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Exploring the microbial communities of ferromanganese concretions and pockmarks in the Gulf of Finland

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Abstract

Ferromanganese concretions are aggregates of precipitated minerals, of which the mechanism of formation is not clear. They are found in aquatic environments and may be formed by abiotic and/or biotic processes. Pockmarks are depressions in the seafloor that can be caused by groundwater breaking through it during a process called submarine groundwater discharge. This work was conducted to assess the bacterial community composition of ferromanganese concretions and pockmark sediments using molecular biology methods and comparing them to seawater and other sediment samples from the Gulfs of Finland and Riga, and Väinameri. The concretion microbiome was significantly different from all the other sample types, as was the sediment microbiome from one pockmark site. Both microbiomes were distinguished by a high abundance of bacteria from the phylum Patescibacteria, which may be involved in the formation of concretions. Pockmark sediments from a different site resembled sediments not affected by groundwater, signifying the inactivity of this pockmark. Different layers of sediment cores were homogenous in their microbial community composition, pointing to the occurrence of sediment resuspension.

Keywords: ferromanganese concretions; submarine groundwater discharge; marine microbial communities.

CERCS: B230 Microbiology, bacteriology, virology, mycology

Ferromangaani konkretsioonide ja väljavoolu süvendite mikroobikoosluste uurimine Soome lahes

Lühikokkuvõte

Fe-Mn konkretsioonid on sadestunud mineraalide kogumid, mille täpne tekkemehhanism ei ole selge. Neid leidub veekogudes ja need võivad tekkida abiootiliste ja/või bioloogiliste protsesside tulemusel. Lehtrilaadsed väljavoolu süvendid on merepõhja lohud, mida võib põhjustada põhjavee või gaaside läbimurde protsessid, mida nimetatakse merealuseks põhjavee väljavooluks. See töö viidi läbi, et hinnata ferromangaani konkretsioonide ja väljavoolu süvendite setete bakterikoosluste koosseisu Soome lahes, kasutades molekulaarbioloogia meetodeid ning võrreldes neid merevee ja teiste setteproovidega Soome ja Liivi lahes ning Väinameres. Konkretsioonide mikrobioom erines märkimisväärselt kõikidest teistest kogutud proovidest, konkretsioonide mikrobioom sarnanes vaid ühe süvendi sette mikrobioomiga.

Mõlemat mikrobioome iseloomustas Patescibacteria hõimkonna bakterite kõrge relatiivne arvukus, mis võivad mängida rolli konkretsioonide moodustumise protsessis. Teise lehtersüvendi setted sarnanesid põhjaveest mõjutamata setetega, mis viitab et see väljavoolu süvend oli muutunud inaktiivseks, st põhjavee väljavool oli selles piirkonnas lakanud. Erinevad setete sügavuskihid olid mikroobikoosluse koosseisu poolest homogeensed, viidates setete aktiivsele segunemisele.

Võtmesõnad: ferromangaani konkreetid; merealune põhjavee väljavool; mere mikroobikooslused.
CERCS: B230 Mikrobioloogia, bakterioloogia, viroloogia, mükoloogia

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TERMS, ABBREVIATIONS AND NOTATIONS

ASV	Amplicon sequence variant
BSA	Bovine serum albumin
MBI	Major Baltic inflow
PCR	Polymerase chain reaction
ROV	Remotely operated vehicle
SGD	Submarine groundwater discharge

INTRODUCTION

Ferromanganese concretions are aggregates of precipitated minerals, which have been found in seas, oceans, and lakes (Belkin et al., 2021; Hayles et al., 2021; Yli-Hemminki et al., 2014). The main components of these concretions are iron oxyhydroxides and manganese oxides, and they often contain alternating layers of these minerals (Glasby et al., 1996).

Both iron and manganese can be oxidized abiotically and with the involvement of microorganisms. At high oxygen concentrations and pH above 5, iron is quickly oxidized abiotically (Ussher et al., 2004), whereas abiotic oxidation of manganese is only fast in environments with pH above 9 (Morgan, 2005). Manganese is thought to be oxidized mostly with the involvement of microorganisms (Jones et al., 2011; Learman et al., 2011).

The mechanism of concretion formation is still not clear. There is a substantial amount of evidence for the involvement of bacteria in this process, and some arguments against it. Manganese oxidizing and iron reducing bacteria (Jiang et al., 2020), and bacteria-like fossils have been found in ferromanganese concretions in multiple studies (Shulga et al., 2022; Yuan et al., 2015). Contrary to this, no microbes were found on ferromanganese precipitates that had formed over 12-15 years (Usui et al., 2020).

Pockmarks are depressions in the seafloor that are caused by water or gases breaking through it (Idczak et al., 2020; von Ahn et al., 2024). Groundwater is one of the water types that can be flowing through the pockmarks. This is referred to as submarine groundwater discharge (Arévalo-Martínez et al., 2023). This flow of water and gases promotes the formation of microbial communities that differ from those usually present in the sediment layer (Idczak et al., 2020; Purkamo et al., 2022).

This work aimed firstly to identify and compare the microbial communities that are present in ferromanganese concretions, pockmark sediment, surrounding seafloor sediment and seawater from the Gulfs of Finland and Riga, and Väinameri with the use of molecular biology methods. When this data was obtained, it was analyzed to determine the possible effect of submarine groundwater discharge on the microbial communities of the seafloor sediment, the potential role of the most abundant taxa in the formation of concretions based on the metabolism of closely related taxa, and the variability of microbial community composition between different layers of sediment cores.

1 LITERATURE REVIEW

1.1 BALTIC SEA ENVIRONMENT

The Baltic Sea is one of the world's largest brackish water basins (Kuliński et al., 2022). The Baltic Sea's catchment area is four times larger than the area of the sea itself (Leppäranta & Myrberg, 2009). Water exchange with the North Sea is limited, so the water retention time in the Baltic Sea is 30-40 years (Meier, 2007). The sea has both a horizontal and vertical salinity gradient and it is permanently stratified (Szymczycha et al., 2018). The surface water is more saline in the Danish Straits, and it gets progressively less saline in the direction of the Gulf of Bothnia (Leppäranta & Myrberg, 2009). A strong halocline in the central Baltic basin separates the more saline and dense water at the bottom of the sea from the less saline surface water (Lass & Matthäus, 2008). Stratification is thought to be a very important reason for the occurrence of vast low-oxygen zones in the sea (Schmidtko et al., 2017).

The deepest areas of the Baltic Sea are permanently hypoxic and the area where hypoxic conditions occur continues to increase due to eutrophication and the increase of water temperature (Conley et al., 2009; Saraiva et al., 2019). Eutrophication continues to be a substantial problem in the Baltic Sea with the highest N and P concentrations occurring in the Gulf of Riga and the Gulf of Finland (Szymczycha et al., 2018). This problem will not be resolved unless the N and P loads going into the sea are significantly reduced, and even then achieving the status of a sea unaffected by eutrophication may take decades or even longer (Murray et al., 2019). According to simulations of future conditions, the increase in temperature due to global warming will not significantly affect the oxygen concentrations in the deeper areas of the Baltic Sea, as long as the nutrient loads being deposited in the sea are small. In this case, the areas affected by hypoxia will eventually decrease. If the nutrient loads continue to be large or even increase, the increase in temperature will exacerbate eutrophication and hypoxia (Saraiva et al., 2019). In anoxic conditions, phosphorus is released from the sediments and this further worsens the eutrophication problem (Ingall & Jahnke, 1994).

Temperature increases may cause the expansion of hypoxic and anoxic zones due to oxygen being less soluble at higher temperatures and the rate of oxygen uptake increasing. In turn, an increase in areas with low or no oxygen will cause an increase in the marine emissions of the greenhouse gas N_2O (thus, accelerating climate change) and negatively affect eukaryote biodiversity in the sea. Only eukaryotes that have developed adaptations, such as high tolerance of H_2S (this compound is toxic to most aerobes and occurs in anoxic sediments and the water column) and depression of their metabolism, can inhabit low-oxygen zones (Breitburg et al.,

2018). Low oxygen conditions also promote the reduction of arsenic (As(V)) to its more toxic form (As(III)) (Li et al., 2018).

In marine environments where oxygen is not present or where its concentration is very low, microbial metabolism dominates (Wright et al., 2012). In such environments, microbial anaerobic denitrification and ammonium oxidation processes occur. These processes cause the loss of bioavailable nitrogen by converting it to N_2 (Breitburg et al., 2018). The microorganisms that are present in large numbers in marine environments with low oxygen belong to the Proteobacteria, Bacteroidetes, Marinimicrobia, Actinobacteria and Planctomycetes phyla (Wright et al., 2012). Anoxic conditions are also favorable for strict anaerobes, such as methanogens and sulfate reducers (Canfield et al., 2005).

1.1.1 Gulf of Finland

As opposed to the rest of the Baltic Sea, there is no halocline in the east of the Gulf of Finland. The salinity in this area is greatly affected by the runoff from the river Neva. This is the river that contributes the largest amount of freshwater to the Baltic Sea out of all the rivers in the sea's catchment area. About 25% of freshwater inflow comes into the Baltic Sea through the Gulf of Finland (Myrberg & Soomere, 2013).

The impact of major Baltic inflows (MBI) (episodic inflows of water from the North Sea) reaches the Gulf of Finland about 9 months after the North Sea water has passed the Danish Straits, and this inflow pushes water from the deep areas of the Baltic Proper into the Gulf of Finland, which increases the salinity and hypoxic areas in the gulf (Liblik et al., 2018; Stoicescu et al., 2019). Oxygen conditions improve in the western Gulf of Finland when MBIs do not occur. But after a few years without MBIs, anoxic areas in the Baltic Proper increase and this water can flow to the western Gulf of Finland, causing short term hypoxia or anoxia (Lessin et al., 2014). In 2016-2017 an average of 15% of the bottom areas of the Gulf of Finland were hypoxic (Stoicescu et al., 2019).

The pH level in the layer below the halocline in the Gulf of Finland in the winter is around 7.2-8.1, whereas in the surface layer it is higher (~7.9-8.2) (Almén et al., 2017). The pH level tends to be lower in the winter than in the summer with the overall average pH being 8.1. This difference could be due to the decrease of CO_2 in the waters because it was used up during the spring algae bloom or because of increased water temperature in the summer which decreases solubility of gasses in water (Brutemark et al., 2011).

