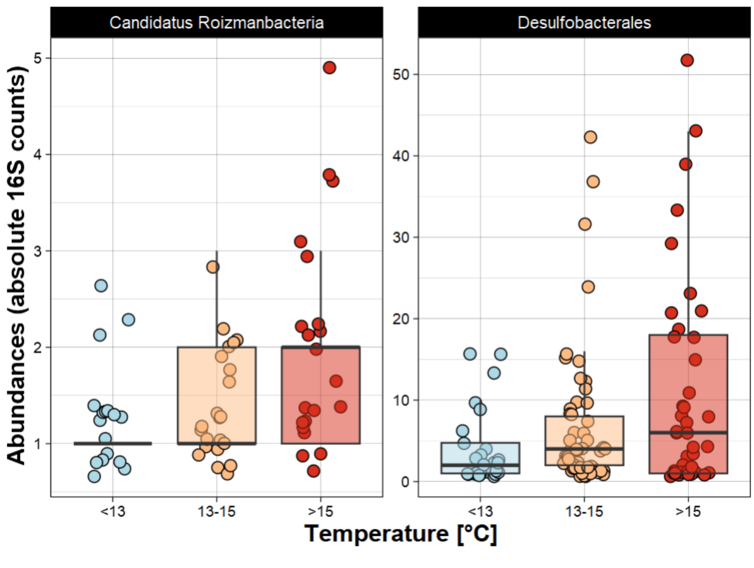

Figure 3.5: Abundance counts of the two orders which showed temperature-related abundance trends. The boxes represent quartiles, the lines represent medians, and dots the sampling sites in the color corresponding to each category.

3.2.3 Core and rare microbiome per sample

Rare species made up 97.64% of all microbial species present (with rare species defined as those ASVs which make up less than 0.01% of the sequences, and occur in less than 20% of the samples, i.e. in less than 63 samples). The core microbiome was defined as species making up more than 0.01% of the community while also being present in at least 80% of the samples, and consisted out of only 2 ASVs fitting this definition. The two ASVs in question belonged to the phylum *Thermoproteota* (family *Nitrosopumilaceae*).

3.3 Impact of temperature on the microbial diversity

3.3.1 Alpha diversity

Alpha diversity per geographical position

A geographical depiction of the Shannon's diversity index shows no clear spatial trend. Specifically, the inner city area, characterized by a significant portion of sealed surfaces and temperatures higher than the surrounding areas, does not exhibit a homogeneous trend in terms of diversity. There was also no clear difference between the aquifer types.

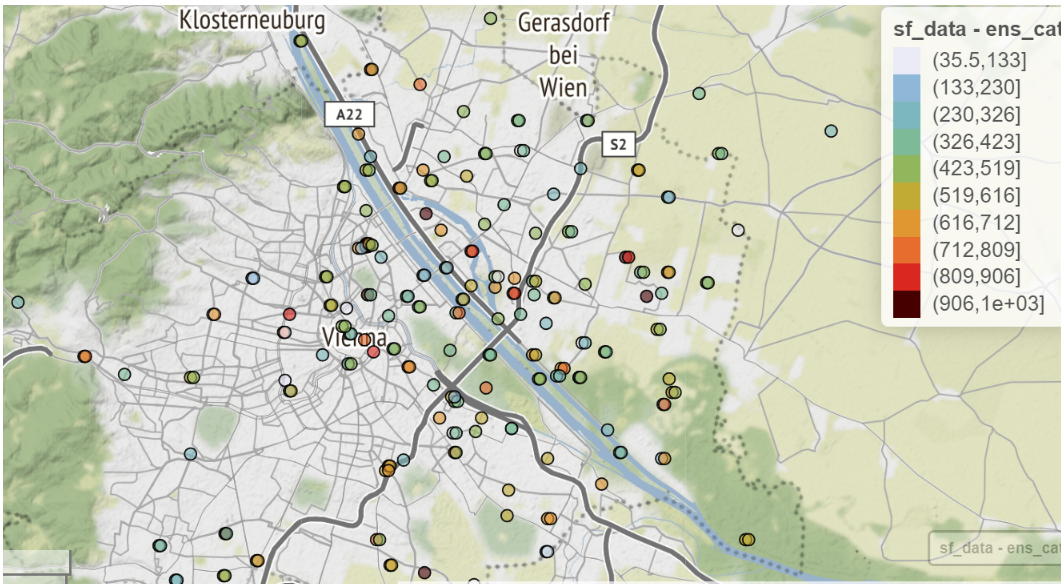

Figure 3.6: Map of Vienna with Shannon diversity index (as effective number of species) segmented into ten equally large intervals, indicated by the color scale. Circles represent each well, with spring wells in foreground, with autumn dots shifted slightly to the left.

In addition to the absence of a clear spatial trend, the diversity also varied from fall to spring. Even the diversity in groundwater from the reference sampling sites does not show complete similarity, with individual wells having medium values, and one well falling on the low side of the diversity spectrum.

Alpha diversity per temperature category

Values of the effective number of species, i.e. the exponential value of the Shannon's diversity index, visibly decreased within the temperature range from 10 to 18 °C. After that, the diversity index of groundwater sampling sites starts increasing. Worth noting are the diversity values of the reference samples, which range from low to moderately high. They are visibly lower than the diversity values of the same temperature urban counterparts (*"10-12"*).

The Shannon's diversity values significantly differ from one temperature group to another ($X^2_{(7)} = 15.57$, $p < 0.05$), Fig. 3.7. However, the post-hoc search for specific differences between categories did not give significant insights, with the dunn test giving all adjusted p-values above 0.05.

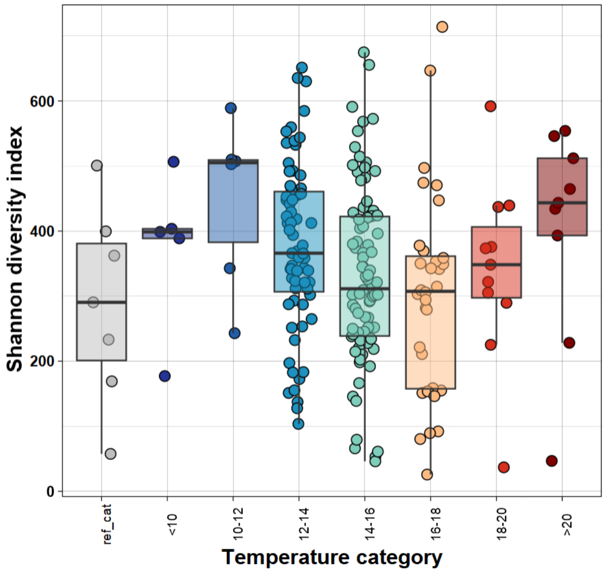

Figure 3.7: Boxplots of Shannon's diversity index of samples by temperature category. The boxes represent quartiles and the lines represent medians, the jittered dots in the color corresponding to each category represent individual sample values. The y-axis is the exponential value of the Shannon's diversity index.

Alpha diversity per aquifer

When analyzing the Shannon's index of groundwater from all wells, they did not reveal any significant correlation to measured environmental variables, aside from the groundwater extraction depth. The deeper the extraction occurred in the well, the lower was the Shannon's index in the well ($R^2 = 0.018$, $F_{(1,234)} = 5.39$, $p = 0.021$). However, the extraction depth does not exhibit a significant relation to any other measured parameters, and is simply the depth of 2 meters below the groundwater table.

The low R^2s of diversity indices and this particular environmental parameter, were reminiscent of the unclear relationships other environmental variables had between themselves. However, a better model was found by accounting for the aquifer type as follows: while an ANOVA performed on absolute diversity numbers per temperature categories was not significant for aquifers ($F_{(2,199)} = 1.012$, $p = 0.365$, as performed for the three aquifers that had more than 5 sampling points), the trends in $\Delta_{diversity}$ in accordance to temperature were aquifer-specific and provided certain regressions (Fig. 3.8).

