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SIMULATED AQUIFER SYSTEM

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## LIST OF ABBREVIATIONS, ACRONYMS, AND INITIALISMS

ANOVA	Analysis of variance
db-RDA	Distance-based redundancy analysis
DIC	Dissolved Organic Carbon
DNA	Deoxyribonucleic acid
DOC	Dissolved Organic Carbon
KEGG	Kyoto Encyclopedia of Genes and Genomes
KO	KEGG Ortholog
LEfSe	Linear discriminant analysis Effect Size
LDA	Linear discriminant analysis
NH <sub>x</sub>	Ammonia/ammonium
NO <sub>2</sub>	Nitrite
NO <sub>3</sub>	Nitrate
PCoA	Principal Coordinate Analysis
PERMANOVA	Permutational multivariate analysis of variance
RNA	Ribonucleic acid

## ABSTRACT

Aquifers are vital resources for drinking water, with approximately one-third of the global population relying on groundwater for domestic use. Microorganisms in subsurface ecosystems play crucial roles in recycling organic matter, degrading contaminants, and improving water quality. However, functional insights into biofilm microbial communities in aquifers remain limited, primarily due to the challenges associated with core subsurface sampling.

To address this gap, our objectives were to (I) investigate the potential functional traits of sessile (biofilm) and planktonic microbial communities and (II) compare their functional profiles across summer and winter seasons. We employed a bioreactor setup simulating an aquifer system to study both communities simultaneously and used metagenomic methods to characterize their potential functional traits.

Our findings revealed that community type significantly influenced all gene relative abundance, with planktonic communities showing higher abundances of genes related to carbon metabolism and core essential metabolism compared to sessile ones. On the other hand, sessile communities exhibited a greater abundance of genes related to xenobiotic degradation pathways and nitrogen metabolism. The seasonal factor had no effect on overall gene abundance; however, during the summer, certain pathways appeared to be enriched, particularly those involved in energy production, stress response, and competition. In contrast, winter conditions seemed to promote a more dormant state, as indicated by the fewer enriched pathways observed.

Our results suggest that although sessile and planktonic communities originated from the same source, they may adopt distinct metabolic strategies to thrive under their respective lifestyles. It is possible that the biofilm effect influences and shapes the potential functional traits of microorganisms living in aquifers.

Furthermore, we believe this project will serve as a foundation for future work to characterize microbial communities and their functions in other aquifers in Canada. This study highlights the potential of subsurface microbial communities to recycle organic matter and metabolize pollutants, contributing to improved water and nutrient balance.

Key words: Biochemical cycling, metabolism, groundwater, functions, bioreactor

## RÉSUMÉ

Les aquifères constituent des ressources vitales pour l'eau potable, environ un tiers de la population mondiale dépendant des eaux souterraines pour un usage domestique. Les microorganismes des écosystèmes souterrains jouent un rôle crucial dans le recyclage de la matière organique, la dégradation des contaminants et l'amélioration de la qualité de l'eau. Cependant, les connaissances fonctionnelles sur les communautés microbiennes en biofilm dans les aquifères restent limitées, principalement en raison des difficultés liées à l'échantillonnage des carottes du sous-sol.

Pour répondre à cette question, nos objectifs étaient de (I) étudier les caractéristiques fonctionnelles potentielles des communautés microbiennes sessiles (biofilm) et planctoniques, et (II) comparer leurs profils fonctionnels entre les saisons d'été et hiver. Nous avons utilisé un bioréacteur simulant un système aquifère pour étudier les deux communautés en même temps et avons appliqué des méthodes métagénomiques pour caractériser leurs traits fonctionnels potentiels.

Nos résultats ont montré que le type de communauté avait un effet significatif sur l'abondance relative de tous les gènes. Les communautés planctoniques présentaient une abondance plus élevée des gènes liés au métabolisme du carbone et aux fonctions essentielles de base, tandis que les communautés sessiles avaient une abondance plus importante des voies de dégradation des polluants et du métabolisme de l'azote. La variable saison n'avait pas d'effet sur l'abondance globale des gènes ; cependant, en été, certaines voies semblaient enrichies, notamment celles liées à la production d'énergie, à la réponse au stress et à la compétition. En revanche, l'hiver semblait favoriser un état plus dormant, comme l'indiquent les voies enrichies moins nombreuses observées.

Nos résultats suggèrent que, même si les communautés sessiles et planctoniques proviennent de la même source, elles peuvent adopter des stratégies métaboliques différentes pour s'adapter à leur mode de vie respectif. Il est possible que l'effet du biofilm influence et façonne les traits fonctionnels potentiels des microorganismes vivant dans les aquifères.

Enfin, nous pensons que ce projet pourra servir de base à des travaux futurs pour mieux comprendre les communautés microbiennes et leurs fonctions dans d'autres aquifères au Canada. Cette étude montre le potentiel des communautés microbiennes souterraines pour recycler la matière organique et métaboliser les polluants, contribuant ainsi à améliorer l'équilibre de l'eau et des nutriments.

Mot clés: Cycle biochimique, Métabolisme, Eau souterraine, Fonctions, Bioréacteur



# 1 INTRODUCTION

## 1.1 Aquifers

Groundwater is the largest source of freshwater on Earth after glaciers. It is stored in aquifers that are geological formations that allow water to circulate through their porous structures (Atangana, 2018). Groundwater mainly originates from surface recharge, typically following rainfall or snowmelt, and gradually infiltrates into these subsurface reservoirs. Over time, it can re-emerge, sustaining rivers, streams, wetlands, and even coastal waters. Globally, groundwater is of critical importance, providing nearly one-third of the world's drinking water supply (Griebler & Avramov, 2015).

Aquifers can be classified based on their structure, texture, lithology, and the mobility of their water, but they are generally mostly classified based on their structure into two main groups: unconfined and confined aquifers (Salako Adebayo & Adepelumi Abraham, 2018). Confined aquifers are those that are covered by a confining layer, usually composed of sandy clay, clay, or silt (Guo et al., 2015). Therefore, this type of aquifer is better protected from contamination and is more stable. On the other hand, unconfined aquifers are those that do not have any confining layer and are generally located near the surface of the ground (Atangana, 2018). Typically, the shallowest aquifers are defined as unconfined aquifers.

Additionally, aquifers can also be classified based on the geological materials that compose them (Charles Willard Fetter, 2001; Earle et al., 2015). Consolidated aquifers are composed of materials such as sandstone, limestone, dolomite, igneous, and metamorphic rocks, while unconsolidated aquifers generally contain granular materials such as sand, silt, clay, and gravel (Charles Willard Fetter, 2001; Earle et al., 2015). Porosity is also an important aspect in defining an aquifer in terms of water quality. Aquifers with low porosity generally have better water quality due to the filtering effect that low porosity can have in purifying groundwater (Atangana, 2018). Permeability is also an important characteristic of aquifers and is closely related to porosity. Permeable aquifers consist of materials with larger pore spaces, while an impermeable aquifer has smaller pore spaces (Earle et al., 2015). Water extraction is also determined by permeability, being easier in permeable aquifers due to the ease of groundwater movement. Because of the growing global population, land use, and industrial expansion, surface water can no longer meet the high demand for fresh water (Chotpantarat et al., 2020). In Québec, groundwater represents a critical water resource. Although about 25% of the population relies on it for drinking water, nearly 90% of the

inhabited territory depends on groundwater as its primary water source (Ministère de l'Environnement du Québec, n.d.). Examples of aquifers in Québec illustrate their importance as sources of fresh water across the province. In the Mauricie region, granular aquifers constitute a major source of domestic water for both urban and rural communities; for instance, the city of Trois-Rivières relies on groundwater for approximately 46% of its supply (Légaré-Couture et al., 2018). In the Basses-Laurentides region, sedimentary aquifer systems provide freshwater to about 23% of the population—nearly 250,000 residents (Cloutier et al., 2006). In Gaspésie, aquifers are found within rock units composed of sandstone, limestone, and conglomerates and currently meet about 48% of the region's water demand. Similarly, on the Îles-de-la-Madeleine, aquifers formed of red sandstone overlain by sandy deposits supply 100% of the drinking water required by the local population (Ministère de l'Environnement du Québec, n.d.).

Overall, groundwater resources represent a generally less contaminated and better-protected alternative to surface water sources. Consequently, the protection, study, and sustainable management of aquifers are essential to preserve this vital resource for future generations.

## 1.2 Microorganisms in aquifers

Not only are aquifers one of the main sources of drinking water worldwide, but they are also involved in nutrient cycling, pollutant degradation, and subsurface weathering processes (Griebler & Avramov, 2015; Sonthiphand et al., 2021). These functions are primarily carried out by the presence and activity of microorganisms thriving in these ecosystems. Indeed, underground environments are known to harbor great microbial diversity, with recent studies estimating that terrestrial subsurface habitats contain up to 40% of the total microbial diversity (Nicholls et al., 2022; Smith et al., 2018).

Moreover, due to the lack of nutrients, oxygen, and light in aquifers, there is metabolic heterogeneity among the microbial groups living there; including heterotrophs, autotrophs, obligate lithotrophs, and facultative lithotrophs, all using different strategies to cope with these oligotrophic conditions (Nuppenen-Puputti et al., 2022; Stevens, 1997). Microorganisms in aquifers can adopt either a planktonic or sessile lifestyle, depending on whether they live suspended in groundwater or attached to solid surfaces, respectively (Berlanga & Guerrero, 2016). The literature reports differences between sessile and planktonic communities. Planktonic communities generally develop more rapidly, exhibit different taxonomic compositions, and often present a dormant or inactive activity, presenting different regulations of genes compared to sessile's (Lehman et al., 2001). Sessile communities, on the other hand, provide more biomass

to aquifer systems, demonstrate greater stability in response to stress factors, participate in the weathering processes and mineral release from rocks, and seem to be more active metabolically (Brewer & Fierer, 2018; Ruiz-González et al., 2021; Samuels et al., 2020). Both types are involved in nutrient cycling, the transformation of carbon and organic matter, and groundwater bioremediation (Nuppunen-Puputti et al., 2022). However, evidence suggests that microbial activity in aquifers is primarily driven by sessile communities rather than by planktonic communities (Griebler et al., 2014; Smith et al., 2018). This difference can be explained by the fact that sessile communities are better protected by living biofilms, and also by the supply of nutrients such as carbon and minerals from sediments and that accumulate on rock surfaces that can be used as carbon and energy sources (Choe & Lee, 2019). In this work, the term “potential functional trait” is used throughout the document to denote the functional potential of the studied organisms.

### 1.3 Microbial ecology studies in aquifers

The study of microorganisms in subsurface environments has gained increasing importance in recent years, due to their diverse metabolic capabilities and crucial roles in biogeochemical processes. Organic matter degradation, nutrient recycling, and involvement in biogeochemical cycles such as carbon and nitrogen are examples of this. Various studies have been conducted on aquifers and groundwater worldwide with the goal of better understanding the role of microorganisms in different cycles and the taxonomic members responsible for them (Nuppunen-Puputti et al., 2022; Villeneuve et al., 2023; Wegner et al., 2019). Viruses, for example, are considered one of the most abundant microbial particles in subterranean ecosystems, playing an important role in the subsurface by controlling microbial populations, nutrient availability, and mediating evolution through gene transfer and metabolic reprogramming in bacterial cells (Cai et al., 2023; Kristopher Kieft et al., 2021; Pan et al., 2017). In the case of eukaryotes, studies have primarily identified amoebae such as Cercozoa and Ciliophora, roundworms like Nematoda, and fungi including Ascomycota and Basidiomycota in groundwater samples (Groult et al., 2023; Herrmann et al., 2020; Inkinen et al., 2019). It is believed that water temperature, water supply networks, and depth are key factors that strongly influence the eukaryotic communities living in groundwater (Inkinen et al., 2019).

Extreme organisms such as DPANN archaea and Candidate Phyla Radiation (CPR), which are poorly understood groups with limited cultured representatives have been detected in groundwater ecosystems (He et al., 2021). Metagenomic profiles of CPR and DPANN have shown potential metabolic roles in nitrate reduction, denitrification, and sulfate reduction, suggesting the involvement of this microbial group in

biogeochemical processes. In a recent groundwater incubation study, extensive 16S rRNA and genome analyses showed that diverse CPR lineages can not only persist but actively grow under both oxic and anoxic treatments. Despite initial declines, CPR abundances recovered significantly over time, with some taxa exhibiting growth rates similar to non-CPR organisms and reaching up to ~30% of the community (Gabashvili et al., 2025).

Bacteria have also been studied in subterranean ecosystems. In a study using microcosms and metagenomics, sessile microbial communities were analyzed (Nuppunen-Puputti et al., 2022). Genes involved in biofilm formation, flagella production, chemotaxis, and glycogen synthesis were detected. Furthermore, genes related to carbon, nitrogen, and sulfur metabolism were identified, indicating a high adaptability of sessile communities to utilize different nutrient sources. Another study using biofilm traps for metagenomic analysis compared planktonic and sessile communities. The results revealed distinct taxonomic compositions between the two, with biofilm communities showing the presence of genes related to biofilm formation, as well as nitrogen/carbon fixation capabilities and hydrogen oxidation (Wu et al., 2017). These results suggest the possibility of a potentially more active metabolism within sessile communities. A study using 16S rRNA gene amplicon sequencing, metatranscriptomics, and flow cells to investigate sessile communities compared biofilm and planktonic communities in a deep subsurface aquifer (Lopez-Fernandez et al., 2023). The results showed that the taxonomic composition differed within each community and across different stages (incubation periods) for the biofilm communities. Additionally, the study identified *Thiobacillus* as the main contributor to processes such as sulfur oxidation and biofilm formation in sessile communities. In contrast, members of *Methanobacterales*, *Desulfobacterales*, and *Campylobacterales* were associated with carbon dioxide reduction in planktonic communities. Hug et al., (2015) also found differences in taxa abundance between sediment and microbial groundwater communities, highlighting distinct microbial compositions between biofilm and planktonic communities.

Moreover, researchers examined planktonic communities in a German aquifer system using metagenomic and metatranscriptomic techniques. They found that the microbiomes were primarily influenced by nitrogen cycling, notably nitrification, denitrification, and ammonia oxidation (Wegner et al., 2019). Moreover, they identified CAZy enzymes, largely produced by the phylum *Proteobacteria*, which play a key role in carbon assimilation. This demonstrated the active metabolic characteristics of planktonic microorganisms in groundwater and their potential collaboration in the recycling of organic and inorganic nutrients within aquifer systems.

Another study using passive samplers compared the functional potential of metagenome-assembled genomes (MAGs) between sessile and planktonic groundwater communities. The authors showed that rock-attached (sessile) populations displayed higher completeness in pathways related to sulfur and iron metabolism, biofilm formation, and other chemolithoautotrophic processes. In contrast, planktonic MAGs exhibited lower completeness in several key metabolic pathways (Sharma et al., 2025).

These studies have shown that both sessile (biofilm) and planktonic microbial communities can contribute to biogeochemical cycling. However, the observed metabolic differences between the two suggest potential functional specialization or activity preferences. Based on previous studies, it is likely possible to think that sessile communities exhibit a higher potential metabolic activity across several biogeochemical cycles compared to planktonic groups. This at the same time can be explained by their biofilm lifestyle, which provides access to stable nutrient gradients, protection from shear stress, and increased opportunities for cell–cell interactions (Choe & Lee, 2019; Shree et al., 2023).

However, this does not imply that planktonic communities lack the capacity to perform these functions. Rather, current studies suggest that planktonic populations tend to be less metabolically versatile because they are more transient, more influenced by groundwater flow, and often subject to nutrient limitation (Sharma et al., 2025).

Furthermore, the taxonomic differences and the generally higher microbial biomass in sessile communities compared to planktonic ones also support the idea that attached microorganisms may contribute more strongly to certain biogeochemical processes within aquifers (Eriksson et al., 2016).

Although sessile and planktonic communities differ markedly in their activity, taxonomic composition, and ecological strategies, they are not isolated systems. Instead, both lifestyles are interconnected and can influence one another. Experimental studies that use bioreactors or passive samplers generally start with groundwater planktonic inocula and show that many planktonic taxa are capable of colonizing mineral surfaces and transitioning into a sessile, biofilm-forming lifestyle. This indicates a continuous exchange of taxa, where part of the planktonic community serves as the source of new biofilm colonizers (Patel et al., 2024; Wu et al., 2017).

On the other hand, biofilms in subsurface environments are dynamic structures: mechanical stress, fluctuations in flow, or nutrient limitations can cause sections of biofilm to detach. These detached cells can re-enter the water column, contributing again to the planktonic fraction. Although direct in situ evidence of this detachment process in aquifers is limited, it remains a widely accepted mechanism in biofilm ecology and is consistent with observations from groundwater mesocosm studies (Flemming & Wingender, 2010).

As described above, sessile communities can promote mineral weathering, releasing solutes and nutrients from rock surfaces. Although this process is difficult to track directly in aquifer studies, these dissolved compounds can be utilized by planktonic microbes suspended in the groundwater. This suggests an indirect functional link between the two lifestyles, where sessile biofilms influence nutrient availability and biogeochemical cycling in the planktonic community (Flemming & Wingender, 2010; Samuels et al., 2020). However, it is important to note that no studies have directly measured metabolite fluxes between sessile and planktonic communities in aquifers. Nevertheless, the functional characteristics of sessile biofilms suggest that they likely release intermediates through their metabolic activity, which can serve as sources of energy for planktonic microbes.

Currently, there are few studies that simultaneously compare the activity or potential activity of sessile and planktonic communities in aquifers. Studying both communities is important because each exhibits distinct characteristics. As described above, sessile and planktonic communities can differ in taxonomic composition, strategies to cope with stress and nutrient availability, and metabolic or functional activity. Investigating both lifestyles allows us to better understand their differences and similarities, particularly in terms of biochemical functions that each community can perform in subsurface systems. This is crucial in aquifers because microorganisms directly influence water quality, contaminant fate, and the overall biogeochemical stability of the system. This information is particularly relevant given that aquifers and groundwater constitute essential resources for human populations, and their chemical and biological characteristics are strongly shaped by microbial activity.

#### 1.4 Seasonal shifts in aquifer ecosystems

No natural habitat is steady in terms of environmental (abiotic) fluctuations, and aquifer systems are not an exception. It has been shown that seasonal changes can influence the microbial activity, composition, and biomass of subsurface ecosystems (Griebler & Lueders, 2009; Karwautz et al., 2022). This is mainly due

to the dependence of aquifers on surface water recharge, which varies seasonally. For instance, rainfall during spring and fall enhances connectivity between surface and subsurface waters, while in winter, frozen soils often limit this exchange. In spring, snowmelt can lead to a rapid influx of surface water, further altering the aquifer's physicochemical conditions and microbial dynamics (Fang et al., 2024; Nygren et al., 2020). Changes in aquifer recharge processes, carbon inputs and nutrient influx driven by seasonal shifts can generate functional and structure variations (Karwautz et al., 2022). A metaproteomics study on planktonic communities from seepage water and groundwater examined the impact of seasonality on microbial functions. The results showed a clear separation of community composition based on the season (Lohmann et al., 2020). In their groundwater samples, Lohmann et al. observed that taxonomic evenness declined significantly from summer to autumn, indicating a strong seasonal influence. Additionally, certain nitrogen-related pathways were more abundant in autumn than in summer. These shifts are likely driven by seasonal changes in hydrological recharge and dissolved organic carbon levels, which in turn favored specific taxa and led to differences in functional profiles.

Another study comparing seasonal variations between groundwater and sediment (mesocosm) microbial communities demonstrated how temporal physical changes can influence community diversity (Zhou et al., 2012). The results showed that Shannon diversity indices in planktonic communities were positively correlated with assimilable organic carbon and were affected by water level fluctuations. In contrast, attached (biofilm) communities remained stable and were not significantly influenced by changes in physicochemical parameters, suggesting that biofilms are more resilient to environmental disturbances. (Villeneuve et al., 2023) tracked microbial communities over the course of one year, focusing on planktonic communities from two aquifers. The findings showed that these communities were influenced by periods before and after groundwater recharge events. Following recharge, noticeable changes in microbial diversity and community composition were observed in both aquifers, highlighting how recharge events—and the associated shifts in abiotic factors—can shape microbial dynamics.

