Microbial Nitrogen Cycling in the Marine Deep Biosphere

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Thesis for the Degree of Philosophiae Doctor (PhD)
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Scientific environment

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Abstract

A considerable fraction of life on Earth is not present in the surface world but is buried in the subsurface - the deep habitable zone supporting the deep biosphere. Subseafloor sediments and the upper oceanic crust are the major compartments hosting microbial life in the marine deep biosphere. The biological activities in this biosphere exert a profound influence on global biogeochemical cycles and the partitioning of elements between the atmosphere, ocean, and deep subsurface. Our current knowledge is largely obtained from organic-rich sediments and seafloor-exposed crust. However, the vast majority of marine sediments are located beneath open oceans and characterized by oligotrophic conditions and most of the oceanic crust is deeply buried below the seafloor. These subsurface environments were largely ignored in the past and only recently explored during international expeditions. Geochemical data from the organic-lean sediments beneath open oceans show extended penetration of oxygen and nitrate, highlighting a markedly different geochemical regime than the one observed in organic-rich regions, and suggest the prevalence of nitrogen cycling processes in large areas of the global sedimentary ecosystem. In addition, nitrogen transformation steps may also prevail in ocean crust, and be linked with sedimentary processes by the diffusion of nitrogen species across the sediment-basement interface. Nonetheless, the microbial nitrogen cycling in the marine deep biosphere has not been well studied.

This thesis, therefore, is dedicated to improve our understanding about microbial life especially those involved in the nitrogen cycling in the subseafloor sediments and oceanic crusts. The samples and data used in this thesis are collected from several oligotrophic ocean regions including the North Atlantic Gyre (sediments and basaltic rocks retrieved at North Pond during the International Ocean Discovery Program (IODP) Expedition 336), the Arctic Mid-Ocean Ridge beneath the Norwegian-Greenland Sea (sediments), the South and North Pacific Gyres (sediments), and the Arabian Sea (sediments). Multiple quantitative approaches, including reaction-transport models, thermodynamic calculations, and functional

gene-based quantitative PCR, were applied to estimate the fluxes, reaction rates, functional group abundances, Gibbs free energy, and power supply of relevant processes of nitrogen cycling in both the sediments and crust. 16S rRNA gene amplicon sequencing was used to complement the quantitative approaches, and profile the community composition and structure with high resolution, to track the dynamic of microbial functional groups with time in the energy-limited deep biosphere.

This thesis is consisted of four individual case studies, each of which is summarized as following.

Paper I is a descriptive study of the sediment-buried basalts at North Pond. Using 16S rRNA gene as the biomarker, cell numbers in the basalts are found to be relatively stable at ~10^4 per gram of rock, with the communities dominated by *Proteobacteria*, especially *Gammaproteobacteria*. The overall community structure in the sediment-buried crust is distinct from that in the overlying sediments, although many of the microbial inhabitants are shared between these two biomes. It provided one of the first reports describing the microbial inventory and quantified the microbial abundances of deeply, buried basaltic crust on a young and cool ridge flank.

Paper II is dedicated to the North Pond subseafloor sediments and highlighted that a substantial fraction of microbes living in the oligotrophic sediments are involved in nitrogen transformation, especially nitrification. Flux calculation suggested that the downward diffusion of nitrate into the underlying crust account for a considerable fraction (up to 29%) of the total nitrate diffusing out of the sediment piles at North Pond. Increases of abundances and diversity (OTU numbers) of nitrifying communities were found at the oxic and anoxic boundaries. Such microbial abundance elevations suggest *in situ* growth of microbes in the subsurface. Their cell-specific rates fall into the same range as those reported for other physiological groups from other locations, supporting the notion that microbes in the sedimentary deep biosphere are sustained by a similar basal energy requirement.

Paper III returns to the sediment-buried basalts at North Pond and is a systematic comparison of ammonia-oxidizing Archaea (AOA) communities (dominated by Nitrosopumilales) found in the bottom seawater, subseafloor sediments, and the underlying oceanic crust, based on phylogenetic marker genes of archaeal 16S rRNA and *amoA*. *In situ* nitrification activity of AOA was suggested by a reaction-transport model, based on the inferred chemical composition in the crustal fluids. Nitrosopumilales communities in the basaltic crust were revealed to be dominated by the Eta clade, with a community structure distinct from that in bottom seawater and surface sediments but similar to that in the basal sediment. This could indicate the potential exchange of microbes between basaltic crust and the overlying sediments and/or the *in situ* growth of the Eta clade in the basaltic basement.

Paper IV presents the quantification of energetic requirements of nitrifiers in marine oxic sediments, based on a compilation of geochemical and microbiological data from seven sediment cores collected at five distinct geographical regions. Substantial differences were observed in these cores, with the oxygen penetration depth varying from centimetres to 42 meters below seafloor (mbsf). By computing the Gibbs free energy, reaction rates, and power supply based on the porewater profiles of relevant solutes, we showed that the power availability to nitrifiers (largely composed by Nitrosopumilales) are mainly controlled by the nitrification rates rather than the Gibbs free energy per reaction. No significant differences were observed between the cell-specific maintenance power requirement of nitrifiers among the sediments from different depths and from locations with various organic matter contents. This study provides an estimate of cell-specific power requirement of nitrifiers in oxic sediments, a valuable parameter to estimate the standing stock of nitrifiers in the seabed based on geochemical profiles.

Taken together, this project provides in-depth insights into the identity, community structure, dynamics, and activity of microbes involved in the nitrogen cycle in the oligotrophic subseafloor sediments and the underlying oceanic crust. It also suggested that nitrifiers in the marine oxic sediments have similar cell-specific power requirement, regardless of the geographic location and oxygen penetration

depth, and therefore, provides constrain on the microbial basal power requirement in the marine sedimentary biosphere.

List of publications

Paper I

Steffen L. Jørgensen and Rui Zhao. Microbial inventory of deeply buried oceanic crust from a young ridge flank. *Frontiers in Microbiology*, 7. doi: 10.3389/fmicb.2016.00820

Paper II

Rui Zhao, Bjarte Hannisdal, Jose Mogollon, and Steffen L. Jørgensen*. **"***Microbial nitrogen cycling in oligotrophic open ocean sediments*". submitted to *PNAS.*

Paper III

Rui Zhao*, Håkon Dahle, Gustavo Ramirez, and Steffen L. Jørgensen. "*Evidence of Active Indigenous Ammonia-Oxidizing Archaea in Subseafloor Oceanic Crust*". Unpublished manuscript, to be submitted to *Environmental Microbiology*.

Paper IV

Rui Zhao*, Desiree Roerdink, Ingunn Thorseth, Ingeborg Økland and Steffen L. Jørgensen. "*Energetics of nitrifiers in marine oxic sediments*". Unpublished manuscript.

Introduction

1 Background

Microbial life is found in almost every corner of the Earth. A considerable fraction of this is located underneath the surface of the planet, the sum of these organisms constitute the so-called deep biosphere. It has been argued that we know more about the surface of Mars than the deep oceans of our Earth. Nevertheless, our understanding has improved significantly in the last half century as a result of extensive oceanographic cruises to various locations around the globe. This thesis revolves around sample materials obtained from one of these expeditions and tries to improve our understanding of microbial life in the marine deep biosphere, with a specific focus on those involved in the nitrogen cycling. Hence, the following sections aim to give a short introduction to microbial life in the marine deep biosphere, their activities, and their role in the nitrogen cycle.

1.1. The compartments of the marine deep biosphere

The exact boundaries of the marine deep biosphere is still under debate (LaRowe and Amend, 2015a), but in this thesis I adopt the definition by Jørgensen and Boetius (2007), for the upper boundary. This definition states that life existing more than one meter below seafloor (mbsf) is part of the deep biosphere, although it is not perfect and does not include the very surface part of the seafloor exposed oceanic crust. The lower boundary is likely defined by the energy availability, temperature (known temperature limit of life, 122^{o}C, Takai et al. (2008)) and pressure (gigapascal range; Sharma et al. (2002)). These parameters are only theoretical, as we have not yet reached the depth limit as so far life have been found in even the deepest samples scientists has analyzed (Inagaki et al., 2015). However, what we know for sure is that there is a large number of living cells in the marine deep biosphere, and that their metabolisms helps to regulate the fluxes between the surface and subsurface of Earth and exert a great influence on a variety of geochemical cycling processes on a global scale.

Subseafloor sediments and upper oceanic crusts are the two important habitable compartments for life in the marine subsurface, and both are introduced with more details below. In addition, the deep ocean is closely associated with the marine deep biosphere, and although it is not defined as being part thereof in this thesis, it deserves a short introduction.

1.2. Microbial Life in Subseafloor sediments

Despite receiving less than 1% of the organic carbon produced in the surface waters, and most of this deposited organic matter being degraded in the surface sediments (Burdige, 2007), the share volume of marine sediments makes them the largest reservoir of organic carbon on our planet (Hedges and Keil 1995). The degradation and burial of organic matter in marine sediments exert a profound influence on global biogeochemical cycles and the partitioning of elements between the atmosphere, ocean, and deep subsurface (e.g. reviewed in Arndt et al. (2013) and Burdige (2007)). Microorganisms, including Archaea, Bacteria and Fungi, are the major dwellers in the marine deep biosphere and have been detected in practically all subseafloor sediments investigated so far (Parkes et al., 1994; Roussel et al., 2008; Orsi et al., 2013a; Ciobanu et al., 2014; Inagaki et al., 2015). This so-called "unseen majority" represents a considerable portion of the total cell abundance on Earth and was estimated to account for 55 to 85% of Earth's prokaryotic biomass and about 30% of the total living biomass (Whitman et al., 1998), and although recent estimates suggests these numbers should be downsized to ~2.9-5.4×10^{29} cells (Kallmeyer et al., 2012; Parkes et al., 2014) ~0.6% of total living biomass, the numbers are still mind baffling.

Microbial life in subseafloor sediments are generally suffering from low energy conditions, and cellular metabolic rates are often more than three orders of magnitude lower than those observed under laboratory conditions (D'Hondt et al., 2002; D'Hondt et al., 2009; Hoehler and Jørgensen, 2013; Lever et al., 2015), resulting in estimated turnover times reaching hundreds to thousands of years (Jørgensen, 2011; Lomstein et al., 2012). However, accumulating evidence suggest

that they still have the potential to be metabolic active over geological timescales (Parkes et al., 2005; Morono et al., 2011; Trembath-Reichert et al., 2017), despite extremely low cellular activity (Hoehler and Jørgensen, 2013; Jørgensen and Marshall, 2016). Their activity is closely linked to the mineralization of organic matter during early diagenesis. In this process, organic matter is re-mineralized by multiple enzymatic reactions coupled to the sequential utilization of terminal electron acceptors, following the order of decreasing energy production per mole of organic carbon oxidized: oxygen, nitrate, manganese oxides, iron oxides, sulfate, and methane (Froelich et al., 1979). This leads to a clearly defined depth zonation of dissolved chemical species known as the redox zonation (Canfield and Thamdrup, 2009). Given that the redox reactions leading to the zonation are mainly mediated by microorganisms, one would expect a corresponding zonation of microbial community, however this link is less obvious.