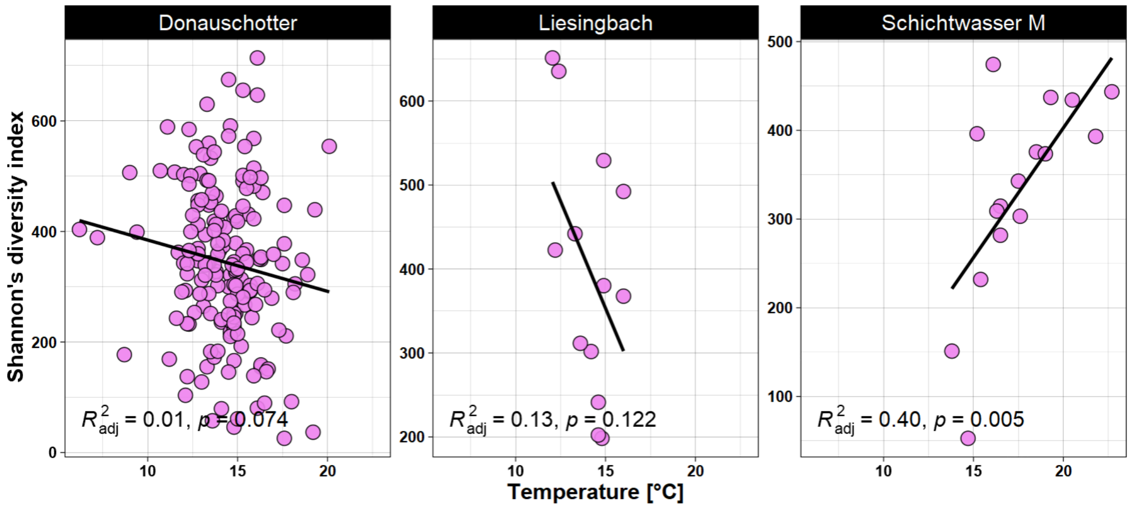

Figure 3.8: Diversity response to heat stress by aquifer. The line represents linear regression, and the R^2 and p-value are written at the bottom of every plot. n(Liesingbach) = 13, n(Schichtwasser) = 16, n(Donauschotter/Danube gravel) = 178.

A decrease in diversity with increasing groundwater temperatures was seen in wells belonging to the Liesingbach aquifer ($p = 0.122$) and those wells embedded in the Danube gravel ($p = 0.074$). In the Schichtwasser system, the trend was increasing. The Shannon's diversity index of this specific aquifer increased with higher groundwater temperatures ($p = 0.005$).

Since the temperature range of the three aquifer types differed, with the Schichtwasser sampling sites exhibiting the highest average temperature, as well as an increasing trend in diversity index accompanying increasing groundwater temperatures, it put the concave trend of the Fig. 3.7 in question. For this reason, the subsets of data along the temperature range and aquifer types were further explored (*3.5. Donauschotter aquifer* section).

3.3.2 Beta diversity

Beta diversity per geographical position

The dissimilarity between the samples was not related to the geographic position of the wells, nor the distance between them (as seen from the absence of a good correlation fit of geographical distances and community distances). Both the spatial distance-dissimilarity relationship for the fall, as well as for the spring samples, provided less than 1% of explained variance. The few samples clustered away from the majority of other wells, shown on the left side of Fig. 3.9, are the same wells referenced in the section *3.3.2 The cluster*. Their uniqueness was not a consequence of physical separation from the rest of the samples, since they disperse along the entire continuum of the physical separation (map supplemented in Appendix, Fig. 6.1.).

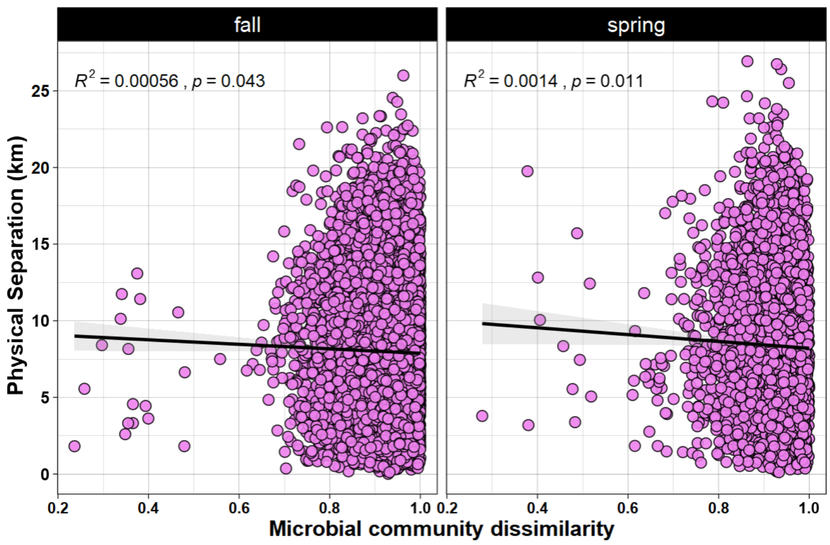

Figure 3.9: Plot of the geographic distance (in kilometers) against the Bray-Curtis distances between fall samples on the left and spring samples on the right. The R^2 and p-value are included in the top left corner of both plots.

Beta diversity per temperature category

The overview of the microbial communities and their similarity to each other within and between the temperature categories, was performed with the Bray-Curtis distance measure of the hellinger-transformed counts and visualized with a non-metric ordination. The ordination was performed on a genus level, in order not to lose information by focusing on species (many ASVs cannot be determined at the species level) and in order not to simplify by analyzing at a higher, too general, taxonomic level.

PERMANOVA of an nMDS did not show a possible grouping by temperature ($R^2 = 0.031$, $F_{(7,211)} = 1.089$, $p = 0.524$, perm = 999), and did not imply compositional differences between the microbial communities of all the groundwater wells (Fig. 3.10, on the left). However, there was a significant difference in the range of within-category dissimilarity (an anova of the betadisper test gave $F_{(7,211)} = 2.222$, $p < 0.05$,). Therefore, although the temperature categories share the taxa and do not have distinct microbial communities, a trend could still be distinguished. The dissimilarity of the microbial communities from warm urban aquifer sampling sites was higher than that of the colder ones. Additionally, the microbial communities' turnover of the reference sampling sites is more comparable to the turnover of the warm end of the spectrum, than to that of the cold one.

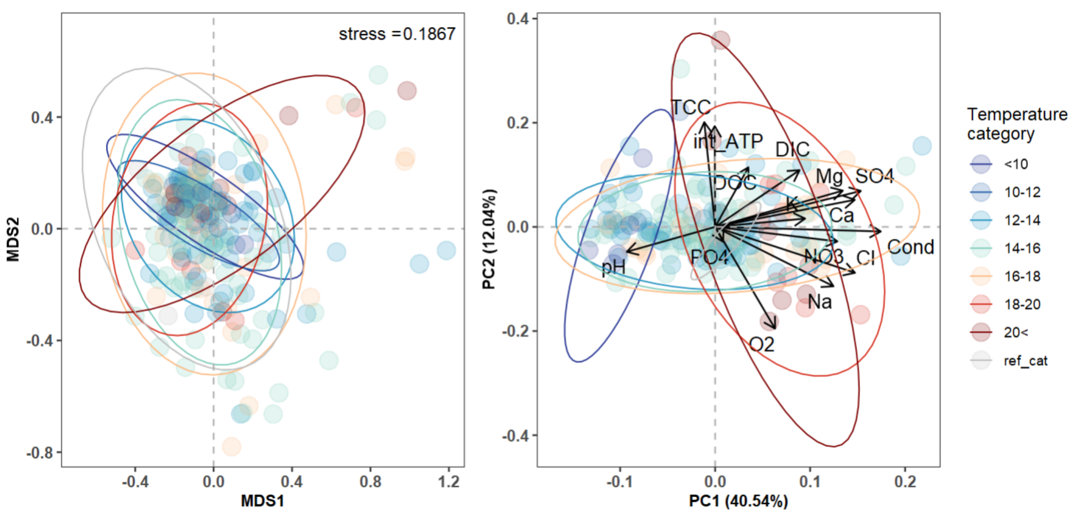

Figure 3.10: Left. NMDS ordination of hellinger-transformed Bray-Curtis dissimilarities of the species counts aggregated on the Genus level. Right. PCA ordination of environmental characteristics for all samples, with PC1 (40.54%) and PC2 (12.04%) axes. Elipses around 95% of data points, in colors corresponding to each of the temperature categories.

The metric ordination (Fig. 3.10, on the right) was performed to connect the possible patterns of environmental variation to the microbial community variation. The metric ordination (a PCA) explains 40.54% of variance on its first axis and 12.04% on its second axis. There are two different clusters in terms of environmental conditions: < 10

°C samples and > 18 °C samples separate along the first PC axis, which was for the most part influenced by pH variation, and various ions/conductivity variation. The pH variation is much more pronounced in the cold samples, than in the warm ones. The high heterogeneity of the microbial community composition of reference sampling sites is highly pronounced, while their environmental measurements show very little variability.

A PERMANOVA with 1000 permutations performed on the Canonical Analysis of Principal coordinates model did not provide statistical support of the presumed connections between the environmental variables and groundwater microbial composition ($R^2 < 0.01$, $F_{(8,187)} = 0.97$, $p = 0.68$). Testing the effect of each variable by performing a PERMANOVA with 1000 permutations that was set to test by margin, gave further insignificant results for each separate continuous variable tested.

