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Microbiological-ecological assessment of Austrian groundwaters - development of a classification scheme and delineation of reference conditions

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Abstract

Groundwater ecosystems play a critical role in supporting terrestrial ecosystems and providing essential services to humans. However, these elusive ecosystems are frequently overlooked and their ecological protection remains significantly underdeveloped in current policies and management strategies. For an appropriate ecological assessment, reliable methodologies are needed. While several approaches utilizing groundwater organisms have been developed in the recent past, critical scientific gaps persist, including: (1) the definition of natural reference conditions, and (2) the development of a biological informed classification system for groundwater ecosystems.

Microorganisms are promising bioindicators due to their pivotal role in groundwater ecosystems. As they are the primary contributors of biomass, intimately involved in the processing of matter and ubiquitously present, microorganisms can provide valuable ecological information. The recently developed D-A-(C) index represents an innovative approach for a microbiological-ecological assessment of groundwater ecosystems. This approach is based on fundamental and easy to measure properties of microbial communities, such as prokaryotic cell density (D) and microbial activity (A). Furthermore, the approach allows the inclusion of additional measures to enhance the sensitivity of the assessment. Thus, the analysis can be supplemented with additional microbial variables or information on the available energy, such as the quantity and/or quality of dissolved organic carbon (C). The D-A-(C) index has been successfully applied on the local to regional scale. So far, the critical step of defining reference conditions has been accomplished by employing expert knowledge and indicators of anthropogenic and surface water influences. However, such measures are not readily available for the identification of reference conditions on a nation-wide scale.

To define microbial reference conditions on a nation-wide scale, more than 3700 groundwater samples were evaluated in this study. These were taken during the national groundwater monitoring program in 2019, coordinated by the Austrian Federal Environmental Agency and provincial state authorities. Measurements of microbial variables were conducted by the Groundwater Ecology working Group, led by Prof. Dr. Christian Griebler at the University of Vienna. Cell density was measured by flow cytometry and quantified as Total Cell Counts (TCC). The overall activity of prokaryotic cells was measured with a cell-lysis luminescence assay and quantified as intracellular adenosine triphosphate (ATP). An additional microbial measure—the proportion of cells with High Nucleic Acid content (HNAP)—was included in the analysis, which is associated with flow cytometric measurements of aquatic samples. HNAP—also known as a cytometric fingerprint—is based on the fluorescence signal of nucleic acid which is stained for cell

enumeration by flow cytometry. This measure potentially contains further information about the physiological status, or the genome sizes, and thus may provide a rough insight into the phylogenetic composition of microbial communities.

As a preliminary step relevant metadata was collected from different sources and plausibility assessments were conducted for the microbial variables. In the first step of the evaluation, a reference dataset was established based on the available hydrochemical Natural Background Values (NBV) for shallow groundwater in Austria. This approach resulted in 586 groundwater samples lacking any sign of human influences regarding the employed hydrochemical criteria. This reference dataset allowed studying the seasonal dynamics and correlations of the microbial properties and to gain insights into the composition of the total ATP pool. In a next step, exploratory analytical techniques were employed to construct a typological foundation. Several categorical factors—expected to affect life conditions in groundwater ecosystems—such as geology, oxygen conditions, composition of the major water ions, distance to surface (indicated by the groundwater table), and land cover, were tested for their influence on the microbial variables in groundwater ecosystems. In addition, potential sampling biases due to the different well & spring types and sampling procedures were evaluated.

The results suggest that under natural conditions microbial properties are mainly affected by geology and the prevalent oxygen conditions. On a supra-regional scale geological features were observed to influence cell density and HNAP. On a regional-to-local scale, oxygen conditions were observed to have a strong effect on the cell density. Furthermore, intriguing insights were gained from the distinct microbial signature of deep (isolated) groundwater occurrences, that showed elevated levels of cell density, HNAP and activity. Small and less conclusive effects were observed for the land cover and distance to the surface as well as for the distinct types of wells and sampling procedures.

Based on these results, a first microbiologically informed classification scheme and reference conditions for the properties of microbial communities were delineated for shallow groundwater in Austria, which can now be used to assess the microbiological-ecological status of groundwater ecosystems on a nation-wide scale. Furthermore, the established classification scheme (typology) allows the application of explanatory analytical techniques on a more nuanced basis, in order to learn about the relationship between environmental factors and microbial properties at a mechanistic and predictive level.

Kurzfassung

Grundwasserökosysteme spielen eine wichtige Rolle für den Erhalt und die Funktionsfähigkeit zahlreicher terrestrischer Ökosysteme und erbringen essentielle Ökosystemleistungen für Mensch und Umwelt. Trotz ihrer Wichtigkeit werden die ökologischen Aspekte von Grundwasserökosystemen zumeist übersehen, mit der Folge, dass diese in aktuellen Richtlinien und Managementstrategien wenig Beachtung finden. Um den ökologischen Zustand von Grundwasser Ökosystemen feststellen zu können, werden verlässliche Methoden benötigt. Auch wenn in jüngerer Vergangenheit mehrere Methoden basierend auf Grundwasserorganismen entwickelt wurden, gilt es—um die ökologische Bewertungen von Grundwasserökosystemen voranzutreiben—folgende Wissenslücken zu schließen: (1) die Definition von natürlichen Referenzbedingungen und (2) die Entwicklung eines biologisch fundierten Klassifizierungssystems für Grundwasserökosysteme.

Aufgrund ihrer zentralen Rolle in Grundwasserökosystemen werden Mikroorganismen als vielversprechende Bioindikatoren für die ökologische Bewertung von Grundwasserökosystemen gesehen. Sie sind allgegenwärtig, tragen wesentlich zum Stoffumsatz bei und stellen den größten Anteil an Biomasse dar. Der kürzlich entwickelte B-A-(E) Index ist eine Methode zur mikrobiologisch-ökologischen Bewertung von Grundwasserökosystemen, die auf grundlegenden und leicht messbaren Eigenschaften mikrobieller Gemeinschaften, wie der Biomasse (B) und Aktivität (A) prokaryotischer Organismen basiert. Um die Sensitivität dieser Methode zu verbessern, kann die Analyse durch zusätzliche Messgrößen erweitert werden. Dies können einerseits weitere mikrobielle Variablen oder Informationen zur verfügbaren Energie (E), wie der Menge und/oder Qualität des gelösten organischen Kohlenstoffs sein. Der B-A-(E) Index konnte bereits erfolgreich auf lokaler und regionaler Ebene angewendet werden. Hierfür konnten Referenzbedingungen durch das Einbeziehen von Expertenwissen, sowie durch die Identifikation Menschen- und Oberflächenwasser-bedingter Störungen ermittelt werden. Da allerdings solch eine Datenlage und Expertenwissen nicht flächendeckend für Österreich verfügbar sind, können Referenzbedingungen auf nationaler Ebene nicht mit derselben Vorgehensweise ermittelt werden.

Mit dem Ziel mikrobielle Referenzbedingungen auf nationaler Ebene zu definieren wurden im Rahmen dieser Studie mikrobielle Variablen von mehr als 3700 Grundwasserproben ausgewertet. Diese waren im Rahmen der regulären Gewässerzustandsüberwachung im Jahr 2019—koordiniert durch das Umweltbundesamt und den zuständigen Landesstellen—erhoben worden. Gemessen wurden die mikrobiellen Variablen von der Arbeitsgruppe Grundwasserökologie, unter Anleitung von Prof. Dr. Christian Griebler, an der Universität Wien. Die Biomasse wurde mit der Anzahl prokaryotischer Zellen mittels Durchflusszytometrie gemessen und als Gesamtzellzahl (GZZ) quantifiziert. Die Aktivität aller prokaryotischer Zellen wurde mit einem Zelllyse-Lumineszens-Assay gemessen und als

intrazelluläres Adenosintriphosphat (ATP) quantifiziert. Eine zusätzliche mikrobielle Variable—der Anteil der Zellen mit hohem Nukleinsäuregehalt (High Nucleic Acid content cells Proportion, HNAP)—die mit durchflusszytometrischen Messungen aquatischer Proben assoziiert ist, konnte extrahiert und in die Auswertung mit einbezogen werden. HNAP—auch bekannt als zytometrischer Fingerabdruck—kann vom Fluoreszenzsignal der Nukleinsäuren abgeleitet werden, die zur Zellzählung mittels Durchflusszytometrie angefärbt wird. Diese Messgröße enthält potenziell weitere Informationen über den physiologischen Status oder die Genomgröße der identifizierten Zellen und könnte so einen (groben) Einblick in die phylogenetische Zusammensetzung mikrobieller Gemeinschaften geben.

In einem ersten Schritt der Auswertung wurde auf Basis der verfügbaren hydrochemischen natürlichen Hintergrundwerte für oberflächennahes Grundwasser in Österreich ein Referenzdatensatz erstellt. Mit diesem Ansatz konnten 586 Grundwasserproben identifiziert werden, die hinsichtlich der angewandten hydrochemischen Kriterien keine menschliche Beeinflussung zeigten. Dieser Referenzdatensatz ermöglichte es, saisonale Dynamiken und Korrelationen der mikrobiellen Variablen zu untersuchen und Einblicke in die Zusammensetzung des Gesamt-ATP-Pools zu gewinnen. In einem nächsten Schritt wurden explorative analytische Methoden eingesetzt, um eine typologische Grundlage für weitere Analysen zu schaffen. Hierfür wurde der Einfluss von Geologie, Sauerstoffbedingungen, Zusammensetzung der Hauptwasserionen, Entfernung zur Oberfläche (gemessen am Grundwasserspiegel) und Landnutzung auf die mikrobiellen Variablen getestet. Zusätzlich wurde evaluiert ob die mikrobiellen Variablen durch die unterschiedlichen Brunnentypen und Protokolle der Probennahmen verzerrt waren.

Die Ergebnisse deuten darauf hin, dass natürliche Einflüsse auf die mikrobiellen Variablen vorwiegend von geologischen und Sauerstoffbedingungen ausgeübt werden. Auf einer überregionalen Ebene konnte gezeigt werden, dass geologische Charakteristika die Gesamtzellzahl und HNAP beeinflussen. Auf regional–lokaler Ebene wurde festgestellt, dass die vorherrschenden Sauerstoffbedingungen einen starken Einfluss auf die Gesamtzellzahl haben. Darüber hinaus konnten unerwartete Einblicke in die mikrobielle Signatur von Tiefengrundwasser—mit erhöhten Werten der Gesamtzellzahl, HNAP und Aktivität—gewonnen werden. Schwächere und weniger aussagekräftige Effekte wurden für die Landnutzung und Entfernung zur Oberfläche sowie für die verschiedenen Brunnentypen und Arten der Probennahme beobachtet.

Basierend auf diesen Ergebnissen wurde ein erstes Klassifikationsschema für die oberflächennahen Grundwasservorkommen Österreichs und Referenzbedingungen für die Eigenschaften mikrobieller Gemeinschaften etabliert. Diese können nun zur mikrobiologisch-ökologischen Bewertung von Grundwasserökosystemen auf nationaler Ebene genutzt werden. Zusätzlich integriert das abgeleitete Klassifikationsschema mikrobiologische Aspekte und kann dadurch eine differenziertere Grundlage für fortführende erklärend-analytische Untersuchungen bieten, um die Beziehung zwischen Umweltfaktoren und mikrobiellen Gemeinschaften mittels mechanistischer und vorhersagender Modelle näher untersuchen zu können.

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Contents

1 Introduction

1.1 Groundwater ecosystems - General background

Groundwater is an invaluable freshwater resource that plays a crucial role in sustaining life and ecosystems above and below the Earth's surface (Gibert et al., 1994; Boulton, 2005; Humphreys, 2006; Griebler and Avramov, 2015). While most people recognize the importance of groundwater as a resource, few are aware of the enormous and fascinating ecosystems that lie beneath our feet. Occurring in void spaces such as pores and fractures beneath the Earth's surface, groundwater forms the foundation of elusive subterranean ecosystems. Shaped by the structural-geological features of aquifers—the groundwater containing layers— these environments are home to unique biological communities, ranging from viruses, prokaryotes and fungi to protists and metazoans (Griebler and Lueders, 2009; Marmonier et al., 2023).

The notion of groundwater as ecosystems, and thus the study about the dynamics and interactions of biotic and abiotic factors, has been rising in the last decades (Danielopol and Griebler, 2008). Several distinct features characterize the dynamics in groundwater ecosystems. Notably, the absence of light together with stable temperatures—with increasing depth, daily temperature fluctuations decrease—create a unique environment. Furthermore, the lack of light impedes phototrophic primary production resulting in energy-poor conditions. Although chemolitoautotrophic processes have been reported to play a role in groundwater ecosystems (Overholt et al., 2022), the primary energy source for most groundwater ecosystems is likely to be the influx of dissolved organic carbon from the surface (Shen et al., 2015). Influx and subsequent transport of nutrients is determined by geological properties such as the size and connectivity of void spaces which determine the quantity and flow velocities of groundwater recharge. Understanding the distinct structural-geological features of aquifers is crucial to comprehend the dynamics of groundwater ecosystems (Weitowitz et al., 2017). A widely used hydrogeological classification system defines three major types of aquifers : (1) Fractured aquifers develop in consolidated hard rocks such as igneous or crystalline rock formations. These aquifers typically have small void spaces with poor connectivity. (2) Karstic aquifers occur in consolidated carbonate and dolomite rocks. Large cavities and thus high flow velocities can be the result of dissolution of rock by CO_2-containing water. (3) Porous Aquifers are composed of unconsolidated sediments transported and desposited by rivers, glaciers or wind. These aquifers have small pore sizes leading to slow flow velocities but high overall permeability resulting in great storage capacities.

Overall the distinct structural-features are important factors determining if and what kind of life is possible. It can range from uninhabitable small pores to large tunnel

systems capable of supporting larger metazoans (Korbel et al., 2019; Aquilina et al., 2023). Although our knowledge about the evolution and ecology of groundwater communities is steadily increasing, it is still relatively limited. Nevertheless, we do know that biological communities play a critical role in maintaining important ecosystems services like the purification of water (Herman et al., 2001; Griebler and Avramov, 2015).

Despite their importance and large scale—groundwater ecosystems are likely the most extensive freshwater environments on the Earth (Ferguson et al., 2021)—these subterranean ecosystems are widely overlooked in conservation policies (Sánchez-Fernández et al., 2021; Mammola et al., 2019). This is of particular concern given that recent research has shown that groundwater ecosystems are particularly sensitive to anthropogenic disturbances, such as pollution, over-extraction and climate-change induced alterations (Mammola et al., 2019; Retter, Karwautz and Griebler, 2021). Consequently, the scientific community is demanding to consider comprehensive ecological criteria to legally protect these vulnerable subterranean environments (Di Lorenzo et al., 2024). While single countries advanced in the protection of groundwater communities, the majority of countries are still lacking legally binding ecological standards for groundwater ecosystems (Griebler et al., 2023).

In 2000, the European Union introduced ecological criteria for the protection of terrestrial aquatic bodies with the enactment of the Water Framework Directive (EU-WFD 2000). However, the establishment of legally binding ecological requirements was achieved for surface waters only but not for groundwater systems. Six years later the European Groundwater Directive (EU-GWD, 2006) acknowledged the ecological status of groundwater ecosystems but only by mentioning that "Research should be conducted in order to provide better criteria for ensuring groundwater ecosystem quality".

Since the enactment of the EU-GWD in 2006, the legal situation has not changed although significant advancements have been made in the fundamental understanding of groundwater ecosystems and several ecological assessment methods have been developed (Hose et al., 2023).

1.2 Ecological assessment of groundwater ecosystems

Currently, in the European Union the assessment of groundwater ecosystems is based on a comprehensive set of physical-chemical parameters and selected microbiological indicators of potential health threats to humans and livestock. Integrating ecological indicators would represent a substantial advancement, as organisms integrate environmental conditions over different time frames and spatial scales, thereby offering a complementing perspective to physical or chemical parameters (Innis et al. 2000).

Different approaches have been developed in the recent past focusing on either microorganisms (Fillinger et al., 2019), metazoans (Hahn, 2006), or a combination of several biotic and abiotic factors (Griebler et al., 2014; Korbel and Hose, 2017). Steube et al. (2009) proposed a stepwise ecological assessment scheme based on four pillars, namely (1) physical-chemical parameters, (2) general microbial variables, (3) microbial community structures, and (4) the groundwater fauna. However, the three biological pillars, each

with its distinct strengths and weaknesses, remain under development and face a variety of challenges (Hose et al., 2023).

Microorganisms offer compelling characteristics for assessing the ecological status of groundwater ecosystems. They are key players in biogeochemical cycling, contribute most of the biomass, and are—in contrast to metazoans, which are often patchy distributed or even absent under anoxic conditions— ubiquitous in groundwater and aquifers (Griebler and Lueders, 2009; Stein et al., 2010; Korbel and Hose, 2017). Moreover, Griebler et al. (2014) identified general microbial properties, such as the overall density (D) and activity (A) of microorganisms as well as the quality or quantity of organic carbon (C), to provide useful information about the ecological status of groundwater ecosystems. Although these microbial variables are integrative sum parameters that do not allow a more detailed determination of the present and active microorganisms, they still offer advantages over molecular approaches. Foremost, the D-A-(C) variables are easy and inexpensive to measure, analyze and interpret and thus are optimal to be integrated in routine groundwater monitorings (Fillinger et al., 2019). Supported by several studies that demonstrate the sensitivity of these variables in water condition monitoring (Hammes et al. 2010; Hammes and Egli 2010; Herzyk et al. 2017; Van Nevel et al. 2017), an ecological assessment tool for groundwater ecosystems namely, the D-A-(C) index, was developed (Fillinger et al., 2019). This has been successfully applied on the local to regional scale (Retter et al., 2021; Noethen et al., 2024). However, for a consequent application on the national scale the delineation of ´pristine´ reference conditions, i.e., state of microbial variables under undisturbed conditions, is still missing.

1.2.1 Research gaps

The D-A-(C) index is a multivariate outlier analysis that allows to distinguish disturbed from undisturbed groundwater samples (Fillinger et al., 2019). For a reliable discrimination, sufficient samples taken from undisturbed sites (i.e., reference samples) need to be included in the analysis (Fillinger et al., 2019; Retter et al., 2021). In previous studies the determination of reference sites was achieved by selecting sites that lacked signs of (1) anthropogenic influences (e.g., Municipal Waste Water Index, MWWI), (2) surface water intrusions (utilizing stable water isotope data) and (3) by employing expert knowledge of the wells (Retter et al., 2021). While this approach proved reliable on the local to regional scale, it can not be directly applied to national-level assessments. The primary limitations are: first, the required data (MWWI, stable water isotope, expert knowledge) is not consistently available on a nation-wide scale and second, scaling up the existent approach would be too labor- and cost-intensive. Consequently a different set of criteria needs to be employed to identify undisturbed reference samples.

Apart from demonstrating the necessity of reference samples, regional differences in D-A-(C) were observed in previous studies (Fillinger et al., 2019; Retter et al., 2021). Although the underlying causes remained unexplored, the authors recommended to classify groundwater ecosystems—based on existing hydrogeological, geochemical, but also ecological classification systems—as a preliminary step for the delineation of specific reference conditions. Despite the existence of various classification systems for groundwater

ecosystems, a critical gap remains—none of these approaches is currently microbiologically informed.

Establishing a reference data set and investigating a microbiologically informed classification scheme are two specific tasks that need to be addressed for a consequent application of the D-A-(C) index on a national scale.

As an open question and ongoing exploration remain the search for further and new microbial criteria that provide sensitive information about the ecological processes active in aquifers. Valuable information could be gained from the nucleic acid content of prokaryotic cells. This variable can be derived from flow cytometric measurements where nucleic acid is stained with a fluorescent dye (Hammes and Egli, 2010). The interpretation of the fluorescence signal allows to discriminate cells from other particles and to count cells. Furthermore, in aquatic samples the clustering of two groups is frequently observed: cells with High Nucleic Acid (HNA) content and cells with Low Nucleic Acid (LNA) content (Gasol et al., 1999). Although early findings suggested these groups to indicate predominantly the physiological state of cells—associating dead, inactive and weakly active cells to the LNA fraction and active cells to the HNA fraction (Servais et al., 2003)—recent findings draw a more complex picture, suggesting a distinct phylogenetic composition of the LNA fraction (Proctor et al., 2018). Consequently, this variable could provide complementing—albeit rough—insights into the phylogenetic composition of groundwater samples, potentially enhancing the sensitivity of the D-A-(C) index.

Despite its limitations, the D-A-(C) index is a cost-effective tool for the microbiological-ecological assessment of groundwater ecosystems that can also be useful in the allocation of resources. More resource-intensive methods, such as the molecular analysis of microbial communities, could be selectively applied when the D-A-(C) assessment reveals anomalies or patterns of particular interest.