Most of the current knowledge about life and biogeochemical processes in marine sediments is deduced from observations on the continental margins, which contain relatively high organic carbon levels. However, the vast majorities of marine sediments are found beneath the open ocean, accounting for more than 70% of the global seafloor, and are typically characterized by oligotrophic conditions (Dunne et al., 2007). These remote places were left almost untouched by (geo)microbiologist, until very recently where a number of expeditions have focused on these regions (D'Hondt et al., 2009; Røy et al., 2012; Ziebis et al., 2012; Orcutt et al., 2013; D'Hondt et al., 2015). These efforts revealed a markedly different geochemical environment relative to organic-rich sediments. One of the most striking differences is that both oxygen and nitrate can penetrate tens of meters into the seafloor in oligotrophic sediments, or even all the way through (Røy et al., 2012; D'Hondt et al., 2015) (Ziebis et al., 2012; D'Hondt et al., 2015), whereas they are depleted within centimeters or less in the continental margin sediments (Revsbech et al., 1980). Moreover, the higher concentration of nitrate in the porewater relative to the bottom seawater seems to be ubiquitous in oligotrophic marine sediments (D'Hondt et al., 2009; Ziebis et al., 2012; D'Hondt et al., 2015; Mewes et al., 2016), indicating that

these sediments are a net nitrate source to the deep ocean. Nitrate, in addition to phosphorus, is a critical nutrient for phytoplankton in the surface ocean and, therefore, serve as a critical control on the primary production of the global ocean (Falkowski, 1997; Tyrrell, 1999). However, such surveys have only been conducted in a very limited number of locations. Given the fact that the majority of the global seafloor is occupied by oligotrophic sediments (Claustre et al., 2008), we clearly need more knowledge about the microbial community composition and their metabolic activities to comprehensively understand microbial life in marine sediments on a global scale.

1.3. Microbial life in oceanic crust

Oceanic crust is part of the outermost layer of Earth's lithosphere and is produced primarily at the oceanic spreading ridges, from where it is transported towards to subduction zones by plate tectonics. Over time and due to the settling of particulate material from the above water masses the crust gets buried by an increasingly sediment cover the further away from the ridges it gets. The young, porous, fractured part of the upper oceanic crust is hydrologically active, allowing seawater circulation driven by hydrothermal heat and pressure (Wheat et al., 2003). Ever since the discovery of hydrothermal vents in 1977, along the mid-ocean ridges, oceanic crust has been postulated to be inhabited by microbes based on the detection of bacterial cells in the hydrothermal fluids (Corliss et al., 1979). Some of the first direct evidence for life related to igneous oceanic crust was reported in 1992 where the presence of microbial life was identified by various approaches, including DNA staining, microbial fossil identification based on morphology, and elemental mapping (Thorseth et al. (1992).

Our understanding of microbial life in the oceanic crust, especially that buried beneath sediments, is very poorly constraint (Edwards et al., 2012a; Orcutt and Edwards, 2014). Regarding the total biomass, it has been argued that oceanic crust could host an even higher number of microbial cells than marine sediments, due to its habitable zone predicted to be 10-fold larger (Heberling et al., 2010; Orcutt et al., 2011a). The distribution of microbial cells in ocean crust is also uneven at different depths: 10^5 – 10^9 cells per g rock (Einen et al., 2008, Santelli et al., 2008) was

generally reported in the seafloor-exposed basaltic rocks, while only up to 10^5 cells per g rock was observed for the sediment-buried ocean crust on the flank of Mid-Atlantic Ridge (Zhang et al., 2016a).

While the newly formed crust is exposed on the seafloor, it eventually gets buried under sediments during most of their lifetime in the movement from ridge-spreading zones to subduction zones. In this subsurface environment the existence of a diverse microbial life has been observed in crustal rocks (Lever et al., 2013; Zhang et al., 2016a), as deep as 1391 mbsf (Mason et al., 2010), and crustal fluids (Cowen et al., 2003; Huber et al., 2006; Nigro et al., 2012; Jungbluth et al., 2013; Meyer et al., 2016), mainly through DNA-based techniques. Due to the oligotrophic conditions, microbes in the crustal fluids have been suggested to contribute a large fraction of dissolved organic carbon to the deep ocean, through chemolithoautotrophic processes (McCarthy et al., 2011; Pohlman et al., 2011). In addition, their functional potentials has been investigated by isotopic labeling incubation (Orcutt et al., 2015; Meyer et al., 2016), stable isotope analysis (Alt, 1995; Lever et al., 2013), and metagenomics sequencing (Meyer et al., 2016; Tully et al., 2018). The main mechanism believed to seed microbes is the hydrothermal circulation of seawater (Schrenk et al., 2010a; Orcutt et al., 2011b; Edwards et al., 2012b). The distribution of microbial life in subsurface crustal rocks has only been investigated by a handful of studies, and several factors, e.g. temperature, redox condition (Baquiran et al., 2016), and host rock lithology (Smith et al., 2011), have been suggested to influence the microbial colonization of crustal rocks. Nevertheless, accumulating data from the last two decades clearly demonstrated the importance of these habitats as compartments of the Earth's total biosphere.

1.4. Microbial life in bottom seawater

While I don't include the deep-water masses as part of the deep biosphere in this thesis, they are closely linked with the above-described subseafloor sediments and upper oceanic crust, and therefore, deserve the following short introduction. The basal part of the open ocean is generally found at a depth that is categorized as either

bathypelagic (1,000 – 4,000 m below surface - mbs) or abyssopelagic (4,000 – 6,000 mbs). Despite the low available energy, microorganisms are present in abundance of 10^3-10^4 cell ml^{-1} (Karner et al., 2001; Sogin et al., 2006), with Archaea accounting for a considerable fraction (up to 39% of the total community; Karner et al. (2001)). There is no light in the bottom seawater and it is completely dark and the sinking particles from the surface waters are considered to be the main carbon source for bathypelagic and abyssopelagic communities (Nagata et al., 2000; Hansell and Ducklow, 2003). However, some studies argue that the organic matter flux is insufficient to meet the demands from the microbes living there, and chemoautotrophic processes has been suggested to be prevailing, especially in the bathypelagic ocean (Middelburg, 2011; Swan et al., 2011).

1.5. Communications between the deep habitats

The three compartments of the marine biosphere mentioned above - the bottom seawater, the sediments and the upper oceanic crust - are closely linked (Figure 1). The fractured, young upper oceanic crust is connected with the bottom ocean through seawater circulation (Fisher, 2005; Edwards et al., 2012b). Seawater entering into the porous crust (dark blue arrows in Figure 1) carries abundant electron acceptors, such as oxygen and nitrate (Orcutt et al., 2013; Meyer et al., 2016), organic matter (Hawkes et al., 2015), and a diverse microbial population (Jungbluth et al., 2016; Meyer et al., 2016; Tully et al., 2018). The latter has been suggested to be the likely inoculum seeding subseafloor crustal biomes (Schrenk et al., 2010b; Orcutt et al., 2011b; Edwards et al., 2012b). Conversely, fluid discharge (focused vents and diffusive discharge; red arrows in Figure 1) may translocate reduced chemical species [e.g. Fe^{2+}, Mn^{2+}, H_2, CH_4 and NH_4^+; see review in Elderfield and Schultz (1996)] and microbes (Lesniewski et al., 2012) from the crustal fluids and back into the bottom ocean. The marine sediments are connected with both the bottom ocean (Hamdan et al., 2013; Walsh et al., 2016a), mainly through sedimentation and diffusion processes (black arrows in Figure 1), and with the fluids in the underlying oceanic

crust(D'Hondt et al., 2004; Wheat and Fisher, 2008; Orcutt et al., 2013; Mewes et al., 2016; Kuhn et al., 2017), primarily through diffusion (green arrows in Figure 1).

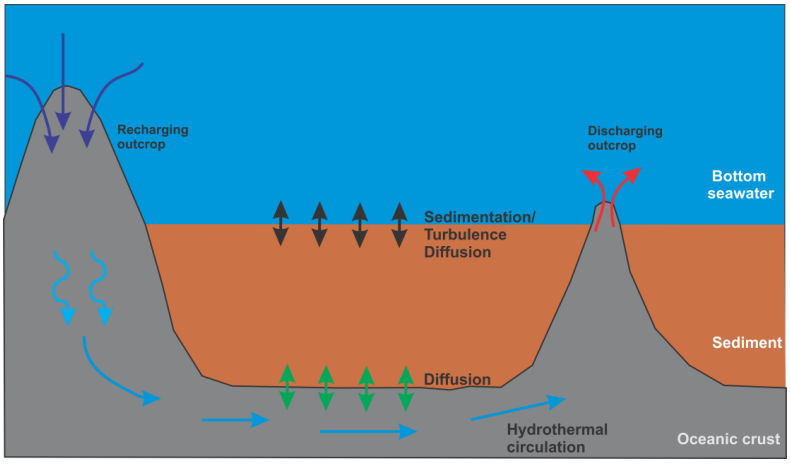

Figure 1. Communication between the three compartments of the deep biosphere. The microbiome in the bottom seawater and sediments are linked by sedimentation/turbulence (black arrows) and is also coupled with the oceanic crust through seawater circulation (blue arrows). There is also communication between the oceanic crust and the overlying sediments, mainly in the form of geochemical diffusion (green arrows) [Modified from Edwards (2012b)].

2 Microbial nitrogen cycling

Nitrogen is the fifth most abundant element in our solar system, and is an essential requirement for the synthesis of vital biomolecules such as nucleic acids and proteins – the two most important polymers of life. Several of the individual steps in the nitrogen cycle outlined in Figure 2, are dependent on redox reactions mediated by microorganisms (Falkowski et al., 2008; Canfield et al., 2010).