Beta diversity per aquifer

The similarity between microbial communities was not significantly impacted by the samples' aquifer type ($F_{(4,197)} = 0.78$, $p = 0.751$, perm = 999). The interaction of temperature and aquifer type was not a significant factor, either ($F_{(27,174)} = 1.05$, $p = 0.062$, perm = 999).

The cluster

Both the non-metric multidimensional scaling, as well as Fig. 3.3 show a distinct cluster, which was not connected to neither the geographic position nor any of the measured environmental variables. There was also no pattern in the sampling nor the molecular processing. Ten samples (the cluster surrounding the [1, 0.4] coordinates in Fig. 3.10. on the left), out of which nine were sampled in autumn, coincided with the cluster described in *3.2.1 Taxonomic overview of groundwater microbial communities.*

3.4 Aquifer characteristics

The most outstanding differences between aquifers with respect to the variables considered in our analysis were in water chemistry. Fig. 3.11 showed differences in mineral ion response to temperature increase for the three aquifers which had sufficient sample sizes. The Schichtwasser system shows a decrease in Ca^{2+}, Cl^-, K^+ (***), Na^+ (***), and Mg^{2+}, as seen clearly also from the decrease in its electrical conductivity (***), while the Donauschotter aquifer showed the opposite patterns (electrical conductivity**, Ca^{2+}**,

Cl^{-}***, K^{+}***, Na^{+}***, Mg^{2+}*). The Liesingbach aquifer had a very narrow temperature range, which made the comparability of trends difficult between aquifers.

Additional to the mineral ions, the significant trends were observed for the sulfate ions, which increased with increasing groundwater temperatures for the Donauschotter aquifer (*), and decreased for the Schichtwasser aquifer (***). This decreasing trend in the Schichtwasser aquifer was fairly pronounced ($R^2 = 0.642$, $F_{(1,14)} = 27.84$, $p < 0.001$).

The pH values varied little between the aquifers. The dissolved oxygen concentrations decreased more rapidly with an increase in the groundwater temperature in the Schichtwasser system than in the other two aquifers, but the decreasing trend of dissolved oxygen concentrations with increasing groundwater temperatures was observed for all aquifers. Furthermore, the behavior of total cell counts (TCC) was not different for individual aquifers. However, the microbial activity increased (ATP) in the Schichtwasser system with increasing groundwater temperatures. The divergent patterns in all the other measured parameters show the complexity in aquifer characteristics and imply the need to analyze the microbial communities as subsets defined by aquifer types. Considering the complexity and differences between aquifers, we proceeded with just the samples from the Donauschotter aquifer which was considered to be the most homogenous in hydrogeochemical properties.

3.5 Donauschotter aquifer

In order to obtain results unaffected by the intricate minerological and hydrological differences of the aquifers, an additional analysis was carried out on a subset of wells, i.e. the ones located in the Danube gravels. The aquifer type Danube gravels exhibited a higher degree of homogeneity in physicochemical characteristics in comparison to the other aquifers.

3.5.1 Temperature influence

Simplifying the dataset to only the Donauschotter aquifer shows a clearer decreasing trend in Shannon's diversity index within the 10-18 °C range ($X^2{}_{(7)} = 14.8$, $p < 0.05$), Fig. 3.12. In comparison to the analysis that takes all the aquifers into consideration (Fig. 3.7.), there is a significant result when performing the post-hoc dunn test (the *"10-12"* and *"16-18"* differ, adj.p < 0.05).

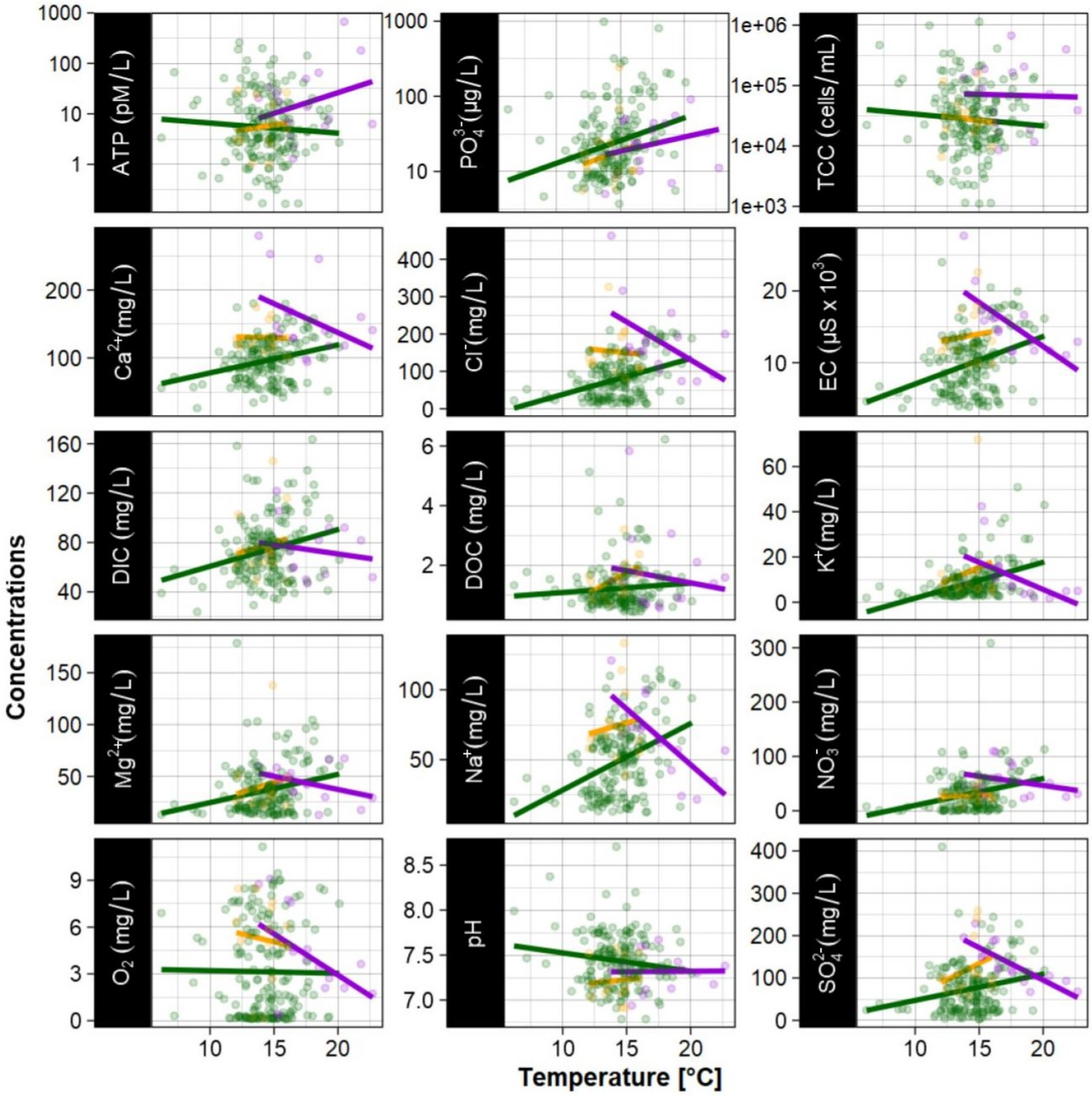

Figure 3.11: Scatter plots of environmental variables according to temperature per aquifer. Points and regression lines in green (Donauschotter), in orange (Liesingbach), in purple (Schichtwasser). Ca^{2+}: calcium (mg/L), Cl^-: chloride (mg/L), EC: electric conductivity (µS), DIC: dissolved inorganic carbon (mg/L), DOC: dissolved organic carbon(mg/L), ATP: cellular adenosine triphosphate (pM/L), K^+: potassium (mg/L), Mg^{2+}: magnesium, Na^+: sodium (mg/L), $NO_3{}^-$: nitrate, O_2: dissolved oxygen (mg/L), $PO_4{}^{3-}$: orthophosphate (µg/L), $SO_4{}^{2-}$: sulfate (mg/L), TCC: total cell counts (cells/mL).

Modelling the diversity response for only one aquifer did not provide different insight into the effect of ions and gases, than what modelling for the whole sampling dataset did. However, the additional measurements performed only in the spring (heavy metals and methane) were considered for this subset, and gave an indication that microbial diversity of urban aquifers could be tied to heavy metals in the environment. Higher levels of Ni in the groundwater samples were accompanied by an increase in Shannon's diversity index ($R^2 = 0.055$, $F_{(1,71)} = 5.167$, $p < 0.05$), while higher Ag concentrations correlated to a decrease in the diversity index ($R^2 = 0.0314$, $F_{(1,71)} = 3.336$, $p = 0.072$). However, the portion of the explained diversity variance falls below 10%. The main factor responsible for the diversity of microbial communities still remains unidentified.