1.3 Aim and Outline

The aim of this study is to delineate reference conditions of microbial properties—i.e., the density and activity of planktonic prokaryotic cells—needed for the microbiological-ecological assessment of Austrian groundwater ecosystems with the D-A-(C) index. To investigate the drivers of formerly observed regional differences in density and activity of cells (Fillinger et al., 2019; Retter et al., 2021), but also to assess if the LNA/HNA variable provides sensitive and additional information, the impact of several categorical factors was assessed. The investigated factors have been either employed to classify groundwater systems—such as hydrogeological features (Weitowitz et al., 2017; Brielmann et al., 2018); distance to the surface, indicated by the groundwater level—the water table (BMLRT, 2020); composition of major water ions (BMLFUW, 2017); composition of dissolved organic matter (Harjung et al., 2023)—or are suspect to affect microbial life in groundwater ecosystems—such as land cover (Retter et al., 2021) and oxygen conditions (Wolters et al., 2022). Additionally, the potential bias of human-made structures (wells and spring) and sampling procedures on measurements of microbial variables was evaluated.

To identify reference conditions, microbial variables of more than 3700 groundwater samples were evaluated. The evaluation contained following steps:

1. A reference dataset was established by employing hydrochemical Natural Background Values (NBV) as selection criteria (Brielmann et al., 2018; Kunkel et al., 2004).
2. The impact of several categorical factors potentially affecting the microbial variables was investigated employing the established hydrochemical pristine reference dataset.
3. Based on these results, a microbiological informed classification scheme was delineated allowing to determine reference conditions tailored to the distinct types of groundwater ecosystems.

2 Materials and Methods

2.1 Study design

2.1.1 Background - monitoring network

A nation-wide microbiological-ecological assessment of groundwater resources in Austria was only possible through the cooperation with governmental authorities. The infrastructure of an existing surveillance monitoring program (GZÜV 2006) was utilized to achieve a comprehensive survey encompassing all possible types of groundwater occurrences. Throughout the last 30 years a monitoring network, consisting of approximately 2000 wells and springs, has been established in order to keep track of the shallow groundwater resources (0.32–86.36 m water table) which are the main source of drinking-water supply in Austria (BMLRT, 2021). The monitoring program—overseen by the Federal Ministry of Agriculture and Forestry in cooperation with the Federal Environment Agency and the nine provincial authorities of Austria—is based on the Water Rights Act 1959 (WRG, 1959) and the Ordinance on the Monitoring of the Quality of Water Bodies (GZÜV, 2006). The latter implements the European Water Framework Directive which requires all groundwater bodies to be monitored regularly in order to keep or reach the requirements of a good chemical and quantitative status of groundwater (EU-WFD, 2000). The classification of groundwater bodies serves as the management unit. The Austrian government has defined 142 groundwater bodies which cover the entire area of Austria (GZÜV, 2006). A comprehensive set of physical-chemical parameters is regularly measured. Results and analysis of more than 200 physical-chemical parameters can be accessed via the public H2O-database.

Monitoring sites are distributed in a way to get a comprehensive representation of all groundwater bodies resulting in an average distribution of approximately one monitoring site per $40 km^2$. Thereby all different kinds of aquifers, geological formations and land cover patterns ranging from the lowlands (114 m) up to the high alpine (2751 m) regions are covered. However, monitoring sites are not randomly and evenly distributed. Natural and anthropogenic factors are taken into account to achieve an appropriate distribution for an optimal assessment. The concentration of monitoring sites is higher in areas of intense groundwater use where the potential for contamination is higher. According to this, monitoring sites can be very concentrated in the porous aquifers of the river valleys and basins of the lowlands with one well per 7.4 km^2 in contrast to one spring per 92.3 km^2 of the karstic and fissured aquifers located in the mountainous regions (Brielmann et al., 2018, S.43) All kinds of wells (i.e., private wells, industrial wells, monitoring wells, water supply facilities) and springs are used. Corresponding to their different properties different types of sampling procedures (e.g. mobile pump, tap, spring) are applied (BMLRT, 2015).

2.1.2 Site description

Austria is located in the southern-central part of Europe. Despite of its relatively small size of approximately 83000 km^2 (Statistik Austria, 2022) it is formed by a diverse set of geological formations. Two different hydrogeological classification systems were employed during this study. The first describes eleven (A-M) geological formations (Brielmann et al., 2018). The second describes three types of aquifers based on structural-geological features (INSPIRE, 2021). In the following both classification systems will be used to give a short overview of Austria's shallow groundwater occurrences. Karstic aquifers, found in the northern and southern part of the Austrian (limestone) alps, are well known because of their importance for drinking-water supply (Fig. 2.1). Infiltrating water dissolves the carbonate-rich, (marine) mesozoic and permian sediments (D) and lead to the formation of large void spaces. Two characteristics of karstic aquifers are high flow velocities and high storage capacities . Porous aquifers are formed by unconsolidated sediments that can contain large volumes of groundwater. In Austria the following two geological formations are associated to this type of aquifer: Quaternary sediments (C) mainly transported and deposited by rivers but also glaciers and wind. These are located in the inner alpine valleys and basins alongside the great rivers like the Rhine, Inn, Salzach, Drau, Mur and Danube supplying large gravel bodies with high storage capacities. Tertiary sediments (B) can be found in the foreland basins of the alps. Filled with sand and gravel bodies alternating with layers of finer clay minerals. Deep and artesian groundwater occurrences are associated with this formation (Fig. 2.1). Fractured aquifers are formed by the weathering of consolidated rocks (other than carbonte-rich rocks). Great in extent but with little storage capacities are the formations, Bohemian massif (A), Eastern Alpine crystalline rocks (H & I) and deep sea sediments in the Flysch Zone (E). Singular groundwater occurrences with greater discharge capacities can be found which are usually of regional importance (Brielmann et al., 2018). The Paleozoic rocks (G), Helveticum and cliffs of the Waschberg-zone (J), the Penninic of the central zone (K), the Subpenninic (L) and Tertiary magmatites (M), are small in extent and have little storage capacities (Fig. 2.1).

2.1.3 Groundwater sampling

A total of 3709 samples were processed during the water quality monitoring program in two sampling campaigns - the first one being in the period of March to July and the second one from September to December in 2019. Samples from 1829 sites in the first campaign and 1880 sites in the second were evaluated. In total samples from 1959 sites were evaluated. Samples for the determination of microbial ATP were filled untreated in pre-washed and combusted 40 mL amber borosilicate vials. For the determination of prokaryotic cells groundwater samples were filled in 15 mL Greiner tubes containing glutardialdehyde (0.5% v/v, final concentration) to fix cells on-site. All samples were stored and transported cooled and in darkness until further processing. Sample collection was executed by the federal state authorities, coordinated by the Environmental Agency, and conducted by individual laboratories in the different provinces.

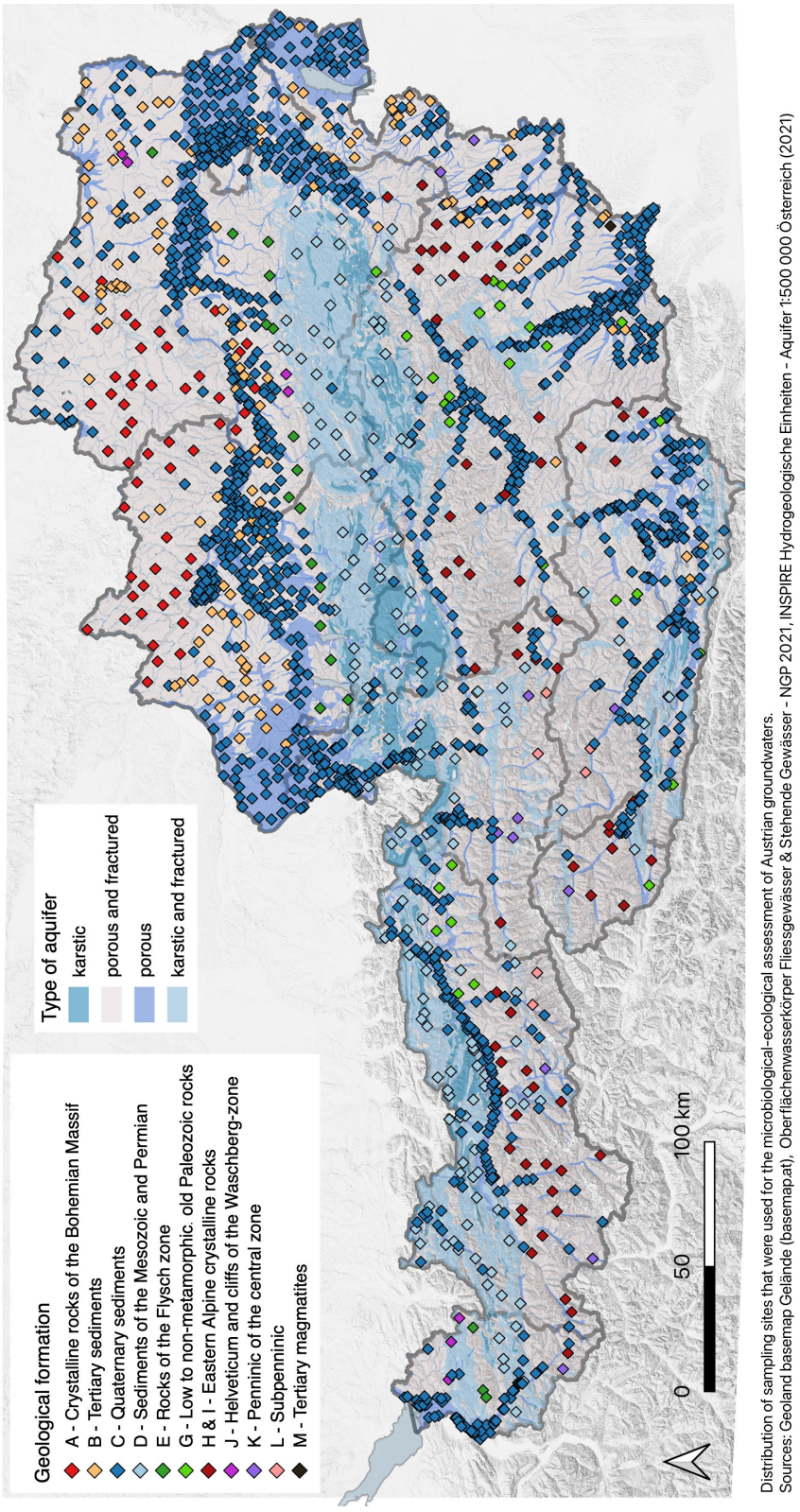


Figure 2.1: Distribution of monitoring sites in Austria.

2.2 Laboratory analysis

2.2.1 Determination of Total Cell Counts and LNA/HNA

The enumeration of planktonic prokaryotic cells, i.e. the Total Cell Counts (TCC), was performed by means of flow cytometry (Cytek® Amnis® CellStream®) applying existing protocols (Kötzsch et al., 2012). Sample aliquots of 150 µL were stained with 15 µL SYBR™ Green I Nucleic Acid Gel Stain (an asymmetrical cyanine dye that binds to DNA) at a ratio of 1:10000 v/v and incubated for 13 min at 37°C in the dark. Samples were excited with an 450 nm LED forward scatter (FSC), an 785 nm Laser side scatter (SSC) and an 488 nm laser and data was recorded for all available Instrument-detector channels i.e. SSC 773/56, 488-528/46-C3, 488-583/24-C4, 488-611/31-C5, 488-702/87-C6, FSC 456/51. Flow rate was set to 14.64 µL/min and all laser intensities to 100%. For the detection of stained cells 488-528/46-C3 was set as the trigger channel with a parameter threshold of 500. Depending on the available sample volume 2-4 measurements were performed on each sample (technical replicates). Measurements with more than 400-500 events per sec were repeated after dilution of the sample. Gates were set following the recommendations and protocols from literature and the Austrian environmental agency (Hammes and Egli, 2005; Zubanovic-Pichler, 2018). To distinguish cells from background noise, a polygon region was defined in dot plots of 528 nm fluorescence (C3) versus 611 nm fluorescence (C5). Gating and subsequent calculation of the total cell counts was done with the Amnis® Cell Stream® Acquisition and Analysis Software Version 1.3.389. Discrimination of Low Nucleic Acid (LNA) and High Nucleic Acid (HNA) containing cells was achieved by setting an additional marker on the 528 nm axis following the protocol of Kötzsch et al., 2012. Calculation of the LNA and HNA subpopulations was done in R using the packages flowCore (Ellis and Haaland, 2017) and flowWorkspace (Finak and Jiang, 2017).

2.2.2 Determination of microbial activity

Adenosine Triphosphate (ATP) concentrations were measured by performing the BacTiter-Glo™ Assay (Promega, Germany) based on the method of Hammes and Egli, (2010). The concentration of intracellular ATP was calculated by subtracting the mean concentration of the extracellular ATP from total ATP. Prior to the measurements, 2 mL of groundwater samples were transferred to a reaction tube and centrifuged at 14000 rpm for 30 min at 4°C. The supernatant was used to determine the concentration of extracellular ATP. Unprocessed groundwater samples were used for the measurement of total ATP. Extracellular ATP and total ATP were measured in triplicates. 180 µL of each sample and 20 µL BacTiter-Glo™ reagent were mixed for 20 sec at 600 rpm and 25°C on a Thermomixer. Luminescence was measured on a GloMax® 20/20. A calibration curve was calculated using an ATP standard with known concentrations (10 nM, 1 nM 100 pM, 10 pM, 1 pM) of ATP in ATP-free water to convert the measured relative Light Units [RLU] to ATP [pM]. Standard deviation of extracellular and total ATP measurements were used to calculate a standard deviation of the intracellular ATP

taking error propagation into account with the following formula:

$$SD_{intracellular ATP} = \sqrt{SD^2_{total ATP} + SD^2_{extracellular ATP}}$$

2.2.3 Plausibility checks

Plausibility of all measurements was assessed to exclude samples with implausible values. Such anomalies might be the result from factors other than natural variation, including improper sample handling and contamination during sampling, or laboratory errors.

Based on existing literature (Hammes et al., 2008) and expert knowledge, values of TCC $<$ 5 x 10^5 cells L^{-1} were considered implausible as a reliable quantification could not be guaranteed. Furthermore, implausible TCC/ATP ratios and strong seasonal dynamics with opposing trends for TCC and ATP were used as indicators. All used criteria are summarized in Table 2.1.

Table 2.1: Criteria applied for the plausibility assessment of the microbial variables Total Cell Count (TCC) and intracellular Adenosine Triphosphate (ATP).

Criterion		implausible	if
	TCC	$<$ 5 x 10^5 cells L^{-1}	-
TCC/ATP ratio	TCC	$<$ 1 x 10^6 cells L^{-1}	ATP $>$ 3 pM
	TCC	$>$ 1 x 10^{11} cells L^{-1}	ATP $<$ 200 pM
	ATP	$<$ 1 pM	TCC $>$ 1 x 10^7 cells L^{-1}
	ATP	$>$ 1000 pM	TCC $<$ 1 x 10^9 cells L^{-1}
Seasonal dynamic	TCC	10-fold increase	opposing trend in ATP
	TCC	10-fold decrease	opposing trend in ATP
	ATP	$<$ 1 pM and $>$ 5-fold greater in other season	TCC $<$ 5-fold greater in other season
	ATP	$>$ 1000 pM and $>$ 5-fold smaller in other season	TCC $<$ 5-fold smaller in other season

Additionally the precision of measurements was used as an indicator for the quality of measurement. High standard deviations derived from the mean concentration of the technical replicates were taken as an indicator for poor precision. Measurements of TCC with an relative standard deviation $>$ 50% were removed. Measurements of total-ATP and extracellular-ATP with a mean concentration $>$ 30 pM and an associated relative standard deviation $>$ 50% were removed.

The revised data of Total Cell Count (H2O database-number: G1191) and intracellular ATP (H2O database-number: G1192) can be accessed at the public H2O database.

2.3 Data overview and external sources

The microbial variables Total Cell Counts (TCC), Low Nucleic Acid/High Nucleic Acid (LNA/HNA) ratio, and Adenosine Triphosphate (ATP) were measured at the University of Vienna. Additional information was obtained from following sources: Data regarding the sampling events and corresponding phyiscal-chemical parameters were derived from the publicly accessible H2O-database. The Environmental Agency of Austria (Umweltbundesamt) provided supplementary information on the classification of the monitoring wells. The digital terrain model of Austria and land cover information were sourced from the public database data.gv.at.

Table 2.2: Overview of data sources.

Source	Keyword	Parameters/Levels
H20 database	Sampling data	Date, temperature
	Field-parameters	Groundwater-temperature, pH, dissolved Oxygen, Electrical Conductivity
	Chemical-analytical	Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, Fe^{2+}, Mn^{2+}, $NH_4{}^{+}$, $NO-2{}^{-}$, $NO_3{}^{-}$, Cl^{-}, $SO_4{}^{2-}$, $HCO_3{}^{-}$, $PO_4{}^{3-}$, DOC
	Metals	B, Cd, Hg, Zn, Cu, Al, Pb, Cr, Ni, Ar, U
	Quantity	Groundwater level, spring discharge
	Type of Aquifer	Porous, karstic-fissured, deep
Umweltbundesamt	Geology	11 geological formations, 48 hydrogeological units
	Groundwater monitoring station type	Unconfined (0-5m, 5-15m, 15-30m, >30m), confined, and karstic and fissured groundwater
	Type of Well	Drilled well, radial collector well, tapped spring, untapped spring, dug well, driven well, monitoring well, groundwater heat pump system
	Type of Sampling	Tap, mobile pump, direct spring sampling, spring capture conduit, spring overflow, other
	Spatial data	X/Y Coordinates
data.gv.at	Digital terrain model of Austria	Elevation
	CORINE-Land Cover 2018	23 Landscape patterns

2.4 Plausibility of chemical parameters

2.4.1 Ion charge balance

A common way of assessing the quality of water analysis is to look at the electrically neutral status of water. For all water sample analysis the electroneutrality condition must be fulfilled. That means that the equivalent concentration of all cations must be equal to the equivalent concentration of the anions. The equivalent concentration of an analyte i is calculated by multiplying the concentration c_i with the corresponding charge number z_i.

$$\sum_{Cations} z_i * c_i = \sum_{Anions} z_i * c_i$$

For the determination of the sum of cations the equivalent concentration of the following analytes were taken into account.

$$\sum_{Cations} = 2[Ca^{2+}] + [Mg^{2+}] + [Na^{+}] + [K^{+}] + [NH_4{}^{+}] + 2[Fe^{2+}] + 2[Mn^{2+}]$$

For the determination of the sum of anions the equivalent concentration of the following analytes were taken into account.

$$\sum_{Anions} = [HCO_3{}^{-}] + [Cl^{-}] + [NO_3{}^{-}] + 2[SO_4{}^{2-}] + x_{pH}[PO_4{}^{3-}]$$

An pH dependent factor x was calculated for the dissociation of $PO_4{}^{3-}$. The following values were used for $x_{pH \leq 6} = 1.12$ and $x_{pH \geq 8} = 1.88$. The values of the factors for samples with pH ranging from 6 to 8 were interpolated with the following formula (Kölle, 2017).

$$x_{pH} = 1.12 + 0.38 * (pH - 6)$$

Deviations from electroneutrality condition can be assessed by calculating the ion charge balance error (IBE). The IBE serves as an indicator of overall measurement quality. In this study the IBE was calculated following the method of Kölle, 2017:

$$IBE = \frac{\sum Cations - \sum Anions}{\sum Cations + \sum Anions} * 200$$

Analysis were seen as incomplete or erroneous when the ion charge balance error was larger than 10%. Deviations can be greater if the total concentration of equivalent ions is ≤ 2 mM Kölle, 2017. In this case an IBE up to 15% were accepted.

2.4.2 Contradicting values

All samples were screened for implausible hydrochemical combinations using plausibility-criteria as outlined by Hölting and Coldewey, 2013. The additional screening served two purposes. First, it provided additional information about the reliability of the hydrochemical analysis, and second, it served as an additional criterion in the selection of references.