1.1.2 Submarine groundwater discharge and pockmarks

Pockmarks are depressions in the seafloor that are caused by water or gasses breaking through it (Idczak et al., 2020; von Ahn et al., 2024). Submarine groundwater discharge (SGD)

is a process where groundwater from aquifers flows into the sea through the seabed (Purkamo et al., 2022). Many of these SGD sites have been identified along the coast of Poland (von Ahn et al., 2024). Pockmarks have also been identified near the Gulf of Finland (Purkamo et al., 2022). Submarine discharge of freshwater mostly occurs close to the coast (<100 m from the coast) (Arévalo-Martínez et al., 2023). This flow of water and gases promotes the formation of microbial communities that differ from those usually present in the sediment layer (Idczak et al., 2020; Purkamo et al., 2022). The SGD water itself carries microbial communities that differ from those in the seawater and sediments (Yanuka-Golub et al., 2024).

About 1-10% of freshwater input into the world's oceans comes from SGD (Abbott et al., 2019; Luijendijk et al., 2020). In the Baltic Sea, freshwater input from SGD is about 4% of the freshwater amount that comes from rivers (Peltonen, 2002). The water involved in SGD can be not only freshwater, but also saline water (Taniguchi et al., 2019). The water in coastal aquifers can be a mix between groundwater and seawater (J. Liu & Du, 2022).

In the southern Baltic Sea, significantly more metals are delivered into the sea via SGD, than from river runoff (Szymczycha et al., 2016). This may also be the case in other areas of the Baltic Sea. There can also be inorganic nutrients, such as nitrate, phosphate, and silicate, and organic matter present in the outflowing groundwater, which can promote eutrophication (Arévalo-Martínez et al., 2023). Nitrate and phosphate in the groundwater generally have the same source as these compounds in river runoff: agriculture and sewage (Bishop et al., 2017). SGD not only promotes hypoxia/anoxia through enhancing eutrophication, but also by direct addition of anoxic water to the sea/ocean (von Ahn et al., 2024).

Aside from groundwater flowing from the pockmarks, there can also be an outflow of gases. For example, a mixture of 76.1% CH_4, 17.6% CO_2, and 0.39% He was found to be seeping out of a pockmark located in the Gulf of Gdansk in the southern Baltic Sea (Idczak et al., 2020). N_2O and CO have also been detected from pockmarks (Arévalo-Martínez et al., 2023).

1.2 IRON AND MANGANESE BIOGEOCHEMISTRY

1.2.1 Iron biogeochemistry

The Earth's crust contains 5.6% iron, making it the fourth most abundant element (behind oxygen, silicon, and aluminum) (Taylor, 1964), while in marine sediments iron makes up about 1-20% of the constituents (Ussher et al., 2004). Iron concentrations are usually much higher in coastal areas than in open oceans (Breitbarth et al., 2010). The Baltic Sea has an iron concentration of 18-360 nM (Pempkowiak et al., 2000; Rydin et al., 2002).

Iron exists mostly in two different oxidation states: divalent (Fe(II)) and trivalent (Fe(III)). At high oxygen concentrations and pH above 5, Fe(II) is unstable and is quickly oxidized (Ussher et al., 2004). Fe(III) hydroxide has poor solubility but it is increased below pH 8 (Breitbarth et al., 2010; X. Liu & Millero, 2002). Dissolved iron is necessary for growth of the main primary producers in the oceans – phytoplankton (Beam et al., 2018). Reactive iron in marine sediments is present in the form of different iron-containing minerals, such as, ferrihydrite (contains iron oxyhydroxides), goethite, hematite, magnetite (contain iron hydroxides), mackinawite and pyrite (contain iron sulfides) (Haese et al., 1997; Raiswell & Canfield, 2012).

It is known that Fe oxidation and reduction is controlled by archaea and bacteria in most environments, and they are known to have the ability to use Fe oxidation and reduction to gain energy (Bird et al., 2011; Weber et al., 2006). Iron is usually easily oxidized abiotically, but in microaerobic conditions (<50 μM O_2 (Druschel et al., 2008)) and environments with low pH abiotic oxidation becomes considerably slower, so more Fe(II) is available for microbial iron oxidation (Bird et al., 2011; Emerson et al., 2010). Microorganisms can oxidize Fe(II) in both oxic and anoxic environments. Fe(II) oxidation in oxic environments at neutral pH is carried out by microaerophilic bacteria that use oxygen as an electron acceptor and are capable of competing with abiotic iron oxidation (Emerson et al., 2010; Weber et al., 2006). In the absence of oxygen, microorganisms couple the oxidation of Fe(II) with the reduction of chlorate, perchlorate and nitrate, or this oxidation can be achieved phototrophically (coupled to reduction of bicarbonate). The most well-known Fe(II) oxidizing bacteria belong to the genera *Gallionella*, *Leptothrix* and *Marinobacter* (Weber et al., 2006). Zetaproteobacteria are a relatively recently discovered (Emerson et al., 2007) group of Fe-oxidizing bacteria that are widespread in marine environments. There are few bacteria that have been isolated thus far, so a lot is still not known about this group but they may provide a significant amount of oxidized iron in marine environments (Beam et al., 2018; Makita, 2018). Iron oxidized by Zetaproteobacteria can form distinct shapes, such as helical or filamentous, or form amorphous precipitates (Makita, 2018).

Fe(III) oxides are mostly present in crystalline form and their reduction is less thermodynamically favorable than that of amorphous Fe(III) oxides but it still occurs (Fredrickson et al., 1998; Kostka et al., 1996; Kostka & Nealson, 1995). Fe(III) reduction can be coupled to ammonium, hydrogen, methane and other organic compound oxidation (Kappler et al., 2021). For *Geobacter* spp., direct contact with Fe(III) is necessary in order for the reduction to occur. Electrons from the bacteria to the iron are transferred through conductive pili – nanowires (Nevin & Lovley, 2000; Reguera et al., 2005). Nanowires are also employed

for iron reduction by other bacteria, such as *Shewanella oneidensis* (Gorby et al., 2006). Some microorganisms do not need to establish direct contact with the iron oxide (with the cell wall or pili) to be able to reduce it. Electrons can be transferred using both endogenously and exogenously occurring electron shuttles. Some examples of exogenous electron shuttles are antibiotics, humic acids, and exudates from plants. It has been shown that some bacteria produce Fe(III)-binding ligands (including siderophores) that help them obtain solubilized Fe(III) (Mawji et al., 2008). Higher concentrations of siderophores are produced at lower iron concentrations (Cabaj & Kosakowska, 2009), and bacteria usually start to produce siderophores at iron concentrations below 1 μM (Neilands, 1995). Common Fe(III) reducing microorganisms belong to the *Geobacter* family and *Shewanella* genus (Bird et al., 2011). Seafloor environments with low oxygen levels and high organic carbon deposition (factors usually present in marine regions with high productivity) are well suited for the iron reduction process to occur (Garber et al., 2021).

Iron-oxidizing and -reducing microorganisms have a mutualistic relationship, but reduced iron is not the only beneficial compound that iron oxidizers get from iron reducers, and vice versa. In a co-culturing experiment, extracellular metabolites from one type of microorganism were shown to promote the growth of the other, and not just by acceleration of the iron cycle. Both species were also producing compounds involved in biofilm formation (Cooper et al., 2020).

The genetics of iron cycling by microorganisms are not yet well understood, with microorganisms in marine sediments being one of the least studied groups (Garber et al., 2021).

1.2.2 Manganese biogeochemistry

The Earth's crust contains 0.1% of manganese, making it the tenth most abundant element (Turekian & Wedepohl, 1961). Manganese is mostly present in three different forms in nature: Mn(II) (reduced), Mn(III) (intermediate), and Mn(IV) (oxidized) (Canfield et al., 2005). There are more than 30 different naturally occurring manganese oxide minerals (Post, 1999).

Manganese is an important element in photosynthesis because it is a part of the water oxidizing complex in photosystem II. It is also necessary as a cofactor for enzymes that protect cells from reactive oxygen species. For example, *Lactobacillus plantarum* uses reduced Mn for this purpose (Archibald & Fridovich, 1981). Some manganese oxidizing organisms get encrusted in the oxides which are hypothesized to protect them from ultraviolet rays, predators, or oxidative stress (Tebo et al., 2004). Mn(IV) is one of the strongest naturally occurring oxidants (Lingappa et al., 2019), and it is the third best electron acceptor for microorganisms after oxygen and nitrate (Vandieken et al., 2014).

Abiotic oxidation of Mn(II) is much slower than that of Fe(II). This reaction becomes much faster above pH 9 (Morgan, 2005). Mn(II) oxidation can also be induced by the presence of other mineral surfaces. Contact with these surfaces lowers the activation energy for the manganese oxidation reaction. For example, kaolinite, montmorillonite and iron oxides can catalyze manganese oxidation when it is adsorbed to these minerals (Yang et al., 2023). But Mn(II) is thought to be oxidized mostly with the involvement of microorganisms (Jones et al., 2011; Learman et al., 2011). Both bacteria and fungi are known to oxidize manganese. So far, there have been no discoveries of archaea species capable of manganese oxidation (Lingappa et al., 2019).

Manganese oxidation coupled to oxygen reduction is carried out by enzymes belonging to the multicopper oxidase and peroxidase cyclooxygenase families. These enzymes are usually attached to the outside of the cell membrane or excreted extracellularly (Lingappa et al., 2019). Multicopper oxidase catalyzes two transfers of one electron from Mn(II) to oxygen to yield Mn(IV) (Butterfield et al., 2013). Mn(III) is an intermediate product in this reaction which forms after the transfer of one electron (Soldatova et al., 2012). Some bacteria are capable of extracellular production of superoxide radical, which oxidizes Mn(II) to form Mn(IV) oxides (Diaz et al., 2013; Learman et al., 2011). Bacteria can also oxidize manganese through the production of hydrogen peroxide (Gounot, 1994).

Initially, it was thought that oxidation of manganese cannot occur without the presence of oxygen (Geszvain et al., 2012; Jones et al., 2011), but it was proven that manganese can be oxidized photosynthetically in anoxic conditions by a mixed species biofilm. The mechanism of this oxidation reaction is not yet known (Daye et al., 2019). Recently, the possibility of manganese oxidation in the absence of both light and oxygen was discovered. A co-culture of *Rhodopseudomonas palustris* and *Geobacter metallireducens* was shown to oxidize manganese in these conditions. *G. metallireducens* produced electrons through oxidation of acetate and transferred them to *R. palustris*, which used them to dissociate water, producing a hydroxyl radical, which then oxidized Mn(II) (Huang et al., 2023). It is unknown how widespread these anaerobic modes of Mn(II) oxidation are in nature.