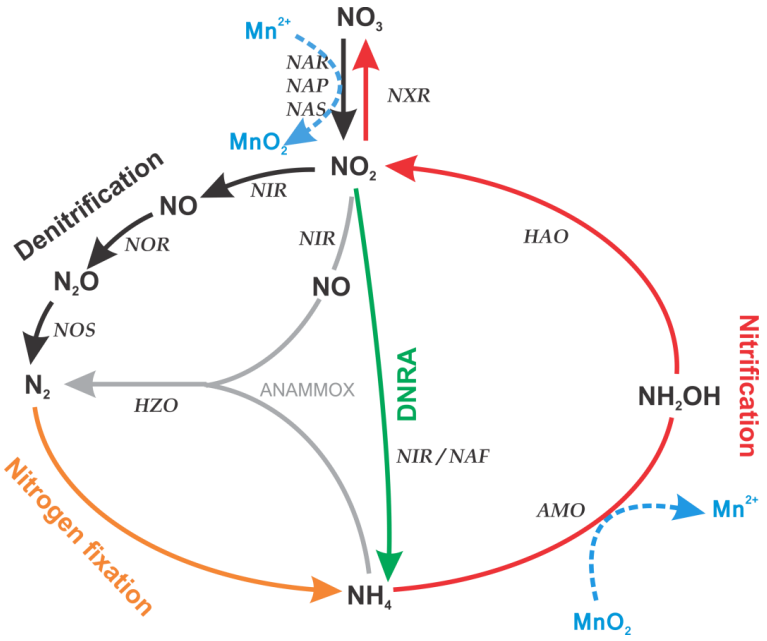

Figure 2. Major pathways in the nitrogen cycling. The cycle consists of nitrification (red colour), denitrification (black colour), anaerobic ammonium oxidation [ANAMMOX (grey colour)], dissimilatory nitrate reduction to ammonium [DNRA (green colour)], and nitrogen fixation (orange colour). Key enzymes are listed between the reactions; AMO: ammonia monooxygenase; HAO: hydroxylamine oxidoreductase; NXR: nitrite oxidoreductase; NAR: membrane-bound nitrate reductase; NAP: periplasmic-bound nitrate reductase; NIR: nitrite reductase; NOR: nitric oxide reductase; NOS: nitrous oxide reductase; HZO: hydrazine-oxidizing enzyme. Also shown is the newly proposed coupling between the nitrogen and manganese cycle (represented by blue dotted lines), namely the denitrifying manganese oxidation and the anaerobic ammonium oxidation coupled to manganese reduction.

Nitrogen is not distributed evenly on Earth, and a large fraction [27-30%; (Goldblatt et al., 2009; Palya et al., 2011)] of the total nitrogen budget is present in the atmosphere in the form of nitrogen gas (N_2). Nitrogen gas is chemically inert and cannot be used by organisms as a direct nitrogen source in the synthesis of cellular build blocks. Although natural lightning fixes N_2, nitrogen-fixing prokaryotes from the bacterial and archaeal domains (diazotrophs) are the vital players who convert N_2 to ammonium (NH_4^+) (orange arrow in Figure 2), and thereby provide bio-available nitrogen to all other organisms (the industrial Haber-Bosch reaction is not considered here). Bacterial nitrogen fixers are primarily found in ocean surface waters (Karl et al., 2002) and as symbionts in rhizospheres and invertebrates (Petersen et al., 2016),

while archaeal nitrogen fixers are confined to methanogens and are found in natural environments such as marine hydrothermal vents (Mehta and Baross, 2006), sediments and cold seeps (Dekas et al., 2009). Some eukaryotes (e.g. legumes and termites) also support nitrogen fixation, but only in symbiotic association with nitrogen-fixing prokaryotes (Petersen et al., 2017).

Nitrogenase, the key enzyme in the nitrogen fixation process, encoded by various *nif* operons, is widely distributed among prokaryotic lineages [reviewed in Boyd and Peters (2013)]. However, most organisms do not contain these genes and cannot fix nitrogen but rather obtain their nitrogen in the form of NH_4^+ (or organic nitrogen) from the environment, or from the reduction of NO_3^- to NH_4^+ through assimilatory nitrate reduction (DNRA; green arrow in Figure 2). The ammonium is released back into the environment when the organism die, and its subsequent fate depend on whether the local environment contains oxygen (O_2) or not. In the absence of O_2, NH_4^+ generally accumulates in the environment, the exception being in the case when NO_2^- is available, as this coupling will return the nitrogen back into nitrogen gas by anammox bacteria from the phylum of *Planctomycetes* (Strous et al., 1999). In the presence of O_2, NH_4^+ is oxidized to NO_3^- by nitrifiers. This process is called nitrification and was first described by Sergei Winogradsky in the late 1800s and is conventionally thought as a two-step process (red arrows in Figure 2). In the first step, NH_4^+ is oxidized to NO_2^- with hydroxylamine as the intermedium, by specific groups of ammonia-oxidizing bacteria (AOB) and archaea (AOA). The key enzyme of this step is the ammonia monooxygenase (AMO), the alpha subunit of which is encoded by the *amoA* gene. This gene is present in both AOA and AOB, and has been widely used as a reliable gene marker to identify nitrifiers in numerous microbiological surveys [as reviewed in Biller et al. (2012)]. In the second step of nitrification, NO_2^- is oxidized to NO_3^- by nitrite-oxidizing bacteria (NOB) using the nitrite oxidoreductase (NXR) (Figure 2). Recently, studies have shown that some members of *Nitrospira*, previously only known as NOB, can perform the complete nitrification, i.e. oxidizing ammonium to nitrite and finally to nitrate, the complete process is now known as Comammox (Daims et al., 2015; van Kessel et al., 2015);

however, these organisms appear to be absent from marine environments (Daims et al., 2015). Nitrifiers can also use the electrons and protons derived from ammonium and nitrite oxidation to fix inorganic carbon (i.e. chemoautotrophy).

In addition to DNRA that convert NO_3^- to NH_4^+, NO_3^- in the environment can also be converted back to N_2 gas under anoxic condition through denitrification (black arrows in Figure 2) which, in the broad sense of the term, is the enzymatic reduction of nitrate coupled to the oxidation of organic matter, reduced metals (e.g. Fe(II) and Mn(II)), methane, or sulfide). Denitrifiers are extremely diverse and include representatives from more than 60 genera of Bacteria and Archaea (Zumft, 1997; Shapleigh, 2013), as well as some eukaryotes e.g., fungi, protozoa, and benthic Foraminifera (Stief et al., 2009) and Gromiida (Demaneche et al., 2009; Pina-Ochoa et al., 2010). The complete denitrification involves four metalloenzymes: dissimilatory nitrate reductase (encoded by *narG* and *napA*), nitrite reductase (*nirS* and *nirK*), nitric oxide reductase (*norB*), and nitrous oxide reductases (*nosZ*). As mentioned above, anammox is an alternative route that converts fixed nitrogen to N_2 (grey arrows in Figure 2). Together, denitrification and anammox close the nitrogen cycle by returning N_2 gas back to the atmosphere.

In addition to these well-known pathways, nitrogen cycling steps have also suggested to be closely linked with the manganese cycling. Particularly, the so-called anaerobic nitrification, NH_4^+ oxidation coupled to manganese reduction (blue dashed arrows in Figure 2), have been proposed in environments like marine sediments [e.g. (Luther et al., 1997)]. In addition, NO_3^- reduction coupled to manganese oxidation (blue dashed arrows in Figure 2) is also a feasible reaction in natural environment [e.g. (Mogollón et al., 2016)].

3 The importance of nitrogen to the marine deep biosphere

Although most of the essential nitrogen pathways have been known for a century and the biochemical mechanisms are well studied, some new pathways (e.g. anammox and comammox) and novel microbial players (i.e. ammonia-oxidizing archaea, comammox Nitrospira, and methane oxidizing nitrate reducers (NC10)) has been discovered over the past two decades (Strous et al., 1999; Venter et al., 2004; Treusch

et al., 2005; Ettwig et al., 2010; Daims et al., 2015; van Kessel et al., 2015). This fact, along with the general limited knowledge about the deep marine biosphere leaves many aspects of the nitrogen cycle poorly constrained in this habitat. However, some recent evidence, do hint to the importance of nitrogen transformation in subseafloor sediments and the upper oceanic crust, as outlined in the following sections.

3.1 Nitrogen transformation in subseafloor sediments

The apparent neglect of the nitrogen cycle in deep ocean sediments, might be due to the earlier tendency to focus on high organic regions in which the inorganic nitrogen transformation is restricted to the top centimeters (Sørensen, 1978). However, with the new focus on oligotrophic regions of the ocean floor, evidence have emerged that varying nitrate concentration can extent to tens of meters below the seafloor or even throughout the entire length of the sediment piles (D'Hondt et al., 2015; Wankel et al., 2015). An increase of porewater nitrate concentration with depth hints to the occurrence of nitrification in subseafloor oligotrophic sediments (Bender et al., 1977; D'Hondt et al., 2009) and nitrogen transformation is supported by dual isotope ($\delta^{15}N$ and $\delta\ ^{18}O$) analysis of porewater nitrate in sediments beneath the North Atlantic Gyre (Wankel et al., 2015). Accordingly, microorganisms involved in nitrogen cycling, especially Thaumarchaeota putatively catalyzing the first step of nitrification, have been detected in a wide variety of deep-sea sediments [e.g. Durbin and Teske (2010, 2011) and Jørgensen et al. (2012)].

Further, incubation experiments suggested that microbes deeply buried in subseafloor sediments preferentially require nitrogen assimilation as compared to carbon substrate for the recovery in vitro (Morono et al., 2011). Moreover, in the organic-rich Peru Margin sediments the transcripts of the functional gene encoding the key enzyme for nitrate reduction, *Nar,* was as abundant as the genes coding for the sulfate reducing enzyme (*Dsr*) (Orsi et al., 2013b), suggesting the role of nitrate as an important electron acceptor.

3.2 Nitrogen transformation in the oceanic crust

In the hydrothermal fluids circulating in the crust at Juan de Fuca Ridge, nitrate reduction pathways including both denitrification and DNRA was detected based on isotopic and DNA-based approaches (Cowen et al., 2003; Bourbonnais et al., 2012b; Bourbonnais et al., 2012a) and at Loihi Seamount, Hawaii (Sylvan et al., 2017). In seafloor exposed basalts, diagnostic genes of various microbial nitrogen cycling pathways, including nitrogen fixation (*nifH* gene), ammonia oxidation (bacterial and archaeal *amoA* genes) and denitrification (*nirS* and *nosZ* genes), were detected (Mason et al., 2009). In the sediment-buried crust, Zhang et al. (2016a) have suggested that nitrogen availability is an important limiting factor for the growth of crustal microbes.

4 Quantitative approaches to understand the microbial nitrogen cycling in the deep biosphere

Despite the above-mentioned importance of nitrogen-based metabolisms in the marine deep biosphere, the composition, distribution and metabolic rates of microorganisms involved in nitrogen cycling are basically unknown. To get a better understanding of the distribution and ecophysiological characteristics one can employ several relevant quantitative techniques and models. The principles and approaches describe below can also be applied to the cycling of other elements, as long as a diagnostic gene can be targeted.