### 1.5 Use of bioreactors to recreate underground ecosystems

Due to the challenges of sampling, the risk of contamination, and the high costs of drilling, various studies have adopted alternative methods to study subterranean ecosystems, particularly biofilm or sessile communities over time. These methods include the use of bioreactors, biofilm traps, and microcosms as alternatives. One example is the use of microcosms to examine the metagenomic profiles of sessile communities (Nuppenen-Puputti et al., 2022). In this approach, mica-schist obtained from the drill core

was sampled and divided into crushed mica-schist and mica-schist slabs to create triplicate microcosms. The crushed or sliced mica-schist was placed in sterilized bottles (i.e., microcosms) and filled with groundwater from the study site. Incubations lasted 8 and 40 months. Afterwards, DNA was extracted from the mica-schist materials for further analysis.

A study in Germany, sessile microbial communities were studied using passive samplers containing crushed rock material. Limestone was crushed to 2–4 mm diameter and sourced from an outcrop within aquifer zone in the study. The passive samplers were deployed in monitoring wells and samples were retrieved at 6, 18, 30, 42, and 54 weeks. The goal of the study was to compare the functionality using the MAGs between sessile and planktonic communities (Sharma et al., 2025)

In another study conducted in Sweden, biofilm traps were used to simultaneously analyze sessile and planktonic communities (Wu et al., 2017). Flow cells (biofilm traps) were directly connected to the boreholes to pump groundwater directly into the system. These flow cells, made of stainless steel and filled with garnet grains and glass beads, were installed for a duration of 33 days. The goal of the project was to promote the direct colonization of microorganisms from groundwater onto the rock surfaces. After the incubation period, the rock materials were removed, and genetic material was extracted for further analysis.

In a study conducted in Tennessee, USA, bioreactors were used to simultaneously investigate sessile and planktonic microbial communities (Wilpieszski et al., 2020). These bioreactors consisted of glass flasks containing eight removable coupons, each holding sediment cores from the studied groundwater well. Additionally, each bioreactor was equipped with a gas inlet and a water jacket to maintain a temperature of 14°C, simulating field conditions. Before incubation, all bioreactors were sterilized in an autoclave to ensure that microorganisms colonizing the sediment cores originated solely from the groundwater samples. Groundwater was pumped into autoclaved reservoir bottles and then into the bioreactors over an 18-day period. During incubation, samples were periodically taken from both the coupons and the bioreactor water to extract DNA and perform amplicon sequencing.

## 1.6 Objective and hypothesis

Despite the increase of studies on aquifer microbial communities in the last few years, there is still limited information about the metabolic functions of aquifer microbial communities. Most of the studies have

focused on planktonic communities and the few related to sessile communities mainly study the taxonomic structure and diversity. It is scarce to find reports employing metagenomics applied to microbial communities in subsurface ecosystems. And there are just few studies comparing the functionality of aquifer sessile and planktonic communities across seasons.

With the goal of advancing on the previous points, the objectives of this project were (O1): Compare the potential functional traits across microbial communities with different lifestyles (sessile vs planktonic). And (O2): Compare functional microbial traits across seasons (summer vs winter).

Previous studies have reported that metabolic functions are different between sessile and planktonic communities in aquifers, which is based on the fact that nutrient availability, stability, and the taxa present in each community is different. That is why we hypothesize that (*H1*) sessile and planktonic communities will have different gene abundances across different pathways as for example in carbon or nitrogen cycling, as well as defense and coping mechanisms required to tolerate environmental stressors (e.g., nutrient limitation, redox fluctuations, and exposure to toxic compounds).

Also, since seasonal and environmental shifts can influence microbial functions, we expect to see differences in the functional gene abundances between summer and winter seasons (*H2*).

The data presented in this thesis will be used to submit a research paper for publishing following the submission to this committee. However, we chose to present the data in a traditional way, and not in the format of a research paper.

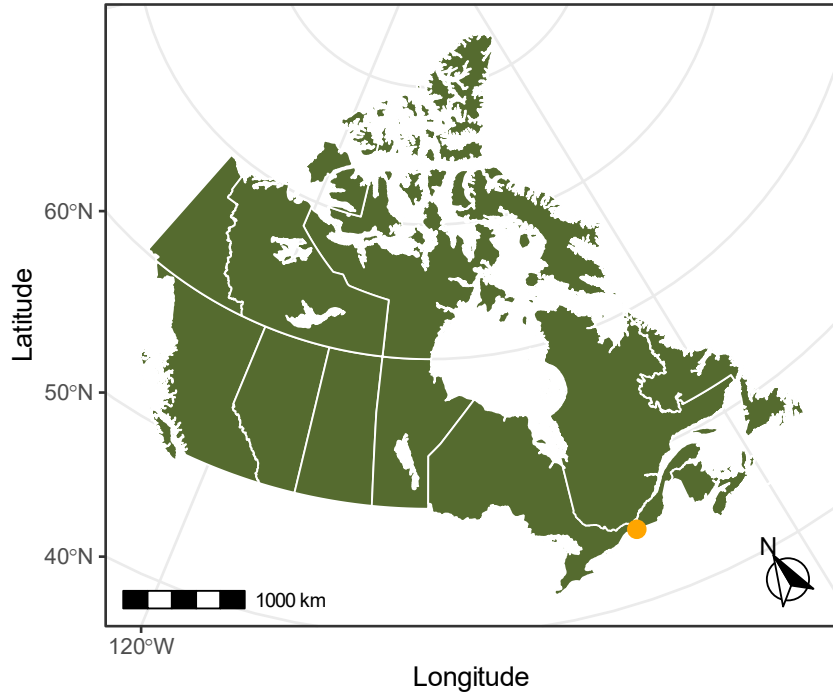
## 2 MATERIAL AND METHODS

### 2.1 Study site and sampling process

The sampling took place in the Basse-Terres of Saint Laurent region in Covey Hill, Quebec (Figure 1). Groundwater was sampled twice from a 1.5 m unconfined aquifer, once during the summer (July 27th, 2023) and once during the winter (March 2nd, 2023). For each sampling event, 10 liters of groundwater were collected in autoclaved amber glass bottles to minimize contamination. The groundwater samples were collected using a manual hand submersible pump. Prior to sample collection, we purged the well by discarding three successive volumes of 10 L each to remove stagnant water and ensure representative groundwater sampling. Salinity, pH, temperature, dissolved oxygen (DO), electrical conductivity, and pressure were measured once on the sampling day using a multiparameter probe (OAKTON PD 450, Cole-Parmer). These parameters were taken once using the calibrated probe. Some of these parameters (temperature, DO and pH) were used to set up the bioreactor's conditions.

Furthermore, on the same sampling days, aliquots of groundwater were collected to measure dissolved inorganic carbon (DIC), dissolved organic carbon (DOC), reduced forms of nitrogen ( $\text{NH}_x$ ), nitrite ( $\text{NO}_2^-$ ), and nitrate ( $\text{NO}_3^-$ ). For DIC and DOC, aliquots were collected on in gas free glass bottles after filtration on 0.45  $\mu\text{m}$  polyethersulfone filters in the field (Sarstedt, USA). For  $\text{NO}_2^-$  and  $\text{NO}_3^-$ , we used plastic bottles and 0.45  $\mu\text{m}$  polyethersulfone filters, and for  $\text{NH}_x$  0.2 $\mu\text{m}$  polyethersulfone filters. For each parameter, aliquots were taken twice to obtain two replicates per variable.

All the geochemical samples were analyzed at the analytical laboratory of the Interuniversity Research Group in Limnology (GRIL, UQAM).



**Figure 1:** Map showing the location of the sampled aquifer point (indicated by an orange circle) in Covey Hill, Quebec, Canada.

## 2.2 Rock samples

The aquifer that was studied in this project is a superficial aquifer composed of deformed and fractured sandstone, outcropping up to the surface (Girard et al., 2015). Therefore, this system allows us not to have to drill the subsurface to obtain aquifer rock material. An outcropping rock slab, from a collection of slabs on the surface around the well, was collected before groundwater sampling. Back in the lab, it was first cleaned with water and neutral soap to remove all plant materials and debris. After that, rock mini plugs were drilled from the rock slab using a bench drill press (18" nova voyager dvr, King Industrial, Canada) and a 1.3 cm diameter diamond drill bit. These plugs were then sawed into rock chips 0.3 cm high (DREMEL 3000, DREMEL, USA). The rock chips were then washed (Soap and sterile H<sub>2</sub>O milliQ) and autoclaved before installing in the bioreactor (Annex 6.11).

## 2.3 Bioreactor assembly

To mimic aquifer conditions, a bioreactor was employed to simulate this ecosystem. For this, a triplicate bioreactor setup was used (CBR 90 Standard CDC Biofilm Reactor, BioSurface Technologies, USA). The system included three 10 L amber glass bottles filled with the groundwater collected in the field, which

was connected to the three bioreactors immediately after sampling. The bottles were placed on magnetic stirring plates to prevent microorganisms and sediments from adhering to the bottle walls or from settling at the bottom. The groundwater was pumped into the bioreactor at a constant flow rate of 0.289 mL/min (IPC, Ismatec, Germany), ensuring a slow flow comparable to natural aquifer conditions. The <sup>1</sup>bioreactor itself was also positioned on a magnetic stirring plate. It contained eight columns, each housing three rock chips, resulting in a total of 24 rock chips within each bioreactor (Figure 2).

The goal was to allow planktonic microorganisms present in the groundwater to colonize the rock chip surfaces once the water entered the bioreactor. As the water slowly flowed through the system, it exited via a pipe and was collected in a separate autoclaved amber glass bottle. In this way, the sessile community developed on the rock surfaces, while the planktonic community was represented by the microorganisms remaining in the collected water. Also, this allowed us to have an open system where water only circulated in one direction, as is in the case in the subsurface. In addition, three gases (N<sub>2</sub>, O<sub>2</sub> and CO<sub>2</sub>) were injected simultaneously at 200 sccm (MCQ GB100 gas mixer, Monkey Industrial Supply, USA) at different concentrations that were established based on the data collected on the sampling date. Furthermore, parameters such as pH and dissolved oxygen (DO) were monitored daily during the incubations to regulate gas concentrations and maintain the pH and DO levels observed in the field. The whole bioreactor system was placed inside an incubation chamber (Annex 6.1) at UQAM, maintained at a temperature similar to that measured in the groundwater on the sampling day.

<sup>1</sup>The entire bioreactor system, including pipes, the bioreactor, bottles, and other components, was washed and autoclaved before starting the incubations.



**Figure 2:** Bioreactor used for the incubations. The image represents one, but other two exactly like this were used for the experiments. The rock chips were installed in the spaces of each column (C). The water exits via the pipe (D) to be collected in another bottle.

#### 2.4 Incubations and sample collection

The incubations lasted 21 days. (Although the incubation period was relatively short, the duration was chosen to be consistent with our methodological constraints. At the established flow rate of the system, a total of approximately 8.4 L of groundwater is circulated through the bioreactor over approximately 21 days). The incubations were performed twice, once in summer (July 27th, 2023) and once in winter (March 2nd, 2023). During the incubations, each 7 days, rock chips and water were sampled from the bioreactor. For the rocks, eight rock chips were taken from the bioreactor, and for the water, approximately 2.8 L of water were collected from the bottle where it was stored after exiting the bioreactor. The process took place under the laminar hood under aseptic conditions in order to minimize the contamination. The rock chips were collected in sterile Falcon tubes and stored at -80 until the next step. The water was filtered on the day it was collected using sterile polyethersulfone membrane filters with 0.2  $\mu\text{m}$  diameter pores (Sartorius, Germany) and a membrane vacuum pump (Welch 2019B-01, Welch, USA). Filters were stored in sterilized Eppendorf tubes at -80 until the next step. In addition, each seven days, water aliquots were collected to measure physical chemical parameters (DIC, DOC,  $\text{NO}_2$ ,  $\text{NO}_3$  and  $\text{NH}_x$ ). The same procedure described above was repeated after 14 and 21 days of incubation.

## 2.5 DNA and RNA extraction

The initial aim of this project was to perform parallel metagenomic and metatranscriptomic analyses of the planktonic and sessile communities. To achieve this, we sampled rock chips and water after 7, 14, and 21 days from each bioreactor, in an attempt to accumulate enough biomass for RNA extractions over the course of one week. Although three identical bioreactors were employed for the incubations, only samples from one bioreactor were used for further analysis.

For the filters, DNA and RNA were extracted using the RNeasy PowerSoil total RNA Kit and the RNeasy PowerSoil DNA Elution Kit (QIAGEN), following the instructions provided by the manufacturer (Annex 6.2). For the rock samples, DNA and RNA were extracted using a modified version of the protocol described by Nuppenen-Puputti et al. (2020) (Annex 6.3). The goal was to dislodge the biomass from the rock surfaces by using silica beads and autoclaved detergents as described in the Nuppenen-Puputti study. This allowed the biomass to be transferred into the liquid supernatant, from which the extraction process could then proceed (Annex 6.2). At the end of each extraction, DNA and RNA from rocks and filters were measured using Qubit DNA and RNA HS assay kit.

To purify the RNA extracted from both rock and filter samples, two protocols were applied. The QIAGEN RNeasy UCP Micro Kit was used for RNA purification, while the Invitrogen RiboMinus Bacteria 2.0 Transcriptome Isolation Kit was employed to deplete ribosomal RNA (rRNA). Both procedures were conducted according to the manufacturers' instructions (Annex 6.4). At the end of the process RNA concentrations were measured again for filters and rock samples. As mentioned before, this process was done for samples in summer and winter. Unfortunately, at the end of these different steps, not enough RNA was present in all samples to be used for shotgun sequencing (Annex 6.9). We then decided to only use the DNA for sequencing, as we had enough in all samples to proceed, apart from 1 sample.

To ensure that the bioreactor system was not a source of microbiological contamination, negative controls were carried out by a lab colleague, as described in her project (Patel, 2023). In these controls, bioreactor incubations were repeated under the same conditions as described above, but using sterile Milli-Q water instead of groundwater. The incubations lasted 24 days, after which rock chips and 300 mL of water were collected from the bioreactor for analysis.

## 2.6 Shotgun DNA sequencing

Shotgun metagenomic libraries were prepared using the NEBNext Ultra™ II FS DNA Library Prep Kit for Illumina (New England Biolabs), following the manufacturer’s protocol. Whenever possible, 50 ng of input DNA was used; however, lower amounts were used for some samples due to limited DNA availability (T1-rocks-winter: 2.6 ng, #T1-rocks-summer: 23.5 ng, #T2-rocks summer and #T3-rocks summer: DNA concentrations were below quantification limits but were nevertheless processed). DNA quantification was performed using the Qubit dsDNA High Sensitivity Assay Kit (Invitrogen). Unique dual indexing was achieved using the NEBNext Multiplex Oligos for Illumina (96 Unique Dual Index Primer Pairs). Library quality control included DNA quantification via Qubit and fragment size estimation using the High Sensitivity D1000 ScreenTape on a TapeStation 4200 system (Agilent). Sequencing was conducted on a NovaSeq 6000 system (Illumina) at the CRCHU de Québec (CHUL) NGS sequencing platform, using a NovaSeq 6000 S2 kit (v1.5) with paired-end 150 bp reads. The number and description of samples sequenced for shotgun sequencing are described (Table 1).

**Table 1.** Overview of sample names, corresponding sample types, and the incubation time performed for each sample.

Sample metadata summary			
Sample Name	Sample Type	Sample Season	Incubation Time
T0_water_winter	Planktonic	Winter	0 days
T0_water_summer	Planktonic	Summer	0 days
T1_water_winter	Planktonic	Winter	7 days
T1_rocks_winter	Sessile	Winter	7 days
T2_water_winter	Planktonic	Winter	14 days
T2_rocks_winter	Sessile	Winter	14 days
T3_water_winter	Planktonic	Winter	21 days

T3_rocks_winter	Sessile	Winter	21 days
T1_water_summer	Planktonic	Summer	7 days
T1_rocks_summer	Sessile	Summer	7 days
T2_water_summer	Planktonic	Summer	14 days
T2_rocks_summer	Sessile	Summer	14 days
T3_water_summer	Planktonic	Summer	21 days
T3_rocks_summer	Sessile	Summer	21 days

## 2.7 Metagenomic workflow analyses

The shotgun sequencing workflow followed an internal protocol developed by members of the lab, which was itself adapted from protocols used in other research groups, including the Baker Lab at the University of Texas at Austin. Analyses were conducted using GNU Bash (version 5.0.17 (1)) and RStudio (version 4.4.0; Posit Team, 2023). Assembly of metagenomic reads was carried out using MetaSPAdes (Nurk et al., 2017), and only contigs longer than 1000 base pairs were retained for downstream analysis. The resulting contigs were translated into amino acid sequences, which were then annotated using BlastKOALA (prokaryote option) (Kanehisa et al., 2016). In total 6291 different genes (Based on K number) were obtained after functional annotation. Functional classification of K numbers into metabolic pathway categories was performed using the KEGGREST package in R (Tenenbaum D, Maintainer B., 2025). Gene counts were normalized by calculating relative abundances. Specifically, the count of each gene in a given sample was divided by the total number of reads obtained for that sample post-assembly.

## 2.8 Statistical analysis for the metagenomic sequencing data

Statistical analysis was performed using RStudio for the metagenomic sequencing data. The normalized count scores were used for all the tests mentioned below.

Beta diversity was evaluated using the Bray–Curtis dissimilarity, calculated with the `vegdist` function from the `vegan` package. PCoA was then performed to visualize the distribution of samples based on season and lifestyle. PERMANOVA was conducted using the `adonis2` function, testing for significant differences in gene

abundance across groups (seasons and lifestyle). Influence of environmental variables on functional diversity was tested using distance-based redundancy analysis (db-RDA). Capscale function from vegan package was used to test the RDA model using bray curtis and Hellinger transformed gene data.

In order to identify the most different pathways in each group (lifestyle and season), Linear Discriminant Analysis Effect Size (LEfSe) from the lefser package (v1.14.0) was applied (Segata et al., 2011). This method used the Kruskal-Wallis test to assess differences in abundance among groups, followed by Linear Discriminant Analysis (LDA) to identify features that contribute most to the differences between the groups.

Additionally, heatmaps were generated to visually compare the abundance of metabolic pathways between lifestyle and season groups (sessile vs. planktonic, summer vs winter) and using the obtained pathways from LEfSe result test. Finally, physicochemical data were visualized using custom plots created with the ggplot2 package (v3.5.1).

To explore the functional relatedness of pathways identified as significant by LEfSe in sessile and planktonic communities, a functional dendogram was constructed based on the KEGG pathway classification hierarchy. Each pathway was assigned to its respective KEGG category and subcategory, and the relationships among pathways were visualized as a dendogram (tree structure), where closely related pathways cluster together. This approach allowed us to assess whether pathways enriched in different sample groups (sessile and planktonic lifestyle) were functionally similar or distinct.

For the comparative analyses, the same set of samples was used to assess both seasonal (winter vs. summer) and lifestyle (sessile vs. planktonic) differences. The samples were grouped accordingly depending on the focus of the analysis, allowing us to examine patterns driven by environmental conditions or community lifestyle using a consistent dataset.

All figures, tables, and maps were created by the author using RStudio and Microsoft PowerPoint. Likewise, all photographs presented in this document were taken by the author and are original and free of copyright restrictions.

Some screenshots included in the annexes were taken from official guidelines provided by the manufacturers of the solutions and reagent kits. These materials are used solely for illustrative and

educational purposes. No copyright infringement or misuse is intended. All scripts used for the analyses can be accessed through my GitHub profile (Annex 6.10).

## 3 RESULTS

### 3.1 Physico- and geo-chemical parameters

Parameters were monitored throughout the incubations conducted during both summer and winter. The measurements taken directly in the field on the sampling days for each season show notable differences, particularly in temperature, with 16°C recorded in summer compared to 4.3°C in winter (Table 2). Dissolved oxygen also varied, with 38.4% in summer and 28.1% in winter, as did pH values, which were 5.49 in summer and 7.82 in winter. In the case of conductivity, it is possible that the probe was not correctly standardized, which may explain the high values obtained during the summer season (Table 2)

Other parameters, such as oxygen concentration and pH, were monitored daily during the incubations (Figure 3AB). Both seasons exhibited higher oxygen levels in the bioreactor than in the field, with summer consistently presenting higher concentrations than winter. pH levels remained relatively stable during incubations, although winter values tended to be slightly more acidic compared to the field.

Additional parameters including dissolved inorganic carbon (DIC), dissolved organic carbon (DOC), ammonium/ammonia ( $\text{NH}_x$ ), nitrite ( $\text{NO}_2^-$ ), and nitrate ( $\text{NO}_3^-$ ) were measured weekly throughout the incubation period (Figure 3). DOC remained relatively stable in both experiments, with slightly higher mean values in summer (34 mg/L) compared to winter (29.8 mg/L). In contrast, DIC levels started higher at day 0 and gradually declined during the incubations (mean: summer, 5.01 mg/L; winter, 7.6 mg/L).  $\text{NH}_x$  showed a similar trend, with a notable decrease following the start of the incubations.  $\text{NO}_2^-$  concentrations were initially very low and dropped to zero after days 7 and 14.  $\text{NO}_3^-$  was consistently undetectable throughout the entire incubation period in both seasons (data not shown).