Table 2.3: Criteria used for the assessment of plausibility according to Hölting and Coldewey, 2013.

if	concentration	implausible
DO	>5.0 mg L^{-1}	Fe^{2+} >0.05 mg L^{-1} Mn^{2+} >0.05 mg L^{-1} NO_2^- >0.05 mg L^{-1} NH_4^+ >0.05 mg L^{-1}
Fe^{2+}	>0.2 mg L^{-1}	NO_3^- >2.0 mg L^{-1}
Mn^{2+}	>0.2 mg L^{-1}	NO_3^- >2.0 mg L^{-1}
pH	>8.5 or <5.5	Ca^{2+} + Mg^{2+} >1 mM

2.5 Censored data

Measurement of hydrochemical parameters is always constrained by technical possibilities. Detection and quantification of components at very low concentrations can be challenging and sometimes might be outside the range of reliable measurement. Such measurements are typically reported to be "below the limit of quantification" (< LOQ) or "below the limit of detection" (< LOD). According to IUPAC, 2019 the LOD is the "smallest measure (...) that can be detected with reasonable certainty for a given analytical procedure". Thus, the LOD provides information on the threshold at which the measured signal can be considered to be significantly larger than the background signal obtained from measuring blanks. Signals above the LOD but to low to be quantified with adequate precision (usually less than 10% relative standard deviation) are reported as < LOQ (IUPAC, 2019).

Data reported to be below the LOD and LOQ are referred to as censored data in statistical analysis. Several approaches have been developed to deal with censored data in hydrochemical and environmental studies (Helsel, 2005). Depending on the amount of censored data but also the desired analysis, the way this sort of data is treated might affect the results. The proportion of censored data can be different for each hydrochemical parameter (Table 2.4. Censored data reached from 0% for Calcium in contrast to 99.9% for mercury).

In this study the concentrations of the hydrochemical parameters were of major importance to assess if the hydrochemical concentrations of the samples exceeded the naturally occuring concentrations (described in more detail in the following section). For this task a conservative approach for the substitution of censored data seemed suitable. Values reported to be smaller than the LOD were set to 0 and values smaller than the LOQ to LOQ/2. For the calculation of the ion charge balance error a similar conservative approach is recommended by Kölle (2017).

Table 2.4: Overview of basic statistics, number of not available (NA) data and censored data—Limit of Detection (LOD) + Limit of Quantification (LOQ)—of the physical-chemical parameters.

Parameter	unit	min	max	mean	median	NA	<LOD	<LOQ	censored
Temp.	[°C]	1.4	92	11	12	77	0	0	0.0%
EC (20°C)	[µS cm^{-1}]	20	5580	551	511	82	0	0	0.0%
pH	[-]	5	10	7.3	7.4	101	0	0	0.0%
DO	[mg L^{-1}]	0	18	7.1	7.8	96	120	130	4.3%
Ca^{2+}	[mg L^{-1}]	0.94	520	77	77	74	0	0	0.0%
Mg^{2+}	[mg L^{-1}]	0.52	830	23	19	72	6	34	0.7%
Na^{+}	[mg L^{-1}]	0.16	1080	18	9.9	72	124	316	7.6%
K^{+}	[mg L^{-1}]	0.14	181	4.2	2.4	72	309	970	22.2%
$NH_4{}^{+}$	[mg L^{-1}]	0.0052	12	0.15	0.017	72	1263	2000	56.5%
$NO_2{}^{-}$	[mg L^{-1}]	0.003	3.5	0.046	0.014	72	3287	1453	82.1%
$NO_3{}^{-}$	[mg L^{-1}]	0.28	358	19	9.9	73	335	219	9.6%
Cl^{-}	[mg L^{-1}]	0.5	559	30	18	78	76	483	9.7%
$SO_4{}^{2-}$	[mg L^{-1}]	0.52	4650	49	27	75	5	23	0.5%
$HCO_3{}^{-}$	[mg L^{-1}]	4.2	2099	274	279	98	0	0	0.0%
$PO_4{}^{3-}$	[mg L^{-1}]	0.006	24	0.1	0.037	72	807	1109	33.2%
Fe^{2+}	[mg L^{-1}]	0.0032	72	1.2	0.024	74	2326	1354	63.8%
Mn^{2+}	[mg L^{-1}]	0.00035	12	0.27	0.036	73	3183	1048	73.3%
B	[µg L^{-1}]	0.0013	3.4	0.053	0.025	72	1032	1069	36.4%
DOC	[mg L^{-1}]	0.2	61	1.5	0.98	93	366	1277	28.6%
Cd	[µg L^{-1}]	0.1	9.7	0.3	0.16	73	5165	265	94.1%
Hg	[µg L^{-1}]	0.016	0.13	0.077	0.087	73	5402	354	99.7%
Zn	[µg L^{-1}]	0.66	7700	87	17	75	432	1100	26.6%
Cu	[µg L^{-1}]	0.5	280	5.1	2.2	75	1599	1792	58.8%
Al	[µg L^{-1}]	1.7	743	18	9	73	3768	799	79.1%
Pb	[µg L^{-1}]	0.1	9	1.6	1.4	73	4912	595	95.4%
Cr	[µg L^{-1}]	0.5	85	1.8	1.3	73	3191	1811	86.7%
Ni	[µg L^{-1}]	0.45	105	2.7	1.5	73	2562	1894	77.2%
As	[µg L^{-1}]	0.21	159	5.2	1.5	74	2818	1682	78.0%
U	[µg L^{-1}]	0.074	216	3.4	1.8	73	1630	1120	47.6%

2.6 Selection of reference samples

2.6.1 Geological natural background values

The establishment of a reference dataset was achieved by selecting samples that were in a natural and near-natural hydrochemical condition. Natural background values (NBV) defined for 27 parameters in 48 hydrogeological units, were used for the selection (Brielmann et al., 2018). For the major components, trace elements and metals an upper limit was determined. An additional lower limit was determined for pH and dissolved

oxygen (DO). Delineation of the background ranges was achieved in several steps and is described in detail in the above mentioned report. In short: (1) All sites suspect to be affected significantly by human activities—based on a catalogue of 14 categories—were excluded. (2) The remaining sites were assigned to homogeneous hydrogeochemical classes. (3) The unaffected normal population of each component, characterised by a lognormal distribution, was determined for each hydrogeochemical unit. (4) The outermost 10% percentiles for the determination of the background ranges were excluded in order to avoid the influence of overlapping populations (suspect to be of anthropogenic sources or hydrochgeochemical anomalies) that could still be found in the uppermost or lowest values of the previously defined normal population. Thus, the upper limit was defined by the 90% percentile value. The lower limit was defined by the 10% percentile value.

Adaptations and additional criteria

To include samples with concentrations slightly surpassing the NBV—due to e.g., potential inaccuracies of measurements—the boundaries were extended. Specifically, 10% were added to the upper limit values and 10% were substracted of the lower limit values of natural concentrations. For example, in the hydrogeological unit A01, the natural concentration of chloride was reported to reach 7.2 mg L^{-1} (90% percentile value). By adding 10% to this value, an upper limit of 7.92 mg L^{-1} was established.

Additionally upper limits for nitrate and dissolved organic carbon which can be important indicators of human activity were defined. An upper limit of 45mg L^{-1} was applied for nitrate. This value was derived from the requirements for groundwater quality defined by the Austrian government (QZV, 2010). For dissolved organic carbon two limits were set depending on the oxygen condition. Naturally occuring, concentrations of DOC up to 2.5 mg L^{-1} in oxic groundwater (DO $>$2 mg L^{-1}) and 4 mg L^{-1} in hypoxic and anoxic groundwater (DO $\leq$ 2 mg L^{-1}) were derived from literature (Regan et al., 2017; Kunkel et al., 2004).

The limits for dissolved oxygen were not taken into account for the selection of references (see Discussion).

2.7 Statistical analysis and data visualization

Statistical analysis was done in R version 4.3.1 (R Core Team, 2023) using the built-in functions of the stats package: wilcox.test(), cor.test(), kruskal.test() for statistical hypothesis testing and lm() for linear regression analysis. To evaluate the Kruskal-Wallis test results the Kruskal-Wallis effect size η^2 (eta squared) was calculated with the rstatix package (Kassambara, 2019) and for post-hoc analysis the DunnTest() from DescTools package (Signorell, 2014) was used.

Scatter plots, violin plots, bar plots, and box plots were created with ggplot2 (Wickham, 2016). The horizontal line of the box plots indicates the median (50th percentile) value. The lower and upper hinge correspond to the 25th and 75th percentiles. The length of the whiskers extend from the hinges to the largest value up to 1.5 of the corresponding hinge.

Values outside the whiskers are considered outliers and plotted individually. Splitted violin plots were created with the introdataviz package (Nordmann et al., 2022).

Maps were created with the free and Open Source QGIS 3.28, (2022). For the generation of maps the following publicly availabe maps were used.

- Elevation - "Digital terrain model of austria, based on airborne laserscanning", 2015,
- Waterways - "Oberflächenwasserkörper Fliessgewässer, NGP", 2021 ,
- Surface waterbodies -"Oberflächenwasserkörper Stehende Gewässer, NGP", 2021,
- Aquifer types - "INSPIRE Hydrogeologische Einheiten - Aquifer 1:500 000 Österreich", 2021

2.8 Delineation of reference conditions

For reference conditions, median values of the microbiological variables were calculated. The 10% percentile and the 90% percentile were calculated to define the lower and upper limits, respectively. This approach aligns with the method employed for the determination of natural background concentrations of chemical components in groundwater as described by Müller et al. (2006) and Brielmann et al. (2018).

3 Results

In the following chapter the study results are presented in four parts: (1) overview of all microbiological measurements; (2) selection and description of a reference dataset; (3) categorical factors impacting the microbiological variables; (4) delineation of reference conditions.

3.1 Measurements

A total of 142 samples were identified as implausible by applying the plausibility criteria for the microbiological variables (Table 2.1). The most frequently exceeded criterion was the lower threshold for TCC. Specifically, 41 samples had fewer than 5.0 x 10^5 cells L^{-1} and 64 samples with intracellular ATP levels higher than 3 pM had fewer than 1.0 x 10^6 cells L^{-1} (Table 3.1).

Table 3.1: Overview of number of samples with implausible microbiological values.

Criteria		Deleted
TCC	Lower threshold	41 + 64
	Upper threshold	0
ATP	Lower threshold	11
	Upper threshold	10
Seasonal dynamic	High & contradictory	8 x 2

3.1.1 Flow cytometer

Total Cell Counts (TCC)

After the plausibility assessment, a total number of 3709 measurements of TCC remained for the evaluation, with 1829 measurements from the spring campaign and 1880 from the autumn campaign. The TCC values ranged from 5.3 x 10^5 cells L^{-1} to 1.4 x 10^{10} cells L^{-1}, with an overall median value of 1.0 x 10^7 cells L^{-1}. In spring, the median TCC was 8.7 x 10^6 cells L^{-1}, which was lower than the autumn median of 1.2 x 10^7 cells L^{-1} (Fig. 3.1). The median relative standard deviation calculated for the mean value of the technical replicates was approximately 10% for both seasons.

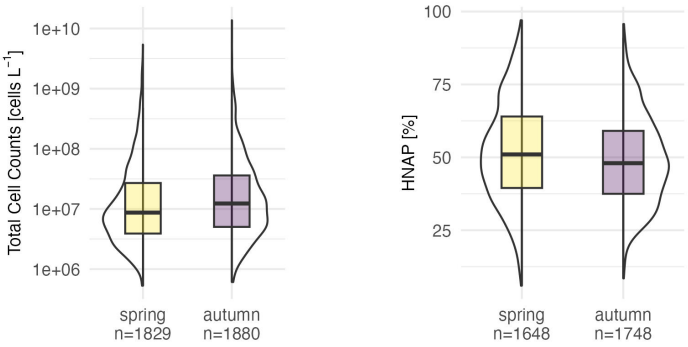


Figure 3.1: Overview of flow cytometric measurements: TCC and HNAP.

Low Nucleic Acid / High nucleic acid content cells ratio (LNA/HNA)

The ratio of cells with LNA/HNA content was calculated for 3396 flow cytometric measurements. For simplicity, only the High Nucleic Acid content cells Proportion (HNAP) will be described. The corresponding proportion of the LNA content cells can be calculated by subtracting HNAP[%] from 100%. HNAP ranged from 6% to 97%, with an overall median HNAP of 49%. The median values differed slightly between the seasons: in spring the median HNAP was 51%, while in autumn, it was 48% (Fig. 3.1). The median relative standard deviation calculated for the mean value of the technical replicates was approximately 4% for both seasons.

3.1.2 Adenosine Triphosphate (ATP)

Total ATP

The concentration of total ATP was measured for 2411 samples, ranging from 0.77 pM to 2712 pM. The median value in spring was 39 pM, with the 10%–90% percentile ranging from 14 pM to 120 pM. In autumn, the median value was 44 pM, with the 10%–90% percentile ranging from 16 pM to 159 pM. The relative standard deviation calculated for the mean values of the triplicate measurements was below 8.2% for the majority (95% percentile) of the measurements in spring. The majority (95% percentile) of the measurements in autumn had a higher relative standard deviation, reaching up to 35% (Fig. 3.2).

Extracellular ATP

The concentration of extracellular ATP was measured for 2411 samples, ranging from 0.26 pM to 499 pM. The median value in spring was 3.5 pM, with the 10%–90% percentile ranging from 1.2 pM to 13 pM. In autumn, the median value was 6.8 pM, with the 10%–90% percentile ranging from 1.9 pM to 28 pM. The relative standard deviation calculated for the mean values of the triplicate measurements was below 13% for the

majority (95% percentile) of the measurements in spring. The majority (95% percentile) of the measurements in autumn had a higher relative standard deviation, reaching up to 35% (Fig. 3.2).

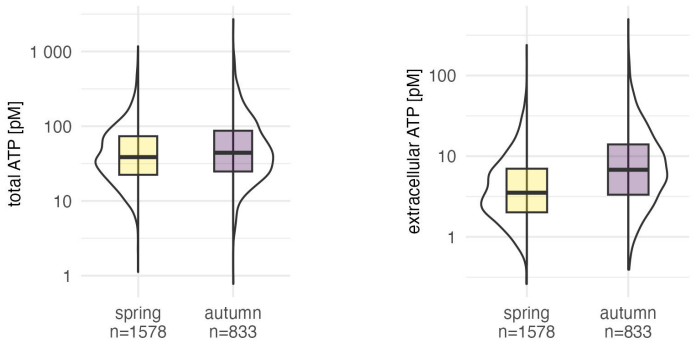


Figure 3.2: Overview of total ATP and extracellular ATP measurements.

Intracellular ATP

The concentration of intracellular ATP, as a measure of microbial activity, was calculated for 2411 samples, ranging from 0.28 pM to 2213 pM. The median value in spring was 34 pM, with the 10%–90% percentile ranging from 11 pM to 111 pM. In autumn, the median value was 35 pM, with the 10%–90% percentile ranging from 10 pM to 136 pM.

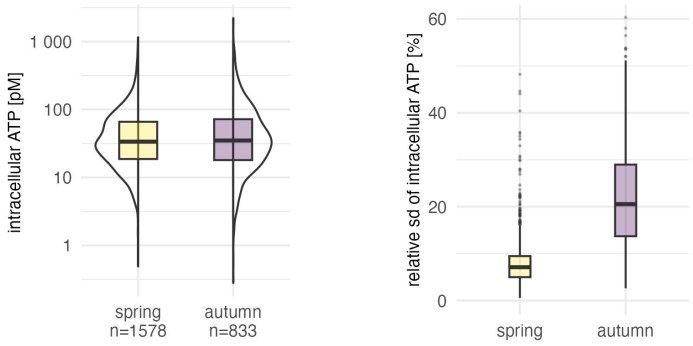


Figure 3.3: Overview of intracellular ATP values and standard deviation.

The relative standard deviation for intracellular ATP was calculated by considering the relative standard deviation of the measurements of total and extracellular ATP, taking the error propagation into account. For the measurements in spring, the majority (95% percentile) was below 15%. In contrast, for the measurements in autumn, the majority (95% percentile) was below 43% (Fig. 3.3).

3.2 Reference data set

3.2.1 Selection of references

Filtering for the groundwater samples with hydrochemical values in the range of natural background values (see Section 2.6) resulted in a reference dataset with 597 samples at 448 sites. Testing for hydrochemical plausibility identified ten samples as implausible. The most frequently exceeded criterion was the presence of dissolved iron at concentrations >0.05 mg L^{-1} together with >5 mg L^{-1} dissolved oxygen and (Table 3.2). One sample exceeded the ion charge balance error criteria defined in Section 2.4 and was removed. After removing samples that showed implausible hydrochemical data, 586 samples from 439 sites remained as reference data (Fig. 3.5).

Table 3.2: Results of the hydrochemical plausibility assessment. Criteria changed after Hölting and Coldewey, 2013.

if	concentration	implausible	samples
DO	>5.0 mg L^{-1}	Fe^{2+} >0.05 mg L^{-1}	7
		Mn^{2+} >0.05 mg L^{-1}	1
		NO_2^- >0.05 mg L^{-1}	0
		NH_4^+ >0.05 mg L^{-1}	0
Fe^{2+}	>0.2 mg L^{-1}	NO_3^- >2.0 mg L^{-1}	5
Mn^{2+}	>0.2 mg L^{-1}	NO_3^- >2.0 mg L^{-1}	1
pH	<5.5	Ca^{2+}+Mg^{2+} >1 mM	0
pH	>8.5	Ca^{2+}+Mg^{2+} >1 mM	0

3.2.2 Comparison of Reference and Non-reference samples

A comparison of the microbiological variables in between the reference samples and the non-reference samples showed significant differences for all three microbiological measures (Fig. 3.4). However, the effect sizes were weak for intracellular ATP and HNAP and moderate for TCC (according to Cohen, 1985, effect sizes $r < 0.1$ are considered weak).

The median value for TCC in the reference data set is 5.9 x 10^6 cells L^{-1}, with the 10%–90% percentile values ranging from 2 x 10^6 to 3.1 x 10^7 cells L^{-1}. This was significantly lower ($W_{Mann-Whitney}$ = 1198746, p = 8.5e-33) than the median TCC value of 1.2 x 10^7 cells L^{-1} for the non-reference sites, with the 10%–90% percentile values ranging from 2.4 x 10^6 to 1.2 x 10^8 cells L^{-1}.

The median value for intracellular ATP in the reference dataset is 29.2 pM, with the 10%–90% percentile values ranging from 10.4 to 92.8 pM. This was significantly lower ($W_{Mann-Whitney}$ = 433143, p = 3.4e-04) than the median intracellular ATP value of 35.2

pM of the non-reference dataset, with the 10%–90% percentile values ranging from 10.7 to 125 pM.

The median value for HNAP in the reference data set is 46.5%, with the 10%–90% percentile values ranging from 29.5 to 72%. This was slightly but significantly lower ($W_{Mann-Whitney}$ = 839461, p = 5.7e-04) than the median HNAP 50% of the non-reference dataset, with the 10%–90% percentile values ranging from 29.5 to 73.5%.

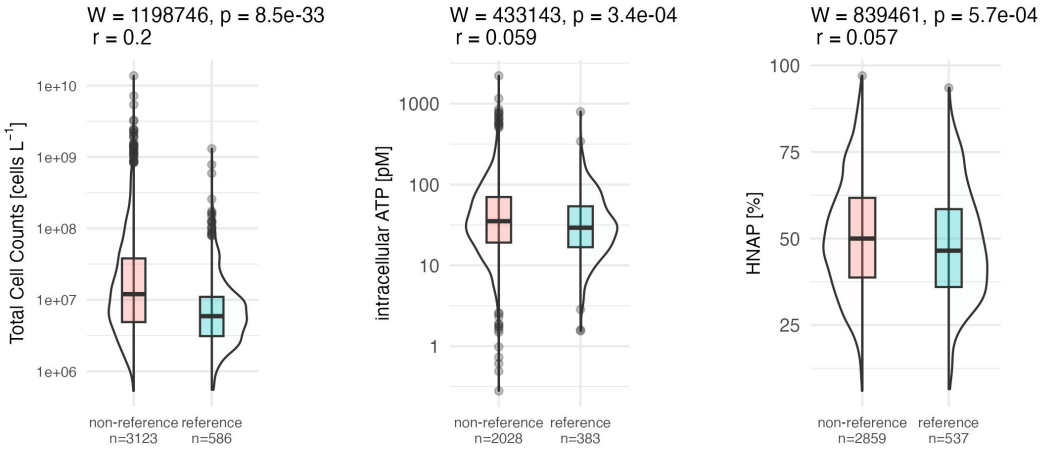


Figure 3.4: Comparison of the microbiological variables—TCC, intracellular ATP, and HNAP—between reference and non-reference samples.

3.2.3 Correlation of microbiological variables

A Kendall's rank correlation test was conducted to assess the relationship between the microbiological variables: TCC, intracellular ATP, and HNAP. None of the three measures showed significant correlations with each other at the 0.05 level. A weak positive correlation was observed between TCC and intracellular ATP ($\tau_{Kendall's}$ = 0.052), which was not statistically significant (p = 1.4e-01). Similarly, the correlation between TCC and HNAP was also weak ($\tau_{Kendall's}$ = 0.035) and not statistically significant (p = 2.5e-01). The two measures of microbial activity, intracellular ATP and HNAP, demonstrated a weak positive correlation as well ($\tau_{Kendall's}$ = 0.016), which was statistically not significant (p = 6.8e-01).