4.1 Reaction rate calculations using Reaction-Transport models

Traditionally, quantification of activity in marine sediments involved mixing samples from the seabed with appropriate amount of substrates, either isotope-labelled or not, incubation under conditions that mimic *in situ* properties, and measuring changes in concentration of the products/reactants over time. Using this approach, a variety of microbial activities, e.g. sulfate reduction (Jørgensen, 1978; Parkes et al., 1994; Parkes et al., 2005; Glombitza et al., 2016), methanogenesis (Parkes et al., 1994; Parkes et al., 2005; Webster et al., 2009), acetate oxidation (Webster et al., 2009; Glombitza et al., 2015), and H_2 oxidation (Adhikari et al., 2016), has been reported from many organic-rich sediment cores. However, the application of this approach in

oligotrophic sediments beneath the open ocean is rarely reported, probably because the rates are too low to be quantified within laboratory timescale, and/or the potential bias occurring due to cross-feeding as a result of long incubation time (Radajewski et al., 2000; Neufeld et al., 2007).

Alternatively, rates of reactions can be calculated based on the porewater concentrations of solutes. Basically, in steady-state (i.e. the considered system is in equilibrium with the surrounding environment), the change of solute concentrations in sediment porewater is the results of transport (including diffusion and advection) and reactions, and can be expressed using a reaction-transport model (Berner, 1980; Boudreau, 1997). This is particular relevant for subseafloor sediments beneath the open ocean, where mass transport is predominantly restricted to molecular diffusion.

A number of numerical programs have been developed in the last two decades to calculate reaction rates in marine sediments. They can be classified into two categories: 1) "Fitting & Calculation" and 2) Reaction network-based simulation. In the "Fitting & Calculation", various algorithms are initially used to provide a best-fit concentration profiles to the measured solute concentrations profile, and then calculate reaction rate as the difference between the overall concentration change and the change caused by transport, with explicit considerations on the variations of downhole sediment properties. Programs applying this principle includes, but not limited to, PROFILE (Berg et al., 1998), NRR [Net Reaction Rates; Wang et al. (2008)], and REC [Rates Estimated from Concentration; Lettmann et al. (2012)], and all have been widely used to calculate the net rates of various reactions in marine sediments from a wide variety of locations (Wang et al., 2008; Wehrmann et al., 2011; D'Hondt et al., 2015; Riedinger et al., 2015; Berg and Solomon, 2016; Meinhardt et al., 2016; Walsh et al., 2016b). It should be noted that the reaction rates obtained from this type of calculation are net rates. With respect to the nitrogen cycle, this is a particular concern since the oxidation and reduction steps are so tightly coupled (Rysgaard et al., 1993; Nunoura et al., 2013; Wankel et al., 2015).

Alternatively, one can use reaction network-based simulation. Applying the same reaction-transport model theory, multicomponent models were developed by

Van Cappellen and Wang (1996) and Soetaert et al. (1996), to simulate the early diagenesis of organic matter and the associated redox reactions in marine sediments. The simulation has some strong advantages relative to the "Fitting & Calculation" models. 1) It takes the reaction network rather than individual solute profiles into account, and its output is validated by comparison against multiple solutes profiles. Therefore, it can provide more comprehensive predictions of *in situ* processes in complex ecosystems. 2) It can predict many individual reaction rates at the same time, once the predicted profile match with the measured ones. For example, the reaction rates for nitrification and denitrification separately, which otherwise would be difficult to obtain using the "Fitting & Calculation" models. 3) It is not susceptible to one-point analytical errors, because it only uses measured profiles to validate the model simulation rather than as an important input. 4) Because the simulation process is independent of the measured profiles, it does not require extremely high-resolution measured data to capture the subtle changes of processes in most active areas, although higher resolution profiles always give better constrains to the simulation results. This simulation method has been used in the calculation of reaction rates in a number of subseafloor sediments [e.g. (Bohlen et al., 2011; Mewes et al., 2016; Mogollón et al., 2016)]. Despite, the many advantages it should be noted that this modelling approach assumes that most reactions follow the Michaelis-Menten and/or bimolecular reaction kinetics and, therefore, might not be very sensitive to abrupt changes of reactions in the subsurface.

Reaction-Transport models have also been proven useful to estimate the reaction rates in the oceanic crust. Although potential rates in seafloor basalts have been successfully measured *ex situ* by substrate addition incubation for extracellular activity (Meyers et al., 2014) and CO_2 fixation (Orcutt et al., 2015), the *in situ* reaction rates in the subsurface crust seems too low to be measured by the isotope-labelling technique (Orcutt et al., 2015). In these cases, numerical models can be useful to decipher the possible biogeochemical processes in the basement. Using a diffusion-advection-reaction model, Orcutt et al. (2013) have estimated the oxygen

respiration rate to ≤1 nmol cm^{-3} d^{-1} in the young and cool basaltic crust on the Mid-Atlantic Ridge.

4.2 Enumeration of functional group abundances

The quantification of any given functional group in the deep marine biosphere is complicated by the facts that most microorganism in this habitat are uncharacterized and that the microbial diversity within a particular functional group is high and therefor challenging to quantify with a limited number of probes/primers. Traditional, microbial quantitative work was restricted to estimating total cell abundance by direct cell counts or most probable number counts (MPN). In the direct cell counts, fluorescent dyes like Acridine Orange and SYBR Green I are used to stain the cells, irrespective of their physiological status - live or dead (Parkes et al., 1994; Schippers et al., 2005; Kallmeyer et al., 2012; Parkes et al., 2014). Later, special protocols involving cell separation (Kallmeyer et al., 2008; Morono et al., 2013), were developed to lower the detection limit and allow the enumeration of extremely low cell densities (~100 cell/cm^3 sediment). Such protocols have successfully been applied on sediment material retrieved 2.5 km below seafloor (Inagaki et al., 2015) and the highly oligotrophic sediments below the South Pacific Gyre (D'Hondt et al., 2015). However, this technique cannot distinguish any particular functional group from the total community. In some cases, one has to assume an arbitrary fraction in the total community for a functional group [~10% of the total cells in the organic-rich sediments are sulfate reducers; Hoehler and Jørgensen (2013)], to estimate the abundance of that functional group based on the total cell counts data. Another alternative is to use Fluorescent In Situ Hybridization (FISH), in which cell counts is based on the visual inspection under microscopy and allows using specific molecular probes to target specific DNA or RNA sequences, and thereby, to quantify specific functional groups (Amann and Fuchs, 2008). The disadvantage of this technique is that it is time consuming, costly, and often limited by low light intensity and hence the results can be affected by the background particles present in samples.

Another method able to quantify functional group abundances is the combination of amplicon sequencing and quantitative polymerase chain reaction (qPCR) of 16S rRNA genes (Widder et al., 2016; Starnawski et al., 2017). Briefly, the total population size is estimated from the qPCR of 16S rRNA gene copy numbers, while the fraction of a particular functional group in the total communities is estimated from the amplicon sequencing, which in itself can be used as a semi-quantitative measure of the abundance of functional groups. However, one of the prerequisites is that the phylogeny of the functional group has been well constrained (e.g. ammonia-oxidizing archaea are only found in the phylum of Thaumarchaeota). This approach has not been widely applied by researchers studying natural ecosystems, likely because most functional groups are not narrowly distributed within a certain taxa and hence taxonomic assignment is not enough to assign function. In addition, many microbial lineages are not characterized and their metabolism unknown.

In addition to the above quantification methods, functional-gene based qPCR was developed (Livak and Schmittgen, 2001) and became a valuable approach to quantify functional groups in complex environments (as reviewed by Lever (2013) for deep subsurface environments). Using this approach, several functional groups has been quantified in subseafloor sediment, including ammonium oxidizers (Roussel et al., 2009; Nunoura et al., 2013), sulfate reducers (Schippers et al., 2005; Nunoura et al., 2009; Blazejak and Schippers, 2011), anaerobic methane oxidizers (Schippers et al., 2012), metal reducers (Schippers and Neretin, 2006), and carbon fixers (Schippers et al., 2012), as well as total bacterial and archaeal cells [e.g. (Schippers et al., 2005; Lipp et al., 2008; Breuker and Schippers, 2013)]. A similar approach have been used on deeply buried crustal rocks, to quantify groups with particular metabolic functions, like sulfate reduction and methane oxidation (Lever et al., 2013). It is worth noting that this technique is particularly useful when applied to microbial groups involved in nitrogen cycling, such as AOA, AOB, NOB, and anammox, primarily because these groups are phylogenetically narrowly distributed, and most of

the sequences of the functional genes are conservative and have common regions that can be targeted by a limited number of primer pairs.

4.3 Cell-specific reaction rates and energy requirements

Cell-specific metabolic rate is an informative indicator of the physiological state of individual cells. By plotting the cell-specific metabolic rates against temperature, Price and Sowers (2004) proposed three distinct metabolic states for microbes: growth, maintenance, and survival. Relative few studies have measured, or tried to estimate the cell-specific metabolic rates of microbes in deep marine sediments, likely because the simultaneously quantifications of reaction rates and functional group abundances are complicated tasks and requires both geochemical and microbiological data (Hoehler and Jørgensen, 2013). Nevertheless, mean cell-specific rates have been reported for sulfate reducers and hydrogen oxidizers in anoxic sediments (Leloup et al., 2009; Hoehler and Jørgensen, 2013; Lever et al., 2015; Adhikari et al., 2016) and for bulk cells in oxic oligotrophic sediments (Røy et al., 2012; D'Hondt et al., 2015). In addition, the recently emerged technique, nanometer-scale secondary ion mass spectrometry (NanoSIMS), offers a valuable approaches to directly measure the single-cell metabolic rates under laboratory conditions (Morono et al., 2011). However, its application may be limited to the microbial assimilation (uptake provided substrates) but not dissimilation (convert substrates without incorporation into cells).

Traditionally, the available energy for a certain functional group can be quantified by the calculation of Gibbs free energy per reaction the microbes mediate (Amend et al., 2011; LaRowe and Amend, 2014; LaRowe and Amend, 2015a). However, their catabolic activities are constrained by, not only the amount of free energy per reaction, but also by the rate that the free energy is released (LaRowe and Amend, 2015a; Lever et al., 2015). LaRowe and Amend (2015a), therefore proposed the notion of power supply, defined as the product of Gibbs free energy per reaction and the reaction rate (LaRowe and Amend, 2015a), and suggested that power supply provides a more reliable proxy than Gibbs free energy when studying the dynamics of

microbial communities from an energetic perspective. The same authors also presented a model that link power supply to catabolic rates, population size and doubling/replacement times of microorganisms in natural settings (LaRowe and Amend, 2015a). In combination with cell counts data, this model was used to estimate the cell-specific power requirement (1.9×10^{-19} W $cell^{-1}$) for the microbes living in the most oligotrophic sediments beneath the South Pacific Gyre (LaRowe and Amend, 2015b). In addition, the cell-specific power requirement of microbes also likely vary under different environmental conditions, e.g. it has been suggested to be up to 30-fold higher in oxic sediments than anoxic counterpart (Lever et al., 2015).