While subsetting for the medium temperature range (a possible proxy for groundwater minimally impacted by the surface water) helped improve the clarity of the decreasing trend, the same subsetting did not provide any improvements with the other environmental variables.

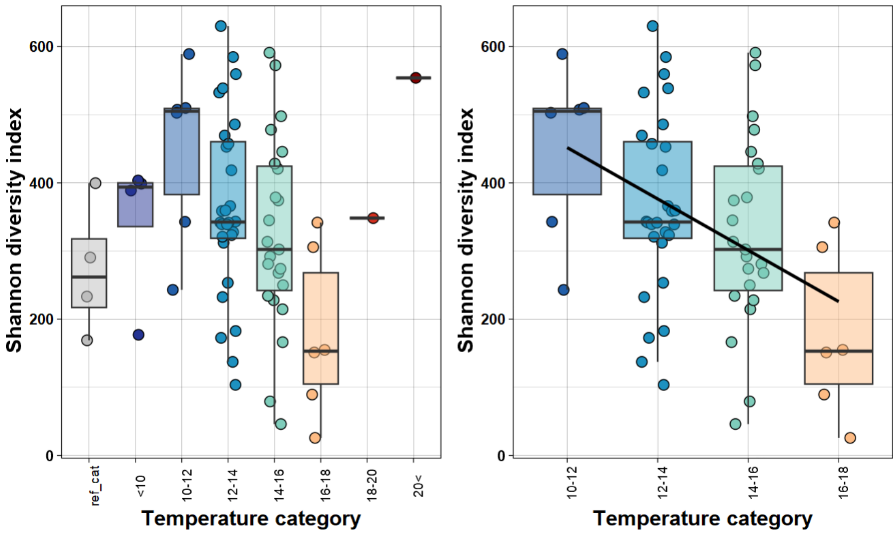

Figure 3.12: Boxplots of Shannon's diversity index of samples by temperature category of the aquifers within the Danube Schotter area. The boxes represent quartiles, the lines represent medians, and dots samples in the color corresponding to each category. Left. temperature categories along the whole temperature range. Right. On the x-axis only the temperature subset which exhibits a trend was shown.

The total cell counts of the groundwater sampling sites showed difference between the urban and non-urban sites, with TCC of the reference category averaging at 1.3 x 10^4, while the *"10-12"* category averaged at 7.1 x 10^4 (Fig. 3.13, left). While all the urban sampling sites had higher TCC, this combination of urbanization and groundwater temperatures close to natural groundwater conditions seemed like the optimal combination for the prokaryotic proliferation. On the other hand, the microbial activity did not differ between the urban and non-urban areas, and decreased in with increasing groundwater temperatures (Fig. 3.13, right).

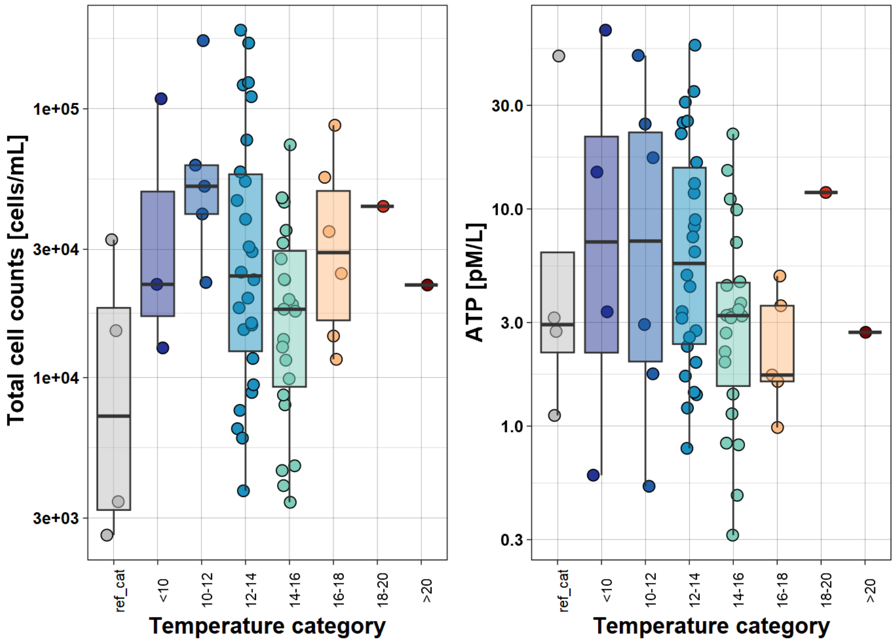

Figure 3.13: Boxplots by temperature category of the aquifers within the Danube Schotter area. The boxes represent quartiles, the lines represent medians, and dots samples in the color corresponding to each category. Left. Total cell counts in cells/mL on the y-axis. Right. ATP in pM/L on the y-axis.

3.5.2 Urban influences

The effect of the individual heat sources and urban indicators on the groundwater microbial diversity and the communities' abundance and activity was explored (Fig. 3.14). The analyses provided insignificant results, except when the opposite was indicated.

The Shannon's diversity index was lower in the sampling sites impervious to the waste water leakage. These sampling sites were also characterized by a higher microbial activity($X^2_{(1)} = 4.27$, $p < 0.05$) and a higher microbial load. The microbial load of sampling sites which were exposed to the influence of surface water was elevated in comparison to the sampling sites not exposed to it ($X^2_{(2)} = 9.03$, $p < 0.05$). The influence of the Danube channel on the groundwater community diversity was exhibited as a decrease ($X^2_{(1)} = 4.27$, $p < 0.05$).

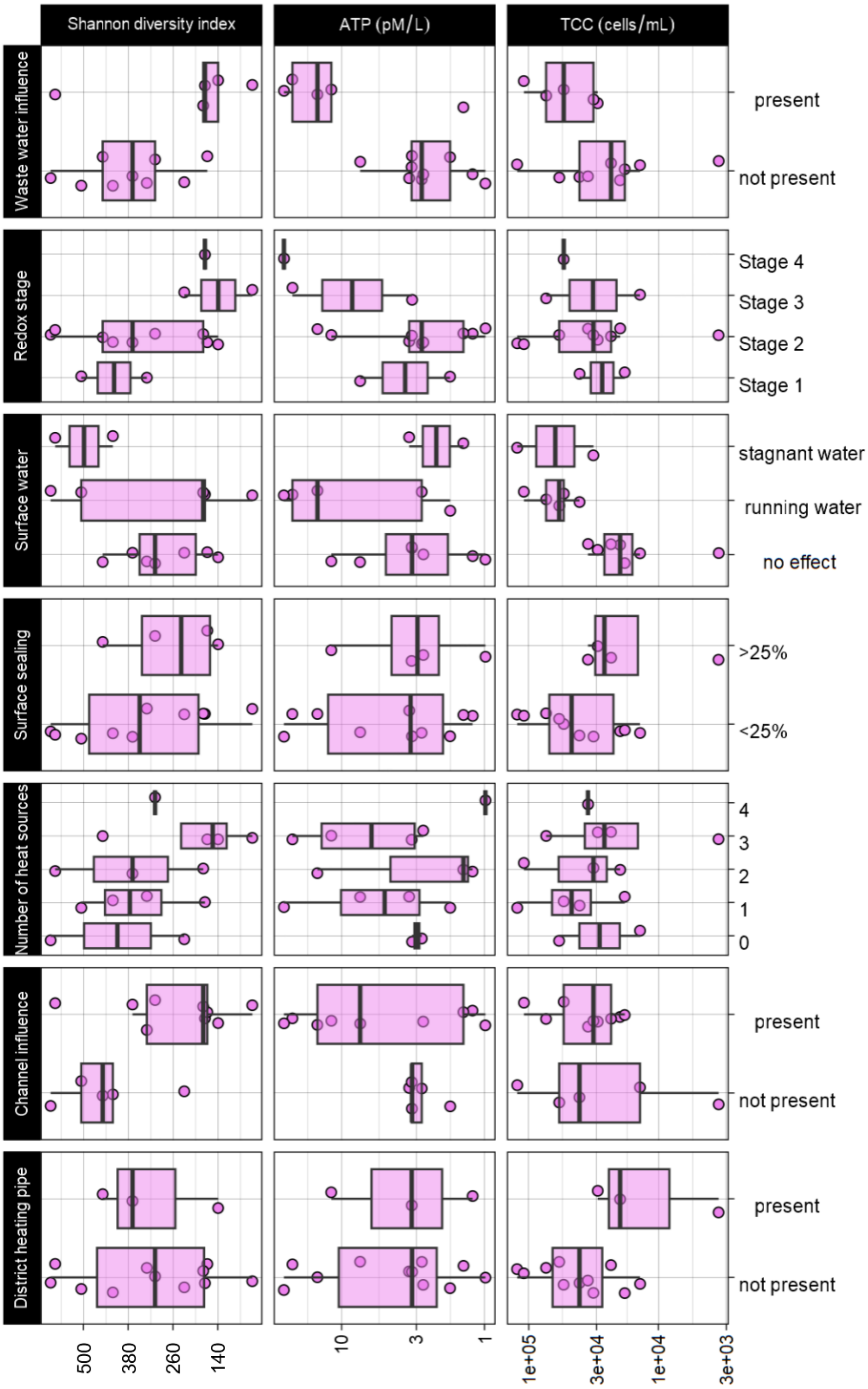

Figure 3.14: Shannon's diversity index, ATP and TCC values across the surface impact categories, wastewater impact categories, phases of redox cascade and urban heat sources. The boxes represent quartiles, the lines medians, and standard deviation shown by the error bar.