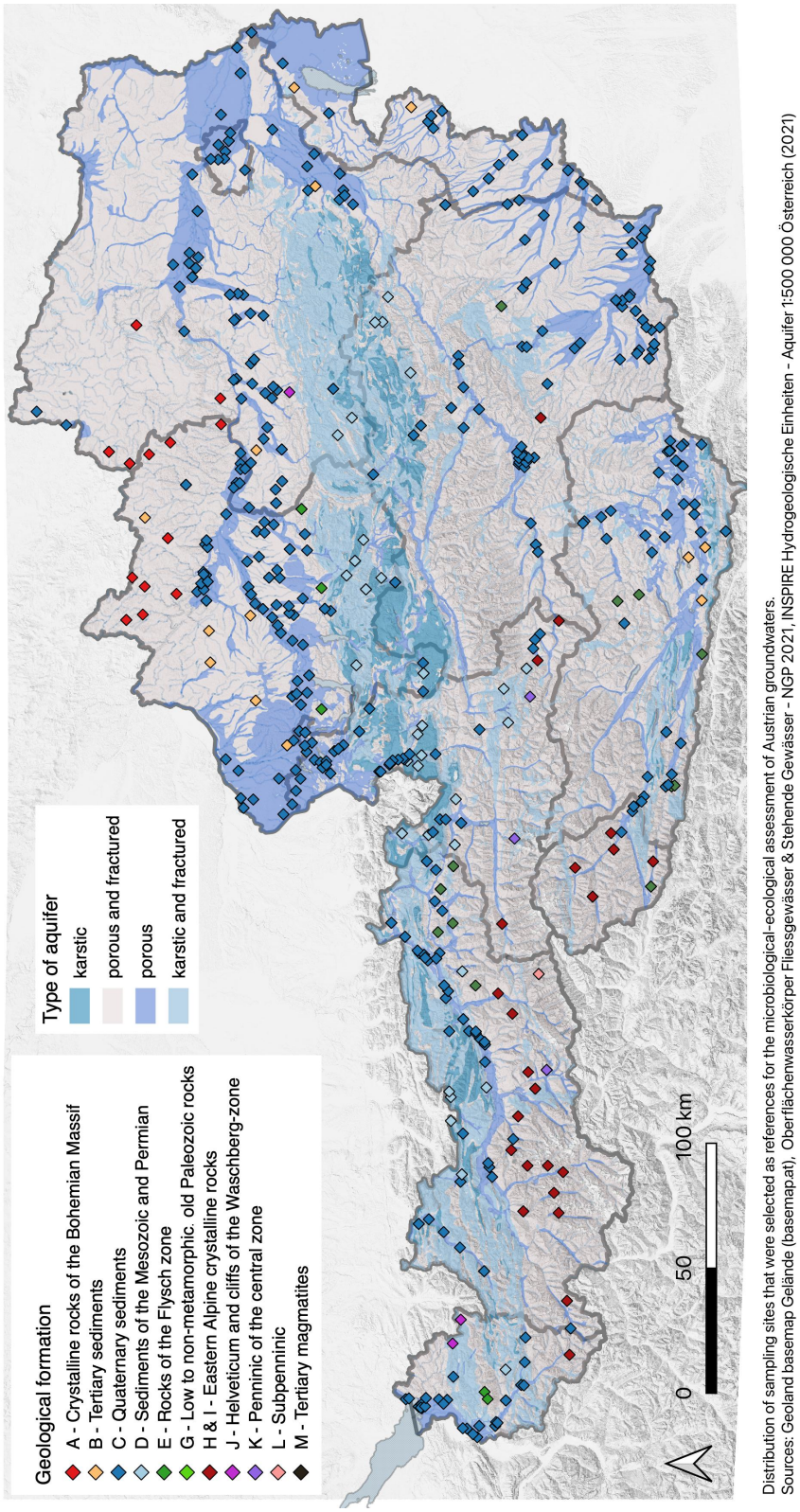


Figure 3.5: Distribution of microbiological reference sites in Austria.

3.2.4 Relationship between the different ATP measures

The relationship between the three ATP measures—total, extracellular and intracellular ATP—were assessed. All ATP measures were logarithmically (log10) transformed to achieve normality and ensure that the assumptions for the calculation of linear regression models are met. A linear regression analysis between intracellular ATP and extracellular ATP (the predictor variable) revealed a significant, though weak, relationship: Y = 1.3 (±0.032) + 0.33 (±0.045)X; R^2 = 0.1313; $F_{1,356}$ = 53.8; P < 0.001. In contrast, the linear regression analysis between total ATP (the predictor variable) and intracellular ATP revealed a much stronger relationship: Y = -0.15 (±0.025) + 1 (±0.016)X; R^2 = 0.9226; $F_{1,356}$ = 4241; P < 0.001. This indicates that intracellular ATP closely mirrors the total ATP concentrations (see also Fig. 3.7). A regression analysis between total ATP (the predictor variable) and extracellular ATP revealed a significant but moderate relationship: Y = -0.46 (±0.084) + 0.66 (±0.053)X; R^2 = 0.3006; $F_{1,356}$ = 153; P < 0.001 (Fig. 3.6).

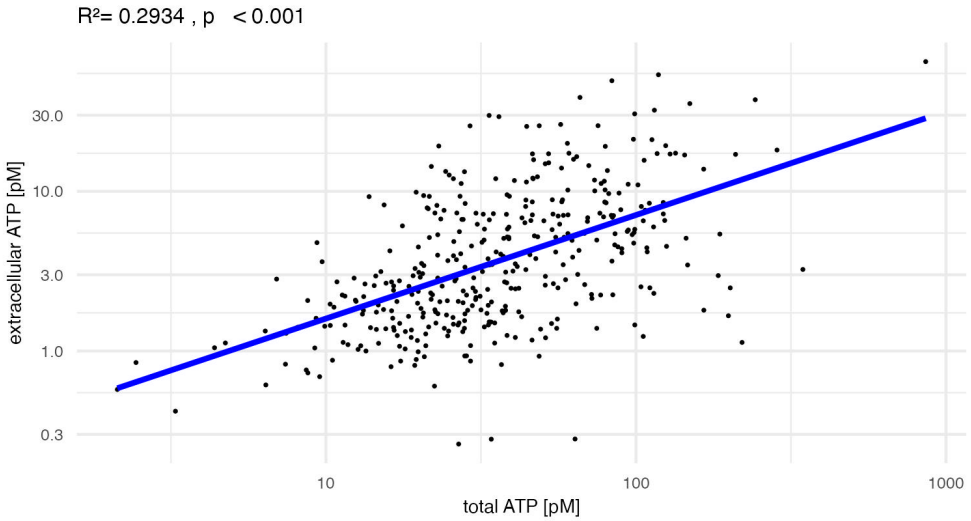


Figure 3.6: Relation of the measured extracellular and total ATP.

Extracellular ATP constituted a fraction of the total ATP, varying from 0.44% to 89%. The median contribution was 11%, with the 10%–90% percentiles ranging from 4.3% to 33%. An accumulation of higher extracellular ATP ratios was observed for samples with low intracellular ATP concentrations (Fig. 3.7).

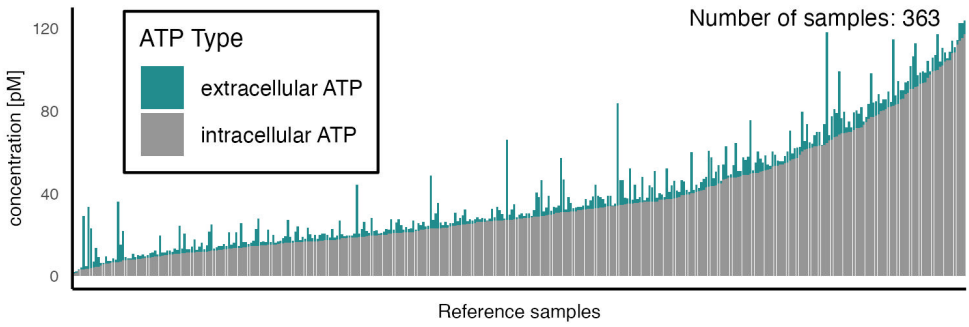


Figure 3.7: Composition of total ATP. The portions of intracellular and extracellular ATP are shown for the majority (95% percentile) of the reference samples.

3.2.5 Seasonal dynamic

To investigate seasonal effects on microbiological variables, sampling sites that were sampled in both spring and autumn were compared. A Wilcoxon signed rank test revealed statistically significant differences for TCC (V = 2481, p = 5.8e-05), with median values of 5.7 x 10^6 cells L^{-1} in spring and 6.1 x 10^6 cells L^{-1} in autumn. No statistically significant seasonal difference was found for HNAP (V = 3610, p = 4.1e-02), with median values of 48% in spring and 44% in autumn (Fig. 3.8) and intracellular ATP (V= 430, p = 0.8), with median values of 29 pM in spring and 30 pM in autumn.

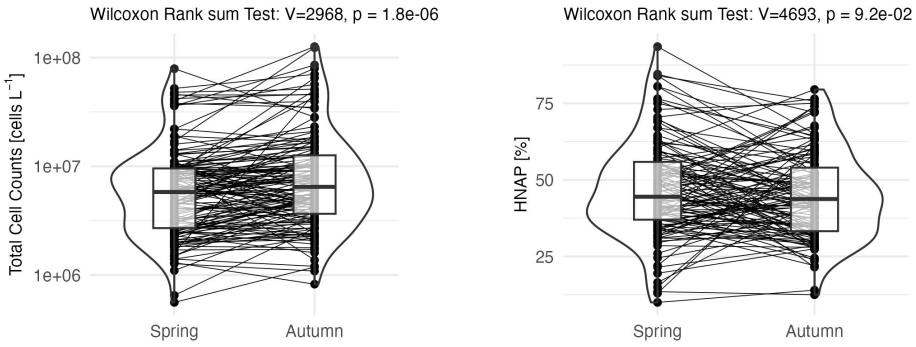


Figure 3.8: The effect of seasons on TCC and HNAP.

Apart from assessing overall seasonal effects, the magnitude of seasonal changes was calculated. For each pair of samples, the difference between seasons was computed. The dynamics of these changes were analyzed for the distinct aquifer types to account for potential geological-structural influences (for further information towards the aquifer types see 3.3.2). Non-reference sites were included in the analysis to compare their seasonal

dynamics with those observed at the reference sites.

TCC

To investigate a potential seasonal effect on TCC, samples from 253 reference sites that were sampled in both seasons were used. The dynamic of TCC was calculated as: $\log10(TCC_{autumn})$ - $\log10(TCC_{spring})$. This difference represents the relative increase or decrease in order of magnitudes. Values around +1 stand for an increase in cell numbers from spring to autumn by one order of magnitude, while a value of -1 indicates a decrease of cell numbers by one order of magnitude.

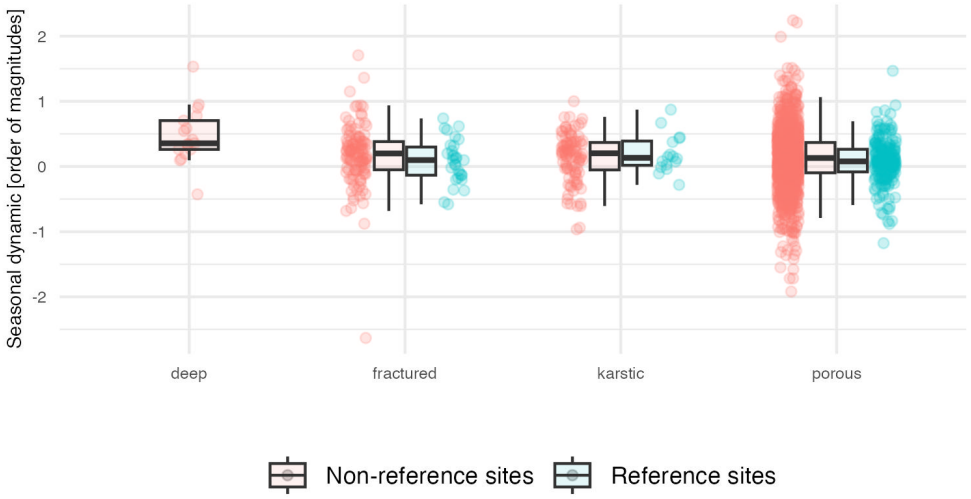


Figure 3.9: Seasonal changes of TCC from spring to autumn.

Throughout all reference sites, a median increase of 0.0796 order of magnitudes was observed for TCC, representing a 1.2-fold increase of TCC from spring to autumn. The 10%–90% percentile ranged from a 0.5-fold decrease to a 3.1-fold increase in TCC. An overview of the seasonal dynamics of TCC for the distinct aquifer types is provided in Fig. 3.9.

In **fractured** aquifers (reference sites) the median seasonal dynamic was also a 1.3-fold increase, with the 10%–90% percentile ranging from a 0.4-fold decrease to a 3.7-fold increase in TCC. In **karstic** aquifers (reference sites), the median seasonal dynamic was a 1.4-fold increase, with the 10%–90% percentile ranging from a 0.8-fold decrease to a 3.6-fold increase. In **porous** aquifers (reference sites), the median seasonal dynamic was a 1.2-fold increase of TCC, with the 10%–90% percentile ranging from a 0.5-fold decrease to a 3-fold increase in TCC. The highest median value of seasonal TCC changes was observed for **deep** aquifers, showing a 2.3-fold increase of TCC from spring to autumn.

ATP

For the assessment of seasonal effects on intracellular ATP, samples from 87 reference sites that were sampled in both seasons were used. The dynamic of ATP was calculated in absolute changes as: ATP_{autumn} - ATP_{spring}. Across all reference sites, a median increase of 4.4 pM was observed for intracellular ATP. The 10%–90% percentile ranged from a decrease of -48.1 pM to an increase of 81.2 pM. An overview of the seasonal dynamics of intracellular ATP for the distinct aquifer types is provided in Fig. 3.10.

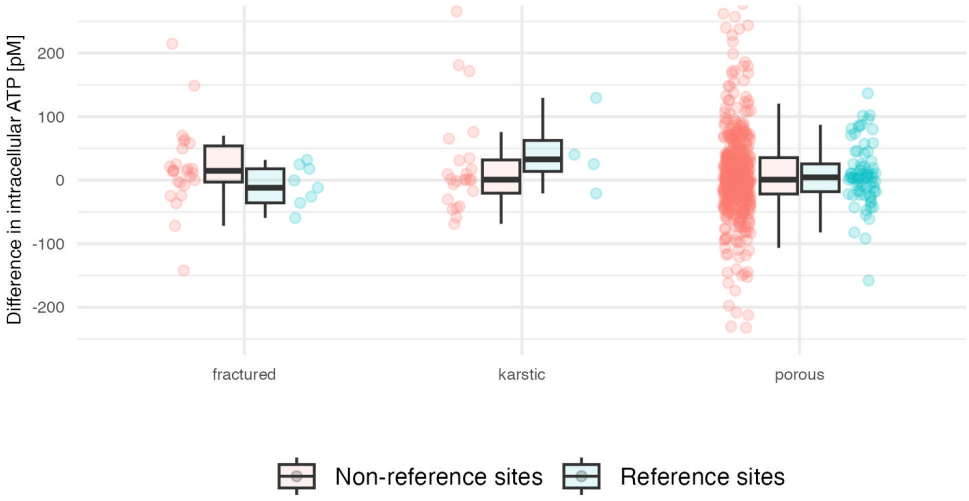


Figure 3.10: Seasonal changes of intracellular ATP from spring to autumn.

In **fractured** aquifers, the median seasonal dynamic of intracellular ATP was a decrease of -12 pM, with the 10%–90% percentile ranging from -200 to 26 pM. Changes in intracellular ATP were observed to go up to several hundred pM between the different seasons (reference sites). The largest dynamic (not displayed in Figure 3.10) was a decrease of -761 pM from spring to autumn in a fractured aquifers. For **karstic** aquifers, the median seasonal dynamic of intracellular ATP could not be assessed due to an insufficient number of paired samples from reference sites. In **porous** aquifers (reference sites), the median seasonal dynamic of intracellular ATP was an increase of 4.4 pM with the 10%–90% percentile ranging from -44.7 to 82.1 pM between the seasons. The highest dynamic registered for non-reference sites was an increase of more than 2158 pM (not displayed in FIGURE 3.10). There were not enough intracellular ATP data points for **deep** aquifers to calculate any reasonable statistics.

HNAP

For the assessment of seasonal effects on HNAP, samples from 215 reference sites that could be analyzed for both seasons were considered. The seasonal changes of HNAP were calculated in absolute values as: $HNAP_{autumn}$ - $HNAP_{spring}$. Across all reference sites, a median decrease of -4% was observed for HNAP. The 10%–90% percentile ranged from a -23.5% decrease to a 16.1% increase in HNAP. An overview of the seasonal dynamics of HNAP for the distinct aquifer types is provided in Fig. 3.11.

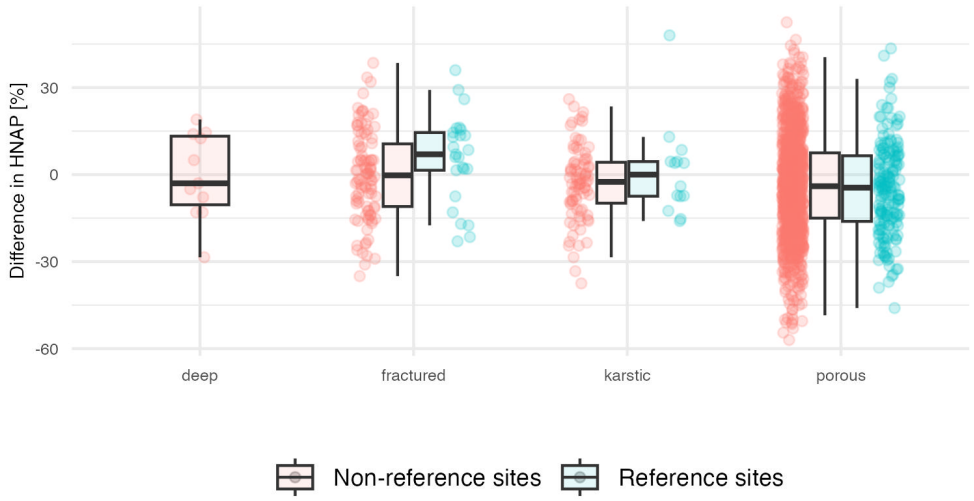


Figure 3.11: Seasonal changes of HNAP from spring to autumn.

In **fractured** aquifers (reference sites), the median dynamic was an increase of 7%, with the 10%–90% percentile values ranging from a -17.3% decrease to a 22.1% increase. In **karstic** aquifers (reference sites), the median dynamic was 0%, with the 10%–90% percentile ranging from a -14.5% decrease to a 11.7% increase in HNAP from spring to autumn. In **porous** aquifers (reference sites), the median dynamic of HNAP was a decrease of -4.5%, with the 10%–90% percentile ranging from a -25.8% decrease to a 15% increase of HNAP from spring to autumn. For **deep** aquifers, the median dynamic of HNAP was a decrease of -3% with the 10%–90% percentile ranging from a -13% decrease to a 14.5% increase of HNAP from spring to autumn.

Extreme values for HNAP changes were around 50% between the seasons. For the reference sites, extreme values ranged from -46% to +48%, while at non-reference sites extreme values ranged from -57% to +52.5%.

3.3 Environmental drivers of microbial variables

The following section presents the results of the investigation into how specific environmental factors influence the microbiological variables. The influence of nine distinct factors, each with multiple levels, was assessed in detail. The factors were grouped as follows: (1) Natural Factors: Geological formations and aquifer types, are generally not influenced by human activities. (2) Mixed Factors: Groundwater monitoring station types, oxygen conditions, DOM-Clusters, water types and land cover can be shaped by natural and anthropogenic influences. (3) Human-made Factors: Well types and sampling types, are predominantly defined by human protocols or activities.

For all factors the normal distribution within groups and homogeneity of variances (homoscedasticity) were assessed. The Shapiro-Wilk test indicated non-normal distribution for most groups, while the Levene test similarly revealed unequal variances for the majority of groups. Additionally, the sample sizes varied significantly, ranging from fewer than 10 to more than 500 samples. To ensure comparability and to accommodate the violation of assumptions (non-normality, heteroscedasticity, and unequal sample sizes) the Kruskal-Wallis rank test was selected for all factors (Kruskal and Wallis, 1952). Although less sensitive than parametric tests, it provides a robust method for initial comparison. The η^2 (eta squared) statistic was calculated to compare the effect sizes across factors (Tomczak and Tomczak-Łukaszewska, 2014). An overview of the conducted tests is provided in Table 3.3, showing the results for all factors where a subsequent Dunn post-hoc test identified significant differences between the distinct levels of each factor. A more detailed analysis of each factor is presented in the subsequent pages.