5 Aims of this project

Given the deficiencies in our understanding regarding the marine deep biosphere especially in remote oligotrophic areas, this project, consisting of four studies, used samples and data from the seven oligotrophic ocean regions shown in Figure 3, to improve our understanding about microbial life especially those involved in the nitrogen cycling in the subseafloor sediments and oceanic crusts. Overall, it employed a wide range of theoretical and experimental (semi-)quantitative approaches including qPCR, reaction rate models, and high-throughput sequencing. The scope of this study includes reaction rates of nitrogen transformation, nitrogen fluxes across the boundaries between the bottom seawater, marine sediment, and basaltic crust, as well as the abundances, community composition and structure, metabolic states, and energy requirement of microbes involved in the nitrogen cycling.

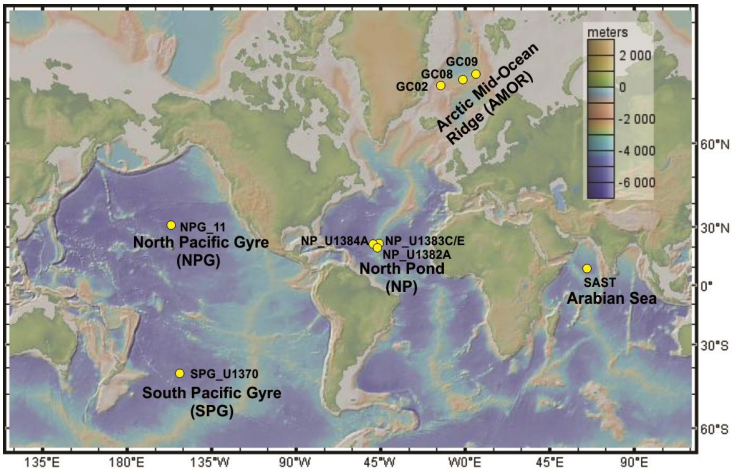

Figure 3. Geographic locations of sediment and rock cores studied in this thesis. Negative values in the inset in upper right indicate seafloor depths in meters. Map was created in GeoMapApp version 3.2.1 (www.geomapapp.org) using the default Global Multi-Resolution Topography Synthesis (Ryan et al., 2009) basemap.

The specific objectives of each study are described below.

Paper I is a descriptive study, focusing on two subsurface basaltic cores retrieved from North Pond, a sediment filled pond on the western flank of Mid-Atlantis Ridge visited during the International Ocean Discovery Program (IODP) expedition 336.

The aim was to employ qPCR to quantify the cell abundances, and used 16S rRNA gene sequencing to describe the microbial inventory in the deeply buried oceanic crust.

Paper II revolves around two sediment cores from North Pond, spanning the entire length of the sediment pile. The aim was to use quantitative approaches to study the vertical distribution of the abundances, reaction rates, community dynamic and metabolic status of nitrifiers and denitrifiers in the oligotrophic subseafloor sediments.

Paper III focuses on the basaltic rock samples from North Pond. The aim was to explore the nitrification potential within the crust and the possible origin of crustal ammonia-oxidizing Archaea (AOA), by a systematic comparison of AOA communities in the bottom seawater, subseafloor sediments, and oceanic crust.

Paper IV is based on a compilation of geochemical and microbiology data from seven (mainly) oxic sediment cores from five distinct oligotrophic regions (shown in Figure 3). We aimed to calculate the energetic requirements of nitrifiers, to test the hypothesis that microbial life in subseafloor oxic sediments are sustained by similar levels of basal power requirement, irrespective of sediment depth and burial age.

6 General Discussions

6.1 Coupling microbiology with geochemical modelling

In this study, reaction-transport models not only suggested the occurrence of nitrification in the oligotrophic subseafloor sediments (Paper II) and subsurface basaltic crust (Paper III), but also provides some quantitative constrains on the *in situ* reaction rates (Paper II and IV). Such information is valuable for microbiological studies focusing on the energy-limited deep biosphere, where microbial biomass is very low (D'Hondt et al., 2009; Kallmeyer et al., 2012) and cellular activities are orders of magnitude lower than what can be measured or observed under laboratory conditions (D'Hondt et al., 2004; Morono et al., 2011; Røy et al., 2012; Hoehler and Jørgensen, 2013; Trembath-Reichert et al., 2017). In particular, in contrast to a feasibility of certain reactions obtained by thermodynamic calculations, the reaction-transport modelling provides a realistic estimation on what processes are occurring and how fast/slow they are. When combined with cell abundance data, the model simulation results can not only sever as a strong indication of *in situ* metabolic activities of functional groups, but also offer some insights into their ecophysiology (Paper II and IV). More sophisticated approaches, exploiting microbial and geochemical models in which the thermodynamic of reactions, growth and mortality of functional groups, and metabolic plasticity are taken into consideration, have recently been developed for investigations focusing on oxygen minimum zones (Reed et al., 2014; Louca et al., 2016) and hydrothermal plumes (Reed et al., 2015). Future application of such coupled modelling approaches to the deep biosphere could shed more lights into the dynamic and physiological characteristics of microbial life in energy-limiting environments.

6.2 Microbial growth in marine sediments and the underlying oceanic crust

The combination of the fine scale community characterization based on the 16S rRNA gene amplicon sequencing, and abundance enumeration of functional genes, provided evidence for the growth of specific AOA clades in the sedimentary oxic/anoxic transition zones (OATZs) (Paper II) and in the basement (Paper III).

Most microbes in marine sediments are generally thought to be persisting under the energy-limiting conditions (Walsh et al., 2016b; Starnawski et al., 2017) with the potential to recover under ideal *ex situ* conditions (Morono et al., 2011). Our results, however, add more details into this general picture by suggesting the *in situ* growth of microbes can naturally occur in the OATZs in deep subseafloor sediments. The inferred growth of AOA may result from the increased energy supply in OATZs, where abundant electron acceptors from the surface meet with electron donor diffusing from below (Canfield et al., 2005).

Net growth rates of functional groups in subseafloor sediments could theoretically be calculated from the depth profile of their abundance when assuming the cell distribution patter are in steady state (i.e. the distribution pattern does not change over the considered time scale) and no significant cell migration on a vertical scale. As such calculation primarily requires cell abundance data and an accurate sedimentation age-model, collecting spatial high-resolution cell abundance data for the functional group of interest in sediments with a clear sedimentation history (hemipelagic) with minimum uncertainty would be the prerequisite to get a first-order estimate of microbial growth rates in subseafloor sediments. However, it's worth noting that these rates would be net rates (the offset between the growth and decay), and could underestimate the *in situ* gross growth rates. Nevertheless, such an approach using numerical modelling might help overcome the challenges related to the measuring low growth rates (Trembath-Reichert et al., 2017), and the difficulties related to subseafloor conditions in the laboratory (Hoehler and Jørgensen, 2013; Lever et al., 2015). Future work applying the above-mentioned sophisticated mathematic models, i.e. the gene-centric models developed for marine water columns as mentioned above (Reed et al., 2014; Reed et al., 2015; Louca et al., 2016), could distinguish microbial growth from decay and thus more accurately characterize the dynamics and turnover of microbial population in subseafloor sediments.

Compared to subseafloor sediments, there is much less available data for cell abundance in subseafloor crust (Salas et al., 2015; Zhang et al., 2016a), not to mention the dynamic (i.e. growth and/or decay over time) of microbial communities

(Tully et al., 2018) within this vast habitable zone on Earth. Our data in Paper I suggested that there is only 10^3-10^4 cells per g rock in the subseafloor basalts at North Pond, generally consistent with other reports of the same area (Salas et al., 2015; Zhang et al., 2016a). Considering that the environmental setting at North Pond is thought to be representative for the majority of habitable ocean crust, these data may indicate that microbial cells abundances in the subseafloor crust are likely at least two orders of magnitude lower that those observed in the seafloor-exposed basalts (10^6-10^9 cell per gram; e.g. (Einen et al., 2008; Santelli et al., 2008)).

Regarding the dynamics of crustal microbial communities, the distinct community structure of AOA (Nitrosopumilales) observed in the bottom seawater, subseafloor sediments, and basaltic crust at North Pond suggested the growth of AOA within the basement (Paper III). Even though *in situ* metabolic activity of these AOA, nitrification, was suggested through modelling the differences of nitrate concentration between different sites, the growth rates of AOA within the basement is difficult to assess when only AOA *amoA* gene abundances data is available, because the gene abundances may have integrated the history of many years of microbial changes (Neira et al., 2016). Nevertheless, the inferred growth of microorganisms in the subsurface basement at North Pond may represent a scenario of the origin of crustal microbes, in which the rare members in the bottom seawater is inoculated into the basement through seawater circulation, and reproduce in the subsurface by harvesting energy from the oxidation of reduced substances like NH_4, Fe(II), S, and H_2 (Bach and Edwards, 2003; Orcutt et al., 2011b; Bach, 2016; Zhang et al., 2016b; Tully et al., 2018).

6.3 The presence of denitrifiers in marine oxic sediments

Our work also suggests that denitrifiers co-exist with nitrifiers in marine oxic sediments (Paper II). In addition to the detection of key functional genes of denitrifiers (*narG*, *nirS*, and *nirK*), our model simulation suggested that nitrate concentration would be higher than the observed concentrations if denitrifiers were absent in the sediments. However, such results could also be explained by the

presence of anammox, however, given our failure to detect anammox bacteria by multiple approaches, this model results provided strong evidence for the presence of denitrifiers in the oxygenated sediments. Although denitrifiers are well known to be facultative, switching between oxygen respiration and denitrification (Zumft, 1997; Chen and Strous, 2013) (Zumft, 1997; Chen and Strous, 2013), they are traditionally considered to perform denitrification only under anaerobic conditions or very low oxygen tension. However, accumulating evidence suggests denitrification activity also under oxic conditions (i.e. the so-called "aerobic denitrification") both in laboratoty cultures (Robertson et al., 1995) and natural environments (Gao et al., 2010; Marchant et al., 2017). It is unclear if the aerobic denitrification in marine sediments occurs when oxygen is present or if the widely appreciated microenvironments found in marine sediments (Lehto et al., 2014) provide anaerobic niches for denitrifiers (Wilson, 1978), while close-living nitrifiers may provide them the necessary substrate - nitrate (Middelburg et al., 1996).