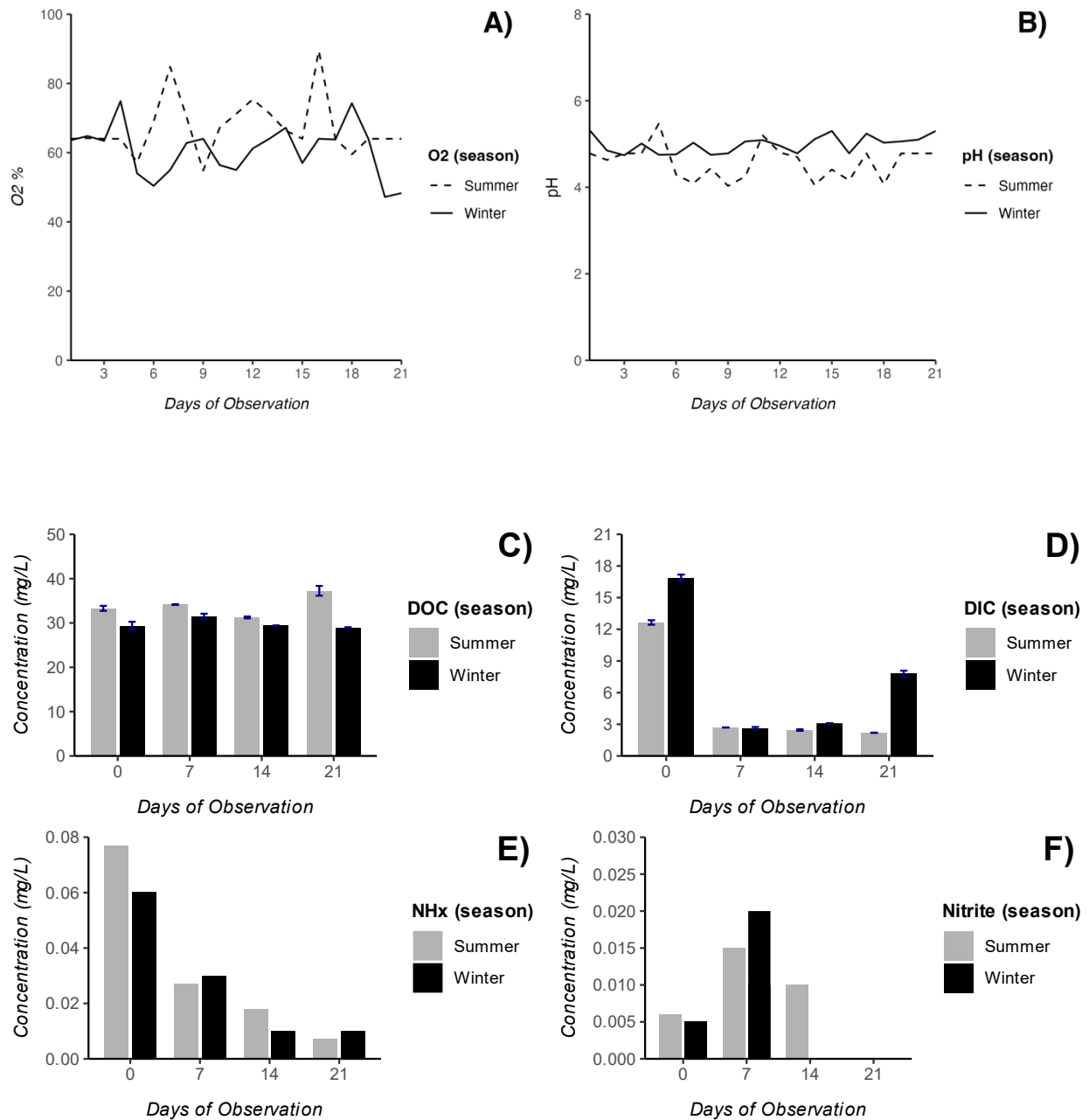
When comparing field measurements with incubation data, notable differences were observed in both dissolved oxygen (DO) and pH. Dissolved oxygen levels during the incubations were consistently higher than those recorded in situ for both seasons. These differences could be explained by oxygen infiltration or contamination in the bioreactor system. Small leaks in the tubing, connections, or headspace between the bottle and the tubing could have allowed oxygen diffusion. In addition, once groundwater is extracted from the aquifer, it becomes briefly exposed to air, which may also increase dissolved oxygen levels before the incubation begins.

In terms of pH, summer values were relatively similar between field and incubation conditions, maintaining an overall acidic character. In contrast, winter field measurements showed alkaline pH values, whereas the corresponding incubation samples shifted toward acidic conditions.

Ammonium, nitrite, and nitrate could not be measured in the field because the available probe did not support these analytes. As a result, these parameters are absent from the in situ dataset.

**Table 2.** Physicochemical parameters measured on field during sampling for winter and summer experiments.

	<b>Summer</b>	<b>Winter</b>
<b>Temperature (°C)</b>	16	4.3
<b>Dissolved oxygen (%)</b>	38.4	28.1
<b>Dissolved oxygen (mg/L)</b>	4.46	3.65
<b>Pressure (kPa)</b>	97.53	96.9
<b>Conductivity (ms/cm)</b>	181.4	0.0312
<b>Salinity (ppt)</b>	0.09	0.01
<b>pH</b>	5.49	7.82

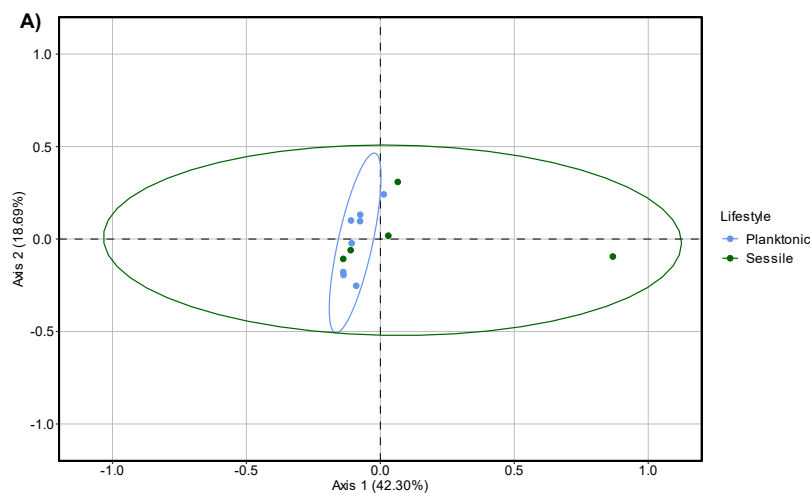


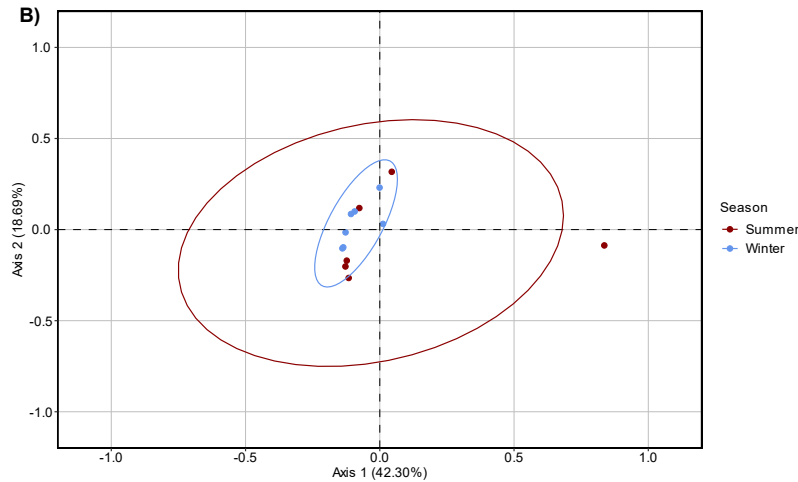
**Figure 3:** Physicochemical parameters measured during incubation experiments for winter and summer conditions. Panels show temporal variation of: (A) oxygen saturation (%), (B) pH, (C) dissolved organic carbon (DOC), (D) dissolved inorganic carbon (DIC), (E) ammonium/ammonia (NH<sub>x</sub>), and (F) nitrite (NO<sub>2</sub><sup>-</sup>). Measurements were taken at multiple timepoints throughout the 21-day incubation period. Each variable was measured twice at each sampling time; the mean of the two readings is shown, with standard deviation (SD) represented by blue error bars.

### 3.2 Functional diversity

Due to low quality following assembly and annotation, one rock-associated sample (summer season, 21 days incubation (T3\_rocks\_summer)) was excluded from downstream analyses. As a result, the final dataset included 13 samples.

A Bray-Curtis dissimilarity matrix was used to evaluate the effect of lifestyle and season on the gene composition of microbial communities. For the lifestyle variable, the PCoA plot revealed an overlap between sessile and planktonic samples, with the planktonic group forming a distinct cluster nested within the broader distribution of sessile samples (Figure 4A). Interestingly, one sessile sample from the summer incubation (T1\_rocks\_summer) emerged as an outlier in the ordination, positioned far from the other sessile communities. Despite this, the PERMANOVA revealed significant differences in gene composition between lifestyles ( $R^2 = 0.14$   $p = 0.039$ ) (Annex 6.5). A similar analysis was conducted for the season variable, and as observed with lifestyle, there was an overlap between summer and winter samples, although the winter group appeared to form a more distinct cluster (Figure 4B). Nonetheless, the PERMANOVA test showed no significant effect of season on gene composition ( $R^2 = 0.089$   $p = 0.345$ ) (Annex 6.5).

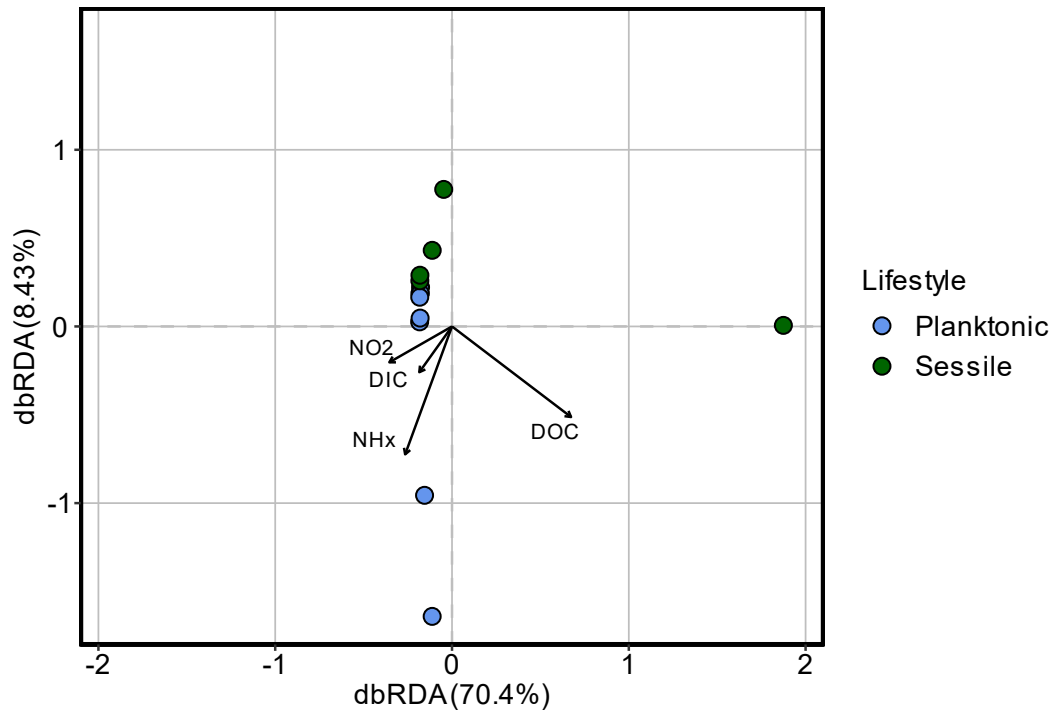




**Figure 4:** Functional beta-diversity of microbial communities grouped by lifestyle (A) and by season (B), based on Bray-Curtis dissimilarity, visualized using Principal Coordinate Analysis (PCoA). Samples are colored by group, with ellipses representing the 95% confidence interval around the centroid of each group.

### 3.3 Beta diversity correlation with environmental parameters

Hellinger and bray curtis distances were used to analyze how physical-chemical parameters could be correlated with dissimilarity matrices. In the analysis all the variables measured in water during the incubations for winter and summer were included (DIC, DOC,  $\text{NH}_x$ ,  $\text{NO}_2$ , pH and DO). The db-RDA was done using planktonic and sessile samples. The included variables explained 84% of the variation in the gene composition across samples. Forward selection identified dissolved organic carbon (DOC) and nitrite ( $\text{NO}_2$ ), dissolved inorganic carbon (DIC) and reduced forms of nitrogen ( $\text{NH}_x$ ) as significant variables explaining variation in the gene composition of samples (ANOVA;  $F = 4.58$ ,  $p = 0.024$ ) (Annex 6.6). The first and second canonical axes explained 70.4% and 8.43% of the variance of the constrained samples. However, permutation test for axes did not reach significance ( $p = 0.078$ ,  $p = 0.352$  respectively). The db-RDA indicated that, based on arrow length, ammonium ( $\text{NH}_x$ ) and dissolved organic carbon (DOC) were the strongest variables shaping on community gene composition (Figure 5). All significant abiotic variables oriented toward the T0 water samples (T0-water-summer and T0-water-winter), suggesting that these early water communities were positively associated with higher concentrations of these factors. In contrast, most sessile and planktonic samples were positioned opposite to the vectors, indicating that they were generally associated with lower values of  $\text{NH}_x$ , DOC, and the other significant variables not measured in the study.



**Figure 5:** Lifestyle distance based bacterial redundancy analysis (db-RDA) using all samples, illustrating the overall variation in gene composition across seasons. Environmental variables, such as DOC, DIC and NH<sub>2</sub> and NO<sub>2</sub>, are visualized with arrows to show their influence on gene structure.

### 3.4 Dominant functional pathways

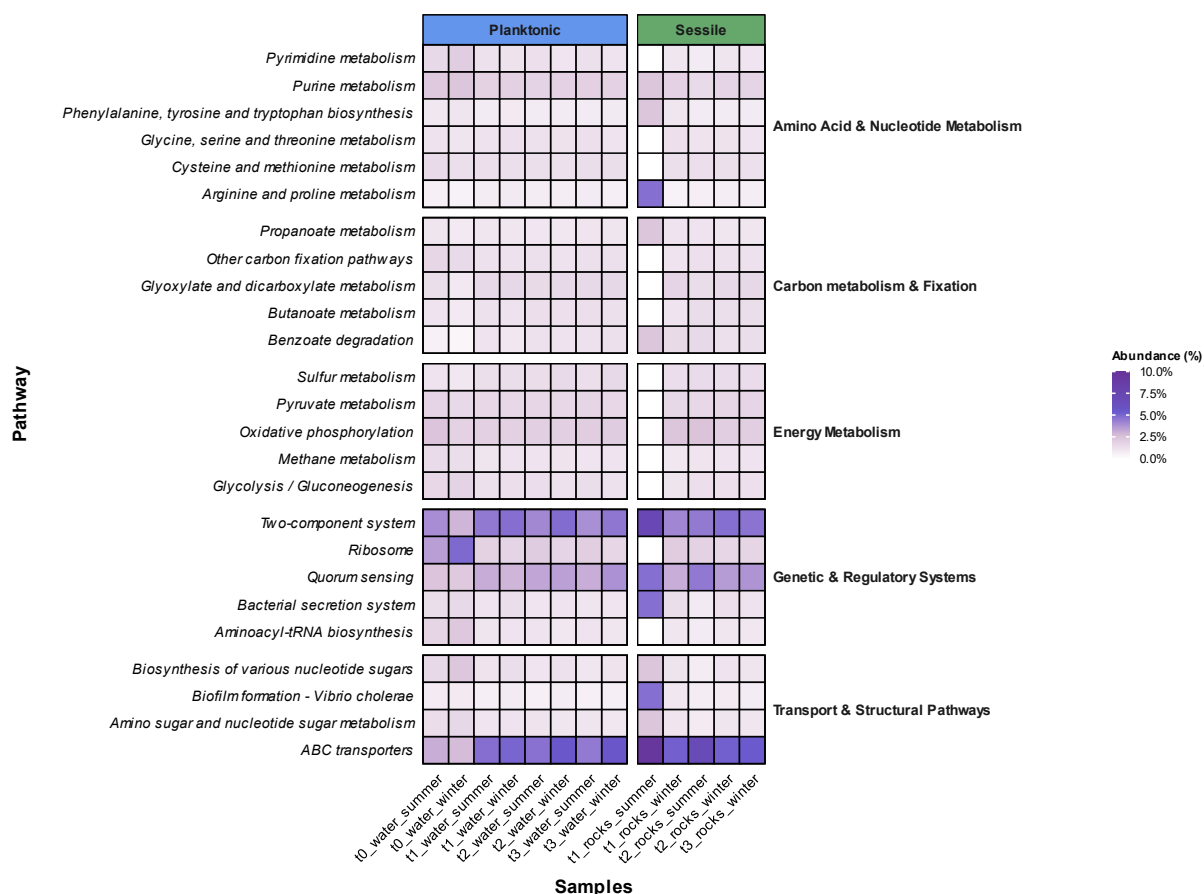
KEGGREST identified 340 different pathways. A heatmap was generated to visualize the relative abundance of KEGG pathway categories across samples, grouped by microbial lifestyle (planktonic vs sessile). The top 25 most abundant pathways were selected based on their relative percentage across all samples. These pathways were organized into five functional categories to aid interpretation: (1) amino acid and nucleotide metabolism, (2) carbon degradation and fixation, (3) energy metabolism, (4) genetic and regulatory systems, and (5) transport and structural pathways (Figure 6).

When comparing pathway abundances between sessile and planktonic communities, no clear lifestyle-based differences were observed in the heatmap. A similar abundance profile was observed for most pathways across the different samples. Interestingly, one sessile sample from the summer incubation

(T1\_rocks\_summer) displayed a markedly distinct functional profile compared to other sessile replicates. This sample showed a reduced abundance of pathways associated with energy metabolism, carbon degradation and fixation, as well as amino acid and nucleotide metabolism. In contrast, it exhibited elevated levels of transport-related and structural pathways.

Among all samples, the most abundant pathways included ABC transporters, two-component systems, quorum sensing, ribosomal proteins, and oxidative phosphorylation, with average percentages ranging from 2% to 5.22% for sessile and planktonic communities.

Although the heatmap did not reveal clear abundance differences and PERMANOVA showed a significant effect of lifestyle on gene relative abundances, slight variations in abundance percentage between sessile and planktonic communities were observed for several pathways. For example, methane metabolism averaged 0.86% in sessile versus 1.2% in planktonic communities. Similarly, biofilm formation was higher in sessile (1.63%) compared to planktonic (0.75%) samples, while ABC transporters averaged 6.39% in sessile and 4.48% in planktonic groups. Other pathways showing differences included two-component systems (5.02% sessile, 4.18% planktonic), quorum sensing (3.94% sessile, 3.05% planktonic), other carbon fixation pathways (0.97% sessile, 1.34% planktonic), bacterial secretion systems (1.89% sessile, 1.23% planktonic), ribosomal proteins (1.49% sessile, 2.46% planktonic), and aminobenzoate degradation (1.27% sessile, 0.34% planktonic). Some of these pathways are not shown in Figure 6 because their average abundance was lower than that of the 25 most abundant pathways.