Table 3.3: Overview of Kruskal-Wallis test results for all factors.

	TCC (n=545)		ATP (n=357)		HNAP (n=498)	
	p	η^2	p	η^2	p	η^2
Geological formation	***	mod.	-	-	***	mod.
Aquifer types	**	small	-	-	-	-
Station Type	***	mod.	-	-	***	mod.
Oxygen condition	***	mod.	-	-	-	-
DOM Cluster	***	small	-	-	-	-
Water type	-	-	**	small	***	small
Land cover	***	mod.	-	-	-	-
Type of Well	***	mod.	-	-	-	-
Type of sampling	***	mod.	-	-	-	-

η^2 effect sizes are $0.01 - 0.06$ (small), $0.06 - 0.14$ (moderate) and ≥ 0.14 (large effect)

p values are $0.01 - 0.05$ (*), $0.01 - 0.1$ (**)and < 0.01 (***)

3.3.1 Geological formation

For the determination of natural background values of nutrients, trace elements and selected metals in Austrian groundwaters, Brielmann et al. (2018) used 52 hydrogeological consistent classes. This classification system was primarily based on lithological and petrographic characteristics, while also considering peculiarities of stratigraphic members and the catchment areas of Quaternary sediments. The geological classes were further grouped into 11 geological formations, representing the next higher classification unit.

For most individual classes, the number of samples that remained after selecting for hydrochemical natural conditions was insufficient to calculate meaningful statistics. Therefore, the next higher classification units, the geological formations, were used to investigate the effect of geology on the microbiological variables. The following list provides an overview of the 11 geological formations present in the dataset, along with the number of classes per formation:

- **A** - Crystalline rocks of the Bohemian Massif (4 classes)
- **B** - Tertiary sediments (4 classes)
- **C** - Quaternary sediments (18 classes)
- **D** - Sediments of the Mesozoic and Permian (6 classes)
- **E** - Rocks of the Flysch zone (1 class)
- **G** - Low to non-metamorphic, old Paleozoic rocks (4 classes)
- **H & I** - Eastern Alpine crystalline rocks (4 classes)
- **J** - Helveticum and cliffs of the Waschberg-zone (1 class)
- **K** - Penninic of the central zone (3 classes)
- **L** - Subpenninic (2 classes)
- **M** - Tertiary magmatites (1 class)

Even at the higher classification unit (i.e., the geological formations), there were not enough samples to calculate any reference statistics for groups, like (L) the Subpenninic, (M) the Tertiary magmatites and (K) the Penninic of the central zone. For (E) the Rocks of the Flysch zone the sample size was also critically small. Further details regarding the characteristics of individual formations can be found in Brielmann et al. (2018).

TCC

The Kruskal-Wallis test revealed a significant effect of the geological formations on Total Cell Counts in groundwater ($\chi^2_{Kruskal-Wallis}$ = 63.4, df = 9, p = 2.92e-10). A Dunn post-hoc test identified TCC in (D) Sediments of the Mesozoic and Permian to differ significantly from the (A) Bohemian Massif (p = 3.69e-03), (G) Low to non-metamorphic, old Paleozoic rocks (p = 1.57e-03) and (H & I) the Eastern Alpine crystalline rocks (p=1.25e-08) as well as the (C) Quarternary sediments to differ significantly from the (H & I) Eastern Alpine crystalline rocks (p = 4.51e-07).

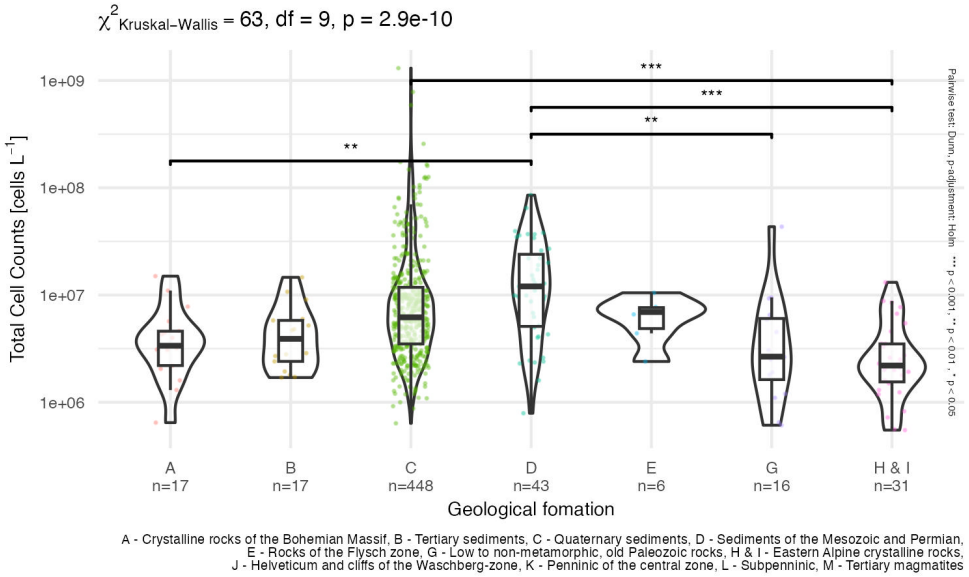


Figure 3.12: Total Cell Counts across different geological formations.

The largest difference in median values of TCC was between (D) the Sediments of the Mesozoic and Permian (1.2 x 10^7 cells L^{-1}) and (H & I) the Eastern Alpine crystalline rocks (2.2 x 10^6 cells L^{-1}) which is a 5.5 fold difference (Fig. 3.12).

ATP

The Kruskal-Wallis test revealed no significant effect of the geological formations on intracellular ATP in groundwater (p >0.05).

HNAP

The Kruskal-Wallis test revealed a significant effect of the geological formations on HNAP in groundwater ($\chi^2_{Kruskal-Wallis}$ = 50.6, df = 9, p = 8.4e-08). A Dunn post-hoc test identified HNAP in (A) the Bohemian Massif to differ significantly from (B) Tertiary sediments (p = 4.79e-05), (C) Quarternary sediments (p = 2.61e-08), (D) Sediments of the Quarternary and Permian (p = 2.21e-05), (G) Low to non-metamorphic, old Paleozoic rocks (p = 2.52e-06), and (H & I) the Eastern Alpine crystalline rocks (p=6.27e-03).

The median HNAP was the lowest in (A) the Bohemian Massif, with 17% and the highest in (G) Low to non-metamorphic, old Paleozoic rocks, with 52% (Fig. 3.13).

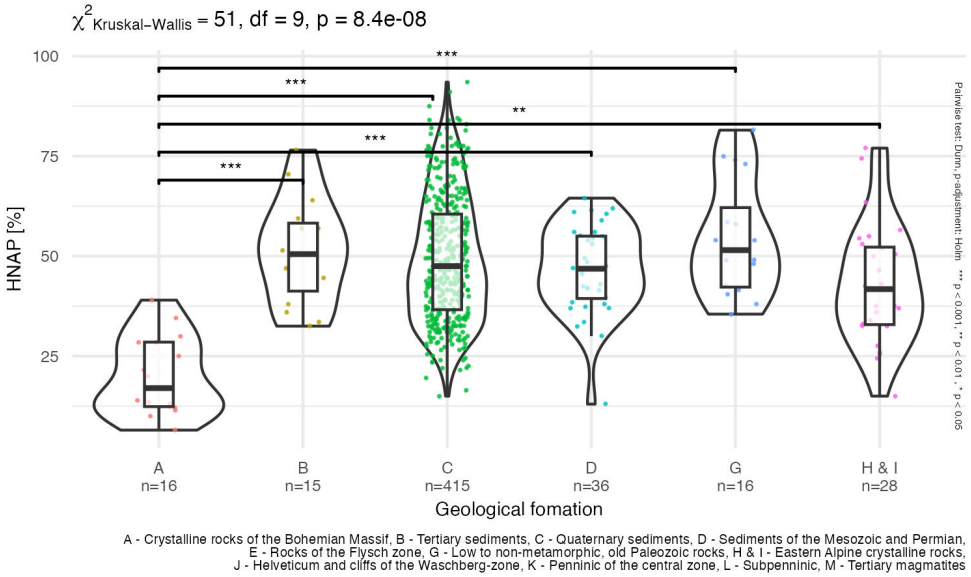


Figure 3.13: The proportion of High Nucleic Acid content cells across different geological formations.

3.3.2 Aquifer type

A hydrogeological classification system for aquifers is used to assess the effect of structural-geological features on microbiological variables. Three different types of shallow groundwater are defined in the Annex of the Austrian Water Methods Regulation (Methodological guidelines in the field of chemistry for wastewater, surface water, and groundwater - MVW, 2019).

- **Karstic** groundwater: "Groundwater in the cavities of karstified solid rocks."
- **Fractured** groundwater: "Groundwater in the fissures of non-karstified solid rocks."
- **Porous** groundwater: "Groundwater in unconsolidated or solid rocks, whose effective flow cavities are predominantly formed by pores."

To assign the sampling sites to distinct aquifer types, the map "INSPIRE Hydrogeologische Einheiten - Aquifer 1:500 000 Österreich", 2021 was used. Additional information about the geological classes (Brielmann et al., 2018) was employed to resolve ambiguous assignments.

A fourth type of groundwater is defined in the water methods regulation (MVW, 2019), based on characteristics other than structural-geological features:

- **Deep** groundwater: "Groundwater in the deeper layers of the Earth's crust, which is extensively overlain by cover layers, has a long residence time, and usually possesses special physical and chemical properties."

Despite the nomenclature, the various definitions of deep groundwater (german: Tiefen-

grundwasser) do not include explicit criteria regarding the depth (Schubert, 2015). In some cases the water-bearing layers may even lie close to the surface (Brielmann et al., 2018). Since natural background values were not determined for deep groundwater in Austria, these samples were not included in the reference dataset. Nevertheless, in this study the microbiological variables were measured for all deep groundwater samples. Only in the subsequent evaluation of the aquifer types, deep groundwater samples were included. Deep groundwater samples were not filtered for natural hydrochemical conditions.

TCC

The Kruskal-Wallis test showed a significant effect of the aquifer types on Total Cell Counts ($\chi^2_{Kruskal-Wallis}$ = 72.8, df = 3, p = 1.1e-15). A Dunn post-hoc test identified TCC in fractured groundwater to differ significantly from karstic (p = 2.56e-11), porous (p = 1.94e-08) and deep (p = 2.46e-12) but also porous from karstic (p = 7.04e-04) as well as deep (p = 1.07e-04) groundwater (Fig. 3.14).

Looking at the reference dataset, the largest difference in median TCC values was between karstic (1.2 x 10^7 cells L^{-1}) and fractured (2.7 x 10^6 cells L^{-1}) groundwater, representing a 4.6 fold difference. The difference between deep (2.1 x 10^7 cells L^{-1}) and fractured (2.7 x 10^6 cells L^{-1}) groundwater was even higher, representing a 7.7 fold difference.

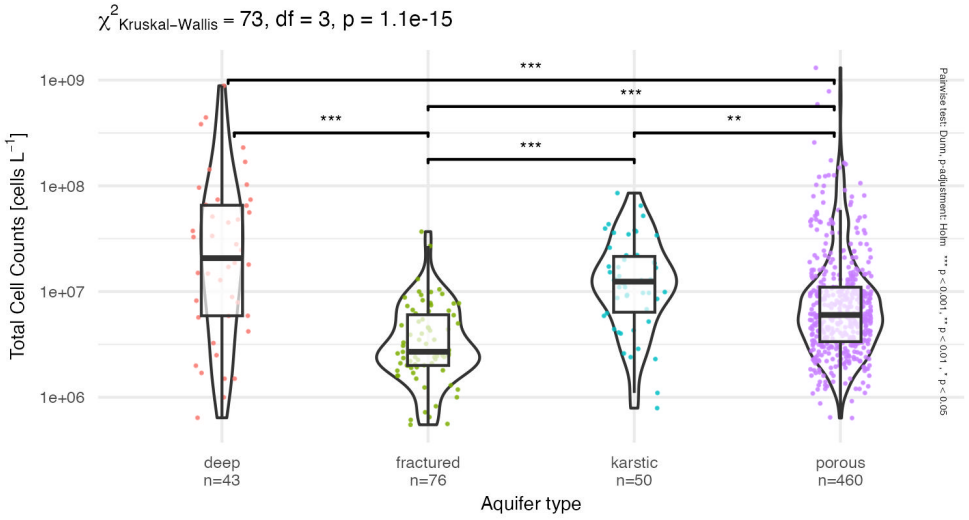


Figure 3.14: Total Cell Counts across different aquifer types.

ATP

Including samples from deep aquifers revealed a significant effect of the aquifer types on intracellular ATP concentrations ($\chi^2_{Kruskal-Wallis}$ = 7.65, df = 3, p = 5.38e-02). A Dunn

post-hoc test identified intracellular ATP in deep groundwater, with a median of 66 pM to differ significantly (p = 4.9e-02) from karstic groundwater samples, with a median 26 pM concentration.

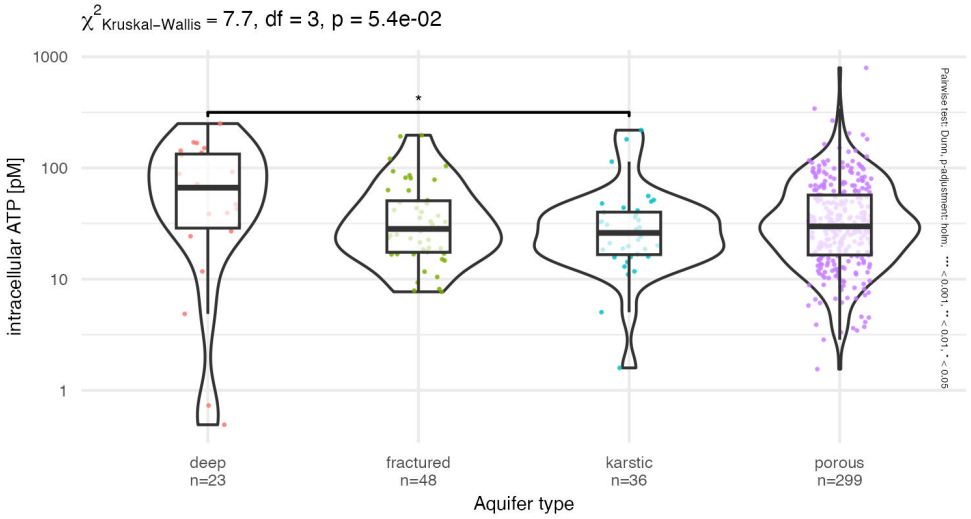


Figure 3.15: The concentration of intracellular ATP across different aquifer types.

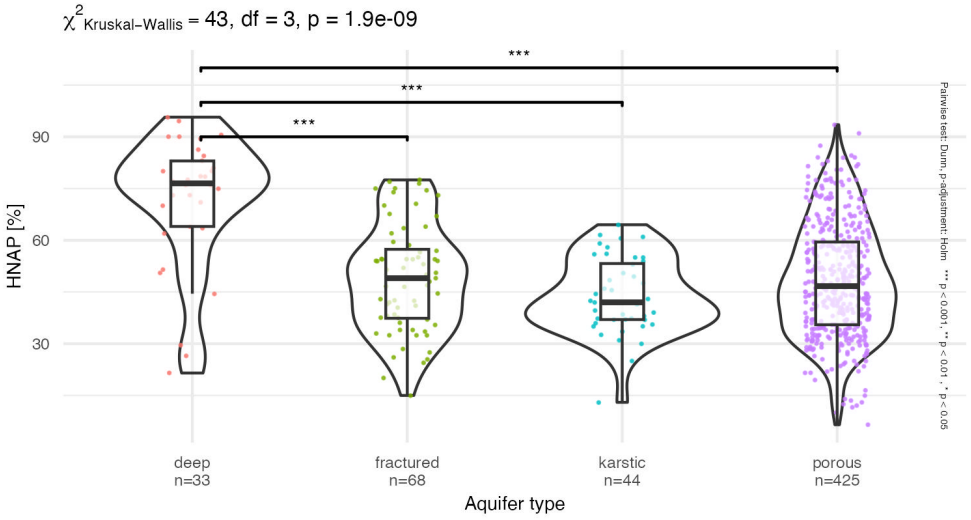


Figure 3.16: The proportion of High Nucleic Acid content cells across different aquifer types.

HNAP

By including deep groundwater samples, a significant effect of the type of groundwater on HNAP ($\chi^2_{Kruskal-Wallis}$ = 43.5, df = 3, p = 1.94e-09) was obtained. A Dunn post-hoc test identified HNAP in deep groundwater to differ significantly from fractured (p = 1.3e-06), karstic (p = 9.68e-09) and porous (p = 2.6e-09) groundwater.

The median value of HNAP was highest in groundwater derived from deep aquifers, with 76% and lowest from karstic aquifers, with 42% (Fig. 3.16).

3.3.3 Water type

Major ions, which include the cations calcium, magnesium, sodium, and potassium, as well as the anions bicarbonate, sulfate, chloride, and nitrate, constitute 99% of the dissolved constituents in groundwater (Kunkel et al., 2004). Based on the composition of these major ions, seven hydrochemically distinct water types were described for Austria (BMLFUW, 2017). The water types in this system are primarily distinguished by the concentrations of elements in the first group of the periodic table—earth-alkaline metals: calcium and magnesium—and those in the second group—alkaline metals: sodium and potassium (Table 3.4). Natural factors shaping the different compositions include the solubility of the water-containing rocks and groundwater residence time.

Shallow groundwater in Austria was predominantly classified as water type 1 (earth-alkaline - bicarbonatic), which is characteristic of groundwater with short renewal times. As circulation slows, the proportion of bicarbonate decreases while other anions, such as sulfate, chloride, and nitrate, increase. According to this, a succession from water type 1 (earth-alkaline - bicarbonatic), through types 2 and 3 (earth-alkaline - (bicarbonatic) - sulphatic), to water type 5 (earth-alkaline - alkaline - sulphatic) with increasing residence time has been described (Kralik et al., 2005).

Water types 4 and 5 (earth-alkaline alkaline) typically occur in crystalline rock formations but can also evolve from water type 1 when influenced by alkaline sources, such as the influx of saline water (e.g., salt deposits) or human activities like fertilizer application or the use of road salt in winter (Kralik et al., 2005).

Water type 1 (earth-alkaline bicarbonatic) was the predominant type in the reference dataset. Alkaline water types 6 and 7 were not represented in the reference dataset. Water type 6, characterized by elevated sodium concentrations, may occur due to the influence of deep groundwater or in regions that were once brackish lakes, such as the Pannonian region in eastern Austria (BMLFUW, 2017). In addition to sodium, the chloride concentration is also elevated in water type 7. It has been described in Austria as occurring only after intense application of road salt (BMLFUW, 2017).

TCC

The Kruskal-Wallis test revealed no significant effect of the water types on TCC in groundwater (p >0.05).

Table 3.4: Hydrochemical water types defined by Furtak and Langguth, 1967.

	Hydrochemical water type	Characteristic	
		HCO_3	SO_4+Cl+NO_3
	Earth-alkaline (Ca + Mg >80 %; Na + K <20 %)		
1	Earth-alkaline – bicarbonatic	>60%	<40%
2	Earth-alkaline - bicarbonatic - sulphatic	40-60%	40-60%
3	Earth-alkaline - sulphatic	<40%	>60%
	Earth-alkaline-Alkaline (Ca + Mg 50-80 %; Na + K = 20–50 %)		
4	Earth-alkaline - Alkaline - bicarbonatic	>50%	<50%
5	Earth-alkaline - Alkaline - sulphatic	<50%	>50%
	Alkaline (Ca + Mg <50 %; Na + K >50 %)		
6	Alkaline - bicarbonatic	>50%	<50%
7	Alkaline - sulphatic	<50%	>50%

ATP

A Kruskal-Wallis test revealed a significant effect of the water types on concentrations of intracellular ATP in groundwater samples of the reference dataset ($\chi^2_{Kruskal-Wallis}$ = 14.9, df = 4, p = 4.9e-03).