There was no visible difference for most of the analyses which included the individual heat sources, i.e. there were no differences of groundwater microbial diversity, abundance and activity between the sampling sites within the 50-meter vicinity of the heat source and the sampling sites outside the 50-meter range. The geothermal energy use and underground construction exhibited similar ranges of groundwater microbial diversity, abundance and activity. Higher Shannon's diversity, lower microbial load and activity of the microbial communities, were implied for the sampling sites in the 50-meter vicinity of the district heating pipe. However, the small sample size of one of the groups (n=3) should be noted. The lower levels of microbial diversity, load and activity were connected to the sampling sites in the areas with more than 25% of the sealed surfaces.

The impact of variables which are not related to temperature (wastewater, surface water, anoxic environments) was more pronounced than the temperature effect. The number of urban heat sources which impacted any given sampling site did seemingly make a difference on the Shannon's diversity index values, with microbial diversity being lower at sampling sites exposed to the influence of three heat sources, in comparison to those exposed to no heat sources, one heat source and two heat sources. The distribution of microbial diversity by the stages of the redox cascade shows that the microbial diversity values of the initial two stages (sufficient levels of dissolved oxygen, and levels lower than 2 mg/L) are not different from each other, but the sampling sites of the third stage (Mn > 0.15 mg/L and Fe > 0.15 mg/L) have lower Shannon index values.

4 Discussion

The aim of this thesis was to test the hypothesis that a change in groundwater temperature in an urban environment is accompanied by a change in microbial community composition. Such insights could also help gain better understanding of the relationship between the groundwater quality changes and the temperature variations. Rising water temperatures in an urban environment are a consequence of geothermal energy use, climate change, and other anthropogenic impacts. Therefore, the urban subsurface environment served as a role model to evaluate the temperature impact on microbial communities under *in situ* conditions, with a temperature range from 7 °C to 27 °C. The impact of temperature on other physico-chemical characteristics, and vice versa, as well as on the microbial productivity, was analyzed. The hypothesis was that the temperature is a major driver of microbial diversity and community composition, if not alone, then combined with other environmental variables and/or stressors. The results did not show a clear relationship between temperature and the microbial community composition, nor were any particular ties to the currently measured environmental variable obvious. A possible role of aquifer type in microbial community response to the groundwater temperature increase was found. The impact of wastewater influence and surface water on microbial communities seem an important influence on the urban microbiome.

4.1 Vienna as an urban sampling site

While other studies report a significant impact of calcium, magnesium, and potassium on the community composition (Brielmann et al., 2009), that was not the case in our study. Almost no fraction of variance in diversity nor composition was explained by the concentrations of major ions, groundwater temperature, pH, groundwater extraction depth, concentration of dissolved oxygen, geographic position, concentrations of organic and inorganic carbon, nor any of their combinations.

The relationships between groundwater temperature and the variables pH, dissolved oxygen, DOC, and sulfate, respectively, were inconclusive and not significant, with less than 2% of variance explained for each individual regression. There was indication that responses of environmental variables to groundwater temperature were aquifer-specific, as was the microbial community diversity response, since most variables acted differently in accordance to temperature in the Schichtwasser aquifer. The interesting notion is that R^2 further decreased when simply removing the Schichtwasser wells from the analysis and focusing on the remaining aquifers with similar trends. Additionally, community trends did not get significantly clearer when performing analyses only for the Donauschotter aquifer, the aquifer type with the most sampling locations. Clearly, the interactions are more complex than simple categorization by aquifer type.

The study on the thermal influences on the urban groundwater in Munich showed that within the same aquifer the identification of the dominant urban heat sources needed to be coupled with the seasonality data and aquifer thickness information, in order to provide results (Böttcher & Zosseder, 2022). The large thickness variation of the quaternary aquifer, the only aquifer in the Munich city area, was a significant variable in the groundwater heat variation. In Vienna, on the other hand, the aquifers dated from few different geological ages, and varied greatly in physico-chemical properties, not only within their aquifer type, but between the types, as well. The Schichtwasser aquifer had large physico-chemical heterogeneity and complex relations with aquifer terraces located beneath it (Grupe et al., 2021), that some sampling sites could not be clearly assigned to neither the Schichtwasser aquifer nor to the aquifers beneath. Furthermore, the three aquifer types which provided a sufficient sample size exhibited different relationships between some measured environmental parameters and the groundwater temperatures. Interesting phenomena presented themselves, such as stable levels of dissolved oxygen even at high groundwater temperatures, and an increase in mineral ion concentrations in some aquifers. The hypothesized decrease in mineral ions (magnesium, calcium) at higher temperatures (Griebler et al., 2016), occurred at only one aquifer, the Schichtwasser aquifer. Similarly, a commonly reported decrease in pH levels at higher groundwater temperatures (Griebler et al., 2016), was not pronounced for all aquifers. Therefore, in the complex geological layout of the city, an equally complex interplay of urban influences occurred, and made the analyses more difficult.

4.1.1 Subsurface urban temperatures

In Vienna, we found high groundwater temperatures, averaging at 15.28 °C and 14.22 °C, in fall and spring, respectively, which is a substantial elevation in comparison to temperatures of undisturbed groundwater, which is usually within the 10-12 °C range for rural areas at comparable latitudes (Menberg et al., 2013; Riedel, 2019; Tissen et al., 2019). Worth noting is the thesis' limitation of working with only a small subset of the ∼700 groundwater wells located in Vienna. When averaging the measurements of the groundwater temperatures from all the wells, as was done within the HBTC project as well, the values resembled the natural averages more, with a fall average of 14.8 °C, and spring average of 13 °C (Steiner et al., 2023). However, it was not possible to analyze the microbiome of all the Viennese groundwater wells.

With the subset available to us, Vienna's groundwater temperature in the city center was predominately higher than 16 °C, making it even warmer than that of some other urban areas (Nürnberg's 13.9 °C center average (Schweighofer et al., 2021), Paris' 14.9 °C city center average (Hemmerle et al., 2019)), or within the same temperature range (Berlin, Munich, Cologne and Karlsruhe exhibited the 13–18 °C range close to the city centers, (Menberg et al., 2013)).

Combining that with the heterogeneity of the city (i.e. Vienna possessing both an abundance of sealed surfaces, as well as a substantial number of green surfaces and surface water bodies), it proved itself to be a role model test site for our study. The impact of localized city trends in Vienna on temperature increase was already stressed in 1998 by Böhm, so the number of sampled wells was 314 in attempts to account for this. Additionally, the high level of urbanization (only 14% of wells resembling conditions of undisturbed groundwater), made it a point of interest for this study.

The pronounced difference between the spring and fall temperatures confirmed the seasonality of the shallow groundwater, and autumn being the warmer of the two also agreed with other reports of groundwater seasonal distributions (Brielmann et al., 2009). However, not all Viennese wells fall in line with the usual yearly groundwater variation, i.e. within the ± 2 °C seasonal fluctuation (Switzerland Federal Office for the Environment, 2022), since there were as many as ten wells which had a temperature fluctuation bigger than 3 °C. This can most likely be tied to the fact that the depth of most wells lies below 10 meters (wells with extreme temperatures, meaning groundwater temperatures higher than 20 °C, averaging at only 3.9 meters), making them more susceptible to surface factors. While it does create fitting conditions for temperature-focused research, because it allows for a wide range of temperatures, it makes controlling for the surface effects on groundwater quite difficult, especially when coupled with the before mentioned hydrogeological complexity of Vienna. This reflected in the results, as most of the microbial community variance remained unexplained.

The locations of extremely warm groundwater clusters are in line with what was hypothesized, i.e. the two subsurface urban heat islands were located in the inner city of Vienna, away from surface waters and underneath the sealed surfaces. Temperature along the Danube's tributaries and its channel did not exhibit extreme temperature highs, even when densely populated. The results confirmed that surface sealing had an effect on warming, and that open landscapes were indeed connected to a lower temperature deviation from the natural groundwater temperature, just as Böttcher and Zosseder (2022), Schweighofer et al. (2021), and Previati et al. (2022) showed. The location of subsurface heat islands did not completely correspond to the urban heat islands above the surface, as proposed in Fig. 1.2. While groundwater wells of the inner city exhibited elevated temperatures, just like the corresponding air sensors did, there was no SUHIs in other densely populated which were not as covered in sealed surfaces (North-East and South part of the city). Additional to surface sealing, the groundwater temperature in Vienna was also elevated at the sampling sites affected by the district heating pipe, subsurface infrastructure, the Danube's channel and the number of heat sources affecting it (Fig. 6.2, Appendix).