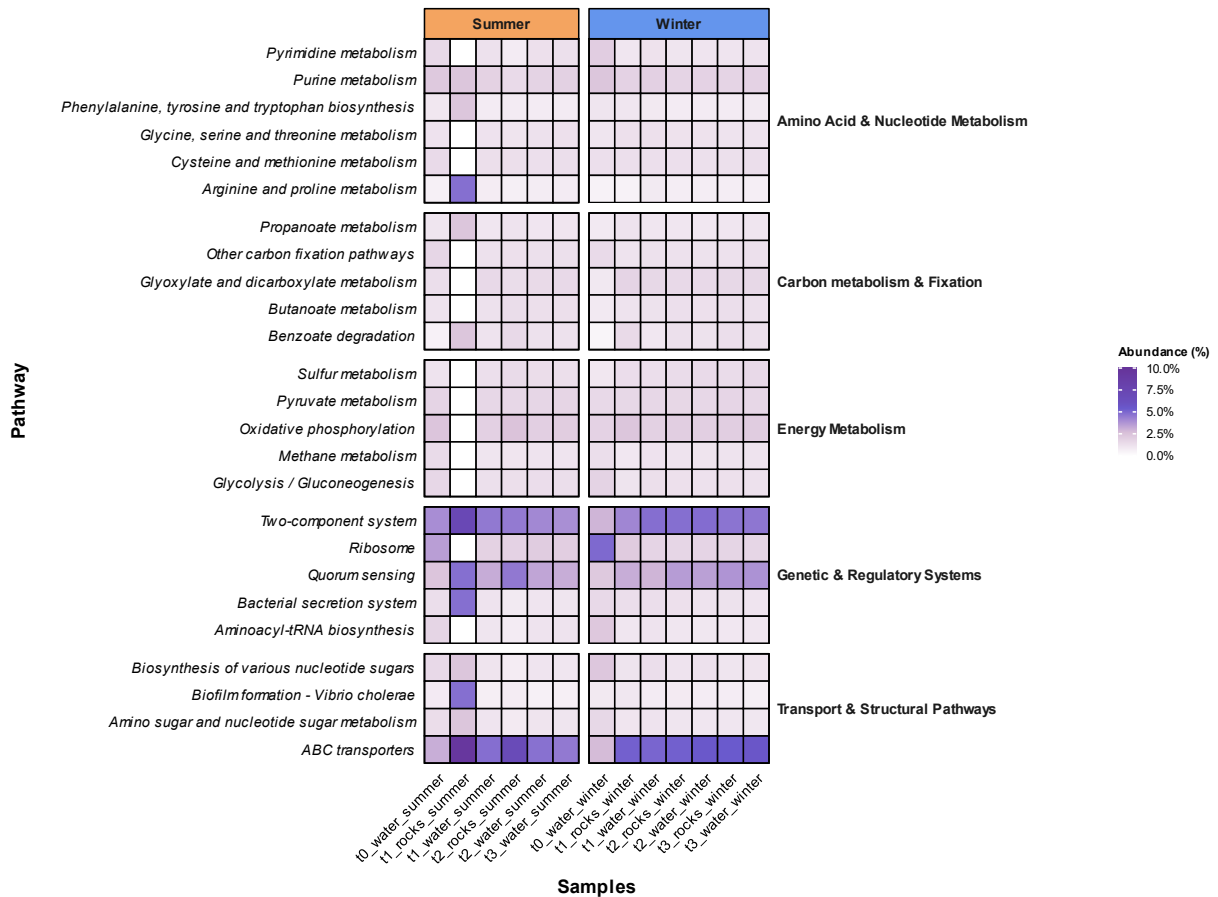


**Figure 6:** Heatmap of KEGG metabolic pathways based on functional annotation. The heatmap shows the relative abundance (in percentage) of the 25 most abundant KEGG pathways across all samples (sessile vs planktonic). Color intensity represents abundance, with dark purple indicating higher values and white indicating lower values (as shown in the scale bar). Pathways are grouped into five functional categories based on shared biological functions.

A similar heatmap was generated to visualize the relative abundance of KEGG pathway categories across samples, this time grouped by season (summer vs. winter) (Figure 7). As with the lifestyle-based analysis, the top 25 most abundant pathways were selected based on their overall relative abundance across all samples, resulting in the same set of pathways being plotted.

As previously mentioned, no major differences were observed when organizing the data by season. However, a distinct pattern emerged for the pre-incubation planktonic samples (T0\_water\_summer and

T0\_water\_winter), which showed higher relative abundance in the Ribosome pathway and lower abundances in pathways related to Quorum sensing and ABC transporters compared to the other samples.



**Figure 7:** Heatmap of KEGG metabolic pathways based on functional annotation. The heatmap shows the relative abundance (in percentage) of the 25 most abundant KEGG pathways across all samples (summer vs winter). Color intensity represents abundance, with dark purple indicating higher values and white indicating lower values (as shown in the scale bar). Pathways are grouped into five functional categories based on shared biological functions.

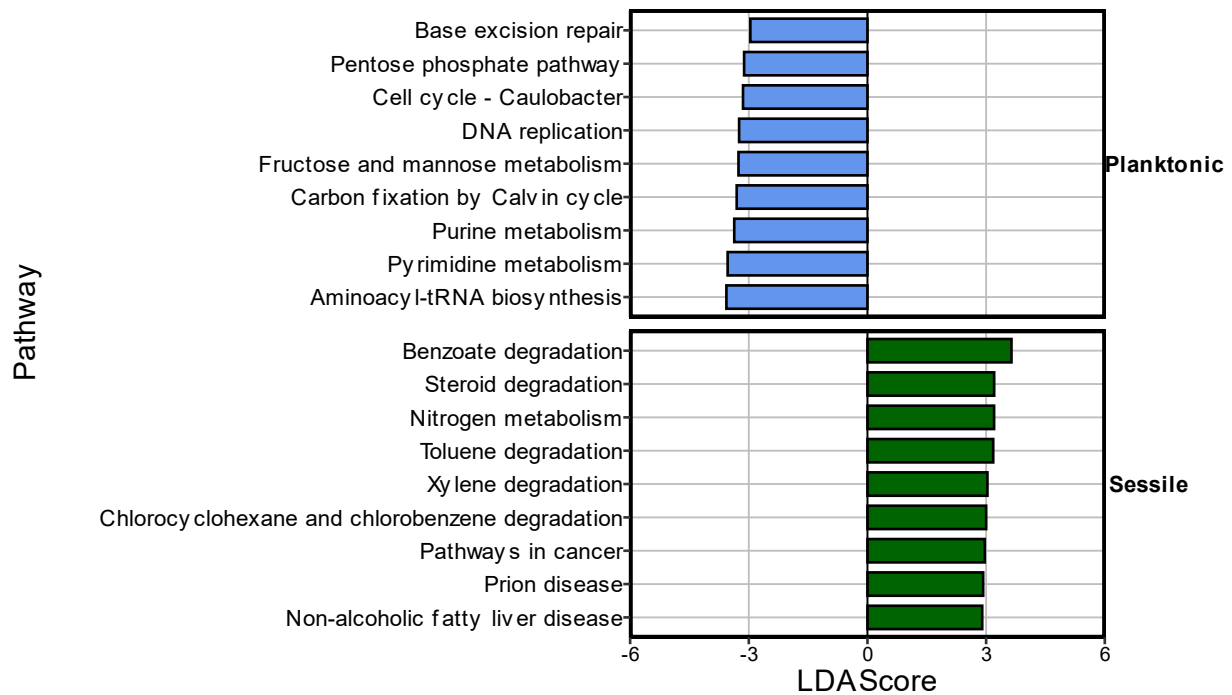
### 3.5 Representative functional pathways between planktonic and sessile groups

To identify functional differences between sessile and planktonic communities, a LefSe analysis was performed using the 340 metabolic pathways detected across all samples. The sample T1\_rocks\_summer was excluded from the analysis due to its atypical metagenomic profile. The analysis revealed 46 pathways

with significant differential abundance: 20 enriched in sessile communities and 26 in planktonic communities. Among these, only pathways with the nine highest LDA score for sessile and planktonic communities were selected for visualization using a horizontal bar plot (Figure 8). The remaining pathways identified in the analysis are listed in Annex 6.7.

In the planktonic communities, the enriched pathways were mainly associated with genetic systems—including DNA replication, base excision repair, and cell cycle, as well as nucleotide metabolism (pyrimidine and purine metabolism), carbon metabolism (pentose phosphate pathway, other carbon fixation pathways, fructose, and mannose metabolism), and amino acid metabolism (aminoacyl-tRNA biosynthesis).

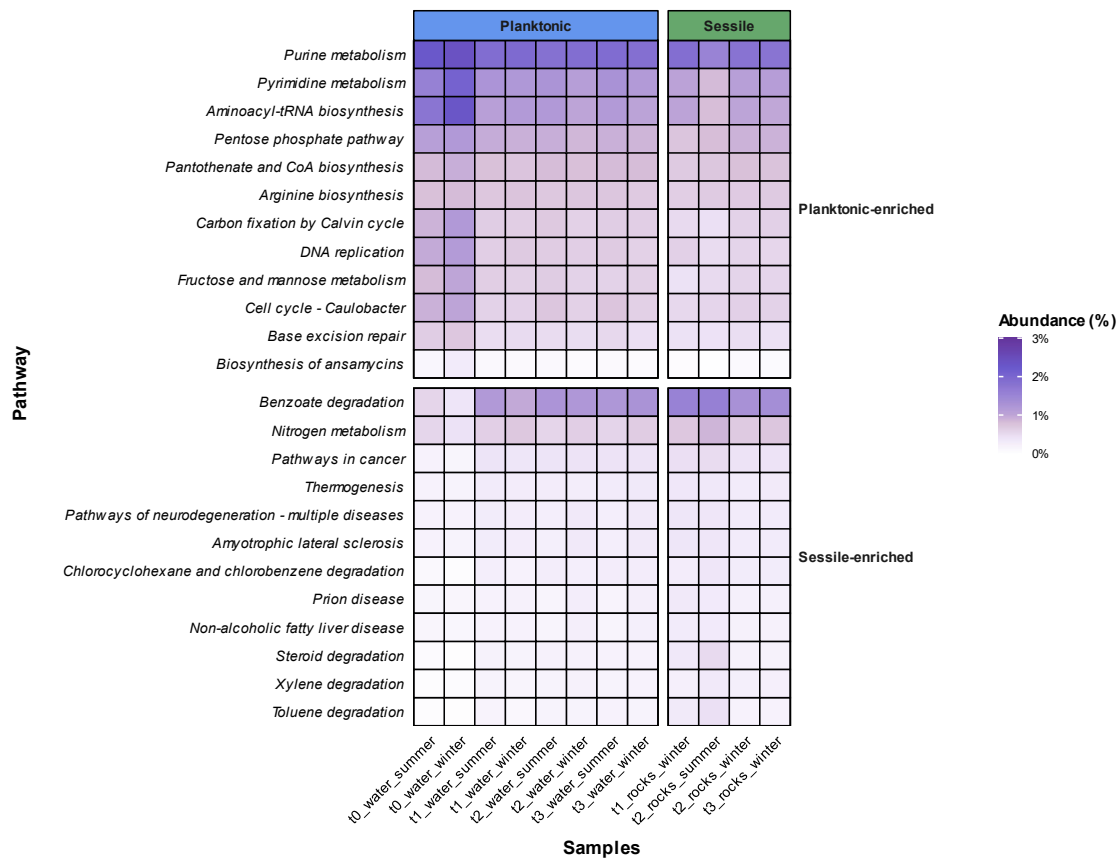
In contrast, sessile communities were enriched in pathways related to nitrogen metabolism and xenobiotic degradation (benzoate degradation, steroid degradation, toluene degradation, chlorocyclohexane and chlorobenzene degradation and xylene degradation).



**Figure 8:** Differential Pathways Between Sessile and Planktonic Communities using LefSe Analysis (LDA Score on X-axis, Pathways on Y-axis). Planktonic enriched pathways are represented in blue, while sessile enriched pathways are represented in green.

A heatmap was generated to facilitate the visualization of differences in pathway abundances between sessile and planktonic communities, based on the 12 pathways with the highest LDA score on each group identified as enriched through analyses (LEfSe). To aid interpretation, these pathways were grouped into two categories: sessile-enriched and planktonic-enriched.

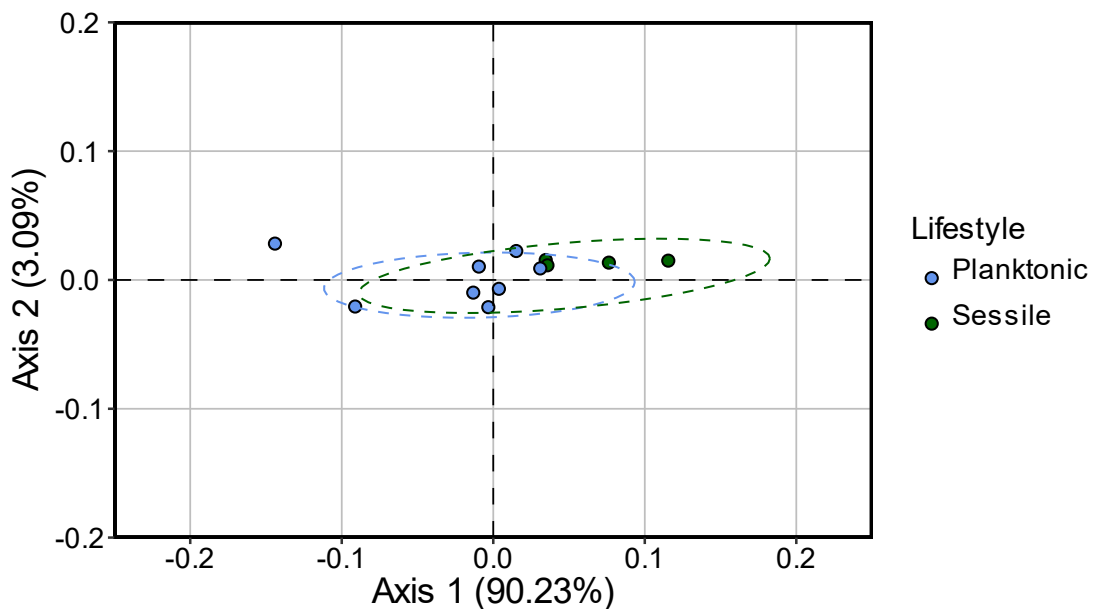
Although the LEfSe analysis identified certain pathways as representative of sessile and planktonic communities, the overall differences in pathway abundances between lifestyles were subtle. The most distinct patterns were observed in the time-0 planktonic samples (T0\_water\_summer and T0\_water\_winter), which displayed pathway profiles that differed from the other sessile samples. Specifically, some pathways in these samples were either much lower or markedly higher in abundance compared to those in sessile communities (Figure 9).



**Figure 9:** Heatmap of KEGG metabolic pathways enriched in sessile and planktonic communities, as identified by LEfSe. The heatmap displays the relative abundance (%) of 20 selected KEGG pathways across all samples. Color intensity corresponds to relative abundance, with dark red indicating higher values and white indicating lower values, as shown in the scale bar. Pathways are grouped by enrichment category (Sessile-enriched or Planktonic-enriched) to highlight lifestyle-specific functional profiles.

### 3.6 Beta diversity of selected functional pathways (lifestyle)

To further explore functional beta diversity by lifestyle (sessile vs. planktonic), we focused on the 46 pathways previously found to be enriched in LEfSe analysis (26 pathways for the planktonic and 20 for the sessile community). Normalized gene abundances were summed per pathway and used to compute a Bray–Curtis dissimilarity matrix. The resulting PCoA axes explained 93.32% of the total variation. Compared to the overall gene abundance profile (Figure 4), this analysis (Figure 10) shows a clearer separation between lifestyles despite overlap between communities. Also, as described before, the time-0 planktonic samples (T0\_water\_summer and T0\_water\_winter) cluster far apart from the other samples, reflecting the differences in pathway gene abundances observed in Figure 10. PERMANOVA test confirmed a significant effect of lifestyle on the selected pathway composition ( $R = 0.27$ ,  $p = 0.0008$ ), supporting the findings from the LEfSe analysis (Annex 6.8).



**Figure 10:** Principal Coordinate Analysis (PCoA) of bacterial functional beta-diversity based on enriched pathways identified by LEfSe. Bray-Curtis dissimilarity was calculated from pathway abundance data, and samples are grouped by lifestyle (sessile vs. planktonic). Points are colored according to lifestyle, and ellipses represent the 95% confidence interval around the centroid of each group, illustrating the functional differentiation between lifestyles.

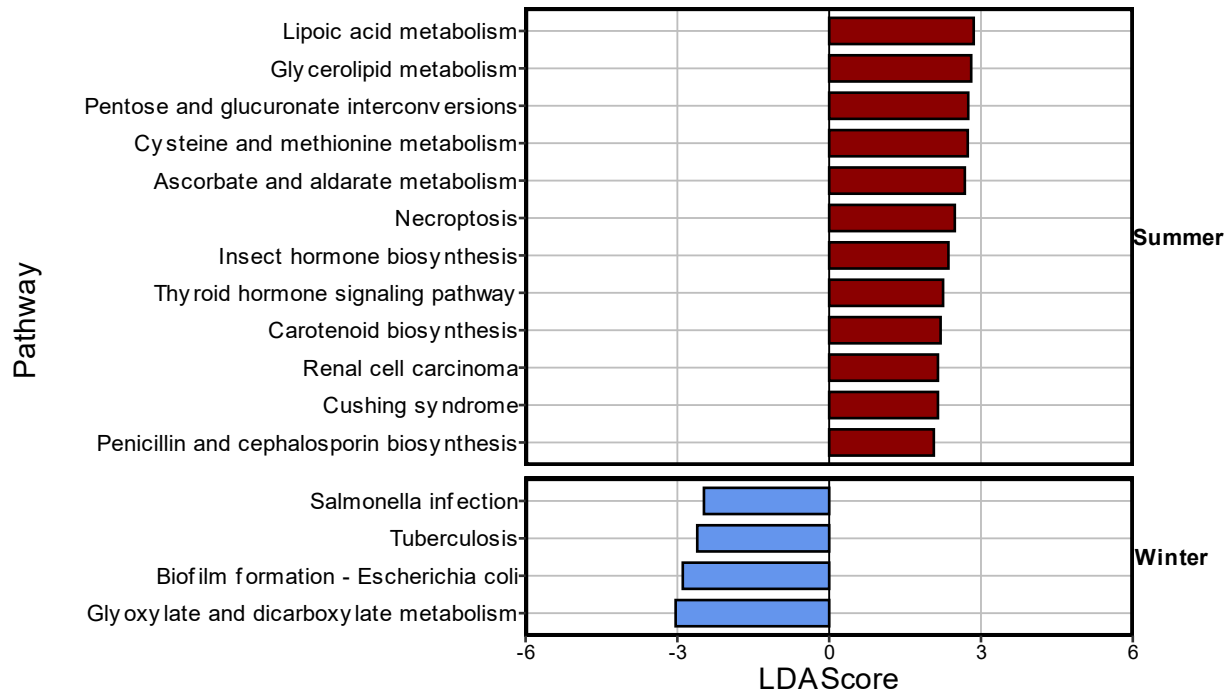
### 3.7 Representative functional pathways between winter and summer season groups

Although, seasonality did not have a significant effect on the overall gene composition (Figure 4B), LEfSe analysis was performed to investigate potential differences at the pathway level between summer and winter samples.

To identify functional differences between summer and winter communities, a LEfSe analysis was performed using the 340 metabolic pathways detected across all samples. The analysis revealed 16 pathways with significant differential abundance—12 enriched in summer communities and 4 in winter communities (Figure 11). Among these, pathways presented an LDA score that ranged from 2.06 to 2.85 in the case of summer group and for winter community a ranged from 2.47 to 3.03.

In the case of summer group, the pathways identified as enriched were involved on nutrient processing as lipoic acid metabolism, glycerolipid metabolism, pentose and glucuronate interconversions, ascorbate and aldarate metabolism, cysteine and methionine metabolism, carotenoid biosynthesis, and antibiotic biosynthesis (Penicillin and cephalosporin biosynthesis).

In the case of winter community, the pathways enriched were related to virulence-associated functions (Tuberculosis, Salmonella infection), biofilm formation, and carbon metabolism (glyoxylate and dicarboxylate metabolism).