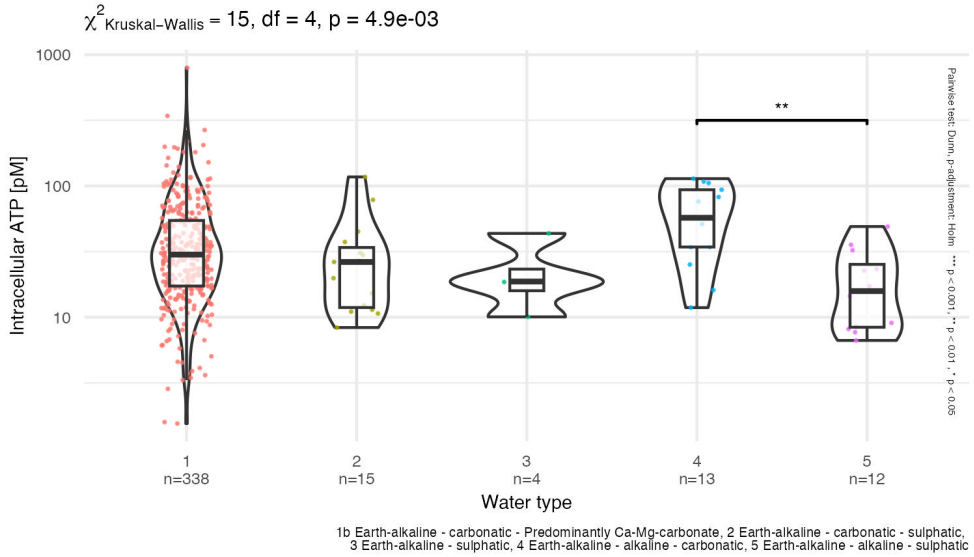


Figure 3.17: The concentration of intracellular ATP across different water types.

A Dunn post-hoc test identified the median concentration of intracellular ATP in water type 4 to differ significantly from that in type 5 (p = 5.4e-03). The median concentration of intracellular ATP was the lowest in water type 5, with 16 pM and the highest in water type 4, with 57 pM (Fig. 3.17).

HNAP

A significant effect of the water types on HNAP in groundwater samples of the reference dataset was observed ($\chi^2_{Kruskal-Wallis}$ = 22, df = 3, p = 6.6e-05). A Dunn post-hoc test identified HNAP in water type 1 to differ significantly from type 4 (p = 7.2e-03) and type 5 (p = 3e-03). Median HNAP was the highest in water type 2, with 47 % and the lowest in water type 5, with 26 % (Fig. 3.18).

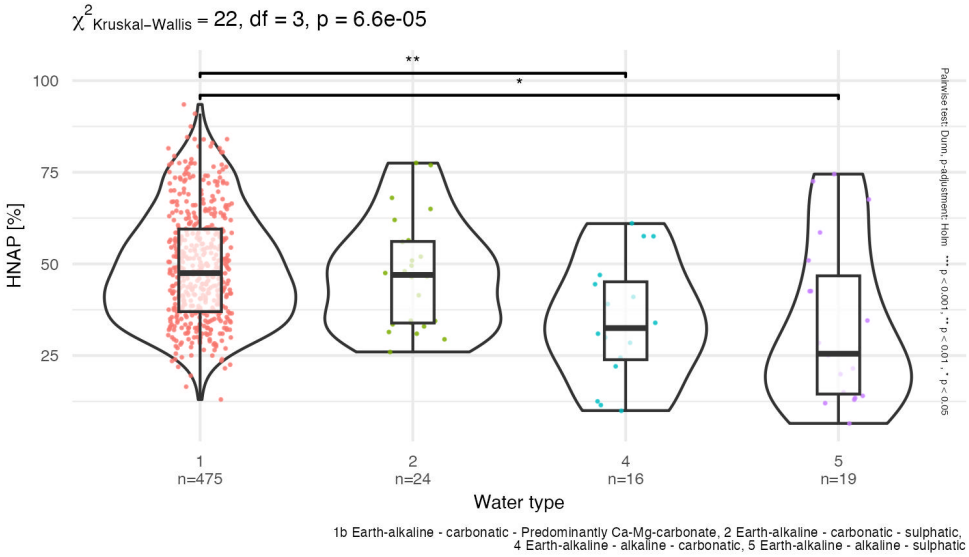


Figure 3.18: HNAP across different water types.

3.3.4 Groundwater monitoring station type

The groundwater monitoring station types were determined for the governmental report: "Nitrate in Soil, Surface Water and Groundwater in Austria (BMLRT, 2020)". Three types of groundwater were described: unconfined, confined, and karstic & fissured groundwater. Unconfined (phreatic) groundwater was further divided into four levels based on the groundwater table, resulting in the following classification levels:

- **0**: Unconfined groundwater 0-5 m (inclusive phreatic springs)
- **1a**: Unconfined groundwater > 5-15 m
- **1b**: Unconfined groundwater > 15-30 m
- **1c**: Unconfined groundwater > 30 m
- **2**: Confined groundwater
- **3**: Karstic and fissured groundwater (inclusive karstic springs)

TCC

A significant effect of the groundwater monitoring station type on TCC in groundwater ($\chi^2_{Kruskal-Wallis}$ = 62.7, df = 5, p = 3.3e-12) was observed. A Dunn post-hoc test identified TCC in 0 (unconfined groundwater, 0-5 m) to differ significantly from 1a (unconfined groundwater > 5-15 m, p = 1.6e-03), 1b (unconfined groundwater > 15–30 m, p = 7.2e-09), 1c (unconfined groundwater > 30 m, p = 2.6e-04), 2 (confined groundwater, p = 2.2e-04) and 3 (karstic and fissured groundwater, p=7.7e-08) as well as 1a (unconfined groundwater > 5–15 m) from 1b (unconfined groundwater > 15-30 m, p = 5.9e-03). The largest difference in median TCC values was observed between 0 (unconfined groundwater, 0-5 m) with 9.3 x 10^6 cells L^{-1}, and the station type 1b (unconfined groundwater > 15-30 m) with 3.6 x 10^6 cells L^{-1}, representing a 2.6-fold difference (Fig. 3.19).

ATP

The Kruskal-Wallis test revealed no significant effect of the station type on intracellular ATP in groundwater (p >0.05).

HNAP

A significant effect of the station type on HNAP in groundwater ($\chi^2_{Kruskal-Wallis}$ = 43.7, df = 5, p = 2.7e-08) was observed. A Dunn post-hoc test identified HNAP in 1c (unconfined groundwater > 30m) to differ significantly from 0 (unconfined groundwater, 0-5 m; p = 3.8e-07), 1a (unconfined groundwater > 5-15 m; p = 7e-05), 2 (confined groundwater, p = 1.6e-05) and 3 (karstic and fissured groundwater, p = 1.2e-05). Additionally 1b (unconfined groundwater > 15-30 m) was found to differ significantly from 0 (unconfined groundwater 0-5 m, p = 6e-03) and 2 (confined groundwater, p = 2.1e-02). HNAP was lowest in 2 (confined groundwater), with 40%, and highest in 1c (unconfined groundwater > 30 m), with 65% (see Fig 3.20).

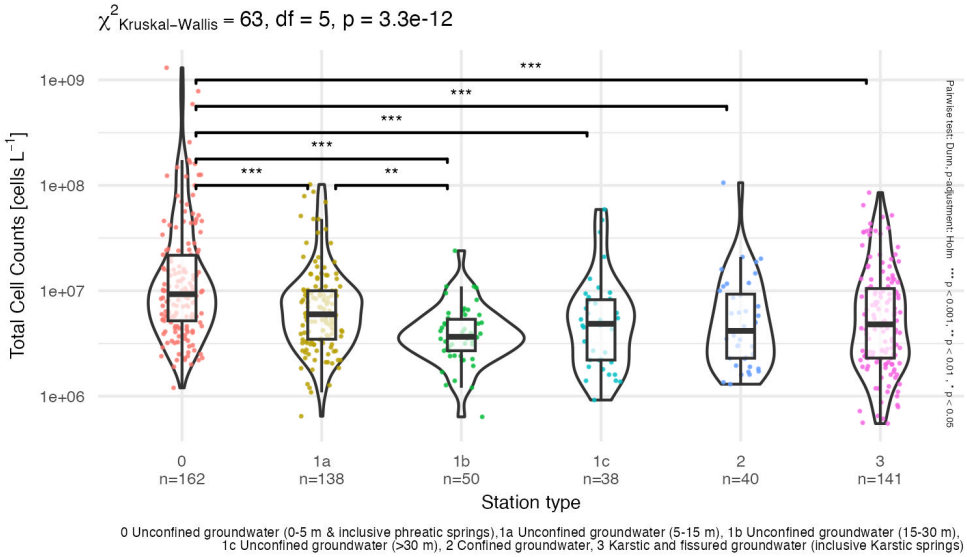


Figure 3.19: Total Cell Counts across different groundwater monitoring station types.

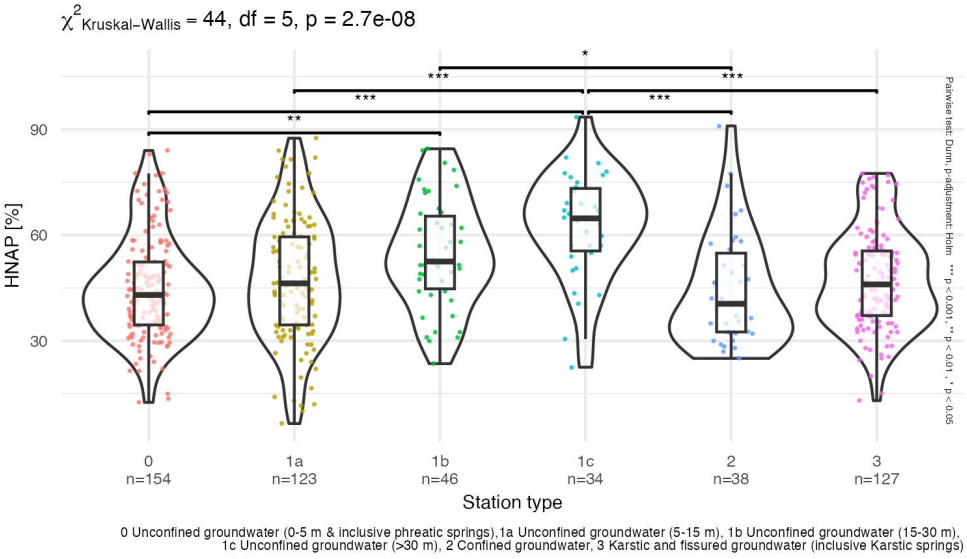


Figure 3.20: HNAP across different groundwater monitoring station types.

3.3.5 DOM Cluster

Organic matter, originating from excretions or the remains of organisms (microbes, plants, fungi, and animals), can occur in both particulate and dissolved forms. In the water column of inland waters, dissolved organic matter (DOM) is the predominant form (Tranvik and Von Wachenfeldt, 2014). DOM is a complex mixture of organic molecules composed primarily of carbon, hydrogen, oxygen, and other elements such as nitrogen, sulfur, and phosphorous (Hartnett, 2018). Various methods exist to analyze DOM, ranging from labour- and cost-intensive techniques, like Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) and nuclear magnetic resonance (NMR) spectroscopy, to more accessible and affordable options like absorbance and fluorescence spectroscopy.

Unlike dissolved organic carbon (DOC), DOM is not routinely assessed, limiting our understanding of both the quantity and quality of DOM in groundwater. During the course of this study, the analysis of DOM was included as a further parameter. Harjung et al. (2023) evaluated groundwater samples from the autumn sampling campaign in 2019. The composition of DOM was analyzed based on the characteristics obtained by fluorescence spectroscopy. An unsupervised cluster analysis revealed six distinct clusters for DOM in Austrian groundwaters:

- **Cluster 1:** Associated with intense land use and higher amounts of nitrate.
- **Cluster 2:** Typically found in the proximity of surface waters or in wells situated in alluvial sediments with lower nitrate levels.
- **Cluster 3 and 4:** Remain undefined and require further research.
- **Cluster 5:** Linked to areas with high precipitation in the karstic Alps and surface water from natural forested regions.
- **Cluster 6:** Located at the highest altitudes, in fissured rocks of the central Alps and characterized by low DOC values.

TCC

A significant effect of the DOM Clusters on Total Cell Counts in groundwater ($\chi^2_{Kruskal-Wallis}$ = 29.3, df = 5, p = 2e-05) was observed. A Dunn post-hoc test identified TCC in DOM Cluster 5 to differ significantly from Cluster 3 (p = 5.9e-04), Cluster 4 (p = 6.9e-03), and Cluster 6 (p = 8.5e-04). The largest difference for TCC was between Cluster 5 groundwater (1.2 x 10^7 cells L^{-1}) and Cluster 6 (3 x 10^6 cells L^{-1}) being an 4.2-fold difference (Fig. 3.21).

ATP

The Kruskal-Wallis test revealed no significant effect of the DOM Clusters on intracellular ATP in groundwater (p >0.05).

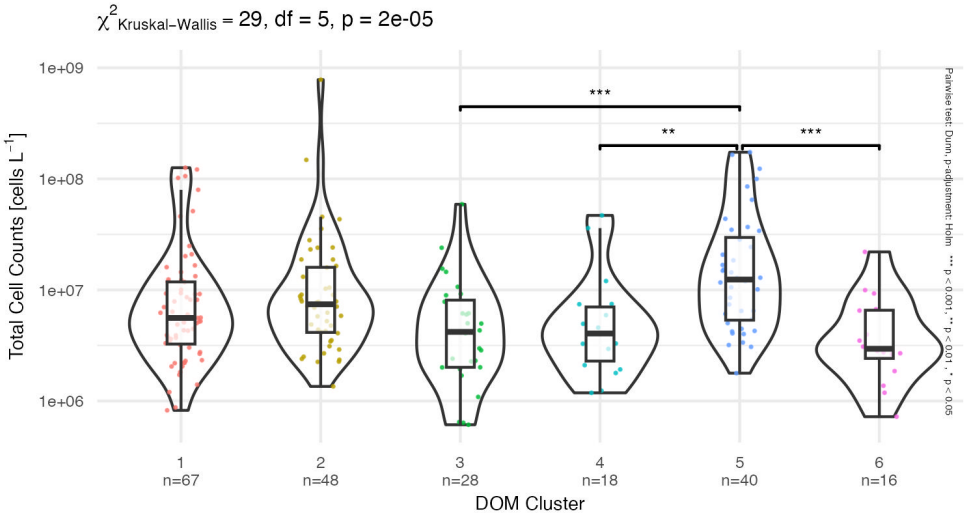


Figure 3.21: Total Cell Counts across different DOM Clusters.

HNAP

The Kruskal-Wallis test revealed no significant effect of the DOM Clusters on HNAP in groundwater ($p > 0.05$).

3.3.6 Oxygen Condition

The effect of dissolved oxygen (DO) was evaluated by assessing the differences in microbial variables across defined levels of oxygen conditions, which encompass environments that support either anaerobic or aerobic microbial processes. A third level was defined to indicate a potential transition zone between these two states. Additionally, the concentrations of bivalent iron and manganese were used as supporting indicators. These metals occur in their reduced forms only when oxygen is absent (Kunkel et al., 2004), making their occurrence useful for identifying anoxic conditions. The inclusion of bivalent iron (Fe^{2+}) and manganese (Mn^{2+}) was chosen to account for the potential unwanted introduction of oxygen during the pumping of groundwater samples. Threshold values for DO, Fe^{2+}, and Mn^{2+} were derived from Wolters et al. (2022):

- **Oxic**: DO concentration > 2 mg L^{-1}, supporting aerobic metabolisms.
- **Hypoxic**: DO concentration ≤ 2 mg L^{-1}, indicating the onset of nitrate reduction.
- **Anoxic**: DO concentrations ≤ 0.2 mg L^{-1} (the lowest LOQ). But also samples with DO concentrations up to 1 mg L^{-1} that contained more than 0.2 mg L^{-1} ferrous iron (Fe^{2+}) and/or more than 0.1 mg L^{-1} of reduced manganese (Mn^{2+}).

TCC

A significant effect of the oxygen conditions on TCC in groundwater ($\chi^2_{Kruskal-Wallis}$ = 59.3, df = 2, p = 1.3e-13) was observed. A Dunn post-hoc test revealed that TCC in anoxic groundwater differed significantly from that in hypoxic conditions (p = 2.9e-02) and oxic conditions (p = 5.3e-10). Additionally, TCC in hypoxic groundwater was significantly different from that in oxic conditions (p = 1.1e-05). The largest difference in TCC was observed between anoxic groundwater, with 5.7 x 10^7 cells L^{-1} and oxic groundwater, with 5.6 x 10^6 cells L^{-1}, representing a 10-fold difference (Fig. 3.22).

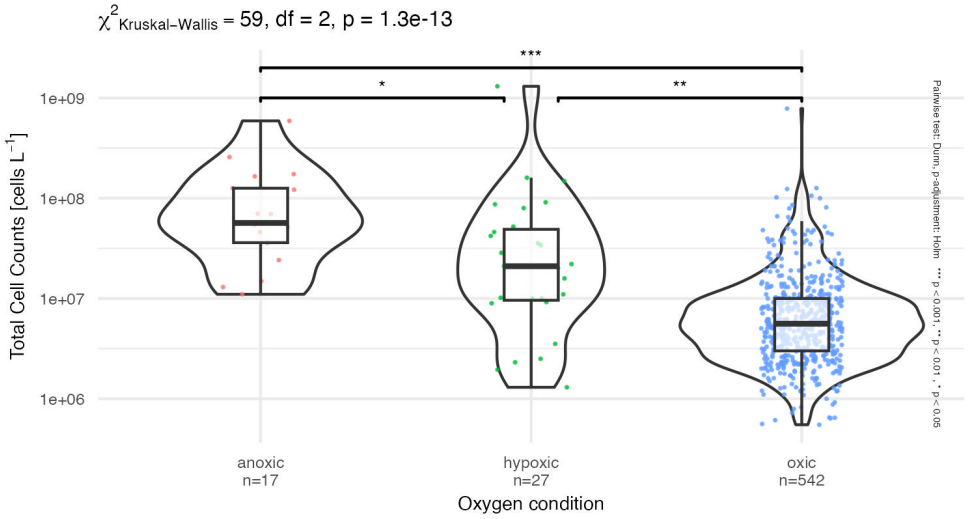


Figure 3.22: Total Cell Counts across different oxygen conditions.

ATP

The Kruskal-Wallis test revealed no significant effect of the oxygen conditions on intracellular ATP in groundwater (p >0.05).

HNAP

The Kruskal-Wallis test revealed no significant effect of the oxygen conditions on HNAP in groundwater (p >0.05).

3.3.7 Land cover

The CORINE (Coordination of Information on the Environment) Land Cover 2018 dataset provides a comprehensive overview of land cover patterns across Europe, based on satellite imagery. The CORINE Land Cover (CLC) nomenclature is structured in three hierarchical levels and 44 thematic classes (land cover patterns). For further information on these

classes and their definitions, the CLC 2018 nomenclature guidelines are available on: https://land.copernicus.eu/.

In Austria, specifically, 23 distinct land cover patterns have been identified. The reference dataset constitutes samples from 9 land cover patterns out of the first three main categories:

1. **Artificial surfaces:** (112) Discontinuous urban fabric; (121) Industrial or commercial units
2. **Agricultural areas**: (211) Non-irrigated arable land; (231) Pastures; (242) Complex cultivation patterns; (243) Land principally occupied by agriculture, with significant areas of natural vegetation
3. **Forest and semi-natural areas**: (311) Broad-leaved forest, (312) Coniferous forest, (313) Mixed forest

TCC

The Kruskal-Wallis test revealed a significant effect of the land cover patterns on TCC in groundwater ($\chi^2_{Kruskal-Wallis}$ = 37.8, df = 8, p = 8.3e-06). A subsequent Dunn post-hoc test identified TCC values in (312) coniferous forest to differ significantly from (311) broad leaved forest (p=3.4e-02), and (211) non-irrigated arable land (p=4.5e-05). Additionally, TCC in groundwater samples assigned to (231) pastures was significantly different from those assigned to (211) non-irrigated arable land (p=3e-04).

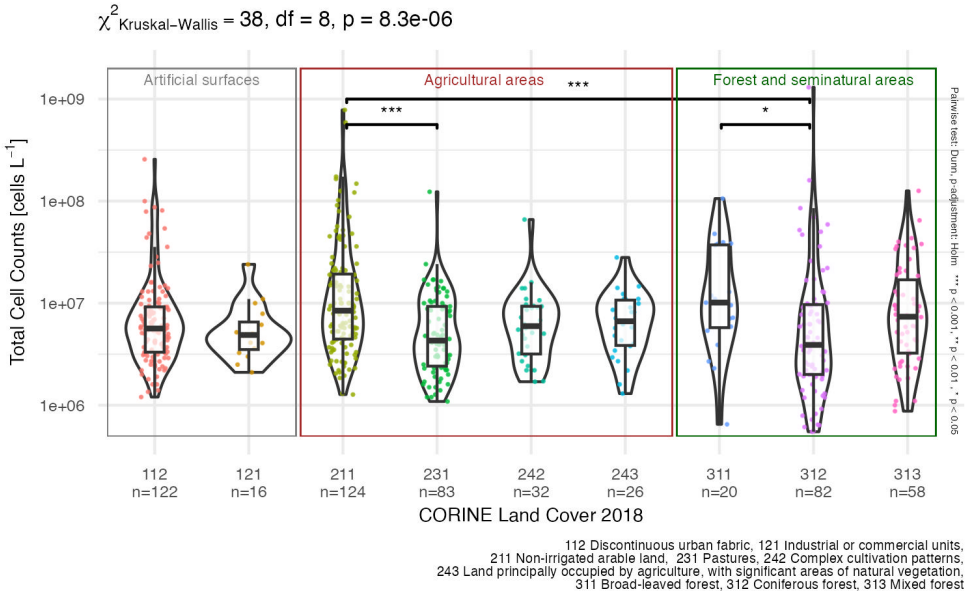


Figure 3.23: Total Cell Counts across different land cover patterns.