4.1.2 Temperature-dependent water quality cascade

A decrease in water quality that comes with higher temperatures has already been described (Bonte, Breukelen, & Stuyfzand, 2013; Bonte, Röling, et al., 2013; Griebler et al., 2016; Riedel, 2019). The rate of biological and chemical reactions increases with temperature, impacting oxygen levels, nutrient cycling, solubility of gases and chemicals, ultimately leading to higher levels of heavy metals/pollutants and reducing conditions. It presents danger to both the groundwater quality and ecosystem and human health, and its risk should be carefully assessed during management of water resources. The question whether this cascade has already been put in motion in Viennese groundwater, does not have a definite answer, considering the different physico-chemical dynamics of each aquifer (Fig. 3.11.). A decrease in groundwater oxygen levels, and a decrease in sulfate which would accompany the transition from iron reduction to sulfate reduction, was present in the highly heterogeneous Schichtwasser, which could imply the cascade effect. There was a significant decrease in sulfate ions with an increasing groundwater temperature, which fits the temperature-related shift to sulfate reduction, as hypothesized.

However, the Donauschotter aquifer wells, which made the majority of sampled wells, showed neither a clear oxygen depletion nor a decrease in sulfate, and actually exhibited an increase in sulfate ions with the groundwater temperature increase. This opposes the hypothesis that a higher rate of sulfate reduction as a result of the temperature impact would mean microorganisms using up sulfate ions as an electron acceptor and converting them to sulfides, and therefore decreasing their concentration (Tripathi et al., 2021). Elevated levels of sulfate in urban groundwater were tied to the building rubble in other European cities, and long-term release of sulfate from construction rubble in urban areas was deemed likely (Abel et al., 2015). It is possible that the elevated sulfate concentrations of the Donauschotter sampling sites were caused by the construction rubble in Vienna as well. However, it should be noted that there was no difference between the sulfate concentrations of sites influenced by surface waters and those which were not influenced, which would be expected if the groundwater sulfate was construction-related as hypothesized.

The elevated microbial load of the urban sampling sites in comparison to the TCC of the reference site presented a possible reason to worry. The results from prokaryotic cell counts implied that urban characteristics impacted microbial communities. The microbial diversity of reference sampling sites, were two times lower than cold/average-temperature samples within the city area. This is consistent with the elevated groundwater TCC in laboratory experiment on stimulated contamination biodegradation (Griebler et al., 2016).

The literature on urban groundwater cell counts *in situ* was not abundant, and should be the focus of future studies. Additionally, given the influence of reduced oxygen levels and elevated heavy metal concentrations in groundwater on the microbial communities of Vienna (Fig. 3.14), it is imperative to carefully observe the behavior of these parameters in the future.

4.2 Diversity

There have been conflicting reports in microbial diversity response to a temperature change. As mentioned before, groundwater microbial community richness and diversity were shown to both increase (Brielmann et al., 2009) and decrease (Metze et al., 2021) with a groundwater temperature increase. The whole range of responses has been recorded in detail (Sharp et al., 2014). Both kinds of trends have been shown to be either linear (Brielmann et al., 2009), or non-linear (Metze et al., 2021; Ruhl et al., 2022). Ultimately, the microbial diversity response cannot be connected solely to temperature, and is rather a result of the interplay of temperature and other site-specific conditions. With the data currently provided, the microbial diversity response of Viennese groundwater appears to be a decreasing linear trend (Fig. 3.7). Along the whole spectrum of groundwater temperature variations, the trend is not uniform, but a pattern emerged when the <10 °C sampling sites were removed from the analysis, due to the likely influence of surface water bodies. A diversity decrease was apparent in the 10-18 °C range. While Shannon's diversity values again increased after the 18 °C point, most of the sampling points with 18+ °C belonged to the distinct aquifer type (all but two sampling points). It is, therefore, possible that we are not observing one non-linear trend with an 18 °C tipping point, but rather the interplay of two conflicting trends from two different aquifers.

The decrease in the Shannon's diversity index with the increasing groundwater temperatures is the characteristic of the Donauschotter aquifer, while an increase in microbial diversity characterized the Schichtwasser aquifer. Worth noting for is the possibility that individual samples taken from the Schichtwasser aquifer differ greatly in physicochemical characteristics, enough to warrant questioning whether they can be regarded as one category. The heterogeneity within this category is so great (Grupe et al., 2021) that the linear relationship between the temperature and diversity could be dubious, and each individual point could be an isolated point. This introduces another layer of complexity to the question whether the microbial diversity trend is attributable to the specific aquifer type or to the urbanization factors. Additionally, this aquifer is partly located with the city center, an area characterized by high percentage of sealed surfaces and recognized as an urban heat island. In light of this, it is plausible that urbanization plays a contributory role in the observed temperature variations, but it is also possible that this particular aquifer type inherently exhibits higher temperatures independently of urbanization effects.

4.2.1 The effect of urbanization on microbial diversity

While efforts to establish a baseline Microbial Community Index which would allow monitoring of anthropogenic impact on groundwater do exist in Asia (Zhong et al., 2023), such an initiative has not yet been significantly backed in Europe. The studies dealing with a comparative overview of urban and rural groundwater communities have been rare for this part of the world, making it hard to put our elevated microbial diversity of urbanized sampling sites into scientific context. Although certain studies deal with just the urban aquifer microbiome (Gruzdev et al., 2023), the diversity indices used are not often comparable across studies. The preliminary results from the comparison of groundwater microbial diversity of the rural Steiermark and the urban Berlin, imply lower Shannon's index in the rural environment (König, J., Unpublished master's thesis, University of Vienna), and confirm our results.

From the urban factors impacting the microbial diversity, the clearest trend was seen in the vicinity of landfills and wastewater. However, the lower microbial diversity of the sampling sites influenced by the waste water is not consistent with the previous findings of increased microbial diversity in the case of wastewater contamination (Cho & Kim, 2000). It is, however, possible that the contaminated sites were not only contaminated by the wastewater, but likely contaminated by the heavy metals. This kind of groundwater contamination would result with the decreased microbial diversity (Cho et al., 2012). Why the overall microbial diversity of all urban sampling sites is elevated in comparison to the reference sites, while also being lower for the sites impacted by the wastewater, remains unclear for now.

Furthermore, the decreased microbial diversity of the groundwater wells influenced by the Danube's channel could be a consequence of the microbial diversity-productivity relationship (Smith, 2007). The increase in nutrient concentration due to the surface water infiltration from the Danube's channel could be a cause for the decreased microbial diversity. However, while the diversity-productivity relationship was confirmed as a general ecological concept, it is not yet proven for the groundwater systems (Griebler et al., 2022), and the microbial diversity decrease as a consequence of surface water infiltration is put into question by the absence of the same trend for the other running water and stagnant water influence (Fig. 3.14.).

4.2.2 The effect of other environmental variables on microbial diversity

Along with the search for the environmental variables which could be singled out as a driving factor for the Viennese groundwater microbial diversity and the species turnover, the possibilities of seasonal influence and the recharge effect should be entertained (Griebler & Lueders, 2009). While our sampling sites were sampled only twice, making the seasonal monitoring harder, the recharge effect is still indicated by our results. When looking at the turnover rates, the ordination showed that higher temperatures coincide with higher dissimilarity of communities, as opposed to the very similar communities of colder wells. The wells with temperature lower than 10 °C were surely impacted by the inflow of colder waters, and the heightened connectivity and lack of dispersal limitation of the said sampling sites is most likely the reason behind the low within group dissimilarity.

The temperature did not drive a grouping by microbial composition, the *">20"* and *"18-20"* samples were dispersed throughout the whole ordination, many of them close to cold samples, as well. The high dissimilarity of the warmer sampling sites is a possible consequence of high variability in conductivity, dissolved oxygen levels and dissolved organic carbon.