6.4 Subsurface microbes do influences nutrient fluxes towards the basement

In terms of sediment biogeochemical cycles and nutrient fluxes, the very surface sediment are often regarded as the most important spot because they harbour the freshest organic matter and the highest abundance of microbial cells, and are responsible for the highest proportion of organic matter degradation. In the oligotrophic sediment columns at North Pond, we observed a higher-than-bottom-water concentration of nitrate throughout the cores, which is a result of the imbalance between nitrification and denitrification (Paper II). Such distribution of porewater nitrate suggests diffusive nitrate flux both upward to the overlying seawater and downward into the underlying basaltic crust. Even though the rates of nitrification and denitrification and cell abundances of nitrifiers and denitrifiers are highest in the top surface sediments, the activity of nitrifiers in the subsurface is critical to maintain the high nitrate concentration in deep sediments and the downward nitrate flux into the basement, although their impact to the fluxes through the sediment-water interface could be negligible.

6.5 The significance of this study

By focusing on the microorganisms putatively involved in the nitrogen cycling, this project characterize the abundance, community composition and structure, nitrogen fluxes, reaction rates, and power requirement of nitrifiers and denitrifiers, by coupling the geochemical modelling with high-throughput sequencing techniques. Overall this study suggests that 1) microbial cell abundances in sediment-buried oceanic crust are on the order of 10^3-10^4 per gram of rock, at least one order of magnitude lower than those reported in seafloor-basalts (Paper I); 2) microbes involved in the nitrogen cycle, especially AOA mediating the oxidation of ammonium, are prevalent in oligotrophic sediments (Paper II) and young and oxic sediment-buried basaltic crust (Paper I and III); 3) the metabolism of nitrifiers in the sediment can contribute to nitrate enrichment in the underlying basement (and the overlying water) (Paper II); 4) *in situ* growth of microbes can occur in the oxic-anoxic transition zone of marine sediments (Paper II) and sediment-buried basaltic crust (Paper III); 5) denitrifiers can be active in well oxygenated sediments (Paper II); 6) the community composition of the prevalent Archaea in oligotrophic settings, AOA, are similar between the basaltic rocks and sediment, suggesting similar selective forces driving the AOA assemblages (Paper III); and 7) nitrifiers persist in oxygenate sediments with similar amount of cell-specific power requirement at different geographic locations with different depths and burial ages (Paper IV).

By integrating findings from previous studies (Wankel et al., 2015; Shah Walter et al., 2018), this project provides an overview of the nitrogen cycling and the associated microbial communities in the sediments and buried basaltic crust at North Pond, as summarized in Figure 4. Similar geochemical processes and microbial communities may be present at other sites of oxic, young mid-ocean ridge flanks.

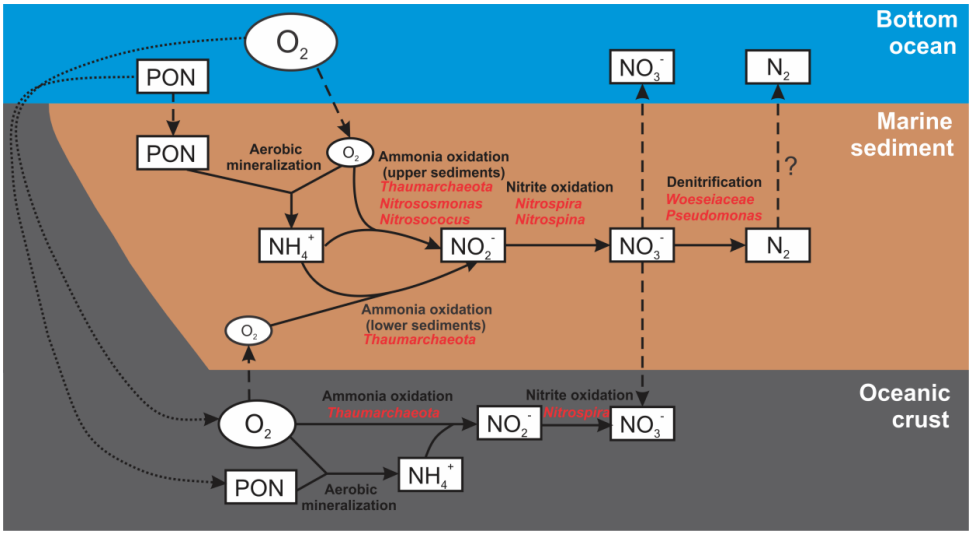

Figure 4. Overview of the microbial nitrogen transformations in the subseafloor sediments and basaltic crust at North Pond. Dashed arrows represent diffusion processes, dotted arrows denote advection processes, while solid arrows designate biological reactions. Major microbial taxa responsible for some of the biological reactions were listed in red. PON, particulate organic nitrogen.

7 Future perspectives

Considering that most of the microorganism in the marine deep biosphere are not cultured and show low similarity to cultured relatives, future efforts aiming to get more knowledge on the physiological nature, such as isolation and integrated omics approaches, will be crucial to reveal the mechanism driving the persisting and active growth of diverse microbial communities in the deep biosphere.

The combination of microbial and geochemical approaches presented in this thesis has demonstrated to be effective to study microbial life in energy-limiting environments. Future efforts improving and applying these approaches to other types of functional groups at sites over larger scales is promising to unravel the essential principles driving the distribution and dynamics of microbial communities in the deep biosphere.

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Microbial Inventory of Deeply Buried Oceanic Crust from a Young Ridge Flank

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The deep marine biosphere has over the past decades been exposed as an immense habitat for microorganisms with wide-reaching implications for our understanding of life on Earth. Recent advances in knowledge concerning this biosphere have been achieved mainly through extensive microbial and geochemical studies of deep marine sediments. However, the oceanic crust buried beneath the sediments, is still largely unexplored with respect to even the most fundamental questions related to microbial life. Here, we present quantitative and qualitative data related to the microbial inventory from 33 deeply buried basaltic rocks collected at two different locations, penetrating 300 vertical meters into the upper oceanic crust on the west flank of the Mid-Atlantic spreading ridge. We use quantitative PCR and sequencing of 16S rRNA gene amplicons to estimate cell abundances and to profile the community structure. Our data suggest that the number of cells is relatively stable at $\sim 10^4$ per gram of rock irrespectively of sampling site and depth. Further, we show that *Proteobacteria*, especially *Gammaproteobacteria* dominate the microbial assemblage across all investigated samples, with Archaea, in general, represented by <1% of the community. In addition, we show that the communities within the crust are distinct from the overlying sediment. However, many of their respective microbial inhabitants are shared between the two biomes, but with markedly different relative distributions. Our study provides fundamental information with respect to abundance, distribution, and identity of microorganisms in the upper oceanic crust.

Keywords: deep biosphere, oceanic crust, geobiology, cell abundance, community structure, endolitihic community

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INTRODUCTION

Every day ~100 billion cubic meters of bottom seawater are transported down into the permeable upper oceanic crust. Within this gigantic aquifer system oxic seawater circulates and reacts with reduced igneous rocks before eventually recharging back into the oceans 10^3–10^4 years later (Wheat et al., 2003; Orcutt et al., 2011). Consequently, the chemical composition of fluids and rocks are strongly altered, with wide-reaching ramifications throughout the marine system (Fisher and Becker, 2000; Bach and Edwards, 2003; Bach et al., 2004). Strong evidence exist for an abundant microbial community residing within this subsurface crustal basaltic aquifer (Giovannoni et al., 1996; Torsvik et al., 1998; Fisk et al., 2003; Lysnes et al., 2004; Orcutt et al., 2011; Nigro et al., 2012; Lever et al., 2013) where microbial activity is believed to influence basalt alteration and mineral dissolution

rates (Thorseth et al., 1995; Fisk et al., 1998; Furnes et al., 2001b; Storrie-Lombardi and Fisk, 2004; Kruber et al., 2008).

Endolithic microorganisms in subsurface basalt were first reported two decades ago from a drilling expedition to the Costa Rica Rift zone (Ocean Drilling Program Leg, ODP Leg 148). The presence and activity of microorganisms were inferred via detection of biosignatures including: (i) microscopic tubular structures in which DNA could be detected by staining (Thorseth et al., 1995; Giovannoni et al., 1996), (ii) targeting and localization of intact and active cells via *in situ* fluorescent hybridization (FISH) (Torsvik et al., 1998), and (iii) site-specific nitrogen and carbon enrichment in the altered tubular structures (Giovannoni et al., 1996; Torsvik et al., 1998). These results were later supported by drilling in the Australian Antarctic Discordance (ODP Leg 187) where, in addition to corroborating textural, geochemical, and molecular observations (Furnes et al., 2001a,b; Thorseth et al., 2003), microbial DNA (16S rRNA genes) from subsurface samples was for the first time successfully amplified and sequenced (Lysnes et al., 2004). Despite limited sequencing depth, this analysis revealed a unique microbial population dominated by the bacterial phyla *Gammaproteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi,* and *Firmicutes*, and different from those in the above sediment and seawater.

Besides the pioneering work outlined above only a few additional microbial studies have directly investigated native subsurface igneous rocks (Fisk et al., 2003; Mason et al., 2010; Lever et al., 2013; Orcutt et al., 2015). Thus, this habitat is heavily under-studied, a fact that can be largely attributed to the immense technical and economic challenges involved in the sampling of deeply buried oceanic crust. Consequently, most of our knowledge about the crustal biosphere originates from samples exposed at the seafloor (Orcutt et al., 2011; Edwards et al., 2012b; Orcutt and Edwards, 2014). Seafloor-derived samples, however, are not representative of the subseafloor crustal environment and constitute only a small fraction of the 10^9 km^3 of the upper oceanic crust that has been suggested to be habitable (Heberling et al., 2010). In an effort to address these concerns, *in situ* subseafloor observatories, installed primarily at Juan de Fuca Ridge (JdFR), have expanded our understanding of subseafloor water–rock–microbe interactions in a more representative setting (Orcutt et al., 2011). Despite such technological advancement, a number of basic questions cannot easily be inferred from subseafloor observatories, including cell abundances and community structure in native material.

The first dedicated microbial investigation of a low-temperature young ridge flank system was undertaken by the International Ocean Drilling Program (IODP) expedition 336 to North Pond in the North Atlantic gyre (Expedition 336 Scientists, 2012e). The basement in North Pond is covered by a sediment layer (up to 300 m) and is characterized by vigorous crustal fluid circulation driven mainly by advection (Edwards et al., 2012a). The fast fluid circulation results in relatively low fluid temperatures (10–15°C) and seawater-like fluid chemistry, such as high dissolved oxygen concentration at discharge zones [55–191 μM] (Orcutt et al., 2013). Further, a recent study revealed an active and distinct bacterial community in the crustal fluids underneath North Pond (Meyer et al., 2016).

In the present study, we analyse the abundance and structure of microbial communities in deeply buried basaltic rocks in a total of 33 different samples retrieved from the basement underneath North Pond and compare these to the communities in the above sediments. We analyse the samples by means of 16S rRNA gene amplicon libraries and quantitative PCR (qPCR). Our results are among the first to quantify microbial abundances in native subsurface basalt, thus guiding biomass constrains for this globally significant system. Furthermore, our data elucidate the taxonomic identity of native microbial inhabitants suggesting a community capable of facilitating a diverse range of redox reactions. Lastly, we provide evidence that the dispersal scenarios of the sedimentary and crustal microbial inhabitants are intertwined and potentially closely linked.