Some genera of bacteria that include species that can oxidize manganese are *Leptothrix*, *Pedomicrobium*, *Hyphomicrobium*, *Caulobacter*, *Arthrobacter*, *Micrococcus*, *Bacillus*, *Chromobacterium*, *Pseudomonas*, *Vibrio*, and *Oceanospirillum* (Gounot, 1994).

Manganese can be reduced abiotically by Fe(II) (Jones et al., 2011; Zuo et al., 2021), organic carbon, methane and sulfide (Jones et al., 2011). Manganese oxides can serve as electron acceptors in anaerobic respiration (Lingappa et al., 2019), allowing microorganisms to obtain energy for growth through manganese reduction reactions (Gounot, 1994). Like iron,

microorganisms are able to reduce manganese in both solid and dissolved form (Reguera et al., 2005). Crystalline manganese oxides are reduced more slowly than amorphous oxides (Gounot, 1994). Some microorganisms are capable of methane oxidation using manganese oxides as electron acceptors (Beal et al., 2009). Hydrogen, acetate and lactate can function as electron donors for manganese reducing microorganisms (Vandieken et al., 2014). For example, Mn(III) complexed with pyrophosphate can be reduced by *Shewanella* species for acetate oxidation (Szeinbaum et al., 2017).

Although electron acceptors, such as iron and sulphate, are more important in anaerobic carbon oxidation in sediments, manganese can re-oxidize these compounds and they can then be used for carbon oxidation again (De Schamphelaire et al., 2007). Most microorganisms that are known to reduce Fe(III) can also reduce manganese (Lovley et al., 2004).

1.3 FERROMANGANESE CONCRETIONS

Ferromanganese concretions (also known as ferromanganese nodules, polymetallic nodules or manganese nodules) were first discovered in 1873 in the Atlantic Ocean (Belkin et al., 2021). The main components of these concretions are iron oxyhydroxides and manganese oxides, and they often contain alternating layers of these minerals (Glasby et al., 1996). In the Gulf of Finland, concretions contain around 10-53% of iron oxyhydroxides and 9-34% of manganese oxides (Anufriev & Boltenkov, 2007; Baturin, 2019; Zhamoida et al., 1996). Concretions that are present on the surface of sediments contain two times more Fe and two times less Mn than concretions that are buried under the sediments (Baturin, 2009).

Manganese oxides tend to be negatively charged, whereas iron oxyhydroxides are positively charged. Thus, these mineral phases attract cations and anions, respectively (Peacock & Sherman, 2007). Concretions with different morphologies have been found to have different concentrations of iron and manganese, which also affects the concentration of cations and anions associated with these compounds. Spheroidal concretions are rich in manganese, while flat/discoidal/crust-like concretions are rich in iron (Bostrom et al., 1982; Zhamoida et al., 1996). Ferromanganese concretions are most commonly 1-5 cm in diameter, but much larger concretions have also been found (Hein & Koschinsky, 2014). Concretions in the Baltic Sea accumulate much faster than those in the deep seas, but their exact formation rate is unknown (Glasby et al., 1996).

Some concretions have formed around a nucleus (a grain of sand or a rock) and others may not have a nucleus (Glasby et al., 1996). Calcareous fossils, fossil fish teeth (Popoola et al., 2021), and microorganisms can also serve as nucleation sites. With microbial nuclei, metal ions first adsorb to the surface of the microorganism and subsequently it is completely encrusted (Lyu et al., 2021).

Concretions usually form in places where the sedimentation rate is very low (Kaikkonen et al., 2019) and at water depths near the halocline (Glasby et al., 1996). In the Baltic Sea, concretions are more likely to occur at the edge of seafloor depressions and in sea areas with high phosphorus concentration, which tends to coincide with high concentrations of other elements (Kaikkonen et al., 2019). According to Hayles et al. (2021), concretion formation rate is accelerated in eutrophic conditions.

There are three groups of marine concretions that can be distinguished based on the source of the manganese and iron that make up the concretions: hydrogenetic, diagenetic and hydrothermal. Hydrogenetic concretions get elements from the water column, diagenetic concretions – from interstitial waters, and hydrothermal concretions – from hydrothermal deposits. Elements in a single concretion usually originate from more than one of the listed sources (Hein & Koschinsky, 2014; Zhou et al., 2022). Mixed concretions of diagenetic and hydrogenetic origin are most widespread (Bau et al., 2014). According to Bau et al., these three concretion groups can be distinguished based on their rare earth element and Y content. For hydrogenetic concretions, the Y anomaly is negative, while the Ce anomaly is positive, and the Nd concentration is high (>100 mg/kg). Concretions of hydrothermal origin are the exact opposite, having a positive Y anomaly, negative Ce anomaly and low Nd concentration (<10 mg/kg). For diagenetic concretions, both the Y and Ce anomalies are negative and the Nd concentration is intermediate (10-100 mg/kg) (Bau et al., 2014). Diagenetic concretions also tend to contain high amounts of Cu, Ni and Zn (Hein & Koschinsky, 2014). Bostrom et al. suggest that in the Gulf of Bothnia, Baltic Sea the elements for concretion formation mostly come from diagenetic sources, but some hydrogenetic contribution may also be occurring (Bostrom et al., 1982).

Hydrogenetic Mn and Fe can be transported from anoxic waters to oxygenated waters where these metals can be oxidized and incorporated into concretions. In the Baltic Sea, water inflows from the North Sea (MBI events) greatly contribute to this movement of dissolved elements. In addition, Fe and Mn also come into the seawater from river and groundwater inflow (Glasby et al., 1996).

The mechanism of concretion formation is still not clear. There is a substantial amount of evidence for the involvement of bacteria in this process, and some arguments against it.

Manganese oxidizing and iron reducing bacteria (Jiang et al., 2020), and bacteria-like fossils have been found in ferromanganese concretions in multiple studies (Shulga et al., 2022; Yuan et al., 2015). Jiang et al. (2019) found that areas of concretions that are rich in manganese contain more microorganism fossils than the surrounding ones, which suggests that microorganisms could have had a role in manganese deposition. They also propose that

microorganisms may have served as a mineralization center to induce ferromanganese aggregate formation. For example, fossils of coccolithophores, which have a calcium carbonate-rich shell, were discovered in the concretions and they were surrounded by a thick layer of iron and manganese. The dissolution of the shells could have released calcium carbonate, which then may have oxidized iron and manganese, starting the concretion formation process. Thus, it is possible that both chemical and biological processes could have contributed to the genesis of concretions.

Bacteria with S-layers were also identified within Fe-Mn concretions. S-layers that serve as templates for mineral formation have been described before, hence it could be possible that Fe-Mn concretions start their formation on these S-layers, making their origin biogenic (X. Wang et al., 2009). High protein content in the concretions also point to the involvement of bacteria in their formation (Zhamoida et al., 1996).

Contrary to all of this evidence pointing to microbial involvement in the formation of concretions, Usui et al. (2020) found no microbes on newly formed Fe-Mn precipitates. In their experiment, artificial plates were placed on the seabed in different locations of the Pacific Ocean near Japan. After 12-15 years, Fe-Mn precipitates a few micrometers in diameter had formed on the plates. Sybr Green I staining revealed no microorganisms on these precipitates. However, there were microbial cells on nearby organic particles that were also on the plate.

2 THE AIMS OF THE THESIS

- to identify and compare the microbial communities that are found in ferromanganese concretions, pockmark sediment, surrounding seafloor sediment and seawater;
- to determine the effect of submarine groundwater discharge on the microbial communities of the seafloor sediment;
- to determine the potential role of the most abundant taxa in the formation of concretions based on the metabolism of closely related taxa;
- to determine variability of microbial community composition between different layers of sediment cores;

3 EXPERIMENTAL PART

3.1 MATERIALS AND METHODS

3.1.1 Collection of samples

The samples for this work were collected in different locations (see Figure 1) in the Baltic Sea on August 17-20, 2023, and October 23-31, 2023. Concretion samples were gathered using a Van Veen grab sampler or an underwater remotely operated vehicle (ROV) claw. Sediment cores were obtained using a Gemax corer or a ROV push corer (9 cm diameter, 50 cm length). Sediment and microbial mat scoops were taken using a ROV pan or Gemax corer. Water samples were taken from 10 m, 1 m, 30 cm, 15 cm, 1 cm above the seafloor sediment. The water samples were filtered using Sterivex™ filter units (pore size 0.22 µm, Merck, Darmstadt, Germany). All samples were collected at locations with a water depth of 5-68 m.

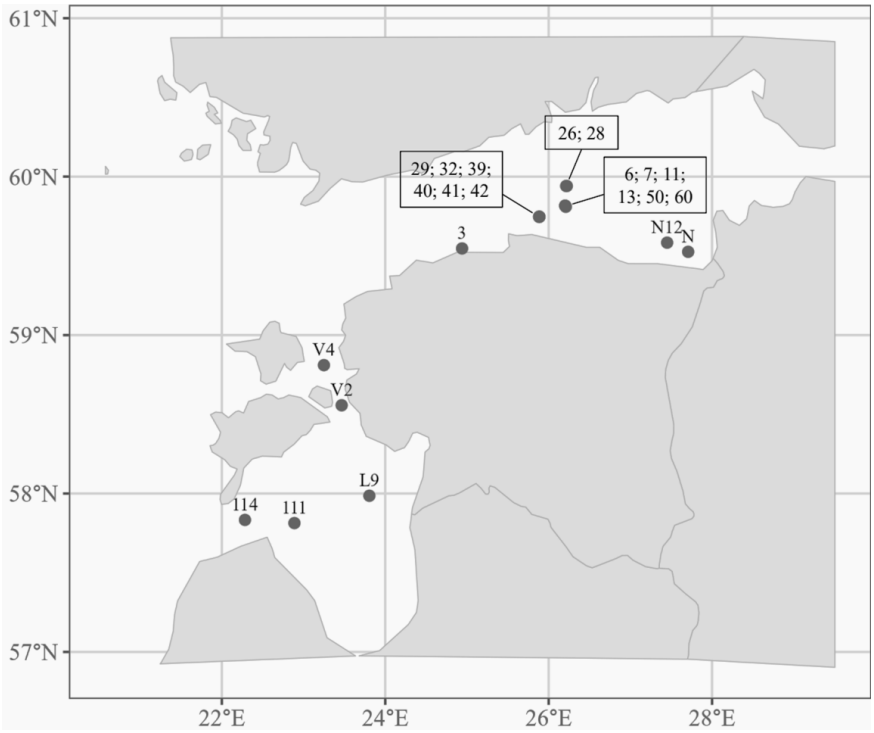

Figure 1 Map of sampling locations in the Baltic Sea. Sample numbers in boxes represent samples that were collected on August 17-20, 2023. Other samples were collected on October 23-31, 2023. If the sampling location is depicted by a number, then further in the text the first (or only) number that appears in a sample name represents the sampling location number.