The microbial diversity trends in accordance to the Ag and Ni concentrations should be considered with caution, since both of the elements were present in concentrations usual for groundwater. In the light of our lack of obvious results, it can not be ignored that we only have insight into the planktonic microbial communities. This is likely to stay a problematic variable in the future of the Viennese groundwater studies, since it was not possible to drill for sediments at 150 locations within a city. Studies analyzing the free-swimming microbiome are likely providing only 1-10% of the microbial cells found in the groundwater ecosystem (Griebler et al., 2022) and are registering the less active part of the community (Smith et al., 2018), which is less likely to show the community reshuffling effect due to the urban contamination, in comparison to the biofilm-attached communities (Colin et al., 2020). Sampling the groundwater sediments and biofilms of Vienna, would perhaps provide additional insights in groundwater response to temperature change, since the active portion of the microbial community is usually tied to the sediment and rocks, and not free-moving in the water column (Griebler et al., 2002), seeing as the attached mode of life provides a more diverse surface and more ecological niches (Griebler & Lueders, 2009). If we sampled the sediments and biofilms we would have most likely bigger organisms, bigger microbial load and higher activity of surface-attached communities (Griebler et al., 2022).

Additionally, the scale on which the microbial communities interact are beyond what we can currently measure. There has been findings that microbial strains are sometimes depth-specific, and will not be present along the whole water column (Griebler & Lueders, 2009). Considering that data about filter depth of a certain portion of groundwater wells are not available, it is visible that we are not in possession of data so precise as we need it in order to deal with microcosm.

Finally, the sampling sites designated as the reference could ultimately be too different from the urban areas to be comparable in terms of their microbial communities, as seen from the differences in microbial diversity of the urban and non-urban sampling sites. An alternative reference category should be considered for future studies. The reference sampling sites located in the Marchfeld area were a good starting point for comparison of the urban groundwater microbiome to microbial communities of the less urbanized sites, but future studies could profit from designating a reference category within the city, and comparing their microbial communities to those of thermally-impacted urban groundwater wells. Considering that the average temperature of the urban groundwater sampling sites is found to be around 2 °C warmer than the rural average (Ferguson & Woodbury, 2007), the reference category should be the sampling sites within the 12-14 °C range.

4.3 Community composition

For the most part, the classes which were present do not differ from the usually reported phyla in groundwater, mainly the various *Proteobacteria*, *Actinomycetia*, *Bacteroidetes*, *Verrucomicrobiota*, *Nitrospirota* (Griebler & Lueders, 2009), *Patescibacteria* (Herrmann et al., 2019), and *Nanoarchaeota* (Retter et al., 2023). The phylum *Firmicutes* which is usually a part of the groundwater microbial communities, was absent in Viennese groundwater. The presence of *Gamma*- and *Alpha-proteobacteria* was not unusual for urban groundwater, considering their ubiquity and presence in almost all conditions and with a wide variety of roles, ranging from the role in the sulfur cycle (Liu et al., 2022; Ágnes Duzs et al., 2018), to carbon cycle (Fukuyama et al., 2020), and nitrogen fixation, degradation of organic matter, and degradation of pollutants (Mukhopadhya et al., 2012).

Unsuprising was the dominance of *Nanoarchaea*, a part of the DPANN superphylum (Dombrowski et al., 2019) whose functional role and metabolic pathways have not been very thoroughly understood, due to their relatively recent discovery (Huber et al., 2002), but it is presumed they are a factor in nitrogen cycling (He et al., 2021). It is not clear what a community dominated by *Nanoarchaea* implies for the stability of the ecosystem. The high relative abundance in this thesis (average 37.9% of relative abundance across all samples), is comparable to the 34.3% of DNA-derived community reported by Retter et al. (2023). The authors performed the analysis on the RNA-derived microbial community, and concluded that in this case, the class *Nanoarchaea* makes only 14.2% of the relative abundance. Considering that the RNA-derived microbial community gives a picture of the active part of the community, future studies could focus on both the DNA and RNA communities pf Viennese groundwater. The success of *Nanoarchaea* and *Patescibacteria* in groundwater is likely due to ultra-small genome (Gios et al., 2023), and the strategy of dependence on other taxa as hosts and investing less resources (Herrmann et al., 2019). It is possible that this evolutionary edge helps establish their presence in the urbanized groundwater, as seen from the classes *Paceibacteria* and *Gracilibacteria* from the phylum *Patescibacteria*, which were the classes found exclusively at urbanized sampling sites.

The second most abundant class *Omnitrophia* comes as a surprise, since their presence is usually not reported in groundwater studies. Their affinity toward anoxic conditions (Glöckner et al., 2010) could be a cause for concern. Our results show a certain portion of taxa which are known to prefer low-oxygen/high-nitrogen conditions (*Omnitrophia* and *Vampirovibrionia*), which is more often a characteristic of highly urbanized and disturbed groundwater ecosystems than the undisturbed ones (Kuroda & Fukushi, n.d.). The presence of *Nitrosphaeria*, the ammonia-oxidizing archaea (AOA), could, on the other hand, be an indicator of low ammonia, and aerobic conditions (Liu et al., 2011), so their stable abundance in both at the reference sampling sites and all other temperature categories, can imply an overall sufficient dissolved oxygen concentrations for the microbial communities of the urban groundwater. However, their frequent findings in wastewater plants should be kept in mind (Park et al., 2006).

Two classes of the *Cyanobacteriota* phylum had substantial abundance in our samples, *Cyanobacteriia*, and the parasitic *Vampirovibrionia*. The stable presence of *Vampirovibrionia*, which feed on other bacteria (Rienzi et al., 2013; Soo et al., 2014), across all temperature categories is not often reported. Its abundance might suggest the shift in microbial interactions, and the rise in the abundance of parasitic taxa could mean that conditions of Viennese urban groundwater are not optimal for aerobic taxa. While the abundance of autotrophic taxa is usually tied to the surface water recharge, the phylum *Cyanobacteria* was surprisingly not very dominant at the surface sampling sites.

A possible improvement of our compositional analysis, in order to identify the possible sources of certain microbial classes, would be source tracking, the strategy of connecting communities to their source of origin (Shenhav et al., 2019), an increasingly popular approach to microbial community analysis. This might help discern groundwater impacted by the Danube and the channel from confined aquifers in a more precise manner, and could shed light on the portion of the community impacted by wastewater organisms. There are already indications that wastewater communities impact the aquifer communities in a very distinct manner, increasing diversity and causing shifts in *Flexibacter-Bacteroides* abundances (Cho & Kim, 2000). Therefore, this method of distinguishing the urbanized wells by biological tracking could give different results than when discerning by environmental variables, and could shed more light on community interactions.

4.3.1 Temperature-dependent compositional trends

The classes which were only abundant (i.e. the relative abundance in the temperature category exceeded 1%) at higher temperatures were *Verrucomicrobiae*, *Cyanobacteriia* and *Actinomycetia*. While the *Cyanobacteriia* abundance could be a consequence of surface water impact (as discussed in the previous section), the *Verrucomicrobiae* were present at only one surface sampling site, and *Actinomycetia* were not present at any of the surface sampling sites. There are conflicting reports for the latter two regarding the temperature optima, reporting their abundance at a wide range of temperatures (Laiz et al., 2003; Stevenson & Hallsworth, 2014), or even having higher diversity at lower temperatures (Chiang et al., 2018).

The classes present at lower temperatures were *Methylomirabilia* (below 14 °C) and *Brocadiia* (below 18 °C). Class *Brocadiia* was the most abundant in the reference samples, and absent not only in hot extremes, but absent in cold extremes, as well. Considering that *Brocadiia* are anammox-related organisms found in wastewater plants (Oyarzúa et al., 2021), their presence at reference sampling sites is not is desirable.

Since distinctions in groundwater microbiome in comparison to the surface taxa were more likely to be detected at finer taxonomic levels (Smith et al., 2018), an analysis for the relationship between the taxa abundance and groundwater temperature increase was performed on the order level, as well. Candidatus Roizmanbacteria and *Desulfobacterales* showed affinity towards groundwater temperatures higher than 15 °C in urban environment. Considering the affinity of the Candidatus Roizmanbacteria to the symbiotic life (Geesink et al., 2020), specifically with the class *Thermodesulfovibrionia*, this could be what provides the possibility of this order to prosper in conditions of temperature stress. The possibility of Candidatus Roizmanbacteria to provide the lactate (Geesink et al., 2020), and the affinity of sulfate-reducing bacteria to lactate (Hao et al., 1996), could tie the *Desulfobacterales* increase to this story, as well. Approaching the microbial community analysis of urban groundwater through taxa networks could provide a more detailed insight in the future.