The largest difference in TCC was between (311) broad leaved forest, with the highest median value of 1.0 x 10^7 cells L^{-1} and (312) coniferous forest, with the lowest median value of 3.9 x 10^6 cells L^{-1}, representing a 2.6-fold difference between these two groups (Fig. 3.23).

ATP

The Kruskal-Wallis test revealed no significant effect of the land cover patterns on intracellular ATP in groundwater ($p > 0.05$).

HNAP

A significant effect of the land cover patterns on HNAP in groundwater ($\chi^2_{Kruskal-Wallis} = 21.2$, df = 8, p = 6.2e-03) was observed. A Dunn post-hoc test did not identify any significant effect of the types of land cover on HNAP (Fig. 3.24).

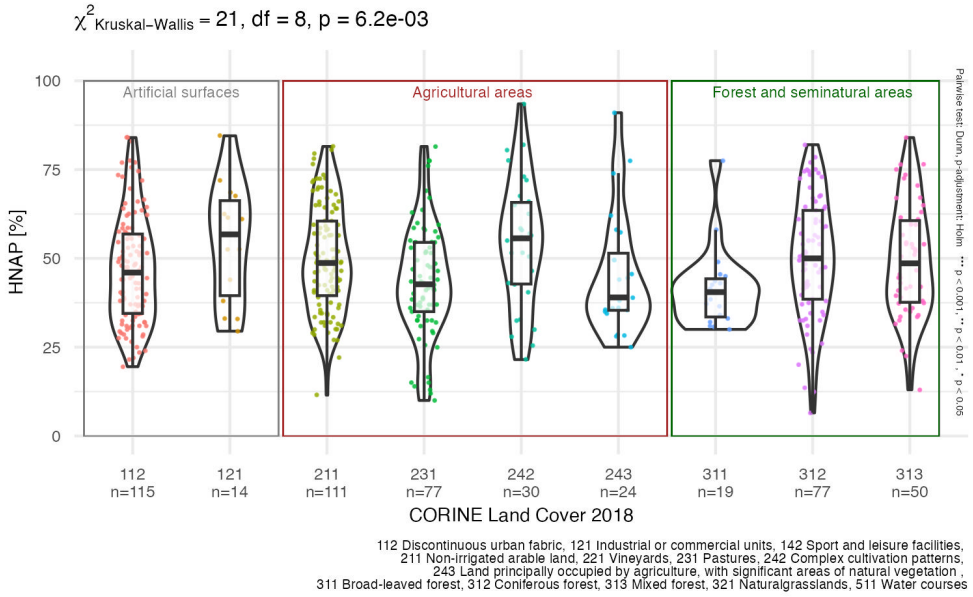


Figure 3.24: HNAP across different land cover patterns.

3.3.8 Wells and spring type

Eight different types of wells and springs have been defined by governmental state authorities. With the exception of untapped springs, all types of wells and springs were constructed or at least modified by humans. Both **untapped springs** and **tapped springs** are associated with karstic or fractured aquifers and some porous aquifers, while all **monitoring wells**, **drilled wells**, and **dug wells** correspond to porous aquifers.

TCC

A Kruskal-Wallis test revealed a significant effect ($\chi^2_{Kruskal-Wallis}$ = 76, df = 7, p = 9e-14) of the type of wells and springs on TCC in groundwater.

A subsequent Dunn post-hoc test identified TCC in groundwater samples derived from monitoring wells to differ significantly from drilled wells (p = 1.2e-08), tapped wells (p = 7.1e-14) and dug wells (p = 2.1e-04). Additionally, TCC in groundwater from tapped springs was significantly different from untapped springs (p=1e-03) and dug wells (p=6.5e-03).

The largest difference in TCC was between monitoring well with the highest median value of 9.8 x 10^6 cells L^{-1} and tapped springs with the lowest median value of 3.7 x 10^6 cells L^{-1}, representing a 2.7-fold difference between these two groups (Fig. 3.25).

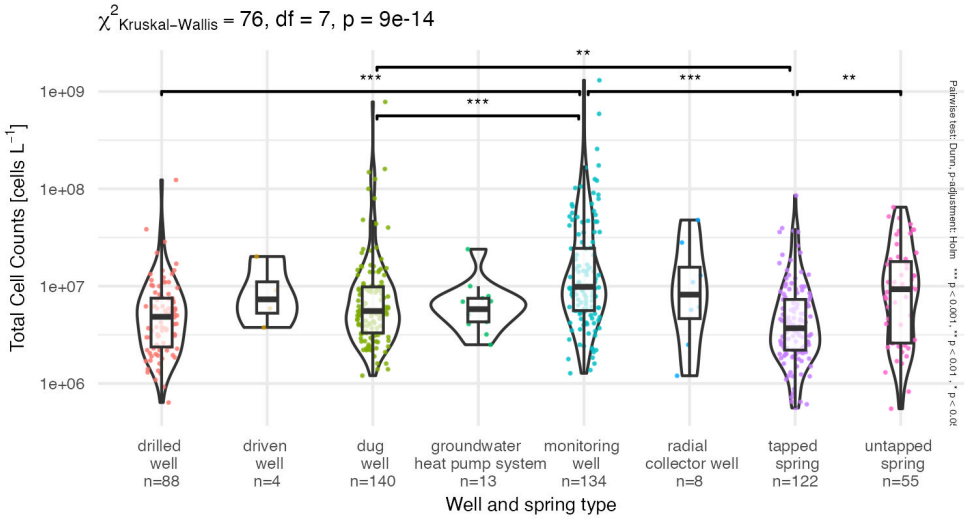


Figure 3.25: Total Cell Counts across different well and spring types.

ATP

The Kruskal-Wallis test revealed no significant effect of the type of wells and springs on intracellular ATP in groundwater (p >0.05).

HNAP

A significant effect of the type of wells and springs on HNAP in groundwater ($\chi^2_{Kruskal-Wallis}$ = 13, df = 6, p = 4.1e-02) was observed. However, a subsequent Dunn post-hoc test did not reveal any significant effect of the different types of wells and springs on HNAP (Fig. 3.26).

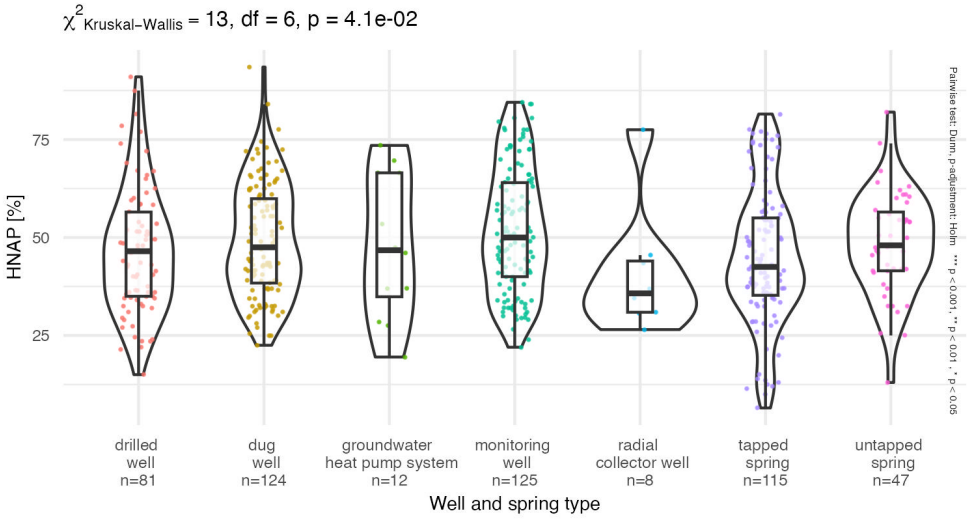


Figure 3.26: HNAP across different well and spring types.

3.3.9 Type of sampling

Based on applied international standards (ISO 5667-14) different types of sampling procedures were employed (BMLRT, 2015). Three main sampling procedures were applied depending on the type of well. For instance, **tap**-sampling was used when the necessary infrastructure was available. A **mobile pump** was employed for all types of open wells, while samples from springs are were taken directly. There are three levels of spring sampling—**direct spring sampling**, **spring overflow**, and **spring capture conduit**—which indicate the specific location at the spring from where the sample was collected.

TCC

A significant effect of the types of sampling on TCC in groundwater ($\chi^2_{Kruskal-Wallis}$ = 80.5, df = 4, p = 1.4e-16) was observed. A Dunn post-hoc test identified TCC in groundwater sampled by a mobile pump to differ significantly from tap (p = 7.7e-12), spring capture conduit (p = 1.3e-10), and spring overflow (p = 3.7e-07) sampling. Direct spring sampling differed significantly from spring capture conduit (p=2.6e-04), tap (p=2.4e-02), and spring overflow (p=2.1e-02) sampling. The largest difference in TCC was between the samples derived from sampling with a mobile pump, with the highest median value of 9.7 x 10^6 cells L^{-1}, and direct sampling at spring capture conduit, with the lowest median value of 3.2 x 10^6 cells L^{-1}, representing a 3-fold difference between these two groups (Fig. 3.27).

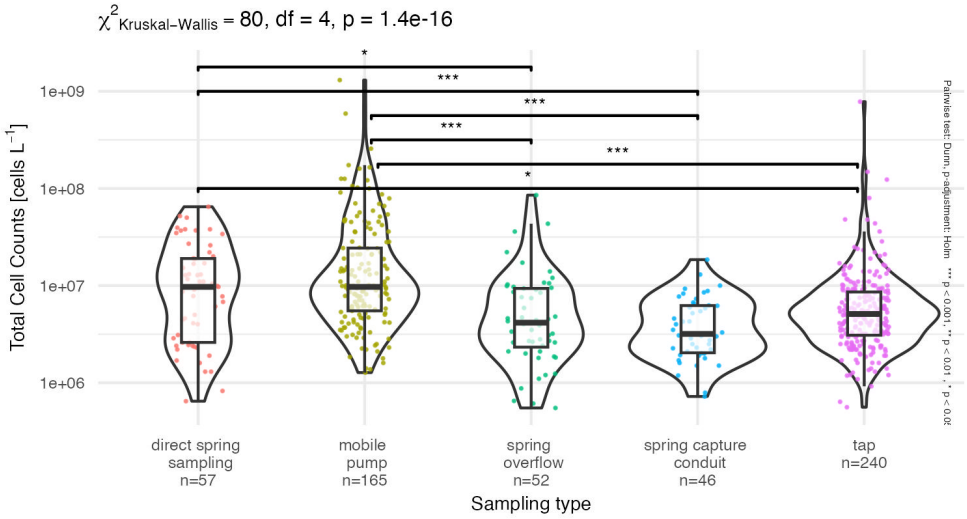


Figure 3.27: Total Cell Counts across different sampling types.

ATP

The Kruskal-Wallis test revealed no significant effect of the sampling types on intracellular ATP in groundwater ($p > 0.05$).

HNAP

The Kruskal-Wallis test revealed no significant effect of the sampling types on HNAP in groundwater ($p > 0.05$).

3.4 Classification and delineation of reference conditions

Using the reference dataset—determined by natural hydrochemical conditions—we were able to observe a significant effect of the geological formations on microbiological variables. For the distinct geological formations, the median values were calculated and similarly to the delineation of natural background values of hydrochemical components in groundwater (Müller et al., 2006; Brielmann et al., 2018), the corresponding 10%–90% percentile values were calculated, summarized in Table 3.5.

Additionally, reference conditions were determined on the basis of a microbiological-ecological classification scheme (Fig. 4.1) which is discussed in the following chapter.

Table 3.5: Reference conditions of Total Cell Counts (TCC), concentration of intracellular ATP (ATP) and High Nucleic Acid content cells Proportion (HNAP) for Austrian shallow groundwaters (135–1910 m.a.s.l., 3–19°C). Values are presented based on two complementary classification systems: aquifer types (INSPIRE, 2021) and Austrian geological formations (A-L, Brielmann et al., 2018).

	TCC [10^6 cells L^{-1}]				ATP [pM]				HNAP [%]			
	n	10%	Mdn	90%	n	10%	Mdn	90%	n	10%	Mdn	90%
Shallow groundwater	586	2.0	5.9	31.3	383	10	29	93	537	30	46	72
Porous groundwater	460	2.2	6.0	35.6	299	10	30	93	425	30	47	72
Oxic groundwater (porous)	416	2.1	5.7	18.8	269	10	29	92	387	29	47	72
Hypoxic groundwater (porous)	27	2.4	21.0	113.9	19	6	34	143	24	30	47	73
Anoxic groundwater (porous)	17	14.1	56.6	207.1	11	8	35	115	14	31	48	70
Karstic groundwater	50	2.8	12.4	38.1	36	12	26	54	44	33	42	59
Fractured groundwater	76	1.2	2.7	9.0	48	11	28	88	68	31	49	73
Deep oxic groundwater[a]	30	1.5	16.1	100.8	15	2.4	67	161	21	50	77	90
Deep hypoxic groundwater[a,b]	4	9.4	57.8	148.7	1	251	251	251	3	34	64	75
Deep anoxic groundwater[a,b]	9	15.6	56.0	531.3	7	19	39	110	9	50	76	92
A - Crystalline rocks of the Bohemian Massif	17	1.5	3.4	9.1	10	8	15	106	16	11	17	32
B - Tertiary sediments	17	1.8	3.9	9.7	12	11	21	111	15	34	50	68
C - Quaternary sediments	448	2.2	6.2	36.0	291	10	30	92	415	31	48	72
D - Sediments of the Mesozoic and Permian	43	2.4	12.0	37.0	29	13	23	42	36	35	47	61
E - Rocks of the Flysch zone[b]	6	3.4	7.0	9.1	3	9	12	28	3	37	41	52
G - Low to non-metamorphic old Paleozoic rocks	16	0.9	2.7	9.4	12	18	45	111	16	39	52	74
H & I - Eastern Alpine crystalline rocks	31	0.8	2.2	7.7	20	17	27	82	28	26	42	59
J - Helveticum and cliffs of the Waschberg-zone[b]	3	2.7	4.0	14.4	3	27	50	185	3	26	28	46
K - Penninic of the central zone[b]	3	2.8	6.2	7.9	2	18	33	47	3	55	68	70
L - Subpenninic[b]	2	4.9	7.1	9.4	1	104	104	104	2	40	46	53

[a] Additional exploratory data

[b] Small sample sizes (n < 10); reference values should be interpreted with caution

4 Discussion

An outstanding feature of this study is that the microbiological-ecological characteristics of shallow groundwater ecosystems in Austria could be comprehensively assessed on a national scale. The assessment of nearly 2000 sites was motivated by the search for reference conditions necessary for the assessment of the ecological conditions of groundwater ecosystems with the D-A-(C) index (see Introduction). The large number of samples allowed to investigate the impact of various environmental factors but also to estimate the potential ranges, dynamics and relationships of the selected microbial variables TCC, intracellular ATP and HNAP to an unprecedented extent.

The chapter will cover (1) an overview of the determined microbial variables and a discussion of potential methodological shortcomings, (2) a discussion of the criteria used for the selection of a reference dataset, (3) an overview of the microbial dynamics and relationships that were investigated using the reference dataset, (4) an overview and comparison of the impact of the various environmental factors that were investigated, and (5) a discussion on how a microbiological-ecological classification scheme could be delineated.

4.1 Overview and quality of all microbiological measurements

Extreme values were observed for all three variables within the lower and upper ranges. The range of TCC observed in this study spanned almost six orders of magnitude (5 x 10^5 to 1.4 x 10^{10} cells L^{-1}), exceeding the reported ranges of other studies by one order of magnitude (Pedersen et al., 2008; Griebler and Lueders, 2009; Griebler et al., 2014). However, when comparing the overall mean TCC to existing national (Griebler et al., 2001; Farnleitner et al., 2005; Wilhartitz et al., 2009; Schönher et al., 2021; Retter et al., 2021) and international (Kötzsch and Sinreich, 2014; Griebler et al., 2014) studies, the values are in the same range (see Table 4.1). The values observed for karstic springs in this study come closest to those of a previous assessment in Austria (Schönher et al., 2021). However, differing sample sizes combined with the use of mean values are not optimal for a comparison since they are easily affected by extreme values. In fact, the overall median TCC in this study was 6–7 times lower than the mean value. Comparing the median values throughout the different studies would allow a more robust comparison but unfortunately such values are not available. All these considerations reinforce the necessity for standardized data analysis and interpretation of flow cytometric analysis in the context of aquatic sciences (Schönher et al., 2021).

A further indication of the necessity of standardization was identified during the calculation of HNAP. As this value was not recorded during the course of the measurements,

it was calculated from the raw data files (.fcs files) in R, together with a recalculation of TCC. Recalculation revealed that TCC values calculated in R differed from those calculated with the software associated to the flow cytometer. The observed deviations did not show a clear trend, and despite of consultations with the manufacturer's technical support, the reason for the deviations could not be determined. Randomly selected samples (data not included) showed only minor deviations (<1%) of HNAP. Both, small deviations and the advantage of reproducibility were the major arguments to proceed with a script-based calculation of HNAP. The observed deviations of TCC once more demonstrated the need for a standardization of flow cytometric measurements. Nevertheless, the stability of the HNAP measurements, allowed to compared it with other studies.

Table 4.1: Total Cell Counts (TCC) and High Nucleic Acid content cells Proportion (HNAP) reported in previous studies that used flow cytometry for the enumeration of prokaryotic cells compared with the results from the water condition monitoring program (Gewässerzustandsüberwachungsverordnung— GZÜV) 2019 in Austria.

		TCC [$10^6 cells\ L^{-1}$]				HNAP [%]
Reference	Groundwater	min	mdn	mean	max	mean
Kötzsch and Sinreich, 2014	shallow	1		43	800	35
Besmer et al., 2017	karstic springs	48			200	
Schönher et al., 2021	karstic springs		15*			40
Schönher et al., 2021	porous		14*			50
Retter et al., 2021	shallow	1		30	150	
Griebler et al., 2014	shallow	1			1500	
GZÜV 2019 (all samples)	shallow	0.5	6	61	14000	50
GZÜV 2019 (all samples)	porous	0.6	3	69	14000	50
GZÜV 2019 (all samples)	karstic springs	0.6	12	15	310	48
GZÜV 2019 (all samples)	fractured	0.5	6	5	20	50

* Estimated median values

The observed HNAP values were similar to those reported by Schönher et al. (2021) and similar patterns—such as lower (<50%) HNAP in karstic, medium (50%) in porous and medium–high (>50%) in deep groundwater— were observed. The similarity of the patterns suggests that HNAP is a robust variable which is easy to determine once the gate is set. It can contribute to the characterization of groundwater ecosystems, by providing additional information on the activity and/or the composition of the microbial community (Servais et al., 2003; Proctor et al., 2018). Furthermore, the relative standard deviation associated to the triplicate measurements could be used as an additional indicator for the quality of the flow cytometric measurements.

An analysis of the ATP measurements revealed a notable discrepancy between the seasons. The relative standard deviation associated to the mean value of the triplicate

measurements of total and extracellular ATP was higher for the autumn season, which can be traced back to different procedure in treatment. Prior to the analysis of the here presented results, all statistical tests (i.e., correlation of microbial variables and the factors impacting intracellular ATP) were conducted separately for the two seasons with similar results (data not included). Since no major differences were observed, it was concluded that the precision of the replicate measurements, but not the accuracy had been affected. However, this aspect was considered throughout the analysis and should be kept in mind for interpretation of the results.

In both seasons the average concentrations of intracellular ATP was slightly lower than total ATP, indicating that intracellular ATP is the main constituent of ATP in groundwater samples. The average concentration of extracellular ATP was one order of magnitude smaller than that of intracellular ATP. These results go along with previous findings (Hammes et al., 2010).