3.1.2 DNA extraction

DNA extractions from concretion, sediment and water samples were done using the DNeasy® PowerSoil® Pro Kit (QIAGEN, Hilden, Germany). For concretion and sediment samples, about 250 mg of the sample was processed according to the manufacturer's instructions. A 5430 R centrifuge (Eppendorf, Hamburg, Germany) was used in this process.

For DNA extraction from water samples, the process was slightly modified. The water samples were stored in Sterivex™ filter units (Merck, Darmstadt, Germany). First, the water was removed from the filter unit with a syringe containing air and the filter unit was opened. For DNA extraction, the filter was cut into pieces and placed into a 5 mL tube with beads. Then, twice the amount of solution CD 1 (1600 µL) was added to the tube, and, after vortexing, it was centrifuged at 7000 × g for 5 minutes using a Sorvall LYNX 6000 centrifuge (Thermo Fisher Scientific, Massachusetts, U.S.A.). After this, each sample was separated into two subsamples and the following steps were performed according to the manufacturer's instructions. The subsamples were combined again in step 8. The extracted DNA was stored at -20 °C until further use. DNA concentration was measured using the NanoDrop spectrophotometer ND-1000 (Nano Drop Technologies, U.S.A.).

3.1.3 Primers

The forward primer sequence used for amplification of the V3-V4 region of the bacterial 16S rRNA was 5'-*ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT* CCT ACG GGN GGC WGC AG-3' and the reverse primer sequence was 5'-*AGA CGT GTG CTC TTC CGA TCT* GAC TAC HVG GGT ATC TAA TCC-3', including Illumina ThruSeq adapters (in *italic*). The V3-V4 region starts at position F341 and ends at position R805 of the bacterial 16S rRNA gene. The indexes used for sample identification and demultiplexing after sequencing are shown in Appendix 1.

3.1.4 Amplification of the bacterial 16S rRNA gene V3-V4 fragment

The bacterial 16S rRNA gene was amplified using PCR. The reaction mix had a total volume of 20 µL and it contained 1 µL each of forward and reverse primers, 0.75 µL 10 µM bovine serum albumin (BSA), 10 µL 2× Phusion Master Mix with HF buffer (Thermo Scientific, Lithuania), 0.5-7.25 µL of DNA sample (depending on DNA concentration in the sample), and the remaining volume was made up by H_2O. If DNA concentration was very low, the sample was cleaned using the Select-A-Size DNA Clean & Concentrator (Zymo Research, California, U.S.A.) according to the manufacturer's instructions. H_2O was used as negative control and a previously successfully amplified sample was used as positive control. The PCR was done using the Eppendorf Mastercycler EP Thermal Cycler Series (Eppendorf, Hamburg, Germany). The amplification conditions were 98 °C for 30 s, 29 cycles of 98 °C for 10 s, 55 °C for 30 s and 72 °C for 15 s, and then 72 °C for 10 minutes. The amplified DNA was stored at -20 °C until further use.

After PCR, gel electrophoresis using a 1.5% agarose gel (Atlas ClearSight Tablets (Bioatlas, Tartu, Estonia)) was performed to see if the templates were successfully amplified. Electrophoresis conditions were 100 V and 400 mA for 20 minutes.

3.1.5 Addition of indexes to the amplified bacterial 16S rRNA gene

To be able to identify the samples after DNA sequencing, a unique index was added to both ends of each amplified sample using PCR. The total volume of the reaction mix was 20 µL and it contained 6.25 µL H_2O, 0.75 µL 10 µM BSA, 10 µL 2× Phusion Master Mix with HF buffer (Thermo Scientific, Lithuania), 1 µL PCR-amplified DNA sample, and 1 µL each of a forward and reverse primer (each reaction mix contained a unique combination of forward and reverse primers with indexes from Appendix 1). The amplified DNA samples were diluted 20× before being added to the reaction mix. H_2O was used as negative control and a previously successfully amplified sample was used as positive control. The PCR was done using the Eppendorf Mastercycler EP Thermal Cycler Series (Eppendorf, Hamburg, Germany). The amplification conditions were 98 °C for 120 s, 12 cycles of 98 °C for 20 s, 60 °C for 30 s and 72 °C for 30 s, and then 72 °C for 5 minutes. To verify successful amplification, gel electrophoresis was performed as described in section 3.1.4. The amplified DNA was stored at -20 °C until further use.

3.1.6 Sequencing library preparation, DNA sequencing, and sequencing data analysis

The DNA sequencing library was prepared by combining each PCR-amplified sample with attached indexes. The quantity of each PCR product to be added was determined based on the intensity of the gel electrophoresis bands. The sequencing library was quantified, quality checked, sequenced, and demultiplexed by Institute of Molecular Microbial Medicine Finland (Helsinki, Finland) using the Illumina MiSeq 2×250 base pairs platform.

Sequencing data analysis was performed using the QIIME 2 bioinformatics pipeline (Bolyen et al., 2019). Quality checking, pairing forward and reverse reads, and removing chimera sequences was performed using the q2-dada2 plugin of the pipeline (Callahan et al., 2016). The 16S rRNA sequences were classified using Greengenes2 release 2022.10 taxonomy with the q2-feature-classifier plugin (Bokulich et al., 2018; McDonald et al., 2023). Before constructing a phylogeny, sequence alignment was performed using the q2-alignment plugin. Then, a phylogeny was constructed using the q2-phylogeny plugin with the FastTree method. The q2-diversity plugin with the method alpha-rarefaction was used to obtain alpha diversity estimates. Finally, the core phylogenetic diversity metrics, including weighted UniFrac dissimilarity matrix, were obtained using q2-diversity plugin (Catherine & Rob, 2005).

Further data analysis was performed using R version 4.1.2 (R Core Team, 2021). The vegan package was used to normalize and filter the data based on abundance (Oksanen et al., 2022). Microbial community data at the species level was rarefied using the function rrarefy from the vegan package. The rarefied data was used to calculate the Chao1 species richness estimate using the function estimate from the vegan package. Analysis of variance (ANOVA) (function aov) and the TukeyHSD (function TukeyHSD) post-hoc test was used to compare the species richness between different sample types. Beta diversity of the samples was assessed using classical (metric) multidimensional scaling with the function cmdscale. Differences between the beta diversities of sample types were assessed using the functions adonis2 from the vegan package and pairwise.adonis from the pairwiseAdonis package (Martinez Arbizu, 2017). Data visualization was carried out using the ggplot2 package (Wickham, 2016).

Sequence data manipulations for phylogenetic comparisons were done using the R package Biostrings (Pagès et al., 2024). Phylogenies were created as described above. Phylogenetic trees were constructed using IQ-TREE software (Nguyen et al., 2015).

3.1.7 Fluorescence microscopy

Prior to microscopy, samples were stained with SYBR Gold nucleic acid stain (Thermo Fisher Scientific, Massachusetts, U.S.A.) on a glass slide. Fluorescence microscopy was performed for all the concretion samples using a ZEISS LSM 900 confocal microscope (ZEISS, Oberkochen, Germany). An excitation wavelength of 511 nm was used.

3.2 RESULTS

3.2.1 Sequencing data

DNA was extracted from samples of concretions (n=6, two biological replicates for each, further - replicates), bulk sediment (n=9, 2-4 replicates), sediment cores (three cores with sediments from 0-7 cm depth (core 11, n=7, 2-3 replicates), 0-11 cm depth (core 32, n=11, 1-3 replicates), 0-3 cm depth (core 42, n=3, three replicates)), and water (water column (n=2, three replicates), bottom water (n=7, 1-4 replicates)).

The total number of sequences obtained from sequencing of 16S rRNA gene amplicon samples (n=45 (111, including replicates) was 2,454,656, on average 22,114 ± 10,442 (mean ± standard deviation) sequences per sample (range 3,287-71,790). A total of 87,287 amplicon sequence variants (ASV) were obtained. The sequencing depths for the different sample groups were quite similar. The highest sequencing depth was observed in the concretion samples (31,720 ± 17,013 sequences), the lowest – in core sediment samples (20,556 ± 6,692 sequences). Water and bulk sediment samples had 20,667 ± 14844 and 22,401 ± 6,907 sequences, respectively. As the rarefaction curves that show the relationship between

sequencing depth and number of ASVs (see Appendix 2) for almost all samples reach a plateau, it can be assumed that the sequencing depth was large enough.

3.2.2 Microbial diversity

The alpha diversity of the different sample types is depicted in Figure 2 using the Chao1 species richness estimate. The concretion samples have statistically significantly lower diversity (78.8 ± 50.2) (mean ± standard deviation) than all other sample types (α=0.05, $p_{adj.}$<0.01), except for the water column samples (208.3 ± 21.8) (α=0.05, $p_{adj.}$=0.08). The bulk sediment samples have the highest diversity (422.9 ± 117.9), but it is not significantly different (α=0.05, $p_{adj.}$=0.07-1.00) from that of core 32 (409.4 ± 94.9), core 11 (405.2 ± 77.0), and bottom water (395.9 ± 84.5) samples.