4.3.2 The compositional outliers

The cluster identified in *3.2.1 Impact of temperature on the community composition* was dominated by *Cyanobacteriia*, *Actinomycetia*, and *Bacteroidia*. We were not able to connect these outliers to any of the measured variables, nor to their geographic position. Furthermore, we were not able to confirm that it is just an artifact, since there was no apparent pattern - the samples were taken with different canisters (8 canisters), different sampling dates (7 different dates), and each sample molecularly processed with a different extraction batch. The samples were further classified as belonging to a different land use category (4 categories), to different aquifer types (5 aquifer types), and to different temperature categories (12-14-16-18-20-20+), with a big well depth range (3 to 20 meters range). Considering the affinity of *Cyanobacteriia* to light availability, their presence shows possibility of contamination by surface water, but the wide range of extraction depths and distances from surface waters makes this implausible. If the cluster was a consequence of wastewater contamination, we would expect a bigger abundance of fecal classes such as *Firmicutes* (Tiwari et al., 2021), which was also not the case. The possibility of uprising thermal groundwater which is reported for several spots within Vienna, impacting the community composition was not checked yet, but future studies with this in mind are planned. At the moment, it is not possible to infer conclusions about the cause of this cluster, and a more detailed analysis must be conducted to determine whether this was an accidental artifact or not. A possible seasonal trend should also be considered.

5 Conclusions

In conclusion, it can be said that the groundwater microbial diversity of Vienna depends on the aquifer type, with a decreasing microbial diversity trend in the sampling sites located in the Donauschotter aquifer, and an increasing microbial diversity trend in the Schichtwasser aquifer, with increasing groundwater temperatures. However, the different temperature ranges between the aquifer types and within-aquifer-type heterogeneity in physico-chemical characteristics make the trends hard to compare. The microbial diversity of urban sampling sites was elevated in comparison to the reference sampling sites, until temperatures crossed 14 °C.

The microbial community composition of urban groundwater of Vienna was consistent with taxa reported in studies of pristine groundwater. There was not a substantial relative abundance of taxa which would indicate increased anthropogenic influence. The analyses at the class level did not provide trends which are likely to be temperature-dependent, but at the order level the Candidatus Roizmanbacteria and *Desulfobacterales* showed indication of temperature-related abundance trends. Further studies of community composition should be done with sediment and biofilm communities in mind, coupled with the source tracking analysis, to better understand community interactions and how communities from wastewater and landfills impact Viennese groundwater. The impact of wastewater on physico-chemical properties of groundwater should also be pursued, considering the observed impact on total cell counts and diversity.

The variation in microbial community composition remained for the most part completely unexplained, and further data collection must be done for this site in order to identify what the main drivers of community dynamics are. The mineral concentrations, temperature, pH, depth, oxygen, geographic position, organic and inorganic carbon concentrations were not clear factors for neither the microbial diversity nor the composition of microbial communities in Viennese groundwater.

Ultimately, the microbial groundwater diversity was influenced by the urbanization, and showed a temperature-related tren, but there was no proof that microbial community composition was impacted by temperature nor the urbanization. However, this thesis provides only the first look into microbial community behaviors in a complex ecosystem of Viennese groundwater, a system where an interplay of numerous urbanization-related variables and numerous aquifer types occurs.

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6 Appendix

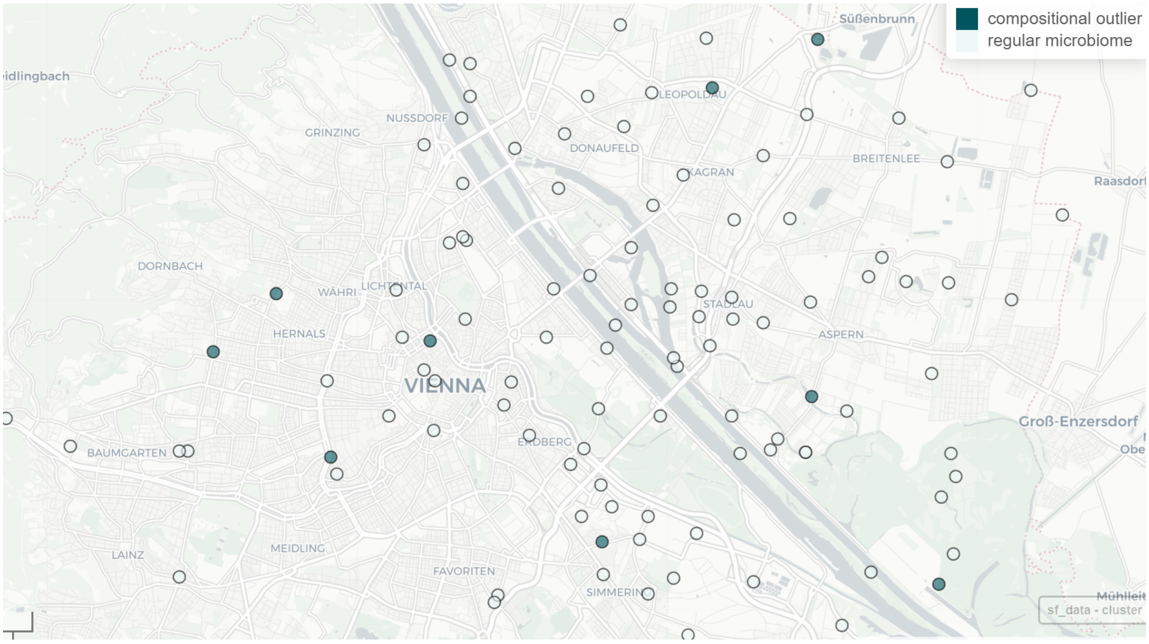

Figure 6.1: Map of Vienna with sampling wells. Circles represent a well colored based on the microbial composition categorization. The microbial composition of "compositional outliers" described in *3.2.1. Overview of groundwater microbial communities at class level.*

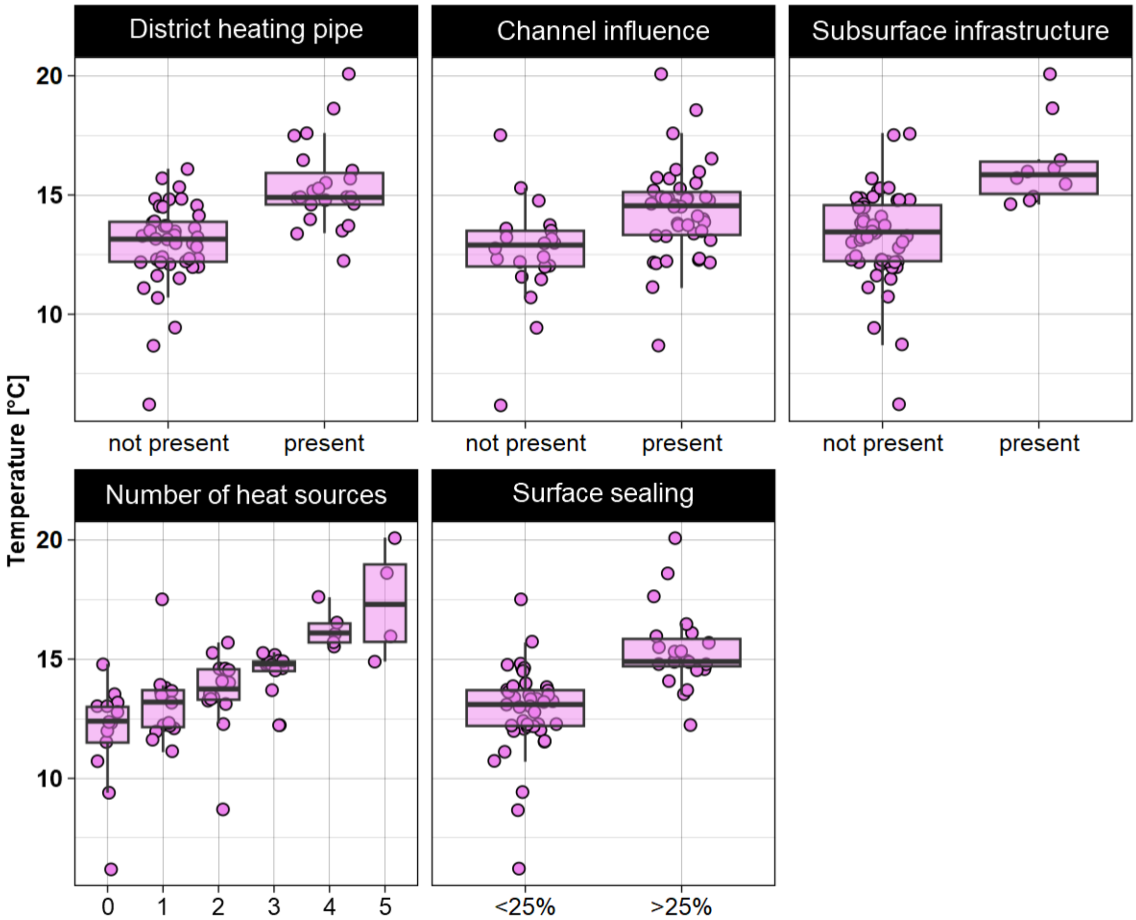

Figure 6.2: Groundwater temperature in °C distributed across the various urban influences. The boxes represent quartiles, the lines medians, and standard deviation shown by the error bar.