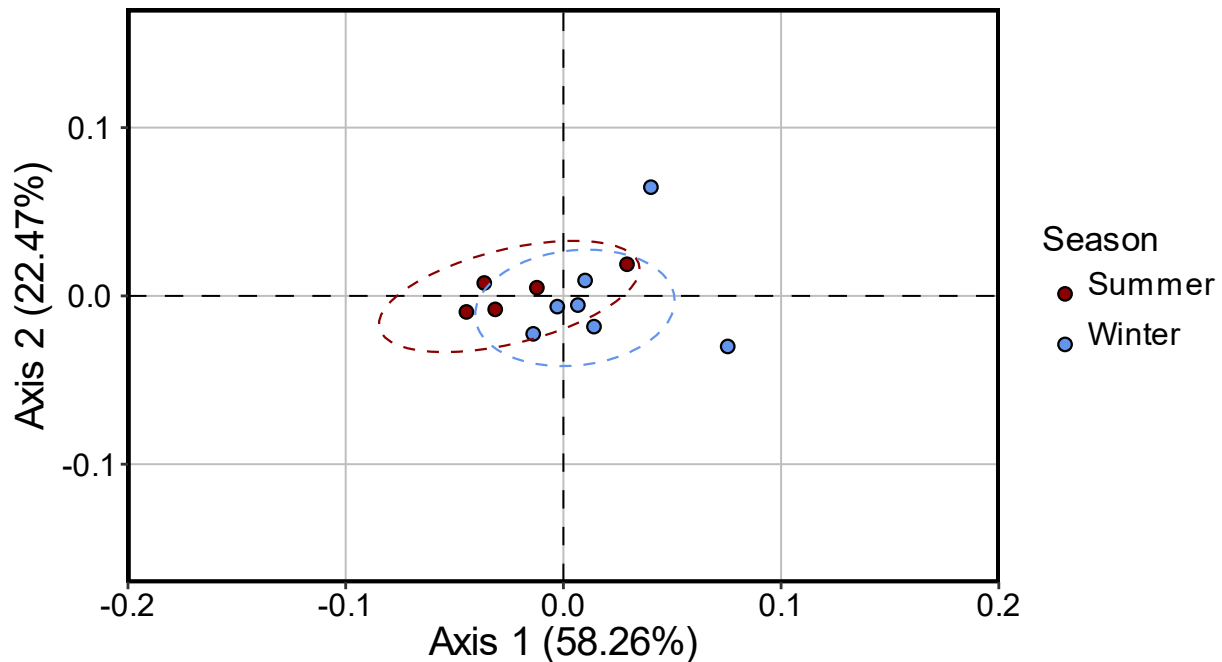


**Figure 11:** Differential Pathways Between Summer and Winter communities using LEfSe Analysis (LDA Score on X-axis, Pathways on Y-axis). Summer enriched pathways are represented in red, while winter enriched pathways are represented in blue.

### 3.8 Beta diversity of selected functional pathways (seasonality)

To better understand the functional differences highlighted by LEfSe, beta diversity was explored using the 16 pathways found to be significantly enriched in either summer or winter communities. Normalized gene abundances were summed per pathway and used to compute a Bray–Curtis dissimilarity matrix. A principal coordinate analysis (PCoA) was then performed to evaluate differences in pathway abundances by season. As seen in Figure 12, axes explained 80.7% of variation in the samples, a higher proportion than that observed in the overall gene-based analysis (Figure 4B). Additionally, in this pathway-focused analysis, samples within each seasonal group clustered more tightly compared to the global gene analysis, while the separation between summer and winter clusters was more pronounced. This suggests that seasonal differences become more evident when focusing on the specific pathways highlighted by the LEfSe analysis, despite no significant separation being observed at the broader gene composition level.

Furthermore, a PERMANOVA test confirmed a significant effect of seasonality on the abundance profiles of the selected pathways ( $R = 0.29$ ,  $p = 0.0185$ ) (Annex 6.8), supporting the differences identified by the LefSe analysis.



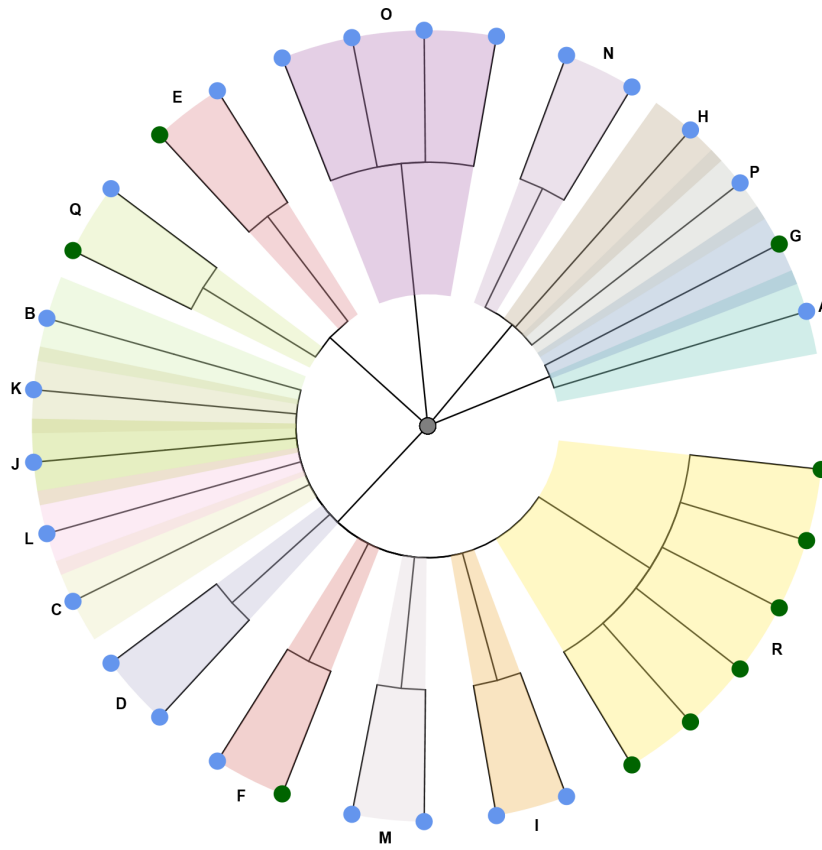
**Figure 12:** Principal Coordinate Analysis (PCoA) of bacterial functional beta-diversity based on enriched pathways identified by LefSe on summer and winter samples. Bray-Curtis dissimilarity was calculated from pathway abundance data, and samples are grouped by Season (summer vs. winter). Points are colored according to season, and ellipses represent the 95% confidence interval around the centroid of each group, illustrating the functional differentiation between lifestyles.

A dendrogram was created to highlight and see the relatedness between the pathways significantly enriched in sessile and planktonic microbial communities, as identified by LefSe. The dendrogram displays the full hierarchy, from broad functional categories at the root (level 1) to more specific categories (level 2), ending with individual pathways at the tips (level 3). Tip points represent the significantly enriched third-level pathways, while the internal nodes correspond to their broader second-level categories. In the dendrogram, the Lifestyle legend indicates the type of community (sessile or planktonic), while the background colors and letters represent the KEGG second-level categories from which the enriched third-level pathways originate (Figure 13).

In the dendogram, the pathways related to human or infectious diseases were excluded. As shown, most of the enriched pathways were associated with planktonic communities (23 pathways), whereas sessile communities showed enrichment in 10 pathways. In planktonic communities, the enriched pathways are primarily related to biosynthesis, core metabolism, and recycling functions. These included pathways involved in amino acid metabolism, carbohydrate metabolism, cell growth, energy metabolism, glycan biosynthesis, lipid metabolism, metabolism of cofactors and vitamins, metabolism of other amino acids, metabolism of terpenoids and polyketides, nucleotide metabolism, replication and repair, signal transduction, translation, and transcription.

In contrast, sessile communities exhibited enrichment in pathways associated with xenobiotic degradation, nitrogen metabolism, and membrane transport.

Despite the differences found, it is notable that most of enriched pathways from sessile and planktonic communities came from different roots highlighting the differences in the potential metabolism between both communities. Just in four cases, sessile and planktonic communities shared types of pathways, which were involved in cell growth and death (E), energy metabolism (F), and transport and catabolism (Q).



**Lifestyle**

- Planktonic
- Sessile

**Level 2 Pathway**

- Aging (A)
- Amino acid metabolism (B)
- Biosynthesis of other secondary metabolites (C)
- Carbohydrate metabolism (D)
- Cell growth and death (E)
- Energy metabolism (F)
- Environmental adaptation (G)
- Folding sorting and degradation (H)
- Glycan biosynthesis and metabolism (I)
- Metabolism of cofactors and vitamins (J)
- Metabolism of other amino acids (K)
- Metabolism of terpenoids and polyketides (L)
- Nucleotide metabolism (M)
- Replication and repair (N)
- Signal transduction (O)
- Translation (P)
- Transport and catabolism (Q)
- Xenobiotics biodegradation and metabolism (R)

**Figure 13:** Dendrogram showing enriched KEGG pathways in sessile and planktonic microbial communities identified by LEfSe analysis.

The dendrogram is structured based on the KEGG pathway hierarchy, displaying three levels: from broad functional categories (center) to specific pathways (tips). Each tip represents a third-level pathway significantly enriched in either sessile or planktonic communities (LDA score > 2.0,  $p < 0.05$ ). Background colors and letters correspond to the second-level KEGG categories from which each pathway originates. Internal nodes represent second-level categories. The Lifestyle legend indicates the type of community in which each pathway was enriched: sessile (green) or planktonic (blue).

## 4 DISCUSSION

The objective of this study was to make one of the first explorations of the potential functional traits of microbial communities in aquifer systems using a metagenomic approach, with a particular focus on lifestyle (sessile vs. planktonic) and seasonal variations. Despite the ecological importance of aquifers, relatively few studies have addressed the metabolic potential of their microbial communities, especially regarding biofilm-associated (sessile) populations and functional shifts across seasons. Our goal was to begin filling these knowledge gaps and to better understand how microbial functional profiles vary between different lifestyles and seasonal conditions. By providing these insights, this information can contribute to a broader understanding of the ecological roles and functional capabilities that aquifer microorganisms (depending on their lifestyle and seasonal variations) may play in subsurface and groundwater ecosystems.

### 4.1 Microbial functional diversity patterns

To assess functional beta-diversity, a Principal Coordinates Analysis (PCoA) based on Bray-Curtis dissimilarity of gene abundances was performed to observe the differences in overall-gene composition across samples according to season and lifestyle. The analysis suggested that lifestyle (sessile vs. planktonic) had a significant effect on overall gene composition, despite no clear group separation in the ordination space. Sessile samples showed greater dispersion in the PCoA graph compared to planktonic samples, suggesting higher variability in gene composition among sessile communities. In addition, one sessile sample (T1\_rocks\_summer) appeared more distant from the others. Because ordination distance reflects dissimilarity, this separation indicates that its gene composition differs from the rest of the sessile samples (Discuss later). In contrast, planktonic communities were more tightly grouped, indicating a more homogeneous gene profile. This homogeneity could be attributed to more uniform environmental conditions or nutrient availability in the water, which may lead to a more consistent functional gene composition across planktonic samples. For sessile communities in particular, the observed variability might also come from differences in rock chip properties or microenvironments, which could influence microbial colonization and the potential functions from sessile communities from one chip to another.

Gene composition differed significantly between the two groups, as shown by the PERMANOVA. This indicates that the functional potential of microbial communities varies between lifestyles. Such divergence is expected given the distinct environmental conditions and nutrient availability experienced by sessile

(biofilm) versus planktonic microorganisms. Nonetheless, some overlap was observed, suggesting that sessile communities still retain gene profiles shared with their planktonic counterparts. Interestingly, these differences in gene composition emerged within only three weeks of incubation. This relatively short timeframe suggests that longer incubations might have revealed an even clearer separation between lifestyles, highlighting the potential for rapid shifts in functional potential despite both communities originating from the same initial microbial source. These findings support the idea that sessile and planktonic communities may play distinct ecological roles within aquifer ecosystems (as discussed later).

In the case of seasonality (summer vs. winter), no clear separation between groups was observed in the PCoA ordination space. Unlike the differences observed between lifestyles, the PERMANOVA test for seasonal variation yielded a non-significant result, showing that seasonality had no observed influence on overall gene composition. Although PERMANOVA did not detect a significant effect of season on overall gene composition, the relatively small number of samples in each group and the degrees of freedom = 1 may have limited the statistical power to detect subtle differences. Additionally, since this analysis was based on the total gene composition (6,291 genes), it is possible that specific pathways or functional categories can be influenced by season and lifestyle as well, even if these effects are not apparent at the global scale (as discussed later). Seasonal trends were also observed on the PCoA graph. Indeed, winter samples tended to cluster more closely together than summer samples, suggesting greater similarity in gene composition during the winter. One possible explanation is that winter abiotic conditions (neutral pH, lower temperatures, lower oxygen and less water recharge) may have induced a dormant state in microbial communities, resulting in a reduced potential metabolism and, consequently, more uniform gene profiles (as discussed later)(Sullivan et al., 2020; Villeneuve et al., 2023; Zhou et al., 2012). In contrast, the summer environment conditions as higher temperature and dissolved oxygen may have supported more active and dynamic microbial processes, leading to greater heterogeneity in gene composition.

Overall, the results highlighted that lifestyle had a significant influence on the functional gene composition of microbial communities. In contrast, no significant impact of seasonality was detected, supporting the idea of functional stability across summer and winter communities. This may reflect a degree of functional redundancy, where different taxa could perform similar ecological roles regardless of seasonal conditions (Yachi & Loreau, 1999). Further investigation using 16S rRNA gene analysis could help clarify whether taxonomic shifts support this hypothesis<sup>1</sup>. However, larger differences between lifestyles or seasons might have been detected if metatranscriptomic data had been available, since this technique captures gene

expression rather than only functional potential. An example of this is provided by a multi-omics study in a subseafloor aquifer, where metatranscriptomics revealed differences in gene expression across depth horizons (Seyler et al., 2021). In particular, transcripts for carbon fixation were more abundant in the middle and deep aquifer samples compared to the shallow horizon. Moreover, in the deeper horizons, where oxygen concentrations decreased, transcripts associated with nitrate/nitrite reduction and sulfur metabolism were more highly expressed, highlighting the spatial heterogeneity of microbial activity within the same aquifer. A similar pattern of functional differentiation could occur between microbial lifestyles or between seasonal communities in this study.

<sup>1</sup>Amplicon sequencing using DNA for the same samples used for this metagenomic analysis were carried out during the spring of 2025, to assess taxonomic composition in the sample. However, as this data was obtained late into this master project, it is not presented in the thesis. It will, however be included in the paper to further investigate the relationship between taxonomic composition and functional potential.

#### 4.2 Effect of environmental conditions on gene community structure

The effect of abiotic factors measured during the incubations on microbial gene composition was assessed using db-RDA. All environmental parameters were measured from water samples. However, since the water was in constant contact with rock surfaces (harboring sessile communities), the db-RDA analysis was applied both to the full dataset (including sessile and planktonic samples).

The  $\text{NO}_2^-$ , DIC,  $\text{NH}_x$ , and DOC vectors pointed toward the T0\_water\_summer and T0\_water\_winter samples (both planktonic), which were positioned closer to the tips of these arrows compared to other sessile and planktonic communities. This may suggest that the functional profiles of these early planktonic samples were possibly associated with higher values of nitrite, inorganic carbon, ammonium, and organic carbon, or that they reflected distinct metabolic potentials related to these variables, compared to later planktonic timepoints.

In contrast, the  $\text{NO}_2^-$ , DIC,  $\text{NH}_x$ , and DOC vectors arrows did not clearly point toward any specific group of bioreactor samples. This may suggest: (i) that their gene composition was shaped by additional abiotic or spatial factors not captured in the current analysis; and (ii) that sessile communities are functionally more stable or buffered against environmental fluctuations. This apparent stability may be linked to the protective nature of biofilms and the physical structure of rock surfaces, which can create microhabitats that shield microbial communities from external environmental changes, as described in the literature (Choe & Lee, 2019). Finally, (iii) the consistently low concentrations of nitrate/nitrite and

ammonium/ammonia over time likely explain why the association was observed only for the early planktonic samples, when nitrogen compounds were comparatively higher than at later timepoints. This could explain the position of most of the samples away from the  $\text{NO}_2$  and  $\text{NH}_x$  vector in the db-RDA plot.

On the other hand, DOC and DIC were measured at relatively high concentrations compared to nitrogen compounds, which may explain the overall high abundance of carbon metabolism pathways across samples (detailed in the next section). Interestingly, the positioning of samples in the db-RDA plot, regardless of season (summer vs. winter) or lifestyle (sessile vs. planktonic), was distant from the DOC and DIC vectors. This pattern likely reflects the widespread presence of carbon cycling pathways across all groups, suggesting that while carbon metabolism is a dominant function, it does not strongly differentiate the microbial communities in this system. Another possible explanation is that bulk DOC measurements represent a highly heterogeneous mixture of organic molecules with diverse reactivities. Therefore, total DOC concentration alone may not sufficiently reflect the specific compounds influencing community gene composition, and a more detailed chemical characterization would be needed to resolve these associations (Muscarella et al., 2019).

Supporting this, a related study using bioreactors with rocks and groundwater collected from the same aquifer system showed that the mineral composition of the rock chips—particularly the presence of quartz and white feldspar—significantly influenced microbial community structure. Quartz, being the most abundant mineral in the chips, may act as a chemically inert yet physically stable surface, promoting the establishment and persistence of specific microbial communities (Patel et al., 2024). While quartz itself is low in nutrients, its structural stability may support the formation of biofilms and microhabitats that enhance microbial resilience under fluctuating environmental conditions. Considering this, it is possible that quartz—being the dominant mineral in the rock chips—also played a role in shaping the gene composition of sessile microbial communities in the present study.

The lower explanatory power of the db-RDA for sessile samples may also be explained since all environmental measurements were taken from the water samples. As a result, these variables are likely more representative of the conditions experienced by planktonic communities and may not fully capture the microenvironmental factors influencing sessile microbial communities. Considering additional environmental factors such as redox potential, sulfate,  $\text{CO}_2$ , ATP availability, and the presence of heavy metals could have improved the explanation of gene composition patterns in both planktonic and sessile

communities. These parameters are known to influence key microbial processes such as methanogenesis, sulfate reduction, microbial activity, and bioremediation (Röling et al., 2001; Zhu et al., 2022).

#### 4.3 Dominant functional pathways

The heatmap of the 25 most abundant pathways across lifestyles and seasons revealed no clear distinction between planktonic and sessile communities. However, some consistent patterns emerged. Notably, several pathways—two-component systems, quorum sensing, ABC transporters, ribosomal proteins, and oxidative phosphorylation—stood out with uniformly high abundances, suggesting that both microbial lifestyles rely heavily on genes involved in these functions. Among these, ABC transporters are a prominent family of membrane proteins that couple ATP hydrolysis to the import and export of solutes across bacterial membranes. They play critical roles in nutrient uptake (e.g., vitamins, ions, amino acids) and in exporting toxins, xenobiotics, and capsular polysaccharides (Akhtar & Turner, 2022; Davidson & Chen, 2004). Supporting this, a metagenomic study from a deep russian aquifer detected ABC transporter genes in Deltaproteobacteria, suggesting their involvement in amino acid, peptide, and carbohydrate transport (Kadnikov et al., 2020). Therefore, it is likely that the microbial communities in this study are actively using ABC transporters to acquire nutrients from the environment or exporting toxins and compounds that can be harmful to microorganisms. This is especially relevant for the bioreactor samples, where ABC transporter pathway abundance was notably higher, possibly reflecting a greater demand for nutrient uptake under the experimental conditions.

Quorum sensing is a bacterial communication system that plays a key role in regulating gene expression in response to cell density. It relies on the production and accumulation of autoinducers, which, once they reach a certain threshold, trigger processes such as virulence, sporulation, biofilm formation, and other community behaviors (Moreno-Gómez et al., 2023). Because autoinducers can readily diffuse away in open water, quorum sensing is more commonly associated with surface-attached or biofilm-forming communities, where signaling molecules are retained at higher local concentrations (Kirisits et al., 2007). This may explain the strong association of quorum sensing pathways with sessile communities in our study. Nonetheless, the detection of quorum sensing genes in planktonic samples suggests that some signaling may still occur in suspended aggregates or microenvironments where diffusion is limited. It is also important to note that our analysis reflects functional potential rather than gene expression; therefore, metatranscriptomic, or proteomic data would be optimal to explore whether quorum sensing activity is indeed more prevalent in biofilm-associated than in planktonic communities.

In Gram-positive bacteria, autoinducers are typically exported via ABC transporters, which may explain the high abundance of both quorum sensing and ABC transporter pathways observed in this study (Windsor, 2020). The high abundance of these pathways may be associated with different roles in the microbial community, such as biofilm formation (especially relevant to sessile communities), nutrient uptake, antibiotic production, or motility — the latter being particularly advantageous for planktonic microbes. Previous studies analyzing quorum sensing in biofilm metagenomes have reported elevated levels of AI-2-type quorum sensing proteins in groundwater environments (Barriuso & Martínez, 2018). The use of AI-2, which serves as a universal signaling molecule, likely facilitates inter-species communication among diverse microbial taxa in such oligotrophic ecosystems.