MATERIALS AND METHODS

Sample Location, Collection, and Description

We investigated a total of 33 subsurface samples (27 from igneous crust and 6 from a sedimentary breccia) collected from North Pond on the west flank of the Mid-Atlantic Ridge. Samples originate from Holes 1382A (22°45.353′N, 46°04.891′W) and 1383C (22°48.1241′N, 46°03.1662′W; **Figure 1**), both were retrieved using rotary core barrel (RCB) coring. Florescent microspheres were added to the drilling fluid in order to assess potential contamination as described in details elsewhere (Expedition 336 Scientists, 2012d). In order to remove potential contamination introduced during drilling operation, all samples were washed three times in sterile seawater on-board before being sub-sampled into smaller pieces using a chisel and hammer under sterile conditions, as described in details elsewhere (Expedition 336 Scientists, 2012c). The presence of fluorescent microspheres in the wash solution was investigated by microscopy after the last wash. Subsamples (∼2 cm^3) were placed in sterile Whirlpak bags and immediately frozen at −80°C before further processing at the home institute.

Samples follow a depth gradient ranging from ∼110 to 200 meter below seafloor (mbsf) in Hole 1382A (16 samples) and 70 to 300 mbsf in Hole 1383C (17 samples). The igneous crust at the two sites likely originates from different volcanic centers fed by a mantle source of variable composition (Expedition 336 Scientists, 2012c). A short overview of sample depths and lithological characteristics can be found in **Table 1**. For a comprehensive description the reader is recommended to consult the IODP proceedings volume 336 (http://publications.iodp.org/proceedings/336/336title.htm). Following is a brief characterization of each site.

In Hole 1382A basement was located 90 mbsf, however the interval from 93 to 99 mbsf are inferred to be sedimentary (Expedition 336 Scientists, 2012e). A total of 32 meter of upper crustal material was recovered between 110 and 210 mbsf (recovery 32%). From this material we analyzed 16 samples covering six of the eight lithological units encountered in this

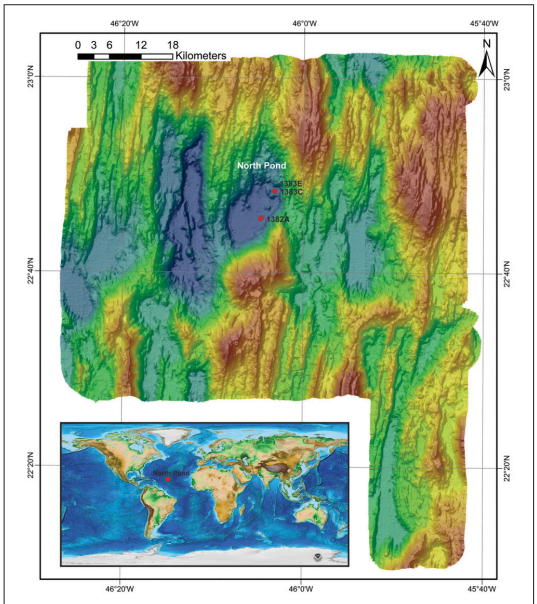

FIGURE 1 | Bathymetric map with the location of the investigated sites. Red circles indicate the geographic location of the investigated sites. Insert: global map indicating the position of North Pond in the Mid-Atlantic Ocean (Source: National Centers for environmental Information - NCEI). Modified from Bach (2012).

Hole (I, II, IV, V, VI, and VII). Unit V consist of sedimentary breccia; likely as a result of a rock slide deposit, whereas all other units are represented by basalt, either as varying volcanic pillow basalt or massive flows with geochemical and petrographic distinct characteristics. Rock alteration can be assigned to low temperature processes.

In Hole 1383C the sediment/basement interface was found at 38.3 mbsf and 50.3 meter of hard rock was recovered from the interval between 69.5 and 331.5 mbsf (recovery 19%). The 17 samples investigated from this Hole are glassy to fine-grained basalts with variable content of phenocrysts, which divides them into three major lithological units (I, II, and III).

DNA Extraction

Small pieces of sample material was pulverized in a flame sterilized steel mortar and ∼0.5 g (0.49–0.91 g) from each sample was subjected to genomic DNA extraction using the FastPrep soil DNA isolation kit (MP Biomedicals) following the manufacturer's instruction with two modifications. First, we used a special bead coating, similar to the G2 DNA/RNA enhancer (Amplicon A/S, Odense, Denmark, available from June 2016) that increases yield, by reducing DNA binding to the beads (Bælum and Jacobsen, 2010; Bælum et al., 2013; Hjelmso et al., 2014). Next, 200 μg of sterile filtered polyadenylic acid (PolyA; Sigma) was added to each lysis mixture prior to bead beating, to avoid DNA binding to the sample matrix (Hugenholtz et al., 1998). Bead beating was performed using the MP-Biomedical FastPrep®-24 for 45 s (speed setting 6). DNA was finally eluted into 75 μl PCR-grade double-distilled water (ddH_2O), and preserved at −80°C until further analysis. In order to assess potential contamination introduced from the extraction kit, two blank extractions were included using the same batch of chemical reagents as for the samples.

Quantitative PCR

Bacterial and Archaeal 16S rRNA genes were quantified individually using quantitative real time PCR applying the StepOne Real Time PCR system (Applied Biosystems). All samples and standards were run in triplicates using SYBR Green Hot Start master mixture (Qiagen) and with the standards, primers, and thermal conditions described in details elsewhere (Jørgensen et al., 2013). In short, a dilution series (10–10^6 target copies) containing *Escherichia coli* PCR amplified full-length 16S rRNA genes and a linearized archaeal fosmid (54d9) was used as bacterial and archaeal standards, respectively. Bacterial SSU rRNA genes were targeted with the primers bac341F (5′-CCTACGGGWGGCWGCA) and 518R (5′-ATTACCGCGGCTGCTGG). For archaeal SSU rRNA gene amplification the primers Un515F (5′-CAGCMGCCGCGGTAA) and Arc908R (5′-CCCGCCAATTCCTTTAAGTT) were used. All R^2 were >0.95 and the amplification efficiency between 90 and 104%.

Ion Torrent SSU rRNA Amplicon Library Preparation and Sequencing

All DNA extracts were PCR amplified in duplicates with the SSU rRNA gene specific primers 519f (5′-CAGCMGCCGCGGTAA) and 805r (5′-GACTACHVGGGTATCTAATCC) in order to generate an amplicon library for subsequent sequencing using the Ion Torrent PGM Personal Genome Machine (PGM) platform technology (Life Technologies). We used a two-step amplification approach as described by Berry et al. (2011), to minimize bias introduced by the long adaptor sequence. The first-round PCR was carried out in duplicate for each sample, to minimize PCR drifting, and each reaction (20 μl) contained 10 μl 2x HotStarTaq® master mixture (Qiagen), 0.2 μl of each primer (100 μM stock), 2 μl template and ddH_2O. The PCR program was initiated with a hot start activation step for 15 min at 95°C followed by an optimized number of PCR cycles (36–37) of 95°C for 30 s, 56°C for 30 s, and 72°C for 30 s. The duplicate PCR products were pooled and purified using QIAquick PCR purification kit (Qiagen). In the second-round PCR attaching the Multiplex Identifiers (MIDs), seven cycles were run, where each reaction (25 μl) contained 12.5 μl 2x HotStarTaq® master mixture (Qiagen), 0.2 μl 806r-B-Key (100 μM stock), and 2 μl 519f MID primer (10 μM stock), with 5 μl of purified PCR products from first-round amplification as the template, according to the Ion Torrent protocol. The PCR amplicons were purified using AMPure XP bead Purification Kit (Agencourt), following manufactures protocol, before all samples were pooled in equimolar concentrations (26 pmol). We note, that due to the PCR and the subsequent equimolar pooling, the

TABLE 1 | General sample description including depth, lithological unit, onboard sample description, and in which group in the hierarchical cluster analysis the microbial community is located.

Sample	Depth (mbsf)	Unit	Specific sample description	Cluster
1382A				
2R_1C	110	I	Massive, minor red-yellow-brown alteration	4
3R_2B	115	I	Massive, yellow-white-brown alteration in vein	4
3R_3A	117	I	Massive, patchy orange-brown alteration	4
3R_4B	117	I	Massive, mostly brown oxidized halo	4
4R_1B	123	II	Aphyric cryptocrystalline basalt, gray-brown alteration	4
5R_1B	133	II	Massive, aphyric, red alteration	4
6R_1A	142	II	Aphyric, cryptocrystalline, less vesicular, patchy alteration	4
7R_2B	153	III	Aphyric, glassy margin, red, and orange-brown alteration	4
8R_1A	161	IV	Ultramafic, pyroxene, evidence of low-temperature alteration	1
8R_1B	161	IV	Porphyritic basalt	4
8R_2F	162	IV	Orange-brown sediment with small (<1 mm) basalt clasts	2
8R_3G	163	IV	Gray-brown + orange-brown sediment with small (<1 mm) basalt clasts	1
8R_4D	163	IV	Sedimentary breccia with basalt clasts, rusty colored, extensive carbon	2
9R_1C	172	IV	Sediment near serpentinized breccia	3
10R_3D	183	V	Medium-grained basalt, massive, porphyritic, pervasive alteration	4
12R_1A	199	VI	Porphyritic basalt, minor alteration	4
1383C				
2R_2E	72	I	Aphyric basalt, highly altered, vein with red alteration, light brown alteration	5
3R_1B	77	I	Aphyric basalt, slight alteration, tan alteration deposits	4
4R_1B	87	I	Light tan micrite breccia with altered glass clasts	4
5R_1B_I	97	I	Aphyric basalt, moderately altered, vein with red alteration	5
5R_1B_II	97	I	Aphyric basalt, moderately altered	5
6R_1A	105	I	Aphyric basalt, slight alteration, altered chilled margin, ochre alteration	6
10R_1A	144	II	Light tan micrite with large clasts of altered glass	5
10R_1D	145	II	Phyric basalt, moderately altered, multiple veins, slight red alteration	5
11R_1C	154	II	Phyric basalt, extensive alteration, orange, and olive alteration	5
19R_1B	212	III	Aphyric basalt, highly oxidized, light brown alteration, relatively brittle	5
19R_1A	212	III	Two small pieces, mostly glass, rust alteration	5
20R_1A	219	III	All basalt glass, rust alteration	5
24R_1B	257	III	Aphyric basalt, oxidized, dark orange-brown alteration, fractured	5
24R_1A	256	III	Aphyric basalt glass with rust alteration, vesicles	5
27R_1A	285	III	Aphyric massive basalt, alteration	4
29R_1A	300	III	Aphyric basalt, highly oxidized, thin carbonate veins	6
30R_1A	304	III	Aphyric basalt, oxidized, some fractures, dark orange alteration	5

*Cluster number corresponds to numbers in **Figure 4**. The sample description presented here is modified from IODP 336 site summaries (Expedition 336 Scientists, 2012a,b).*

number of reads do not reflect the original concentration of DNA, which based on gel band intensity after PCR was much higher in the samples than in the blank extractions. Raw reads generated in this study were deposited at the NCBI Sequence Read Archive under the project number SRP070121.