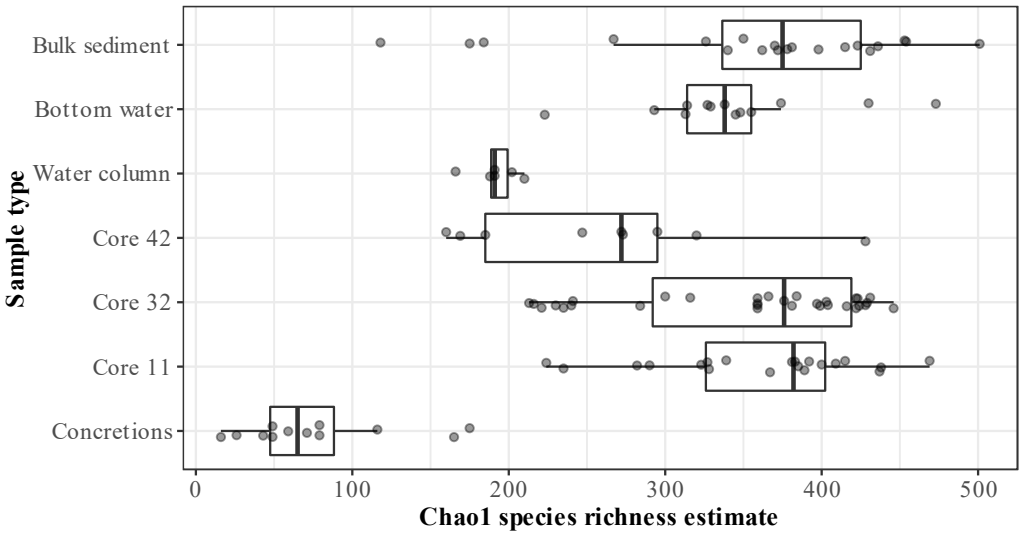

Figure 2 Alpha diversity of bottom water, water column, core sediment (core 11, core 32, core 42), concretion and bulk sediment samples depicted using Chao1 species richness estimate. The box starts at the 1st quartile and ends at the 3rd quartile. The line within the box represents the median value. The whiskers extend to the furthest point within 1.5 times of the inter-quartile range. Points outside the whisker range are outliers.

The beta diversity of the samples is shown in Figure 3. Most concretion samples are very different from all the other samples (α=0.05, $p_{adj.}$=0.02), and the concretions are not all similar to each other. All bulk sediment samples cluster together, apart from four outliers. Sediment cores 11 and 32 are also similar to most of the bulk sediment samples (α=0.05, p_{adj}=0.06-1.00). Core 42 is noticably different from the other sediment cores (α=0.05, p_{adj}=0.02), but does not differ significantly from bulk sediments (α=0.05, p_{adj}=0.42). Some bottom water samples are similar to core 42, and others – to the samples in the cluster of bulk sediment and cores 11 and 32. The bottom water doesn't differ significantly from core 42 (α=0.05, p_{adj}=1.00) and bulk sediments (α=0.05, p_{adj}=0.44), but it differs from core 11 and core 32 (α=0.05, p_{adj}=0.02). The

water column samples are also not significantly different from the bottom water ones (α=0.05, p_{adj}=0.38).

There is no significant difference between the beta diversity of different layers of each of the sediment cores (α=0.05, p_{adj}=0.30-1.00), so all layers of each core can be grouped together in analyses.

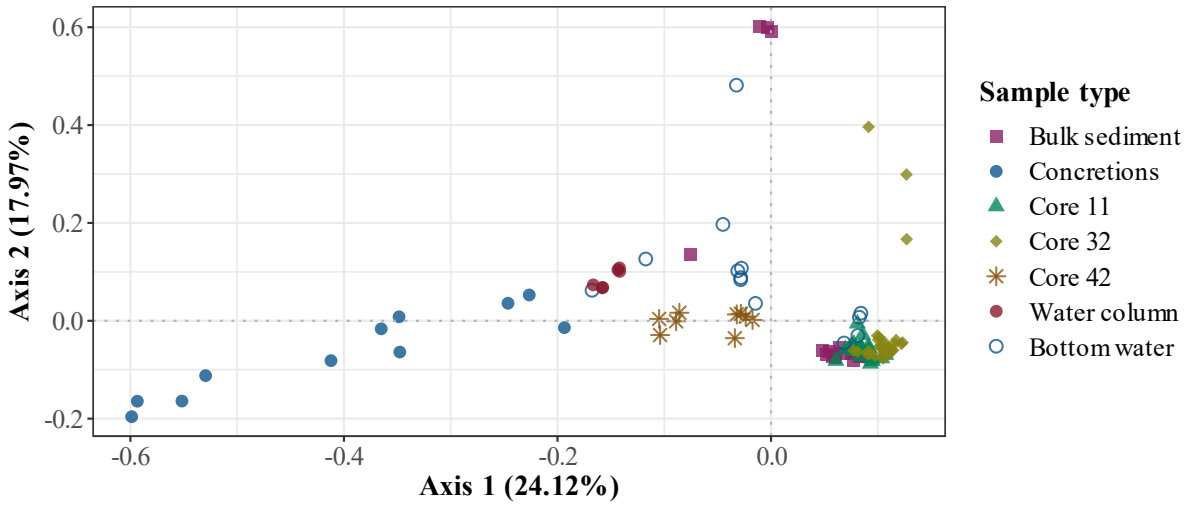

Figure 3 Beta diversity of bottom water, water column, core sediment (core 11, core 32, core 42), concretion and bulk sediment samples depicted using principal coordinates analysis with a weighted UniFrac dissimilarity matrix. Percentage of the total variability that each axis represents is shown in the axis titles.

3.2.3 Microbial community composition

The most abundant phylum in all sample types, except for concretions, was Actinobacteriota (see Figure 4). This phylum made up 33% in bulk sediments, 40% in bottom water, 57% in the water column, and 41, 39 and 35% in sediment cores 11, 32, and 42, respectively. Chloroflexota was the second most abundant phylum in bulk sediments, core 11 and core 32, comprising 20, 31 and 31%, respectively. For bottom water (19%) and core 42 (20%) samples, the second most abundant phylum was Cyanobacteria. There was also a considerable amount (14%) of Campylobacterota in bulk sediments. The concretions stood out, having a high abundance of Proteobacteria and Patescibacteria, which make up 51 and 29% of all bacteria, respectively. The Patescibacteria are represented in the concretion samples by the classes Microgenomatia, Dojkabacteria, WWE3, ABY1 (see Figure 5). The second highest abundance of Patescibacteria (after concretions) was in sediment core 42 (7%), while the second highest abundance of Proteobacteria (14%) was present in the water column samples. The water column samples stood out with the highest abundance of Planctomycetota (19%).

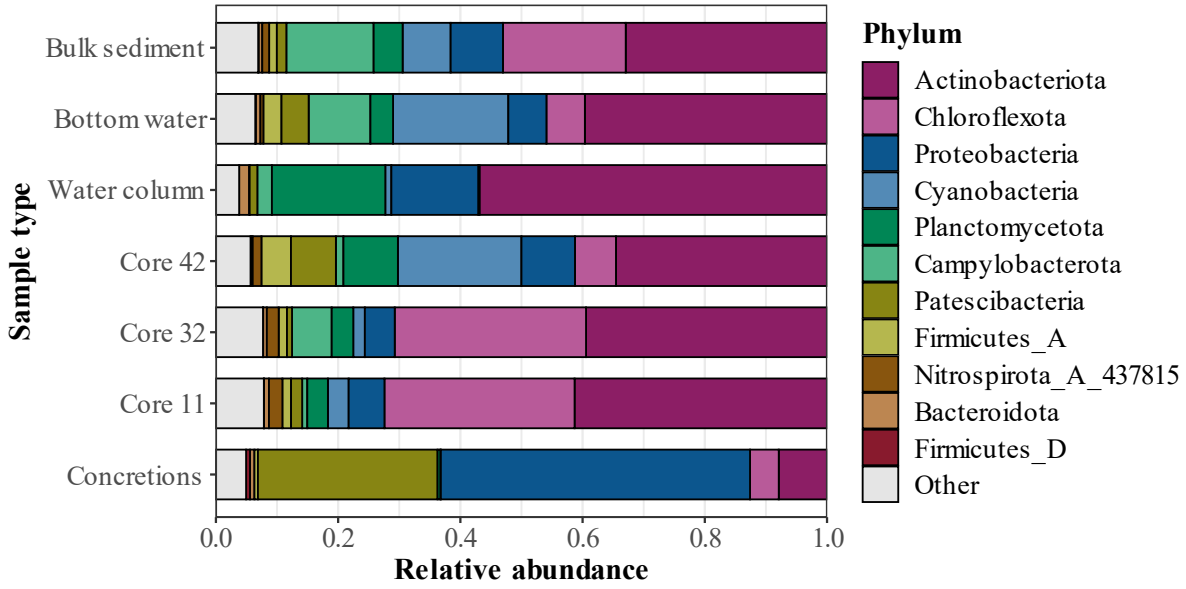

Figure 4 Relative abundance of bacteria belonging to different phyla in bottom water, water column, core sediment (core 11, core 32, core 42), concretion and bulk sediment samples. Phyla whose relative abundance is at least 0.03 in at least one sample are shown. Less abundant phyla are grouped together as *Other*.

By far, the most abundant genus in concretions was *Jidaibacter* (31%) of the class Alphaproteobacteria (see Figure 5). In one sample this genus made up as much as 89%. Bacteria from an unidentified genus belonging to the family *Xanthobacteraceae_503485* that is also from the class Alphaproteobacteria was the second most abundant in concretion samples (10%). Multiple members of the class Microgenomatia were relatively abundant in the concretions: the genus *GWC1.42.9* (5%), an unidentified genus from the family *UBA8517* (4%), and an unidentified genus from the family *PJMF01* (3%).

Mycobacterium, belonging to the class Actinomycetia, was the most abundant genus of bacteria in all the core sediments (core 11 – 13%, core 32 – 11%, core 42 – 19%) and in bottom water samples (17%). Making up 30%, the genus *UBA3006* of the class Acidimicrobiia_401430 was the most abundant genus in water column samples. This was followed by *UBA969* (13%) of the class Planctomycetia and *Planktophila* (7%) of the class Actinomycetia.

The genus *Sulfurimonas* (class Campylobacteria) was the most abundant in bulk sediment samples (11%). In some bulk sediment samples this genus made up 70-74% of all genera present, while in others it was present only in negligible amounts. *Sulfurimonas* were also very abundant in all replicates of sample 32_1, which is from the top layer of sediment core 32 (28-47%) and in one bottom water sample 42A (53%).