The two-component system (TCS) is a key regulatory mechanism that allows bacteria to sense and respond to external stimuli such as pH, nutrient availability, osmotic pressure, redox state, quorum-sensing signals, and antibiotics (Liu et al., 2019). This system operates through a phosphorylation cascade that ultimately triggers changes in gene expression. In the study, the TCS pathway was found in high abundance, like quorum sensing, suggesting a possible synergistic relationship between the two systems. Both pathways appear to be actively used by planktonic and sessile microbial communities to coordinate complex responses such as stress adaptation and biofilm formation (Bhagirath et al., 2019). Notably, these pathways were also detected at high abundances. These findings suggest that the microbial communities in this study have the capacity to detect environmental changes or stress factors and respond rapidly through regulatory and communication networks, an ability that is crucial in aquifer systems where nutrient fluctuations and stress conditions are common.

Ribosomal proteins were also found in high abundance. These proteins play a key role in stabilizing the ribosome and regulating ribosomal activity during protein biosynthesis (Nikolaeva et al., 2021). This likely indicates that the microbial communities are actively using ribosomal genes to support high levels of protein production. This finding aligns with the high abundance of pathways related to purine and pyrimidine metabolism and amino acid biosynthesis, which are essential for DNA replication and peptide/protein synthesis. Altogether, this suggests that both sessile and planktonic communities are likely metabolically active and possibly growing. Interestingly, previous metagenomic studies on aquifer systems have not reported high abundances of ribosomal protein genes in biofilm or planktonic communities, making this a potentially novel observation of microbial potential functions within aquifer environments.

Finally, oxidative phosphorylation was also found in high abundance. These energy metabolism-related pathways are essential for ATP production. The high abundances found in this study may suggest that microbial communities are actively using a variety of electron donors and nutrients to generate energy. As previously discussed, both sessile and planktonic communities appear to be metabolically active. The high abundance of oxidative phosphorylation may be functionally linked to other key pathways also abundant in the communities, including sulfur metabolism, carbon fixation, methane metabolism, glycolysis/gluconeogenesis, and pyruvate metabolism, suggesting the use of different electron acceptors and donors to produce energy.

In our metagenomes both sulfide-oxidation and sulfate-reduction pathways were well represented, indicating a highly dynamic sulfur cycle in the aquifer. Genes for sulfide oxidation such as *sqr* and *fccAB*, and the complete *sox* gene cluster (*soxABCDXYZ*) were detected in both planktonic and sessile communities; which encode the Sox multi-enzyme system and associated enzymes that oxidize sulfide, sulfur and thiosulfate to sulfate (Ghosh & Dam, 2009). Additionally, thiosulfate-oxidation markers (*TST*, *tsdA*, *doxD*) and sulfite-oxidation genes (*sorA/sorB*, *soeABC*, *SUOX*) were also found, supporting multiple oxidative routes. On the reductive side, canonical sulfate-reduction genes (*sat*, *aprAB*, *dsrAB*) were present together with genes for thiosulfate/tetrathionate reduction (*phsA/psrA*, *ttrB*) and elemental-sulfur reduction (*hydA/B/G*), indicating potential for anaerobic sulfur respiration (Vigneron et al., 2021). We also detected the *dsrMKJOP* accessory components (*dsrM*, *dsrK*, *dsrJ*, *dsrO*, *dsrP*), which form a membrane-associated electron transfer complex that supplies electrons to the *DsrAB* catalytic subunits during dissimilatory sulfate reduction (Ghosh & Dam, 2009; Girard et al., 2015).

This highlights the versatility of the microbial communities, which are capable of transforming sulfur compounds through both oxidation and reduction reactions, indicating their potential role in regulating sulfur levels in aquifer systems. The detection of numerous genes involved in diverse sulfur pathways suggests the presence of a substantial source of sulfur compounds in the aquifer, which could explain the high presence of these genes to meet energy demands.

Although numerous genes mapped to methane metabolism pathways were detected, many correspond to general metabolic functions that share intermediate metabolites with methane metabolism (e.g., glycolysis, C1 carbon transfer, formaldehyde transformation) that are not exclusive to methanogenesis or methane oxidation. However, key marker genes involved directly in methane cycling, such as *xoxF*, *mch*,

*mtdB*, and *fdh*, were also present in low abundances, indicating a potential active methane transformation in the microbial communities (Semrau et al., 2018). The presence of general metabolic genes highlights the integration of methane metabolism with central cellular pathways, which supports efficient energy conservation and biomass synthesis.

Rubisco (*rbcL/rbcS*), the enzyme that fixes CO<sub>2</sub> into organic carbon, was detected in nearly all samples (except for T1\_rock\_summer), indicating widespread potential for Calvin–Benson–Bassham (CBB) cycle activity (Michelet et al., 2013). Other CBB genes such as *prkB* (phosphoribulokinase) and sedoheptulose-1,7-bisphosphatase were also present, supporting the capacity to regenerate ribulose-1,5-bisphosphate and produce organic carbohydrate intermediates. Genes of the Wood–Ljungdahl pathway (e.g., *fhs*, *folD*, *metF*, *acs*) were identified as well, suggesting potential for reductive acetyl-CoA (autotrophic) metabolism (Köpke et al., 2013). Almost all markers of the reverse (reductive) TCA cycle, including *kor*, *frd*, *acn*, and *por*, were also detected (Arnold & Finley, 2022). Finally, acetyl-CoA carboxylase (*acc*), a CO<sub>2</sub>-fixing enzyme associated with the 3-hydroxypropionate bicycle, was found, although propionyl-CoA carboxylase (*pcc*) was not detected; thus the 3-HP bicycle appears incomplete in the samples (planktonic and sessile) (Hügler & Fuchs, 2005). Overall, results indicate that microbial communities (sessile and planktonic) have the potential of fixing CO<sub>2</sub> by different strategies. This is crucial for microbial communities by not depending just on organic carbon to cope with energy needs. Also, this is important by guaranteeing a balance ecosystem between inorganic and organic carbon on aquifer systems.

Similar metabolic capabilities have been observed in other metagenomic studies of planktonic and sessile microbial communities from aquifer systems, particularly involving the cycling of sulfur and carbon (Wegner et al., 2018; Wu et al., 2017).

Processes such as carbon fixation, glycolysis, and gluconeogenesis have also been consistently reported in sessile and planktonic groups from aquifer systems (Nuppenen-Puputti et al., 2022; Wegner et al., 2018).

The findings suggest that both microbial lifestyles and seasons possess the metabolic potential to participate in sulfur, methane, and carbon cycling processes that are crucial for maintaining biogeochemical homeostasis and contributing to bioremediation in subsurface ecosystems such as aquifers.

Furthermore, several other pathways were found in high abundance, including butanoate metabolism, benzoate degradation, propanoate metabolism, and glyoxylate metabolism. Butanoate metabolism, involves the use of butyrate, a fermentation product, as an energy source (Singh et al., 2022). Given that the groundwater in this study was collected from a shallow aquifer where oxygen levels are reduced but not fully anoxic, the presence of this pathway is noteworthy. It may suggest either the presence of fermentation processes or the utilization of fermentation-derived products by certain microbial populations. A study using microcosms from a contaminated aquifer (Struchtemeyer et al., 2011) suggested a potential syntrophic interaction between butyrate (butanoate) oxidation, sulfate reduction, and methanogenesis. These processes are likely mediated by sulfate-reducing bacteria and methanogens, which utilize the hydrogen and acetate produced during butyrate oxidation as electron donors or carbon sources. In the present study, such interactions may also be occurring, as indicated by the high relative abundances of pathways involved in sulfur and methane metabolism pathways, supporting the presence of similar anaerobic microbial consortia.

Benzoate degradation, on the other hand, is associated with the breakdown of aromatic compounds, many of which are considered to be xenobiotics or have antimicrobial properties. The detection of this pathway suggests the possible presence of benzoate or structurally related aromatic compounds in the aquifer and highlights the microbial community's capacity to tolerate and metabolize such compounds (Walczak-Nowicka & Herbet, 2022). This may reflect environmental exposure to organic pollutants, with microbial populations adapting to perform detoxification and energy production through this degradation pathway. Additionally, the high abundance of ABC transporter genes may support this interpretation, as these transporters are known to facilitate the export of toxic compounds like benzoate, helping microbes survive in chemically challenging environments.

Propanoate (propionate) metabolism was also found in high abundance and is particularly interesting due to its known association with sulfate-reducing bacteria and methanogenic archaea. The co-occurrence of carbon fixation and sulfur metabolism pathways further supports the potential presence of both microbial groups in the bioreactor and possibly in the aquifer system as well. According to previous studies, propionate can be oxidized to  $\text{CO}_2$  and  $\text{H}_2$  by sulfate-reducing bacteria under sulfate-rich conditions. In turn, methanogens can use these products to generate methane, suggesting a metabolic partnership between the two groups (Westerholm et al., 2022).  $\text{CO}_2$ , on the other hand, can be used via carbon fixation pathways to produce organic carbon and energy. While sulfate,  $\text{CO}_2$ , and  $\text{H}_2$  concentrations were not

measured in this study, the detection of these metabolic pathways suggests that such syntrophic interactions may be occurring or are at least metabolically possible in the system.

Another pathway found in high abundance was glyoxylate and dicarboxylate metabolism. This pathway enables bacteria to utilize glyoxylate when sugars are scarce, producing intermediates such as succinate and malate, which can feed into gluconeogenesis, the TCA cycle, or other biosynthetic processes (Dolan & Welch, 2018). Additionally, the ability to metabolize dicarboxylic acids (e.g., malate, fumarate, succinate) provides an important source of energy and carbon skeletons for biosynthesis. The high abundances of these pathways suggests that both sessile and planktonic microbial communities are not solely relying on fermentation products, glucose, sulfur compounds or inorganic carbon as energy sources. Instead, they also appear to employ alternative metabolic strategies to maintain carbon balance and generate precursors for essential cellular functions. This includes the potential to store carbon and redirect it toward the biosynthesis of amino acids, purines, and peptides, as supported by the high abundances observed in these pathways.

Although nitrogen pathways were not among the 25 most abundant pathways, they were detected across almost all lifestyles and seasonal samples (except for T1\_rock\_summer). This is somewhat surprising given the low concentrations of nitrogen compounds measured both at sampling and during incubations. Genes involved in nitrogen processes as nitrogen fixation (*nifDFK*), dissimilatory nitrate/nitrite reduction (*narGHL*, *napAB*, *nirBD*, *nrfA*) were detected. As well, genes implicated in assimilatory nitrate reduction (*nasABCDE*). Regarding nitrification, only two genes (*hao* and *hcp*) were detected, suggesting that this pathway might be incomplete or not the primary nitrogen transformation route used by these microbial communities. Additionally, denitrification genes (*nirA*, *nirK*, *norB*, *norC*, *nosZ*) were present. It is notable how the microbial communities appear to harbor a nearly complete nitrogen cycle, utilizing different chemical forms of nitrogen, from  $N_2$  to  $NO_3^-$ . This reflects the diversity and functional potential of microbes in aquifers, including nitrogen fixers, nitrifiers, and denitrifiers working together to maintain a nitrogen balance in groundwater and subsurface ecosystems. Given the connectivity between aquifers and surface waters, maintaining such a nitrogen-balanced environment is crucial for primary producers like plants, algae, and fungi, which rely on specific bioavailable nitrogen forms for their metabolism (Könneke et al., 2014; Mosley et al., 2022).

The biofilm formation pathway was also found in high abundance across both sessile and planktonic samples. This result is not unexpected, as biofilm-related genes are essential for sessile microorganisms to initiate surface attachment and biofilm development when the organisms are in a planktonic state, as reported in other studies on aquifer microbial communities (Nuppunen-Puputti et al., 2022). However, the observation that this pathway showed similar abundance levels in both planktonic and sessile communities suggests that, as previously indicated by beta diversity patterns, microbial functional differentiation may not have fully occurred at the time of sampling. This implies that sessile microorganisms may still share a functional gene profile like their planktonic counterparts. This trend is also reflected in the other 24 most abundant pathways, reinforcing the idea that microbial communities may still be in a functional differentiation phase, with limited clear functional divergence between lifestyles. It is also noteworthy that planktonic samples harbor these genes in high abundance. However, as these results are based on metagenomic data, they may reflect only the potential for these functions, rather than their actual expression.

One sample, T1\_rocks\_summer (collected 8 days after incubation during the summer season), exhibited a distinct gene abundance profile on different pathways compared to the other sessile samples. This deviation is consistent with the beta-diversity analysis, where the sample appeared as an outlier, positioned further away from the cluster of other sessile communities in the PCoA plot. A possible explanation for this discrepancy is the considerably lower number of reads obtained after the assembly process for this sample (729,672 vs. an average of ~46.8 million for the other sessile samples). Since gene abundances were normalized based on the total number of reads per sample, such a low sequencing depth likely introduced some bias. Specifically, with fewer reads, relative abundances become unstable—some pathways may appear artificially inflated, while others may be underrepresented or entirely absent. As a result, this sample could disproportionately influence multivariate analyses and heatmaps, even if the observed variation does not reflect a true biological signal. That is why this sample was excluded from lefse analysis for further analysis.

#### 4.4 Discriminant pathways between sessile and planktonic communities, and during the winter and summer seasons

LEfSe analysis was performed to identify the most representative pathways across lifestyles and seasons. For lifestyle, the planktonic group showed pathways primarily associated with fundamental cellular processes. One example is base excision repair, a mechanism that bacteria use to detect and correct various

types of DNA damage, such as replication errors or damage caused by reactive nitrogen and oxygen species (Wozniak & Simmons, 2022). Since DNA damage can compromise essential processes like replication, transcription, and translation, maintaining efficient repair systems is crucial for microbial survival and proper cellular function. For planktonic cells, a higher abundance of DNA repair genes may be necessary, as these free-living microbes are more directly exposed to environmental DNA stressors such as ultraviolet radiation and oxidative compounds. In contrast, sessile communities covered within a biofilm benefit from the protection of the extracellular polymeric substance (EPS) matrix, which can buffer cells against these damaging agents and thereby reduce the need for such extensive DNA repair mechanisms. (Flemming & Wingender, 2010).

Another pathway enriched in the planktonic group was cell cycle – *Caulobacter*, which represents a dimorphic cycle. It begins with a motile swarmer cell equipped with a flagellum and pili, allowing environmental exploration but not DNA replication. Under favorable conditions, the swarmer differentiates into a stalked cell (sessile cell), which attaches to surfaces and initiates DNA replication. Division of the stalked cell produces two distinct daughters: a new stalked cell that continues replication and a swarmer cell that remains in G1 until triggered to differentiate again, thereby completing the cycle (Fatima et al., 2022). This type of cell cycle requires extensive regulation, which may explain why planktonic cells are enriched in DNA damage control pathways such as base excision repair. What makes this cycle particularly interesting in our study is that planktonic communities could have the genetic capacity to transition into sessile or biofilm-forming communities with relative ease.

Although the sessile community in this system originated from the planktonic community, the fact that planktonic microbes already possessed the genes and molecular tools to switch to a sessile lifestyle could explain why the functional profiles of other pathways are so similar between the two lifestyles. This rapid transition, potentially taking place in as little as seven days, may explain the similar pathway abundance profiles between planktonic and sessile groups. Such a shift might involve only limited genetic or functional adjustments, suggesting that microorganisms can readily alternate between lifestyles without extensive metabolic changes. At the same time, it is well known that sessile cells within biofilms can detach through both active and passive mechanisms. Passive detachment can result from external forces or abiotic stressors, whereas active dispersal is a regulated response triggered under specific stress conditions, such as changes in water flow, temperature fluctuations, or nutrient limitation (Flemming & Wingender, 2010). During active dispersal, bacteria can upregulate genes associated with motility, including flagellar

machinery, enabling them to transition back to a planktonic, free-living lifestyle. This dynamic process facilitates lifestyle transitions between planktonic and sessile communities, enabling planktonic cells to colonize new surfaces.

When comparing the general cell cycle pathway to the cell cycle – *Caulobacter* pathway, the latter was found in higher relative abundance in both sessile and planktonic communities (data not shown). This suggests a possible preference for this type of dimorphic metabolism and the associated flexibility it offers to microbial communities to transfer from planktonic to sessile lifestyle. It is also possible that, over time, weeks or even months later, the sessile community could shift its pathway abundances compared to the planktonic community by becoming more specialized in the environment. This remains a hypothesis, and longer-term studies would be needed to explore and confirm it.

It is important to note that this study was conducted in a bioreactor designed to replicate an aquifer system, and differences from natural conditions could have influenced the metabolism of both lifestyles and seasonal communities. Furthermore, it remains uncertain whether true sessile communities, those naturally attached to sediments and rocks in the aquifer, would display functional profiles as like planktonic communities as observed here. As stated in the introduction, sampling core sediments from underground aquifers is costly and logistically challenging. Nevertheless, previous studies have managed to do so, reporting small differences, such as higher sulfur metabolism in sessile (sediment) communities compared to groundwater (Anantharaman et al., 2016). In contrast, our results showed the opposite trend, with sulfur metabolism slightly higher in planktonic communities (1.33%) than in sessile ones (1.15%). However, this small difference was not detected by the LEfSe analysis, suggesting only minimal variation in the abundance of this pathway.

Furthermore, carbon fixation via the Calvin cycle was enriched in planktonic communities compared to sessile ones, suggesting a possible preference for CO<sub>2</sub> fixation among planktonic microbes. This pattern could be explained by a possible higher exposure of planktonic communities to dissolved carbon sources in the water, unlike sessile communities that are more rock substrate-bound. Consistently, pentose phosphate and fructose and mannose metabolism pathways were also enriched in planktonic communities, reinforcing their reliance on both organic and inorganic carbon for energy.

Planktonic communities were enriched in pathways related to purine and pyrimidine metabolism, DNA replication, and tRNA biosynthesis. This pattern may suggest that planktonic cells could grow more rapidly and invest more in developing genes machinery compared to sessile communities. Although literature on aquifer systems generally reports that sessile or biofilm communities contain a higher proportion of total biomass in aquifers than planktonic cells, their growth rates may differ (Hug et al., 2015b; Ruiz-González et al., 2021b). As described above, a plausible scenario is that a higher concentration of dissolved nutrients in the water column provides planktonic microbes with easier access to these resources, allowing them to metabolize them efficiently and achieve faster growth rates. In addition, planktonic cells may grow faster because they do not need to invest as much energy into producing extracellular polymeric substances (EPS), biofilm proteins and polysaccharides required for biofilm formation and maintenance (Grinberg et al., 2019). In the study, relative average abundances of biofilm pathways were higher on sessile communities (1.03 % vs 0.64%), suggesting change of genes profiles from planktonic compared to sessile group. However, LEfSe did not detect this change as significant pathway feature on sessile communities, which can support the previous idea stayed where no strong functional changes are needed to switch between lifestyles.

It is reasonable to hypothesize that the metabolic profiles of planktonic communities may confer an advantage for rapid growth and population development. In the other hand, sessile communities, being more physically protected and generally more resistant to environmental stress, may be able to accumulate greater biomass over extended periods, as reported in previous studies (Lisle, 2020; Smith et al., 2018). In the present study, neither total biomass nor active biomass was measured, but incorporating such measurements in future experiments could provide important insights into the relationship between growth rate and biomass accumulation in aquifer microbial communities.

Sessile communities, on the other hand, were enriched with pathways involved in xenobiotic degradation such as benzoate, toluene, xylene, chlorocyclohexane, and chlorobenzene. These xenobiotics, which include hydrocarbons, aromatic hydrocarbons, and other aromatic compounds, can strongly impact soil, surface water, and groundwater. Sessile communities showed a higher abundance of these pathways compared to planktonic communities. This is relevant in biotechnology since microbial communities have the potential to metabolize these compounds. In more detail, genes involved in aerobic benzoate/benzene degradation were detected, including *catB*, *dmpK*, *fadA*, *pcaD*, *dmpC/D/B/H*, and *mphE/F*, while anaerobic degradation involved *ubiX*, *badA*, *bcr*, *fadA/J*, *paaF/H*, and *acat* (R. Zhang et al., 2024a). Genes related to

xylene degradation, such as *xyI/X/Y/L/G/F/C/K/Q/J*, were also identified, representing part of the two operons typically responsible for xylene breakdown (Svenningsen et al., 2016). However, this pathway appeared incomplete, as some genes were missing. In the case of toluene, *tmo*, *dmp*, and *tom* genes were found (Parales et al., 2008), while for chlorobenzene degradation, *catA/B/E* and *dmpL* were detected (Li et al., 2025; X. P. Zhang et al., 2022). Previous studies have reported complete aerobic and anaerobic benzene degradation pathways in groundwater communities (R. Zhang et al., 2024b). Other work has similarly shown that both sessile and planktonic communities can partially metabolize phenol, xylene, toluene, and naphthalene when tested in bioreactors designed to study xenobiotic degradation in contaminated aquifer systems (Yavari-Bafghi et al., 2023). In addition, metatranscriptomic and metagenomic studies have confirmed the presence and expression of toluene degradation genes in groundwater from industrially contaminated aquifers (Taş et al., 2018). Together, these findings suggest that it is possible that a source of aromatic hydrocarbons and related pollutants are likely connected to the aquifer and groundwater. The results also highlight the capacity of sessile microbial communities to differ from planktonic ones metabolically, particularly in their potential to metabolize these contaminants. Furthermore, biofilm properties could allow it to accumulate solids and solutes, including xenobiotics, which enhances their availability to sessile communities and may explain the higher abundance of these pathways (Flemming & Wingender, 2010). For future studies in this aquifer area, or in related research focusing on functional aspects of microbial communities, it would be important to measure the concentrations of benzene, aromatic compounds, or other hydrocarbons in the groundwater, as this would allow for a better interpretation of the metabolic capacities of the microbial communities.