4.2 Reference dataset

4.2.1 Selection criteria

The reference dataset, consisting of 586 samples (i.e., 16% of the whole dataset), was established by applying carefully selected hydrochemical criteria. Natural Background Values (NBV) of 27 hydrochemical components, determined by Brielmann et al. (2018) for Austrian groundwaters, were the main criteria. However, since the NBV had been determined separately for every hydrochemical component and hydrogeological unit, it was assumed that the NBV did not account for complex relationships and interactions of hydrochemical components. An additional plausibility assessment was carried out to take this into account. Overall 14 samples with implausible hydrochemical combinations were identified and excluded from the reference dataset. A common reason for the observed implausible combinations—i.e., reduced metal species (Fe^{2+} and Mn^{2+}) together with elevated levels of dissolved oxygen or nitrate—can be the mixing of different groundwater sources during sample pumping (Kölle, 2017).

An undesired effect was observed with the application of the NBV of dissolved oxygen. The determined lower threshold values (the 10% percentile) of the NBV ranged from 0.2–10.2 mg L^{-1} for the distinct hydrogeological units. On one hand it is useful to set a lower limit of oxygen, to indicate deviations from naturally expected conditions since the depletion of oxygen can be the result of human activities (Riedel, 2019) with potentially severe effects on groundwater organisms (Becher et al., 2022) and the mobility of certain problematic components like iron, manganese, nitrite, arsenic or copper (Brielmann et al., 2018). On the other hand, anoxic conditions do not only indicate human influences but do also occur naturally like in the porous aquifers of northern Germany that are composed of fine unconsolidated sediments (Kunkel et al., 2004). When applying the NBV of DO as a selection criterion, all anoxic groundwater samples (DO$<$0.2 mg L^{-1}) would have been excluded even if all other NBV were in the range of natural conditions. A further argument to reconsider the use of the NBV of DO as a criterion was the high seasonal

variability of DO, as observed in shallow groundwater located in the Lobau in Vienna, a part of the Danube floodplain national park (unpublished). Both, the high variability and low concentration of DO known to occur naturally in groundwater ecosystems led to the decision to ignore the NBV of DO as a criterion for the selection of hydrochemically natural reference samples.

4.2.2 Overall microbiological properties

The established reference dataset was employed to investigate the seasonal dynamics and relationships of the microbiological variables, on the assumption that the observed variations would reflect natural variations. Having selected hydrochemically natural samples, it was assumed that anthropogenic influences were either absent or at least only moderate. All three microbial variables—TCC, intracellular ATP, and HNAP—were significantly lower in the reference dataset compared to the sample subset that did not qualify as hydrochemically natural (Fig.3.4). Surprisingly, no significant correlation between the microbial variables were observed. Several studies have shown significant correlations between the microbiological measures TCC and total ATP (Siebel et al., 2008; Eydal and Pedersen, 2007; Pedersen et al., 2008; Fillinger et al., 2019) and between intracellular ATP and HNAP (Sousi et al., 2020). Many studies report the relationship of these variables in the context of drinking water supply, with a smaller amount of samples and sources and in higher temporal resolution (Hammes et al., 2008; Sousi et al., 2020). The missing correlation found in this study indicate that such observed patterns can not be generalized and easily transferred to the different environmental conditions encountered in natural occurring groundwater ecosystems. Changes in the microbial variables might correlate during a major disturbance event as shown by Nescerecka et al. (2016). However, the present result suggest that under natural conditions, microbial variables are found to be largely independent from each other, supporting a similar observation made by Fillinger et al. (2019).

Investigating the relationship of the distinct ATP measures revealed a strong correlation between total and intracellular ATP, but also a significant though weaker correlation between total and extracellular ATP. Usually the concentration of extracellular ATP is not used for any other purposes, than to calculate the concentration of intracellular ATP. This might be due to the fact that little is known about the origin and fate of extracellular ATP (Nescerecka et al., 2016). A first survey throughout different types of groundwater resources showed that extracellular ATP can account for 0–96% of the total ATP pool (Hammes et al., 2010). Nevertheless, the results of this study showed that elevated values of extracellular ATP are rarely encountered. In combination with the results of Nescerecka et al. (2016) it can be assumed that high levels of extracellular ATP indicate a major disturbance. Extreme levels of extracellular ATP have been observed to occur after chlorination, where an initial extracellular concentration of 80 pM (6% of total ATP) rose up to several hundred pM and constituted up to almost 100% of the total ATP pool (Nescerecka et al., 2016). In the reference dataset the median was 11% and ranged up to 89% in rare cases. Although little is known about the origin and fate of extracellular ATP, it's relative abundance might give use valuable information about the

microbial community (e.g., elevated values of extracellular ATP may indicate disturbances like virus-induced cell lysis). Furthermore, it could be used as an additional criterion for the selection of natural groundwater samples.

Another potential variable indicating natural conditions could be the seasonal dynamics. Groundwater ecosystems are assumed to be nutrient-poor (oligotrophic) and comparably stable environments (Griebler and Lueders, 2009). Therefore, less seasonal dynamics is expected in these ecosystems when compared to surface habitats. The only exception would be very shallow groundwaters. On the other hand, strong dynamics could be an indicator for disturbance. Such disturbances do not necessarily indicate human influences but can also be caused by natural phenomena such as the influx of surface water after floods or heavy rain events. The signs of such events are expected to disappear over time and groundwater ecosystems will return to stable conditions with little dynamics. Although strong dynamics may have natural causes, using samples that exhibit such signs should not be used as reference samples, as they likely represent the exception rather than the norm. The investigation of the seasonal dynamics of the microbial variables has the potential to provide a baseline for the range of dynamics observed in undisturbed, natural groundwater ecosystems. Overall, TCC was observed to increase slightly (1.2-fold) from spring to summer with the majority (10%–90%) ranging from a slight 0.5-fold decrease to a maximum 3-fold increase. Although this variable seems promising, the calculations of the dynamics of intracellular ATP showed that it can not be simply delineated. Rather high changes in ATP were observed for the reference dataset indicating that still remaining extreme values were biasing the results. More reasonable ranges of the seasonal dynamics, could be derived by (1) defining the natural occurring ranges of intracellular ATP for the distinct types of aquifers (2) excluding all samples trespassing these ranges and (3) calculating the relative changes based on the remaining samples. Furthermore, longer time series and larger sample sizes for the distinct types of aquifers will be needed to get robust results.

4.3 Environmental factors affecting the microbiological variables

In addition to studying the dynamics and relationships of the microbial variables the reference dataset was employed to assess potential effects of environmental factors. The impact of nine different factors on the microbiological variables was investigated. Each factor was selected either to evaluate natural variations or to assess remaining anthropogenic influences that would not be detected by the previously applied hydrochemical criteria. Correspondigly, the assessed factors were grouped into (1) natural (2) mixed (3) and human-made factors, thereby providing a better overview and at the same time indicating the potential source of the observed pattern. An overview of the groups and the test results of all factors is given in Table 3.3.

Natural factors, like the geology, were observed to have significant and large effects on the microbial variables. For the two geological classification systems investigated in this study, similar patterns were observed. Regarding the types of aquifers, the biggest

difference in TCC was between fractured and karstic aquifers (4.6-fold difference in median TCC). Similarly, the greatest differences in median TCC (up to 5.5-fold) was between the geological formations that can be assigned to fractured aquifers—i.e., the Bohemian Massif; Low to non-metamorphic, old paleozoic rocks and the Eastern Alpine crystalline rocks)—and karstic aquifers (i.e., the Sediments of the Mesozoic and Perm). The Clusters of Dissolved Organic Matter (DOM) that were determined for Austrian groundwaters by Harjung et al. (2023) reflect different influences—such as land use, proximity to surface waters, precipitation, geology and vegetation—on the quality and quantity of DOM. The differences observed between the DOM Clusters resemble those of the geological factors but were smaller. Median TCC in DOM-Cluster 6—which is associated with fractured aquifers— was 4.2 times smaller than in Cluster 5—which is associated with karstic aquifers. The resemblance of the observed pattern might be because the composition of DOM in groundwater is strongly influenced by the geological setting, which determines various abiotic factors (e.g., groundwater recharge and residence time) and biotic factors (vegetation encountered at the surface). The analysis of DOM can provide insights towards the origin and hence the quality of the available organic matter (Harjung et al., 2023). The evaluation of the effect of DOM Clusters on TCC suggests that the composition of DOM affects the prokaryotic community. For a more detailed investigation, it will be necessary to move from DOM Clusters to evaluating the effect of single fluorescence indices and components on TCC.

Assessing the types of wells/springs and sampling procedures (both human-made factors) revealed minor effects on TCC. For the different types of wells and springs the largest difference (2.7-fold) was found between monitoring wells and tapped springs. The lowest median TCC value was observed for tapped springs. However, the median TCC of untapped springs was in the range of the samples derived from monitoring wells suggesting that the tapping of the springs does have an effect on the TCC. This pattern was only observed for porous groundwater. For karstic groundwater no significant difference was observed between tapped and untapped springs. Regarding the sampling type TCC values were 3 times higher when sampled by mobile pumps than directly at the spring capture conduit. However, since the geology determines which type of well or spring is encountered, and subsequently also the sampling procedure, the observed differences might again reflect geological features. This study did not allow a systematic evaluation of this matter. Throughout all assessed factors, the largest differences in TCC were observed for the distinct oxygen conditions. Anoxic groundwater samples had a median TCC that was 10 times higher than that of oxic groundwater samples. Notably, all hypoxic and anoxic groundwater samples were located in porous aquifers of a single hydrogeological formation, namely the Quatenary Sediments. Minor effects were observed for the distinct groundwater monitoring station types and land cover patterns, with maximum 2.6-fold differences in median TCC values between the different groups. Higher values in TCC were found in groundwater located below arable land and broad-leafed forests and lower TCC values below pastures and coniferous forests. Regarding the station type, higher values and variance of TCC was observed for groundwater samples that were closest to the surface.

Contrary to this trend, HNAP increased with the distance to the surface, thereby indicating either that cells were more active or had larger genome sizes at lower water tables. Furthermore, an effect of the geology factors on HNAP was observed. The lowest median value (17%) was observed in groundwater samples derived from the Bohemian Massif located in the northern part of Austria. This geological formation is composed of crystalline rocks, such as granites and gneiss, whose weathering results in fractured aquifers and acidic (pH <7) conditions (Brielmann et al., 2018). The observed HNAP signature indicates either a lower activity (Servais et al., 2003) or a microbial community with a higher abundance of small-sized genomes (Proctor et al., 2018) related to this specific environmental conditions. Differences in HNAP that were observed between water type 1 (earth-alkaline) and water types 4 & 5 (earth-alkaline alkaline) reflect the geological formations. Water type 4 and 5 (earth-alkaline alkaline) typically occur in fractured aquifer systems with higher groundwater residence times (BMLFUW, 2017).

Between water type 4 (earth-alkaline alkaline bicarbonatic) and 5 (earth-alkaline alkaline sulphatic) significant differences in intracellular ATP were observed. Median intracellular ATP levels were the highest in water type 4, with 57 pM. However, since it is known that human activities (e.g., the application of salt on roads) can cause a shift towards more alkaline water types (BMLFUW, 2017), additional indicators are needed to decide whether the determined water type reflects natural variation or human influences. Half of the samples that showed elevated levels of intracellular ATP (>100 pM) were located in Vienna. This observation raises the question if shallow groundwater located in urban areas should be considered as reference samples. Apart from this questionable effect of the water types, no significant effect on intracellular ATP was observed for any of the other factors. Only the comparison of the reference dataset with the measurements of the deep groundwater samples revealed a potential effect.

Since NBV were not determined for deep groundwater (Brielmann et al., 2018), deep groundwater was not considered in the selection of reference samples. However, deep aquifers are characterized by their isolated state, thus it can be assumed that human influences are not present or reduced to a minimum (Schubert, 2015). Surprisingly, intracellular ATP levels were significantly higher in deep groundwater samples, with a median concentration of 66 pM, indicating a higher microbial activity in these isolated groundwater ecosystems. Furthermore higher median values of TCC but also HNAP were observed, compared to the reference dataset of shallow groundwater. A higher density of cells has been reported recently for older groundwater (Ruff et al., 2023) and Schönher et al., (2021) found higher HNAP values for Austrian deep groundwater. Although, the goal of this study was to assess the microbiological-ecological conditions of shallow groundwater, the distinct microbiological signature of deep groundwater demands for further investigation. Furthermore, since deep groundwater can discharge into shallow groundwater (BMLFUW, 2017) it should be part of (or at least mentioned in) a microbiological-ecological informed classification scheme.

4.4 A microbiological-ecological classification scheme

Investigating the impact of environmental factors clearly showed that natural differences exist, which should be taken into account for the delineation of specific and sensitive reference values. In the following, the acquired knowledge is used to outline a microbiological-ecological classification scheme of shallow groundwater occurrences in Austria. In order to achieve a classification scheme for groundwater ecosystems that reflects natural variations, it is essential to base it on factors that are resilient to human activities. Grouping the factors into natural, mixed and anthropogenic categories served not only to provide a better overview but also to introduce a criterion for the selection of appropriate classification units.

The classification units of natural factors—such as types of aquifers and geological formations—represent inherent envrionmental characteristics that are largely unaffected by human activities. These units are assumed to be robust to human activities, allowing us to assume that observed patterns reflect natural variations.

Mixed factors—such as the groundwater monitoring station type, water type , DOM cluster, oxygen condition, and land cover pattern—are assumed to be less robust, because they can reflect both, natural and human influences. However, mixed factors revealed influences that were not captured by the natural factors and significantly affected the microbial variables. The observed differences caused by the distinct oxygen conditions, groundwater monitoring station types (depth of water table/distance to the surface), and land cover patterns are examples of local phenomena. It can thus be assumed that local variations can be more adequately captured through these factors. While valuable information is gained by including these factors, it is crucial to carefully assess whether human activity has influenced these classifications—an issue that was addressed previously in the discussion of water types.

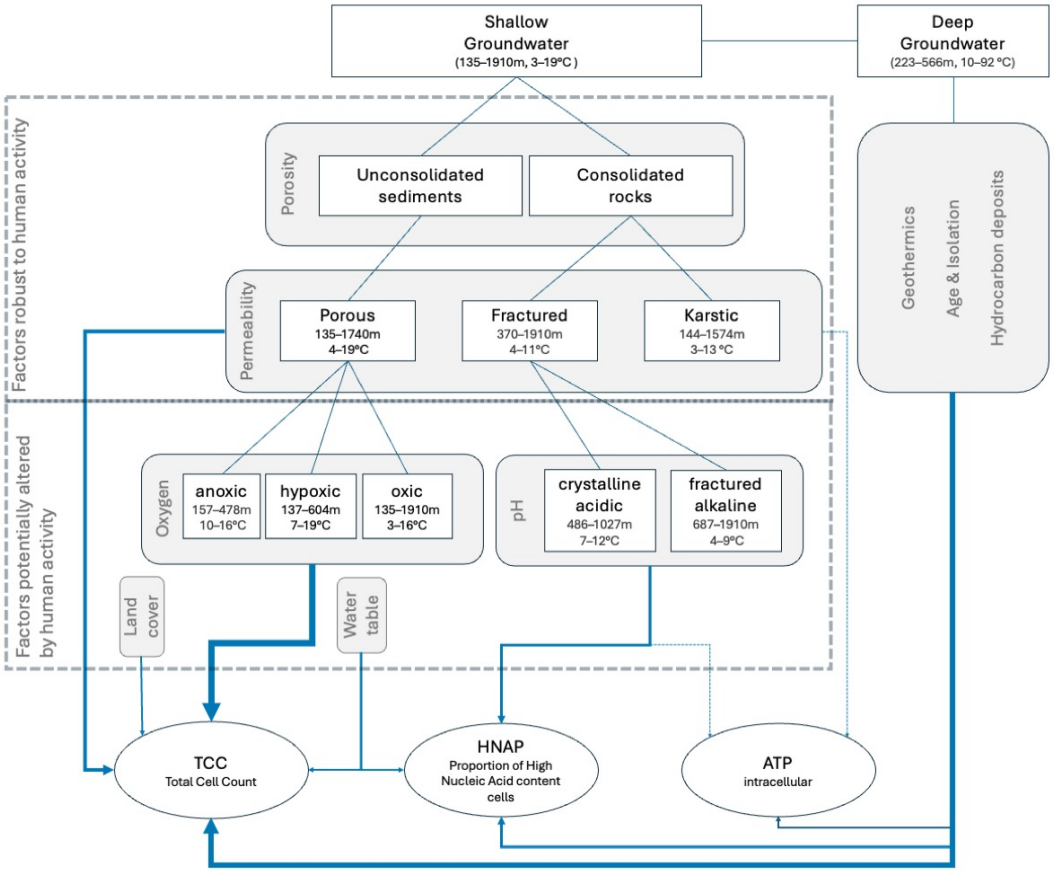
With all this aspects in mind a classification scheme for shallow groundwater ecosystems was drawn up based on the above-mentioned factors (Fig. 4.1). The distinct types of aquifers were used as the basis for this classification scheme. This hydrogeological classification system is well-established and widely used, unlike the system of hydrogeological classes/formations proposed by Brielmann et al. (2018). Additionally, broader insights might be gained through a more abstract classification system and the delineated microbiological values can be compared with similar studies in other countries.

4.5 Microbiological-ecological reference conditions

Reference conditions of TCC, HNAP and intracellular ATP were delineated for the two complementary hydrogeological classification systems mentioned above (Table 3.5). Reference conditions delineated for the aquifer types could find a broader application beyond national borders. Nevertheless, attention should be paid to the fact that reducing complex hydrogeological conditions to three categories is associated with inaccuracies and loss of information. For example, the clear differences in HNAP between the crystalline rock formations, i.e., the Bohemian Massif and Eastern Alpine crystalline, could not

be found in the analysis based on aquifer types. In this case, the geological formations (Brielmann et al., 2018) provided a better basis. In the context of a stepwise ecological assessment procedure, it would be ideal to define the microbiological-ecological reference values for the hydrogeological classes (instead of geological formations), as hydrochemical NBV are available for these classes only. While, this could not be achieved with the existing dataset, it remains a worthwhile goal for future projects.

Figure 4.1: Microbiological-ecological classification scheme of shallow groundwater ecosystems in Austria (altitude of monitoring sites: 135–1910 m, groundwater temperature: 3–19°C). White boxes represent the classes of shallow groundwater ecosystems including porous (anoxic, hypoxic and oxic), fractured (crystalline acidic and alkaline) and karstic systems. Grey boxes and vertical letters represent the factors that were observed to affect the microbiological variables —Total Cell Count (TCC), High Nucleic Acid Content cells Proportion (HNAP) and intracellular ATP significantly. The arrows indicate which factors affected the microbiological variables, with arrow thickness representing the estimated effect size based on the variations observed in the investigated reference dataset (Table 3.5). Factors were categorized as either potentially altered by human activity or robust to human activity, as indicated by the dashed boxes. The white ellipses represent the assessed microbiological variables. Deep groundwater ecosystems (223–556 m, 10–92°C) were not investigated systematically and thus were pooled into a single group.



5 Conclusions

5.1 Conclusions

The findings of this study mark a further step towards the potential application of the D-A-(C) Index for the ecological assessment of Austria's shallow groundwater ecosystems. Microbiological-ecological reference values—defining natural conditions— were missing for an appropriate application. The creation of a corresponding reference dataset (based on hydrochemical criteria) has been now achieved. This dataset allowed to investigate the natural ranges, dynamics, and correlations of three microbial variables: Total Cell Counts (TCC), intracellular ATP, and the High Nucleic Acid content cells Proportion (HNAP). Thereby, two factors were identified as particularly worthy of further investigation: seasonal dynamics and the proportion of extracellular ATP in the total ATP pool. These factors may serve in future as valuable indicators of the ecological condition. Moreover, the impact of various environmental factors on individual microbial variables was investigated. The density (D) of prokaryotic cells, quantified as TCC, emerged as the most sensitive variable, significantly influenced by hydrogeological factors and prevailing oxygen conditions. In contrast, the activity (A) of microorganisms, quantified as intracellular ATP, was largely unaffected by the different environmental influences. The third microbiological variable HNAP was found to be influenced by lithological conditions, particularly those associated with crystalline acidic rocks. Although it appears to be a promising candidate for characterizing groundwater ecosystems, this variable has not been previously used in the context of the D-A-(C) Index. To evaluate it's usefulness as an ecological criterion, further investigations will be needed.

The observed patterns were used to outline a microbiologically informed classification scheme (Fig. 4.1), which provides a starting point for future investigations at a mechanistic and predictive level. Based on this scheme, microbiological reference values, tailored to the various types of groundwater ecosystems, were derived (Table 3.5). Additionally, reference conditions for the different geological formations in Austria were derived (Table 3.5), which, in conjunction with hydrochemical Natural Background Values, could provide a more specific basis for the assessment of Austrian groundwaters.

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