The genus *Ilumatobacter_A* was quite abundant in bulk sediment samples (9%), core 11 (6%), and core 32 (7%). Some members of the class Cyanobacteriia were relatively abundant

in bottom water and core 42 samples: the genera *Dolichospermum* (9% in core 42), *SIO2C1* (5% in core 42, 10% in bottom water), *Cyanobium_A_31520* (3% in core 42), and *Synechococcus_D* (4% in bottom water). The genus *SIO2C1* was especially abundant in sample 11 (34%), while the genus *Dolichospermum* was very abundant in all biological replicates of sample 42_1, which is from the top layer of core 42 (13-30%).

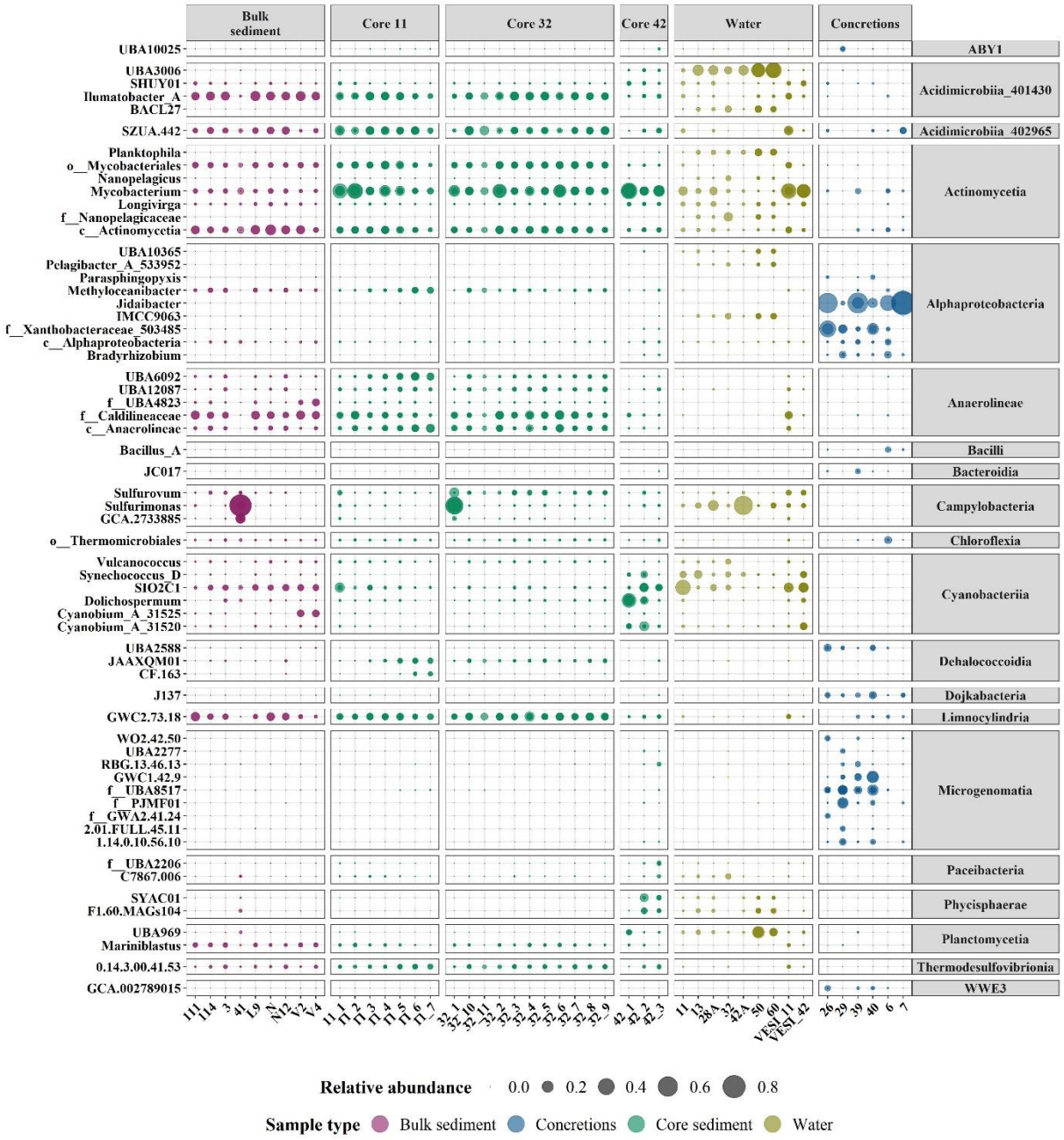

Figure 5 Relative abundance of different genera (left) of bacteria and the classes (right) that they belong to in water (samples 50 and 60 are from the water column; others are bottom water), core sediment (core 11, core 32, core 42), concretion and bulk sediment samples. Biological replicates are plotted on top of each other. Genera whose relative abundance is at least 0.03 in at least one sample are shown. If the genus was unidentified, the next lowest taxon is shown instead. f__ - family, o__ - order, c__ - class.

The microbial community composition of different layers of the sediment cores is relatively homogeneous, except for the aforementioned high abundance of *Sulfurimonas* and *Dolichospermum* in the top layer of cores 32 and 42, respectively.

3.2.4 Phylogenetic comparison with other relevant studies

Phylogenetic trees were constructed using 16S rRNA gene sequences from this work and from other studies that were done in similar environments and from which the sequences of the same region of the 16S rRNA gene were available.

The ASVs from this work that had at least 300 copies present in the samples were compared to 988 DNA sequences from a study which assessed the microbial community composition of concretions from the Gulf of Finland (Yli-Hemminki et al., 2014). They also had sequences from sediment samples, so all of the similar sequences are not from concretions. The ASVs from this work that belonged to the orders *Kiloniellales*, *Gemmatimonadales*, *Phycisphaerales*, *Thermodesulfovibrionales*, *Limnocylindrales*, families *Caldilineaceae* (two different ASVs), *Bryobacteraceae*, *Ignavibacteriaceae_785640*, *Coleofasciculaceae_23353* (two different ASVs), *Zambryskibacteraceae_A* (two different ASVs), and the species *Terrimicrobium sacchariphilum* had very high similarity (≥99%) to sequences from the study by Yli-Hemminki et al. (2014).

ASVs that belong to the class Microgenomatia of the phylum Patescibacteria, which is abundant in concretions and core 42, were compared to sequences from a study by Westmeijer et al. (2022) who analyzed groundwater samples from aquifers below the Baltic Sea. Westmeijer et al. (2022) had identified 10678 sequences that belong to Microgenomatia, while in this work there were 467 ASVs from this group. There were few ASVs that had high similarity (≥95%) with the sequences from Westmeijer et al. (2022). These were ASVs that belonged to the family *UBA8517* (one ASV unidentified at the genus level, two belonging to the genus *RBG.13.46.13*).

Some ASVs that belonged to the genus *Sulfurimonas* remained identified at the genus level and did not have good matches at the GenBank database (less than 92% match with isolates. Therefore, 16S rRNA gene sequences of *Sulfurimonas* isolates for the comparison were obtained from GenBank and a phylogenetic tree was constructed. The most abundant unidentified *Sulfurimonas* ASV was phyogenetically closest to *Sulfurimonas indica*.

3.2.5 Fluorescence microscopy

Fluorescence microscopy was performed for concretion samples to determine if there are intact cells on them that harbor the DNA that was extracted from the samples. Figure 6 shows fluorescence microscopy images of the concretions.

There were bacteria identified on all the concretion samples, but their abundance varied. Samples 26 and 39 were densely covered with cells, while for the other samples cells were less abundant.

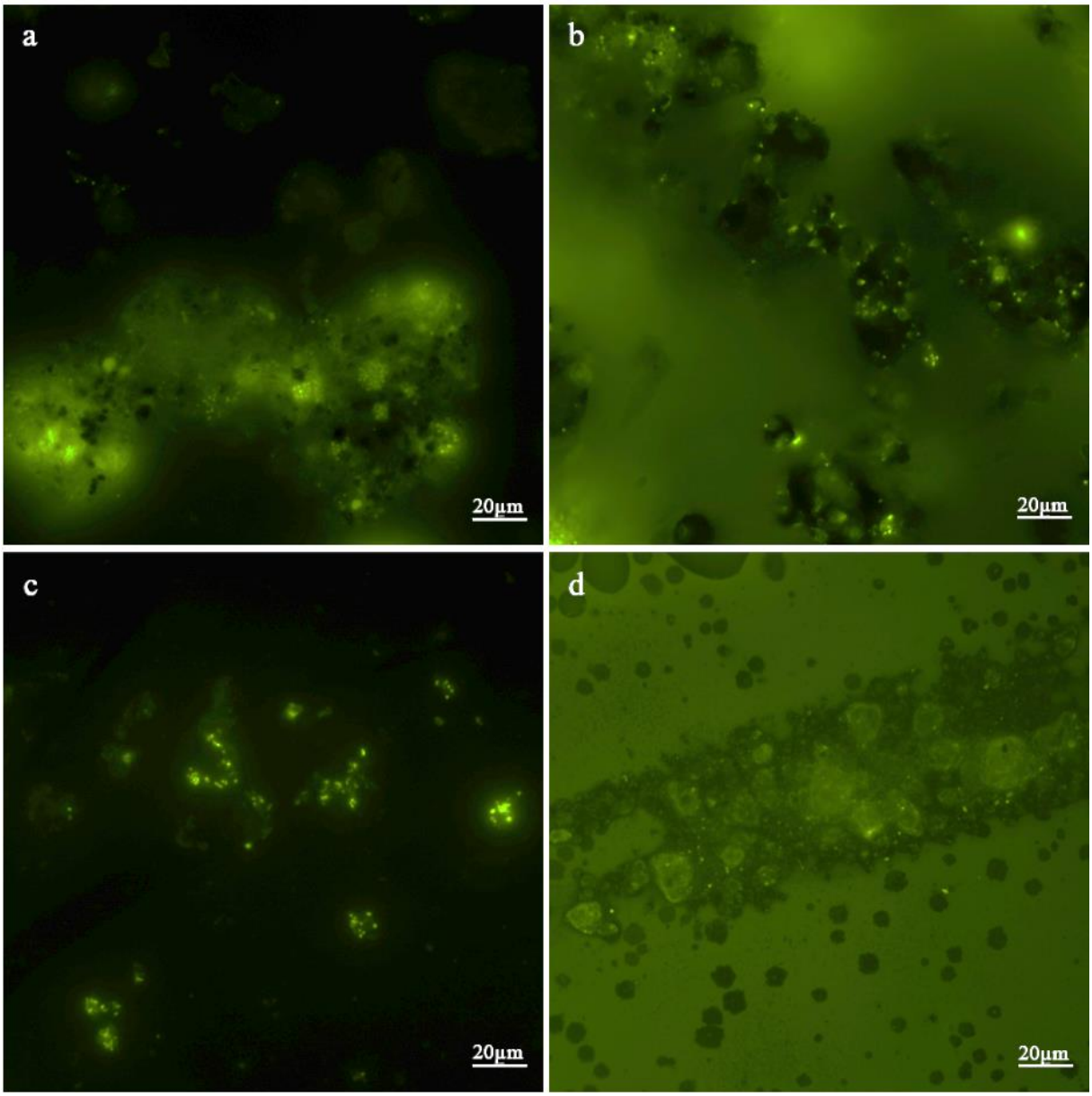

Figure 6 Fluorescence microscopy images of concretion samples. a) sample 39, b) sample 26, c) sample 6, d) sample 29.