Sessile communities were also enriched in steroid degradation pathways. Steroid contamination in aquatic environments can come from multiple sources, including agriculture, industry, and pharmaceutical residues, but the primary source is human excreta (Chiang et al., 2019). The presence of genes involved in steroid degradation is important because it may suggest the removal of these compounds from aquatic systems, preventing disruption of endocrine systems in fish, other eukaryotes, and even microbial communities themselves. Additionally, some steroids can trigger microbial growth, toxin production, or virulence, influencing infection dynamics; therefore, their removal can be considered a natural bioremediation mechanism. Steroid degradation is also biotechnologically relevant, as microbes can modify steroids to produce medically important drugs (vom Steeg & Klein, 2017).

The biofilm lifestyle may promote the horizontal gene transfer, which was not detected as enriched pathway on sessile communities but may facilitate the transfer of genetic information as steroid degradation genes, nitrogen metabolism genes (later explained) and xenobiotic degradation, facilitating metabolic capacities that are not enriched in planktonic communities. Some genes as *virB* operon involved conjugation and *pilA* and *recA* involved in transformation were detected on the study across sessile samples, suggesting possible gene transference (B. J. Arnold et al., 2021; Mullany et al., 2018). In this way, it is possible that some microorganisms can metabolize steroids, potentially generating compounds into CO<sub>2</sub> fixation or other energy yielding processes, highlighting both the ecological and functional significance of steroid degradation in biofilm communities.

Sessile communities were also enriched in genes involved in nitrogen metabolism, an interesting result given that the rocks used in the bioreactor incubations did not contain nitrogen, as confirmed in a previous study using the same rock type (Patel et al., 2024). This suggests that the enrichment is not due to nutrients directly supplied by the rock matrix. A more plausible explanation is that biofilm formation on rock surfaces creates microenvironments that promote nitrogen cycling processes such as nitrification and denitrification. In addition, mineral surfaces may adsorb nitrogen compounds (e.g., ammonium, nitrate) from the groundwater during incubations, making these nutrients more readily available to attached communities. Similarly, biofilms themselves can trap dissolved or particulate nitrogen compounds from the water, increasing local nutrient concentrations. Finally, the structured nature of biofilms allows for metabolic complementarity, where different microbial groups carry out distinct steps of nitrogen transformations, enhancing the overall capacity for nitrogen metabolism. This functional division of labor is less feasible in planktonic communities, which are typically composed of free-living, individual cells.

Furthermore, sessile communities showed enrichment in pathways classified by KEGG under “human diseases.” Although these categories are typically associated with pathogenesis, many of the genes mapped to them are also involved in fundamental cellular processes such as stress response, DNA repair, signaling, and protein quality control. KEGG annotations can therefore reflect a bias, since homologous genes in bacteria may be categorized as part of disease pathways simply because they are implicated in infection processes in humans, even though their roles in microbes are primarily metabolic or protective. For example, genes assigned to cancer pathways often participate in cell cycle regulation and apoptosis, while those mapped to prion disease are commonly related to chaperones and protein folding. Similarly, pathways labeled as non-alcoholic fatty liver disease frequently include genes for oxidative

phosphorylation and energy metabolism. Also, it is possible that the higher number of pathways enriched on these functions in sessile communities may reflect the need to cope with stronger environmental stresses, including nutrient limitation, oxidative stress, and competition among taxa. Thus, the apparent enrichment of “disease-related” pathways likely highlights the importance of stress response and metabolic possibilities in sessile communities, rather than direct links to human diseases. However, it cannot be completely excluded that some of the genes mapped to disease-related pathways may also reflect functions relevant to pathogenic microorganisms, suggesting a potential (though limited) risk for human health. Nevertheless, among the 340 pathways identified across sessile and planktonic communities, the disease-related pathways detected by LEfSe were not within the top 80 most abundant (data not shown). This indicates that, although certain disease associated pathways appeared enriched, their overall gene copy numbers were very low. Therefore, their ecological significance in these aquifer communities is likely related to core metabolic or stress response functions, rather than representing a major risk of human infection.

$\beta$ -diversity was assessed using only the gene abundances of pathways enriched in sessile and planktonic communities identified by LEfSe. The stronger cluster differentiation observed likely reflects the focus on specialized functions that best capture metabolic differences between lifestyles. Nonetheless, some overlap remained between the two groups. This overlap may suggest that the functional differentiation between sessile and planktonic communities is relatively modest, which is consistent with the small differences in gene abundances observed for these pathways in the heatmap.

To summarize the LEfSe results by lifestyle, sessile communities were enriched in pathways associated with environmental resistance, stress response, and nitrogen metabolism, whereas planktonic communities were more strongly linked to core cellular processes, rapid proliferation, and carbon cycling. The dendrogram from the LEfSe analysis illustrates the relationships among pathways enriched in each lifestyle. In sessile communities, xenobiotic degradation and environmental adaptation pathways formed distinct roots that were not shared with planktonic communities, underscoring their specific association with the sessile lifestyle and potential role in coping with environmental stress. By contrast, planktonic communities displayed separate roots enriched in core metabolic processes such as translation, DNA replication, amino acid metabolism, and signal transduction—functions typically linked to rapid growth and high cellular activity. Some functional categories, including energy metabolism, cell growth, and catabolism, were

positioned between both groups, suggesting that these pathways are broadly utilized to support essential metabolic requirements across lifestyles.

For seasonality, LEfSe analysis was performed even though no significant differences were detected in the overall gene composition between summer and winter communities. The objective was to identify potential functional differences that might exist between microorganisms from the two seasons. In summer microbial communities, the analysis highlighted lipoic acid metabolism as the most enriched pathway. Lipoic acid plays a critical role as a cofactor for key enzyme complexes involved in the Krebs cycle, energy metabolism, and sulfur metabolism. Its involvement in these processes supports essential cellular functions such as energy production, nutrient cycling, and enzyme regulation, which are fundamental for cell growth and metabolic adaptability (Cao et al., 2018; Cronan, 2024). Glycerolipid metabolism was also enriched as well, a pathway essential for the recycling of glycerolipids. These compounds play key roles in maintaining membrane integrity, contributing to bacterial cell structure, and serving as energy storage molecules (Han et al., 2024). The pentose and glucuronate interconversion pathway, although is not widely studied in bacteria, especially in environmental communities, likely plays an important role in carbon utilization. Based on available literature, this pathway enables bacteria to convert pentose sugars and oxidized sugars such as glucuronate into intermediates that feed into central metabolic routes, including the Krebs cycle, glycolysis, gluconeogenesis, lipogenesis, and the pentose phosphate pathway. (Lin et al., 2011; Wushensky et al., 2018). Ascorbate and aldarate metabolism were also enriched in the summer microbial communities. This pathway enables microorganisms to use ascorbate (vitamin C) and aldarate sugars as sources of carbon and energy (Levy et al., 2025; Stack et al., 2020). To date, no studies have reported the presence or role of this pathway in groundwater or aquifer systems. Interestingly, carotenoid biosynthesis pathways were also detected. In bacteria, carotenoids can contribute to photosynthesis by absorbing light, thereby enhancing energy harvesting. They also play protective roles, including preventing oxidative damage, providing temperature resistance, and stabilizing membrane proteins (Hashimoto et al., 2016; Saubenova et al., 2024). Although aquifer systems are typically dark, this finding may suggest that certain bacteria such as cyanobacteria, purple non-sulfur, and green sulfur bacteria might still use carotenoids to help to generate energy, potentially coupling with sulfur metabolism, which was also detected in this study. Similar to ascorbate and aldarate metabolism, no previous studies have reported the presence of carotenoid biosynthesis in aquifer microbial communities. The enrichment of antibiotic biosynthesis pathways in summer might reflect competition within both planktonic and sessile microbial communities. In nutrient-limited systems like aquifers, producing antibiotics could help certain microbes

eliminate their competitors. Supporting this idea, other studies have found antibiotic resistance genes in groundwater, suggesting that microbial communities may use antibiotic production as a way to cope with stress and compete for limited resources (Zainab et al., 2020). Human-related disease pathways were also enriched in the summer group. However, as suggested by the lifestyle LEfSe results, this may reflect functional homology and shared metabolic functions rather than a direct association with humans.

The summer group showed a higher number of enriched pathways according to LEfSe analysis compared to winter season. This pattern could be influenced by several factors. Abiotic conditions, such as higher temperature, increased recharge input from rainfall, elevated oxygen levels, and slightly acidic pH, could explain these events. Increased water recharge may dilute nutrients, leading to nutrient limitation. Microorganisms can respond by increasing copies of certain metabolic genes to cope with this deficit, resulting in the functional heterogeneity observed in the LEfSe results for summer season, including pathways related to nutrient cycling, stress response, competition, and energy production. This effect has also been observed in river and lake samples, where summer communities showed higher abundances of genes related to energy metabolism (Wilhelm et al., 2014). Although lakes and aquifers are different ecosystems, similar seasonal patterns may occur in groundwater. During winter, surface waters and aquifers experience lower recharge, leading to more stable nutrient conditions and less nutrient movement, whereas higher recharge in summer may dilute nutrients and may stimulate microbial communities to increase metabolic heterogeneity and activity.

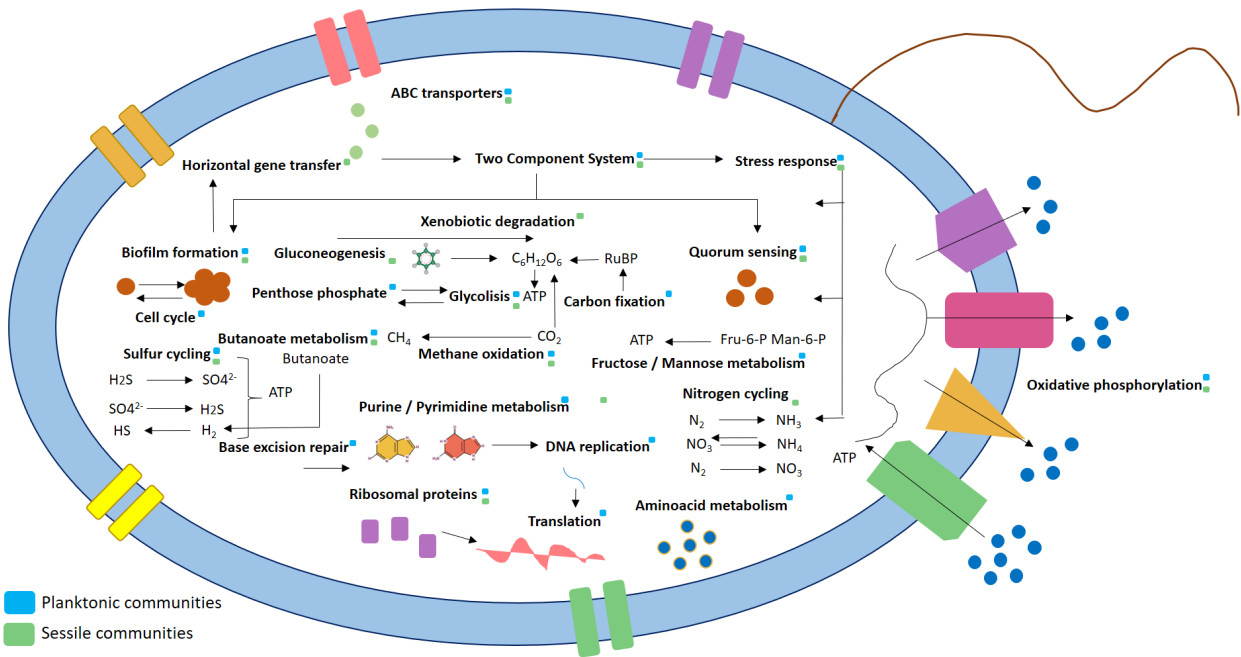
In contrast, winter conditions may induce a more dormant state in microbial communities, leading to similar gene profiles and less specialization in specific pathways. This is supported by the LEfSe results, which show fewer enriched pathways during this season. Enrichment of biofilm formation pathways could indicate a stress response and resource retention, but it may also reflect a dormant or low-activity lifestyle, as lower temperatures, reduced oxygen, and limited nutrient movement can trigger microbes to slow growth and persist over time using biofilm strategies. Although microbial activity may be reduced in winter, stress is still present, and the enrichment of pathways such as glyoxylate and dicarboxylate metabolism suggests strategies to cope with carbon limitations and other nutrient environmental stresses.

Finally,  $\beta$ -diversity was assessed using only the pathways identified by the LEfSe analysis for summer and winter samples. Although some overlap between groups remained, it was reduced compared to the overall gene abundance analysis. This suggests that the pathways highlighted by LEfSe contribute to, but do not

fully explain, the functional differences between seasons. As noted in the lifestyle analysis, the short sampling period and low number of samples may not have been sufficient to capture substantial functional shifts, which could explain the absence of pronounced metabolic differences between summer and winter.

#### 4.5 Discussion remarks

The results of this project indicate that microbial communities in the aquifer system can employ diverse metabolic strategies to assimilate carbon, nitrogen, sulfur, and other compounds. The coexistence of CO<sub>2</sub> fixation pathways alongside organic metabolisms such as glycolysis, gluconeogenesis, and the degradation of propanoate, benzoate, and butanoate and others, suggests that these communities integrate both inorganic and organic carbon sources to sustain their metabolism. This metabolic flexibility likely reflects the coexistence of possible autotrophic, heterotrophic, and mixotrophic microorganisms across both sessile and planktonic lifestyles and between seasons (summer and winter). Notably, functional differences were more pronounced when comparing lifestyles than when comparing seasons. Sessile communities, in particular, exhibited potential functional traits associated with nitrogen metabolism, environmental adaptation, and the metabolism of contaminants (Figure 14). In contrast, planktonic communities were more strongly associated with core cellular processes such as replication, translation, carbon metabolism, and growth (Figure 14). While abiotic factors alone may not fully explain the observed variation in community functions, seasonal conditions appear to shape some gene compositions. Summer conditions may promote an increase in gene abundances related to stress response, competition, and energy production, whereas winter conditions may favor a more dormant state, reflected in fewer differences in gene and pathway abundances.



**Figure 14:** Functional overview of community-level metabolic pathways based on the most abundant pathways and those identified by LEfSe analysis.

Blue and green markers beside each pathway indicate whether it is associated with planktonic or sessile communities, respectively. Arrows illustrate either the metabolic products inferred from the genes detected in each pathway or the connections between related metabolic processes. Note that only the most relevant pathways are shown; not all abundant pathways are represented in this figure.

The functional profiles across samples highlighted the potential roles of microbial aquifer communities in subsurface ecosystems and are consistent with previous findings. This study shows that these communities could contribute to the cycling of both organic and inorganic matter, playing key roles in carbon, nitrogen, and sulfur cycles. Such functions are not only crucial for maintaining subsurface nutrient balance but also could influence surface waters through aquifer–surface connections. Moreover, the microbial communities appeared to play potential key roles in bioremediation and in the metabolism of contaminants, xenobiotics and steroids, processes that are critical for improving water quality.

Aquifers, as described at the beginning, are vital sources of freshwater. Understanding their chemical, biological, and microbiological characteristics is crucial, especially considering future periods when surface water availability may become limited.

## 5 CONCLUSIONS

This study provided new insights into the functional diversity of microbial communities inhabiting aquifer systems.

$\beta$ -diversity analyses revealed that lifestyle (sessile vs. planktonic) shaped overall gene composition, while seasonal effects were not clearly observed. However, db-RDA indicated that environmental factors such as inorganic and organic carbon, nitrite, and ammonium influenced functional diversity, particularly in early planktonic samples.

Functional profiling showed that microbial communities harbored a wide range of genes involved in carbon metabolism (both inorganic and organic pathways), along with genes related to sulfur metabolism, stress response, and signal transduction (quorum sensing, biofilm formation, two-component systems, and ABC transporters). These pathways were consistently detected across all samples.

LEfSe analysis highlighted contrasting functional roles between lifestyles. Sessile communities were enriched in pathways associated with xenobiotic degradation and nitrogen metabolism, likely reflecting the nutrient adsorption properties of biofilms. In contrast, planktonic communities were enriched in pathways related to core metabolism and carbon cycling, consistent with their access to dissolved nutrients in the water column.

Although seasonal differences were not evident in overall gene composition, LEfSe identified that summer communities were more enriched in pathways related to energy production, stress management, cellular integrity, and inter-taxa competition. Winter communities, by contrast, showed fewer enriched pathways, suggesting either greater stability or more uniform gene abundances under colder conditions.

Overall, these findings demonstrate that microbial communities from aquifer systems, when studied under bioreactor conditions, possess a strong potential for driving the biogeochemical cycling of carbon, nitrogen, and sulfur. At the same time, they play a critical role in the detoxification of contaminants and xenobiotics. This functional versatility shows their potential in maintaining groundwater quality and highlights the need for continued investigation of aquifer systems. Future studies incorporating higher sequencing depth and

complementary approaches (e.g., transcriptomics or proteomics) will provide deeper insights into the active processes driving microbial roles in these environments.

## 5.1 Limitations

There are some limitations in this project that are important to mention. First, time was a significant factor in the study. As described earlier, longer bioreactor incubations could have yielded different results or revealed clearer differences in gene profiles between communities and across seasons. In addition, it is important to note that this project was designed to replicate aquifer conditions, and therefore the microbial metabolism and functional traits observed, particularly in sessile communities may differ from those naturally occurring in the aquifer environment. It is important to note that the results obtained in this study cannot fully replicate natural aquifer conditions. First, no artificial system such as a bioreactor can reproduce a natural environment with complete accuracy, even when abiotic and biotic parameters are adjusted to match field conditions. Second, although the bioreactor approximated several key physical and chemical variables, it still cannot fully mimic the complexity of in situ aquifer processes. As a result, the microbial responses and the potential functions observed during the incubations may differ from those occurring in the aquifer itself.

Initially, it was planned to use replicates from the two additional bioreactors, which could have helped capture larger differences between conditions. Unfortunately, due to financial and logistical limits, only samples from one bioreactor were analyzed. It was also planned to perform metatranscriptomic and proteomic analyses on both rock and water samples. However, proteomics was not pursued because of the difficulty in recovering proteins from rock surfaces after extraction, and metatranscriptomics was not possible due to low RNA concentrations obtained after extraction, purification, and tRNA depletion. These two complementary omics approaches could have provided higher-resolution insights, particularly regarding gene expression.

Another limitation of this study was the relatively low number of samples analyzed, which may have influenced the results. A larger number of replicates could have provided different outcomes and increased the robustness of the statistical analyses. In addition, the sample types were not fully independent. The same samples were analyzed both by lifestyle (sessile vs. planktonic) and by season (summer vs. winter), meaning that community and seasonal categories were derived from the same dataset rather than from

separate replicates. This overlap may have limited the ability to detect stronger differences between groups.

## 5.2 Perspectives

There are some perspectives planned for the future of this project. First, 16S rRNA amplicon sequencing data from the same samples studied in this project, will be analyzed to gain insights into the taxonomic composition of lifestyle and seasonal communities. This will help identify the most representative members within each group. In addition, metagenome-assembled genomes (MAGs) will be analyzed to establish links between functional potential and specific taxa.