3.3 DISCUSSION

3.3.1 Variability of bacterial abundance and composition on the concretions

Previous studies have identified microorganisms and their fossils inside concretions (Jiang et al., 2019, 2020; Shulga et al., 2022). Jiang et al. (2019) found that in some areas of concretions bacteria were more abundant. These were manganese-rich areas in particular. The porosity of concretions decreases in the direction of their center, and there are more microorganisms in the areas with larger and more abundant pores (Jiang et al., 2020). Hayles et

al. (2021) describe a concretion structure that has zones of different chemical composition and structure (an iron-rich zone, a radial growth zone with alternating iron and manganese layers, a low-density manganese-rich zone). These zones are likely inhabited by different taxa and the abundance of microorganisms between the zones should be variable.

Fluorescence microscopy was performed on all the concretion samples to confirm presence of intact cells on them, and variability of bacterial abundance within a concretion and between different concretions from different locations. This would mean that the DNA that is extracted from these samples is coming from cells, rather than free DNA molecules in the environment. Cells were identified on all the concretions, but their distribution was patchy on most of them. This variable distribution of cells could be explained by differences in the chemistry and structure of the concretions. While visual differences between different concretions and areas within a single concretion could be observed (color differences between different parts of a concretion, layered structure in some concretions), there were no chemical composition or structural analyses performed in this work, so it is not possible to say what differentiates the cell-rich areas from the others.

The concretion samples had the lowest microbial diversity of all the studied sample types. Previous studies also found that the microbial diversity of concretions is lower than that of sediments (Blöthe et al., 2015; Cho et al., 2018), which had overall the highest diversity among the samples in this work. Beta diversity of the concretions shows that they are different from all the other samples, but they are also different from each other. There is also considerable variability between the two replicates of each concretion. This shows that not only does the abundance of microbes vary in different parts of the same concretion, but the microbial community composition does as well.

3.3.2 Microbial communities in the concretions

There is one study in which the microbial communities were identified in concretion samples from sampling sites nearby to the locations from which concretion samples were obtained for this work (Yli-Hemminki et al., 2014). The same as in this work, Yli-Hemminki et al. (2014) found that half of the bacteria found in concretion samples belonged to the Proteobacteria phylum. While in their study Alpha-, Beta-, Gamma- and Deltaproteobacteria were almost equally abundant, in this study only Alphaproteobacteria and a minor fraction of Gammaproteobacteria were present. In the study by Yli-Hemminki et al. (2014), about a third of the bacteria in their samples were not assigned to any phylum. In this study, only 4% of the bacteria on average belonged to an unidentified phylum (these sequences were excluded from the analysis). Their analysis did not show any Patescibacteria, which were very abundant in this

work. Actinobacteriota and Chloroflexota were also identified in both studies with a notable abundance.

One possible explanation for the differences in microbial community composition could be the time of collection of samples. Although it is unknown at what time of year the samples for the study of Yli-Hemminki et al. (2014) were collected, the samples for this work were collected at the end of the summer. At this time of year, the oxygen concentration is lower than earlier in the year due to algal blooms which use up a lot of oxygen (Stoicescu et al., 2019) and the increase in temperature, which decreases the solubility of oxygen (Brutemark et al., 2011). Hence, aerobic bacterial groups may not be abundant during this time of year.

Phylogenetic comparison showed that there are 14 ASVs that have a very high sequence similarity with the sequences from Yli-Hemminki et al. (2014). None of these ASVs were very abundant in concretions. One of the ASVs that is present in concretions was assigned to the order *Kiloniellales*, which are facultatively anaerobic bacteria that commonly associate with different marine flora and fauna but can also be found in sediments and seawater. The type species of the family *Kiloniellaceae* is able to reduce nitrate and assimilate sulfate (Wiese et al., 2020). This family of bacteria have been identified on concentrations before (Shulga et al., 2022). Another ASV was identified as *Gemmatimonadales*, which belongs to the phylum Gemmatimonadota that can be found in many different environments, including aquatic. The bacteria from this phylum can utilize complex carbon sources, some are capable of CO_2 fixation, and they may be able to use N_2O and NO as terminal electron acceptors (Xiaowei et al., 2022). Another closely matching ASV, *Bryobacteraceae* are a family of bacteria with the ability to reduce iron and nitrate (Dedysh et al., 2017). The reason for few matching sequences could be that Yli-Hemminki et al. (2014) had them clustered in operational taxonomic units.

The genus *Bacillus_A*, which was identified in some concretion samples in minor amounts, could be involved in metal cycling. One *Bacillus* strain has been identified on concretions before and it has been proven to be capable of reduction of iron and manganese oxides (de Castro & Ehrlich, 1970). The species *Bacillus megaterium*, *Bacillus cereus* and *Bacillus subtilis* are able to oxidize iron (Hajiali et al., 2022; Khan et al., 2022; S. W. Wang et al., 2009). Some *Bacillus* species are also able to oxidize manganese (Gounot, 1994).

Proteobacteria are the most abundant phylum present in concretions. This phylum was mostly represented by the genus *Jidaibacter* (unidentified at the species level). They are endosymbiotic bacteria of amoebas, although they have many genes that overlap with those of free-living bacteria. *Jidaibacter* belong to the family *Midichloriaceae*, which is poorly studied and has no cultivated members (Giannotti et al., 2022; Schulz et al., 2016). Since the bacteria of this genus are obligate endosymbionts, it can be assumed that amoebas are present in

concretions. Due to being poorly studied, the metabolic capabilities of *Jidaibacter* are not known, thus it is not possible to determine if they have any role in concretion formation. Bacteria from the order *Rickettsiales*, to which *Jidaibacter* belong to, have been identified on concretions before (Shulga et al., 2022).

No taxa which are well-known manganese or iron oxidizers (mentioned in sections 1.2.1 and 1.2.2) were identified in the concretions (except *Bacillus*). These metal oxidizers were also not abundant in the study of Yli-Hemminki et al. (2014). This could indicate that the oxidation is carried out by lesser-studied taxa, that the metal oxidizers are less abundant in oxygen-limited conditions, or that extracellular electron transfer could be involved in the oxidation process.

Some bacteria can transfer electrons extracellularly, both to other cells (can be different species) and minerals, including those that contain iron and manganese in oxidized and reduced states. This transfer is achieved by using electroconductive pili, outer membrane cytochromes, or synthesizing extracellular electron shuttles (Dong et al., 2020). The well-represented class of Microgenomatia, which belongs to the Patescibacteria phylum could be one of the groups that contribute to extracellular electron transfer in concretions.

3.3.3 Influx of SGD-derived taxa to the marine environment

Patescibacteria, which are very abundant in concretions, are bacteria with small cells and a reduced genome that are adapted to the anaerobic environment. These bacteria have been previously found in groundwater (an oligotrophic environment) and sediments. Since they lack many basic metabolic functions, they are dependent on symbiotic relationships with other microorganisms. Patescibacteria were found to have pili that connect them to other microorganisms. These pili may serve as a means of nutrient and electron transfer (Luef et al., 2015; Tian et al., 2020). Thus, these bacteria may contribute to metal cycling in concretions through electron transfer.

Cores 32 and 42 were collected from pockmarks, so they are influenced by groundwater, which should also be reflected in the microbial community composition. But only core 42 contains the Patescibacteria that are characteristic to groundwater (Westmeijer et al., 2022). Core 32 instead seems to be more similar to core 11 and bulk sediment (except sample 41), which are not from a pockmark. This similarity also shows in the beta diversity of these cores. Only the top layer of core 32 (32_1) is different from core 11 and bulk sediment (as evidenced by the beta diversity). This difference comes from the large abundance of the genus *Sulfurimonas* in all the replicates of this sample. This genus also had an extremely high abundance in bulk sediment sample 41 that came from nearby the location of core 42. As it is so different from the rest of the bulk sediment, this sediment sample may also have been reached by the influence of groundwater discharge. This bacterial genus is present in a variety of

environments, including marine sediments and the water column (Henkel et al., 2021). These bacteria oxidize sulfur to gain energy. Most of them can oxidize hydrogen and reduce nitrate and oxygen. Some also perform autotrophic carbon fixation (Han & Perner, 2015). A recently isolated *Sulfurimonas* species in the Baltic Sea is also able to reduce manganese oxides (Henkel et al., 2021). The most abundant *Sulfurimonas* ASV was unidentified at the species level, but it shows the highest sequence similarity to *Sulfurimonas indica*, which was first isolated from a hydrothermal sulfide chimney and is able to oxidize hydrogen and sulfur (Hu et al., 2021).

The area where the sediment core samples were taken is on the northern edge of the Baltic Artesian Basin, where aquifers containing glacial palaeogroundwater that is mixed with meteoric groundwater are shallowly buried (<100 m depth). The water in the aquifer is under pressure (Raidla et al., 2009; Vaikmäe et al., 2021), thus it can escape through pockmarks. We can assume that cores 32 and 42 are affected by SGD from the Baltic Artesian Basin.

Westmeijer et al. (2022) assessed the microbial community composition of groundwater near the coast of Sweden, an area that is also at the edge of the Baltic Artesian Basin. They found that Proteobacteria and Patescibacteria are the most abundant phyla in deep biosphere groundwater, together making up at least 50% in all samples. Both of these groups are present in core 42, but their combined abundance is only 16%. In core 32 only Proteobacteria are present in a significant amount. The most abundant phylum in both of the cores is Actinobacteriota, which is also similarly abundant in core 11 that is not from a pockmark. This means that bacteria from this phylum inhabited the sediments before pockmark formation, and they were able to adapt to the changed environment, so they remained abundant. The same can be said about the phylum Chloroflexota that is equally abundant in cores 11 and 32. Through phylogenetic analysis, three ASVs from this work were found to have sequence similarity with Microgenomatia (phylum Patescibacteria) sequences from the study of Westmeijer et al. (2022). This could mean that the groundwater sources in the Gulf of Finland and near the coast of Sweden are connected to some extent, since these closely related taxa are not typically present in marine sediments.

Idczak et al. (2020) analyzed the microbial community of pockmark sediments from the Gulf of Gdansk (Idczak et al., 2020). This location is also within the Baltic Artesian Basin, but the thickness of sediments covering the aquifer is much larger than it is in the north of the basin (Vaikmäe et al., 2021), thus the water seeping from this pockmark probably doesn't come from the same source as in this work. In the study by Idczak et al (2020), Proteobacteria (>40%) were the dominant phylum, followed by Cyanobacteria, Actinobacteriota, Chloroflexota, and Bacteroidota (all 5-10%). All these phyla are present in cores 32 and 42 as well, but their abundance is very different. Proteobacteria are not nearly as abundant (5% and 9% in cores 32

and 42). The abundance of Bacteroidota is negligible, while Actinobacteriota are much more abundant in both cores. Cyanobacteria in core 42 and Chlorofexota in core 32 are more abundant than they were in the study by Idczak et al. (2020).

Purkamo et al. (2022) studied pockmark sediment in the Gulf of Finland, but their study location was outside the Baltic Artesian Basin, thus the groundwater coming from these pockmarks should be different (Purkamo et al., 2022). They found Campylobacterota in sediments from an inactive pockmark, but these bacteria were almost completely absent from active pockmark sediments. There was a higher abundance of this group in the surface layer (~8%), than in a deeper layer (~3%). This same trend is visible also in core 32, although the abundance of Campylobacterota is extremely high in this core's surface layer (46%) and very low in the other layers. On the other hand, just like the sediments from active pockmarks in the study by Purkamo et al. (2020), core 42 has only a minor presence of Campylobacterota (1%). Purkamo et al. (2020) also found that the alpha diversity of inactive pockmark sediments was higher than that of active pockmark sediments. In this work, the alpha diversity of core 32 was higher than that of core 42. These similarities could point to the pockmark in the location of core 32 not being active or having a negligible SGD rate.

3.3.4 Microbial communities in sediments and seawater

All sediment cores were separated into different depth layers before DNA extraction, so depth-related microbial community differences could be observed. Based on beta diversity analyses, there is no significant difference between any of the layers of the cores. The homogeneity of the microbial community composition of the sediment cores points to the occurrence of sediment resuspension.

Generally, the phyla composition of the bulk sediment is similar to that of core 11. The biggest difference is the high abundance of Campylobacterota in bulk sediments, which is due solely to this phylum being dominant in all the replicates of sample 41. Some variance in the abundance of the other phyla is visible, but all of the same phyla are present in the bulk sediment and core 11. The genera composition of these samples is also quite similar. The sediment samples were collected from the Gulfs of Riga and Finland, and Väinameri. This demonstrates that sediments that are not affected by SGD have similar microbial community composition even when they are located far apart.

In terms of alpha diversity, bottom water samples had significantly higher diversity values than samples from the water column. This could be because the water column samples were taken at the same location at different depths, while the bottom water samples were from multiple different locations. Some of the bottom water samples were taken directly from the surface of sediment cores, so these water samples were mixed with some resuspended

sediments. This could also have increased the microbial diversity of these samples, since the sediment samples had the highest alpha diversity. This mixing between sediments and water also explains why some of the bottom water samples are close to core sediments in terms of beta diversity. The beta diversity of the water column and bottom water samples did not differ significantly, but there are some differences between the most abundant taxa in these samples. The biggest difference is that the water column contains a sizeable community of Planctomycetota (19%), while the bottom water contains the same amount of Cyanobacteria (19%).

3.3.5 Limitations and future perspectives

This study cannot account for describing all of the microbial diversity in the studied sites because bacteria-specific primers were used, thus Archaea and Eukaryotic microorganisms could not be detected. Analyses of geochemical parameters of the studied samples are not yet completed, so their contribution to the microbial community composition cannot be assessed at this point. If this information becomes available, then perhaps it could explain why the microbial community of one pockmark sediment is different from that of non-pockmark sediment and another one is not. It could be possible that the SGD from the pockmark in the location of core 32 doesn't have a high enough volume to affect the microbial community composition. To verify this, the volume of SGD should be measured. Further work should also be done to assess seasonal differences in microbial community composition of concretions from the same area. Collecting data on the oxygen concentrations at sampling sites would be needed, so the role of redox conditions in microbial community composition can be determined.

4 SUMMARY

This work was conducted to assess the bacterial community composition of ferromanganese concretions and pockmark sediments using molecular biology methods and comparing them to seawater and other sediment samples. The concretion microbiome was significantly different from all the other sample types. A marked difference could also be observed between the bacterial communities of one pockmark sediment core and the other sediment samples, the most significant difference being the increase in the abundance of bacteria from the phylum Patescibacteria. Another pockmark sediment core was more like sediments that are not affected by groundwater, which could point to this pockmark being inactive. The role of the most abundant bacterial taxa in concretion formation could not be accurately determined due to the lack of research on these taxa. The results indicate that the process of concretion formation may be seasonal. Bacteria of the class Microgenomatia may have a part in iron and manganese oxidation through electron transfer. The microbial community composition of sediment cores was mostly homogenous between their different layers, signifying the occurrence of sediment resuspension. Further work is needed to be able to elucidate the mechanism of concretion formation and determine the seasonal and geochemical effects on microbial community composition.

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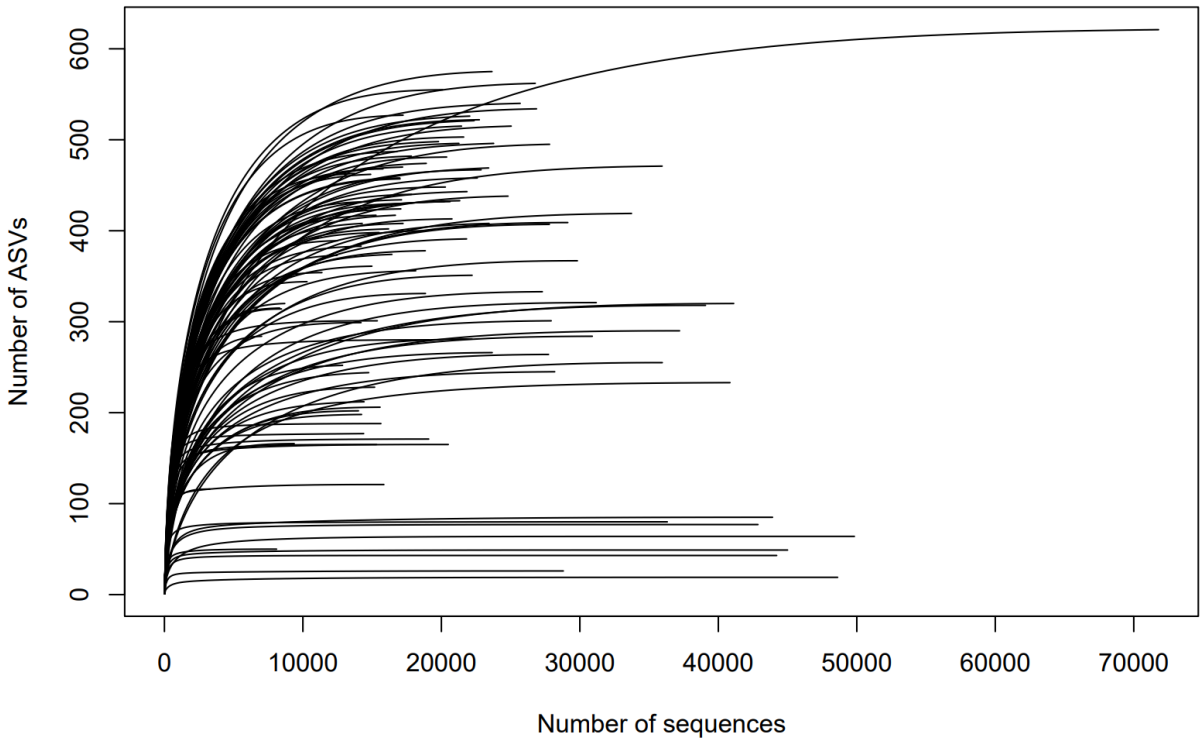
APPENDIX

Appendix 1 Sequences of indexes used for sample identification and demultiplexing after sequencing.

Reverse		**Forward**	
Index name	**Sequence (5′–3′)**	**Index name**	**Sequence (5′–3′)**
R501	AGGCTATA	F501	TATAGCCT
R502	GCCTCTAT	F502	ATAGAGGC
R503	AGGATAGG	F503	CCTATCCT
R504	TCAGAGCC	F504	GGCTCTGA
R505	CTTCGCCT	F505	AGGCGAAG
R506	TAAGATTA	F506	TAATCTTA
R507	ACGTCCTG	F507	CAGGACGT
R508	GTCAGTAC	F508	GTACTGAC
R701	CGAGTAAT	F701	ATTACTCG
R702	TCTCCGGA	F702	TCCGGAGA
R703	AATGAGCG	F703	CGCTCATT
R704	GGAATCTC	F704	GAGATTCC
R705	TTCTGAAT	F705	ATTCAGAA
R706	ACGAATTC	F706	GAATTCGT
R707	AGCTTCAG	F707	CTGAAGCT
R708	GCGCATTA	F708	TAATGCGC
R709	CATAGCCG	F709	CGGCTATG
R710	TTCGCGGA	F710	TCCGCGAA
R711	GCGCGAGA	F711	TCTCGCGC
R712	CTATCGCT	F712	AGCGATAG

Appendix 2 Rarefaction curves that depict the relationship between sequencing depth and number of amplicon sequence variants (ASVs).



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