Future research on microbial aquifer communities is needed to better understand their biochemical processes and ecological roles in groundwater and subsurface environments. Additional chemical measurements, including carbon dioxide, major ions, hydrocarbons, methane, and sulfur species (sulfate, sulfide, hydrogen sulfide), would help strengthen the links between abiotic factors and microbial functional diversity, as these compounds are involved in many of the key metabolic pathways observed in the microbial communities of this study. In addition, CPR bacteria and DPANN archaea represent promising targets for further investigation to improve our understanding of these ultra-small microorganisms. Previous studies have demonstrated their considerable diversity and functional potential, suggesting that focused investigation of these communities could provide novel and complementary insights beyond current knowledge of groundwater microbial ecosystems. To deepen this understanding, complementary omics approaches such as metatranscriptomics and proteomics could be applied to capture the metabolic activity and gene expression of these communities.

Finally, performing an in-situ omics study on biofilm or core sediment samples from aquifer zones near the one studied here would be highly valuable. Such studies could provide real time results, reinforce the findings of this work, and uncover new insights by directly comparing both communities.

We strongly believe that the findings of this study will serve as a solid basis for future investigations and encourage further exploration in this area of research.

6.1 Closed incubation chamber

Figure S1: Closed incubation chamber used for bioreactor experiments.



## 6.2 DNA and RNA extraction kit used for filters (water samples)

### Procedure

1. Add up to 2 g of soil to the 15 ml PowerBead Tube (provided). Please refer to the Troubleshooting Guide for information regarding the amount of soil to process.
2. Add 2.5 ml of PowerBead Solution, 0.25 ml of Solution SR1 and 0.8 ml of Solution IRS.
3. Add 3.5 ml of phenol/chloroform/isoamyl alcohol (pH 6.5–8.0, [User supplied]). Cap and vortex the PowerBead Tube to mix until the biphasic layer disappears.
4. Place the PowerBead Tube on a Vortex Adapter (cat. no. 13000-V1-15) and vortex at maximum speed for 15 min.
5. Remove the PowerBead Tube and centrifuge at 2500 x g for 10 min.
6. Transfer the upper aqueous phase (avoid the interphase and lower phenol layer) to a clean 15 ml Collection Tube (provided). Discard the phenol/chloroform/isoamyl alcohol.  
**Note:** The biphasic layer will be thick and firm in soils high in organic matter and may need to be pierced to remove the bottom phenol layer.
7. Add 1.5 ml of Solution SR3 to the aqueous phase and vortex to mix. Incubate at 2–8°C for 10 min and then centrifuge at 2,500 x g for 10 min at room temperature.
8. Transfer the supernatant, without disturbing the pellet (if there is one), to a new 15 ml Collection Tube (provided).
9. Add 5 ml of Solution SR4 to the supernatant in the Collection Tube and invert or vortex to mix. Incubate at room temperature for 30 min.  
**Note:** Previous protocol instructions were to incubate at –20°C. If you have achieved good results for your soil type using the previous protocol, you may continue to follow it.
10. Centrifuge at 2500 x g for 30 min.

11. Decant the supernatant and invert the 15 ml Collection Tube on a paper towel for 5 min.
12. Shake Solution SR5 to mix and add 1 ml to the 15 ml Collection Tube. Resuspend the pellet completely by repeatedly pipetting or vortexing.  
**Note:** If the pellet is difficult to resuspend, place the tube in a heat block or water bath at 45°C for 10 min, followed by vortexing. Repeat until the pellet is resuspended.
13. Prepare one JetStar Mini Column (provided) for each RNA isolation sample:
  - 13a. Remove the cap of a 15 ml Collection Tube (provided) and place the JetStar Mini Column inside it. The column will hang in the Collection Tube.
  - 13b. Add 2 ml of Solution SR5 to the JetStar Mini Column. Allow it to completely gravity flow through the column and collect in the 15 ml Collection Tube.  
**Note:** Do not allow the column to dry out before loading the RNA isolation sample.
14. Add the RNA isolation sample from Step 12 onto the JetStar Mini Column and allow it to gravity flow through the column into the 15 ml Collection Tube.
15. Add 1 ml of Solution SR5 to the JetStar Mini Column and allow it to completely gravity flow into the 15 ml Collection Tube.
16. Transfer the JetStar Mini Column to a new 15 ml Collection Tube (provided). Shake Solution SR6 to mix and then add 1 ml to the JetStar Mini Column to elute the bound RNA. Allow Solution SR6 to gravity flow into the 15 ml Collection Tube.
17. Transfer the eluted RNA to a 2.2 ml Collection Tube (provided). Add 1 ml of Solution SR4. Invert at least once to mix and incubate at –15°C to –30°C for a minimum of 10 min.
18. Centrifuge the 2.2 ml Collection Tube at 13,000 x g for 15 min to pellet the RNA.
19. Decant the supernatant and invert the 2.2 ml Collection Tube onto a paper towel for 10 min to air dry the pellet.
20. Resuspend the RNA pellet in 100 µl of Solution SR7.

## Procedure

1. Transfer the RNA Capture Column from step 16 of the RNeasy PowerSoil Total RNA Kit (cat. no. 12866-25) to a 15 ml Collection Tube (provided).
2. Add 1 ml of Solution SR8 to the RNA Capture Column to elute the bound DNA into the 15 ml Collection Tube. Allow Solution SR8 to gravity flow into the Collection Tube.
3. Transfer the eluted DNA to a 2.2 ml Collection Tube (provided) and add 1 ml of Solution SR4. Invert at least once to mix and incubate at  $-15^{\circ}\text{C}$  to  $-30^{\circ}\text{C}$  for 10 min.
4. Centrifuge the 2.2 ml Collection Tube at  $13,000 \times g$  for 15 min at room temperature to pellet the DNA.
5. Decant the supernatant and invert the 2.2 ml Collection Tube onto a paper towel for 10 min to air dry the DNA pellet.
6. Resuspend the DNA pellet in 100  $\mu\text{l}$  of Solution SR7.

**Note:** Although RNA carryover does not occur with most soil types, certain soils high in organic matter may present unique carryover situations. When the absence of RNA contamination is critical, an RNase treatment of the isolated DNA is recommended; please refer to the Troubleshooting Guide for instructions.

### 6.3 DNA and RNA extraction kit used for rock samples

1. In the Falcon tube with the rocks chips, add 1g of zircon/silica beads<sup>1</sup> with 2mL of 2 $\times$ AE buffer.
2. Vortex at full speed for 30sec.
3. Add 500 $\mu\text{L}$  of SDS 20%.
4. Vortex at full speed for 1min.
5. Centrifuge at 8 000g for 5min.
  - o If the supernatant is turbid, repeat the centrifugation for 3min.
6. Take the chip out with sterilized pliers. Place the rock in a 15mL Falcon.
7. Use the supernatant and follow the protocol used for filters samples.

## 6.4 RNA purification and rRNA depletion protocol

### Procedure

1. **Cells:** Harvest a maximum of  $5 \times 10^5$  cells as a cell pellet or by direct lysis in the vessel. Add 350  $\mu$ l Buffer RULT and homogenize.  
**Tissues:** Disrupt and homogenize  $\leq 5$  mg tissue in 350  $\mu$ l Buffer RULT using the TissueRuptor<sup>®</sup> or Tissuelyser instrument. Centrifuge the lysate for 3 min at maximum speed. Carefully remove the supernatant by pipetting, and use for step 2.
2. Add 1 volume of 70% ethanol to the lysate and mix well by pipetting. Do not centrifuge. Proceed immediately to step 3.
3. Transfer the sample, with any precipitate, to an RNeasy UCP MinElute spin column in a 2 ml collection tube (supplied). Close the lid and centrifuge for 15 s at  $\geq 8000 \times g$ . Discard the flow-through.
4. Add 350  $\mu$ l Buffer RUWT to the RNeasy UCP MinElute spin column. Close the lid. Centrifuge for 15 s at  $\geq 8000 \times g$ . Discard the flow-through.
5. Add 10  $\mu$ l DNase I stock solution to 70  $\mu$ l Buffer RDD. Mix by inverting the tube. Add the DNase I incubation mix (80  $\mu$ l) directly to the RNeasy UCP MinElute spin-column membrane. Place on the benchtop (15 to 25 °C) for 15 min. Add 350  $\mu$ l Buffer RUWT to the RNeasy UCP MinElute spin column. Close the lid and centrifuge for 15 s at  $\geq 8000 \times g$ . Discard the collection tube.
6. Place the RNeasy UCP MinElute spin column in a new 2 ml collection tube (supplied). Add 500  $\mu$ l Buffer RUPE to the spin column. Close the lid and centrifuge for 15 s at  $\geq 8000 \times g$ . Discard the flow-through.
7. Add 500  $\mu$ l of 80% ethanol to the RNeasy UCP MinElute spin column. Close the lid and centrifuge for 2 min at  $\geq 8000 \times g$ . Discard the collection tube.
8. Place the RNeasy UCP MinElute spin column in a new 2 ml collection tube (supplied). Open the lid of the spin column and centrifuge at full speed for 5 min to dry the membrane. Discard the flow-through and collection tube.
9. Place the RNeasy UCP MinElute spin column in a new 1.5 ml collection tube (supplied). Add 14  $\mu$ l RNase-free water directly to the center of the spin-column membrane. Close the lid gently and centrifuge for 1 min at full speed to elute the RNA.

### Prepare 1X Hybridization Buffer

1. Pre-heat 2X Hybridization Buffer at 50°C for at least 5–10 minutes to bring any precipitated salts into solution.
2. Prepare 200  $\mu$ L of 1X Hybridization Buffer for each RNA sample by diluting 2X Hybridization Buffer with an equal volume of nuclease-free water.

## Hybridize probe mix and total RNA

1. Mix the components in the order listed in the following table in a sterile PCR strip tube or 96-well plate.

Component	Volume
2X Hybridization Buffer (yellow cap)	50 $\mu$ L
RiboMinus™ Pan-Prokaryote Probe Mix (green cap)	1 or 3 $\mu$ L <sup>[1]</sup>
Total RNA (100–5,000 ng)	x $\mu$ L
Nuclease-free water	up to 100 $\mu$ L

<sup>[1]</sup> Use 1  $\mu$ L of RiboMinus™ Pan-Prokaryote Probe Mix for samples with 100 ng to <1,000 ng total RNA input, or 3  $\mu$ L for samples with 1,000–5,000 ng total RNA input.

2. Mix by gentle vortexing, then centrifuge briefly.
3. Incubate the plate/strip in a thermal cycler using the following conditions.

**Note:** Set ramp rate at 100%, and heated lid to default at 105°C.

Step	Temperature	Time
Denaturation	10 minutes	70°C
Hybridization	20 minutes	37°C

**Note:** Do not place samples in cold water. Do not allow samples to cool to room temperature.

4. During incubation for hybridization, proceed to “Prepare RiboMinus™ Magnetic Beads”.

After the hybridization step is complete, leave the reactions at 37°C, until ready to add sample to prepared RiboMinus™ Magnetic Beads.

## Prepare RiboMinus™ Magnetic Beads

1. Resuspend the bottle (blue cap) of RiboMinus™ Magnetic Beads thoroughly by vortexing.
2. For each sample, add 500  $\mu$ L of bead suspension into a sterile, RNase-free 1.5-mL microcentrifuge tube.
3. Place the tubes with the bead suspension on a magnetic stand for 1 minute or until the solution clears. Gently aspirate and discard the supernatant without disturbing the beads.
4. Remove the tubes from magnetic stand.
5. Wash the beads with 500  $\mu$ L nuclease-free water by dispensing the water down the side of the tube where the beads are collected. Alternatively, mix by gentle vortexing, then centrifuge briefly.
6. Place the tubes on a magnetic stand for 1 minute or until the solution clears. Gently aspirate and discard the supernatant without disturbing the beads.
7. Repeat steps 5–7 one more time.
8. Resuspend the beads in 200  $\mu$ L of 1X Hybridization Buffer.
9. Place the prepared beads in a 37°C heat block for at least 5 minutes. The beads can stay at 37°C until ready to use with the hybridized RNA/probe mix.

## Capture and remove rRNA-probe complexes

1. Briefly centrifuge the RNA/probe mix in the PCR plate/strip tube to collect the contents at the bottom.
2. Transfer each RNA/probe mix (100  $\mu$ L) sample to a tube containing prepared RiboMinus™ Magnetic Beads (200  $\mu$ L). Mix well by pipetting or by low speed vortexing.
3. Incubate tubes at 37°C for 15 minutes using a heat block or incubator.
4. Centrifuge the tubes briefly, then place the tubes on a magnetic stand for 1 minute or until the solution clears.
5. Transfer the supernatant (300  $\mu$ L) containing the rRNA-depleted RNA to a new tube.

## Concentrate rRNA-depleted RNA

- Mix the components in the order listed in the following table in a sterile, RNase-free 2-mL microcentrifuge tube.

Component	Volume
Nucleic Acid Binding Beads (clear cap)	10 $\mu$ L
Binding Solution Concentrate	400 $\mu$ L

- Mix thoroughly by gentle vortexing, or pipetting up and down.
- Add the entire volume (300  $\mu$ L) of bead supernatant containing the rRNA-depleted RNA to the microcentrifuge tube, then mix thoroughly by gentle vortexing, or pipetting up and down.
- Add 1 mL of 100% ethanol to the microcentrifuge tube. Ensure the cap is securely closed, then mix well by inverting the tube.  
**Note:** The mixture should look homogeneous at this point.
- Incubate at room temperature for 5 minutes. If any sample is retained in the cap, centrifuge the tube briefly to collect the contents at the bottom before proceeding.
- Place tube onto a magnetic stand for 3–5 minutes or until the solution clears. Aspirate and discard the supernatant without disturbing the beads.
- Remove the tube from the stand, and wash the beads by dispensing 300  $\mu$ L of prepared Wash Solution down the side of the tube where the beads are collected. Alternatively, mix by gentle vortexing, then centrifuge briefly.
- Replace the tube on the magnetic stand, allow solution to clear. Aspirate and discard the supernatant. Carefully remove any remaining supernatant with a 20- $\mu$ L pipettor without disturbing the bead pellet.
- Keep the tube on magnetic stand for 2 minutes to allow the beads to air dry, then remove the tube from the stand.
- Add 12–50  $\mu$ L of pre-heated (70°C) nuclease-free water to the beads and incubate for 1 minute at room temperature to elute the RNA.  
**Note:** Use an elution volume suitable for your downstream application.
- Place the tube on the magnetic stand and carefully collect the supernatant in a new microcentrifuge tube.

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**IMPORTANT!** The supernatant contains the eluted rRNA-depleted RNA (RiboMinus™ RNA).

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## 6.5 Permanova results on all gene composition matrix (beta diversity)

Table S1: PERMANOVA test results evaluating the influence of season (summer and winter) on functional gene composition.

	<b>Df</b>	<b>SumOfSqs</b>	<b>R<sup>2</sup></b>	<b>F-value</b>	<b>P-value</b>
<b>Model</b>	1	0.17712	0.08958	1.0824	0.3452
<b>Residuals</b>	11	1.80006	0.91042		

Table S2: PERMANOVA test results evaluating the influence of microbial lifestyle (planktonic vs. sessile) on functional gene composition.

	<b>Df</b>	<b>SumOfSqs</b>	<b>R<sup>2</sup></b>	<b>F-value</b>	<b>P-value</b>
<b>Model</b>	1	0.28631	0.1448	1.8626	0.0394
<b>Residuals</b>	11	1.69088	0.8552		

#### 6.6 Anova results of environmental variables on functional gene composition.

Table S3: ANOVA test results evaluating the effect of DIC, DOC and NH<sub>x</sub> and NO<sub>2</sub>, the variables selected as significant on functional gene composition.

Model: RDA (Formula = functions\_bray\_curtis ~ DOC + NO<sub>2</sub> + DIC + NH<sub>x</sub>, data = metadata)

	<b>Df</b>	<b>Variance</b>	<b>F-value</b>	<b>P-value</b>
<b>Model</b>	4	0.82294	4.5816	0.024
<b>Residual</b>	8	0.35924		

Testing the axes:

	<b>Df</b>	<b>Variance</b>	<b>F-value</b>	<b>P-value</b>
<b>CAP1</b>	1	0.83228	27.6481	0.078

CAP2	1	0.09965	3.3104	0.352
Residual	6	0.18062		

## 6.7 Metabolic pathways identified by LEfSe analysis.

Table S4: Metabolic pathways identified by LEfSe analysis as significantly enriched in sessile and planktonic communities.

<b>Sessile enriched</b>	<b>Planktonic enriched</b>
Benzoate degradation	Aminoacyl-tRNA biosynthesis
Steroid degradation	Pyrimidine metabolism
Nitrogen metabolism	Purine metabolism
Toluene degradation	Carbon fixation by Calvin cycle
Xylene degradation	Fructose and mannose metabolism
Chlorocyclohexane and chlorobenzene	DNA replication
Pathways in cancer	Cell cycle - Caulobacter
Prion disease	Pentose phosphate pathway
Non-alcoholic fatty liver disease	Base excision repair
Amyotrophic lateral sclerosis	Pantothenate and CoA biosynthesis
Thermogenesis	Biosynthesis of ansamycins
Huntington disease	MAPK signaling pathway - plant
Cardiac muscle contraction	Protein processing in endoplasmic reticulum

Dioxin degradation	N-Glycan biosynthesis
Adipocytokine signaling pathway	Longevity regulating pathway
Efferocytosis	Plant hormone signal transduction
Shigellosis	MAPK signaling pathway - fly
Ferroptosis	Coronavirus disease - COVID-19
Axon regeneration	Biosynthesis of various antibiotics
	Prolactin signaling pathway
	Selenocompound metabolism
	FoxO signaling pathway
	African trypanosomiasis
	Lipoarabinomannan (LAM) biosynthesis
	Autophagy - yeast

6.8 Permanova results on selected pathways detected by LEfSe.

Table S5: PERMANOVA results testing the effect of microbial lifestyle (planktonic vs. sessile) on pathways enriched and selected by LEfSe in lifestyle communities.

	<b>Df</b>	<b>SumOfSqs</b>	<b>R<sup>2</sup></b>	<b>F-value</b>	<b>P-value</b>
<b>Model</b>	1	0.016783	0.39453	6.5161	0.0191
<b>Residuals</b>	10	0.025756	0.60547		

Table S6: PERMANOVA results testing the effect of microbial season (summer vs. winter) on pathways enriched and selected by LEfSe analysis on season communities.

	<b>Df</b>	<b>SumOfSqs</b>	<b>R<sup>2</sup></b>	<b>F-value</b>	<b>P-value</b>
<b>Model</b>	1	0.0043525	0.29103	4.1049	0.0185
<b>Residuals</b>	10	0.0106032	0.70897		

### 6.9 RNA concentrations after extractions and RNA depletions

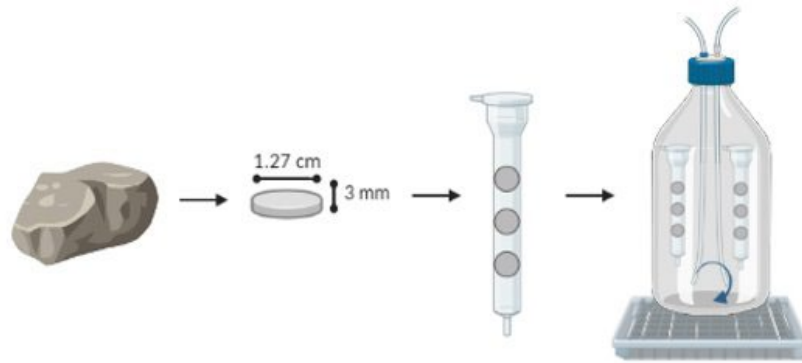
RNA samples		
Sample name	RNA concentration (qubit)	RNA concentration (qubit) after rRNA depletions
T0_water_winter	16.7 ng/ul	Too low
T1_water_winter	8.97 ng/ul	Too low
T2_water_winter	15.6 ng/ul	Too low
T3_water_winter	7.4 ng/ul	5.68 ng/ul
T0_water_summer	66.7 ng/ul	18.8 ng/ul
T1_water_summer	25.7 ng/ul	4,90 ng/ul
T2_water_summer	15.5 ng/ul	2.3 ng/ul
T3_water_summer	9.27 ng/ul	too low
T1_rocks_winter	1.31 ng/ul	Too low
T2_rocks_winter	5.68 ng/ul	Too low
T3_rocks_winter	10.3 ng/ul	Too low

T1_rocks_summer	7.40 ng/ul	Too low
T2_rocks_summer	13.1 ng/ul	2.00 ng/ul
T3_rocks_summer	9.32 ng/ul	Too low

6.10 Scripts for figures and statistical analysis

[GitHub/nickysu50](https://github.com/nickysu50)

6.11 Rock chips illustration



**Figure 15:** Diagram illustrating the preparation of rock chips: a rock slab (left) was cut into smaller chips, which were installed in the column and subsequently placed in the bioreactor.



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