OTU Filtering, Clustering, and Taxonomic Assignment

Sequence reads obtained from the Ion Torrent sequencing were cropped at 220 bp and quality filtered with a 0.5 quality cut-off, chimera checked, and Operational Taxonomic Units (OTUs) clustered (97% similarity) using UPARSE/USEARCH (Edgar, 2013). The resulting OTUs were taxonomically assigned using the CREST software, with a lowest common ancestor algorithm implementing the SilvaMod reference database (Lanzen et al., 2012). A fasta sequence file of the represented OTUs can be found in the Supplementary Material (Data sheet S2).

Contamination Assessment of Sequence Pool

Contamination issues have previously hampered progress in deep marine research. In an attempt to avoid such obstacles this study applies several measures to prevent and assess the degree of potential contamination, as outlined in the sample handling and collection section above. Further, as a broad reaching contamination control for drilling protocols we extracted and

sequenced DNA from the drill mud and a recovered microsphere bag (exposed to bottom seawater, drill fluid, and mud). The purpose of this control is to address potential inadvertent contamination of samples introduced during standard IODP drilling protocols. The results allow estimating the ratio of the inferred natural community that is likely to arise from contamination. Any OTU present in the control and in the native sample material was removed from the dataset. We note that true overlap between communities in the control and native samples may exist, which would lead to culling of legitimate sequences. Additionally, in order to assess potential contamination in the 16S rRNA gene amplicon preparation procedure two blank extractions (no sample material) were subjected to the same amplification protocol as the samples. Amplified DNA from these blanks may represent contamination originating from DNA extraction kit and/or PCR mix reagents (Champlot et al., 2010; Lusk, 2014; Salter et al., 2014). Therefore, any OTU found in both the extraction blanks and the native sample material was removed prior to any further downstream analysis, with the exception of OTUs that was found to be more than 50 times as abundant in the basalts than in the above-described controls. These were retained in the dataset, due to the plausible scenario of cross-contamination from controls to samples. This approach is similar to that described by Lee et al. (2015) with modified increased stringency addressing the assumed lower biomass in our sample set. However, in order to evaluate the impact of this stringent filtering, ordination, and clustering were likewise performed on the full dataset prior to the above described filtering.

Ordination and Hierarchical Clustering Analysis

The relative abundances of individual OTUs in each sample were clustered based on unconstrained Bray-Curtis, Jaccard, and Dice dissimilarity index using the software PAST version 3.08 (Hammer et al., 2001), before and after removal of potential contaminant reads. The basalt-hosted microbial communities were compared to those in the overlying sediments (Hole 1383E), using non-metric dimensional scaling (NMDS) applying Bray-Curtis dissimilarity. The data from the sediment samples was generated following identical protocols (e.g., the same primers, extraction kit, PCR mix), sequencing platform and downstream analysis, thereby enabling a valid comparison. The concentrations of major and trace elements from rocks provided by the IODP data report (Expedition 336 Scientists, 2012a,b) originating from the same core sections, but separated from the samples used for microbiology (between 20 and 70 cm distance), were used in cluster analysis using Bray-Curtis and Jaccard.

RESULTS

16S rRNA Gene Abundance

The total abundance of 16S rRNA gene copy numbers (Archaea plus Bacteria) estimated by qPCR varies between 0.3 and 8.3 $\times$ 10^4 copies per gram in Hole 1382A and 0.6–3.9 $\times$ 10^4 copies per gram in 1383C (**Table 2**), with the majority of all samples (80%) falling within the range of 1.9–5.8 $\times$ 10^4 copies. Three samples from the sedimentary breccia between 162.8 and 163.8 mbsf in Hole 1382A (8R-2F, 8R-3G, and 8R-4D) had notably lower numbers (0.3–0.6 $\times$ 10^4 copies per gram) than the remaining samples from this site. Based on our quantification the 16S rRNA genes are predominantly of bacterial origin, comprising between 92.6–100% and 91–100% in Hole 1382A and 1383C, respectively (**Table 2**). Assuming an average copy number per genome of 4.2 for Bacteria and 1 for Archaea (Stoddard et al., 2015), these copy numbers suggests cell abundances ranging $\sim$0.1–2 $\times$ 10^4 per gram of sample material (average 0.71 $\times$ 10^4).

Sequence Reads, Filtering, and OTU Clustering

The total number of sequence reads per sample after filtering and potential contaminants removal (OTUs present in the four controls) varied between 8598 and 25,841 with an average of 17,649 (**Table 2**). A total of 1,804 OTUs (>97% sequence similarity) were found across all samples, of which 1,643 OTUs (91% of total OTUs) were assigned to the bacterial domain, 44 OTUs (2.5% of total OTUs) to the archaeal domain, while 60 OTUs (3.4% of total OTUs) were Eukaryotic. The remaining 57 OTUs were classified as "no hits" which means that the sequence is <80% similar to any in the database (Data sheet S1). The number of OTUs in the individual samples varied between 16 and 371 (**Table 2**). Blank extractions contained a combined total of 221 OTUs of which 142 were removed according to the criteria outlined in the Materials and Methods Section. An additional 50 OTUs were removed from the original data, as they were present in either the drill mud and/or on the recovered microsphere bag (12 and 46 OTUs, respectively).

Microbial Community Composition

As the microbial communities are spread across 41 different phyla, 73 classes, 155 orders, and 218 families, it is far beyond the scope of this work to address the community composition in all samples in detail. Therefore, only the results of the most abundant groups are listed here. However, a full list of OTUs present and their taxonomic assignments can be found in the Supplementary Material (Data sheet S1). In general the communities are relatively homogenous on higher taxonomic level across all samples, however in the sedimentary breccia the diversity of microorganisms and number of OTUs are extraordinarily low, especially in the middle section of that lithological unit (**Figure 2** and **Table 2**), in many ways causing these samples to deviate substantially from the rest.

All samples are dominated by *Proteobacteria* (35–99% of the total communities) of which the class of *Gammaproteobacteria* is the most abundant, followed by *Alphaproteobacteria*, *Deltaproteobacteria,* and *Betaproteobacteria*, respectively (**Figure 2A** and Data sheet S1). Only very low abundances, if any, were assigned to the class of *Epsilonproteobacteria* and *Zetaproteobacteria*. Analysing the different classes of *Proteobacteria* with higher taxonomic resolution show that many (average 60%) of the *Gammaproteobacteria* could not be assigned below class level (**Figure 2B**). The majority of these were represented by OTU5, which showed high similarity (100%) to *Pseudomonas* when performing NCBI blast search. The limited taxonomic resolution of OTU5 in our analysis is

TABLE 2 | General molecular characteristic of the samples investigated.

Sample ID	Depth (mbsf)	Unit	16S rRNA copies ($\times 10^4$)	% Bacteria		Reads after filtering	OTU #	Shared with sediment	
				qPCR	Amplicon			OTUs	Reads of total %
U1382A									
2R_1C	110	I	5.7	98	100	8,598	371	198	72
3R_2B	115	I	8.3	100	100	12,966	174	114	81
3R_3A	117	I	2.7	100	99	16,574	96	66	80
3R_4B	117	I	2.4	100	100	11,349	224	133	81
4R_1B	123	II	4.8	96	97	15,330	215	128	78
5R_1B	133	II	3.9	92	99	19,517	279	168	83
6R_1A	142	II	2.1	100	100	20,437	175	101	69
7R_2B	153	IV	3.8	100	100	10,968	193	127	85
8R_1A	161	V	1.8	100	100	23,251	42	23	80
8R_1B	161	V	4.1	95	100	12,495	261	142	80
8R_2F	162	V	0.3	100	100	19,858	16	10	99
8R_3G	163	V	0.5	100	100	19,145	25	18	22
8R_4D	163	V	0.6	100	86	25,028	27	20	31
9R_1C	172	V	4.2	92	99	16,873	314	185	82
10R_3D	183	VI	5.8	97	99	18,080	283	148	79
12R_1A	199	VII	3.0	92	98	19,506	273	153	75
U1383C									
2R_2E	72	I	2.8	99	100	19,163	319	165	63
3R_1B	77	I	3.1	98	100	18,240	141	75	75
4R_1B	87	I	1.9	98	97	19,144	124	72	72
5R_1BI	97	I	3.1	91	96	15,164	177	124	77
5R_1BII	97	I	2.6	99	99	19,553	330	168	75
6R_1A	105	I	2.2	100	100	23,627	243	146	71
10R_1A	144	II	2.1	90	100	25,841	158	99	86
10R_1D	145	II	2.9	98	100	17,447	326	161	83
11R_1C	154	II	1.7	99	100	24,686	204	116	87
19R_1B	212	III	3.0	98	100	19,107	264	130	84
19R_1A	212	III	3.3	99	100	18,946	126	81	88
20R_1A	219	III	2.9	100	100	3916	148	90	81
24R_1B	257	III	1.9	100	100	12,775	162	99	86
24R_1A	256	III	3.2	100	100	20,900	213	121	86
27R_1A	285	III	3.9	99	100	9,811	162	113	87
29R_1A	300	III	0.6	100	98	11,218	69	48	82
30R_1A	304	III	3.3	100	100	22,903	229	117	88

Including gene copy numbers of total prokaryotic 16S rRNA gene copies per gram of sample material. Percent of the total community related to the domain Bacteria reported both from the qPCR and the amplicon library. The total number of reads after filtering. Number of OTUs in each sample (97% similarity). The number of OTUs and the % of total read that is shared with the samples in the above sediment from North Pond (1383E).

likely due to the high stringency used by the lowest common ancestor algorithm applied to assign taxonomy. Most of the reminding *Gammaproteobacteria* in Hole 1382A belonged to the two orders *Oceanospirillales*, mainly affiliated with the SAR86 clade and *Pseudmonadales*, (largely divided between the families *Moraxellaceae* and *Pseudomonadaceae*). In contrast, the most abundant Gammaproteobacterial order from Hole 1383C is *Alteromonadales*, whereof most can only be assigned to family level (*Alteromonadaceae*) and to a lesser extent *Marinobacter* (<1% of total community). Although, abundance-variation between samples is present within the *Alphaproteobacteria*, as for all taxonomic groups, *Rhizobiales*, *Rhodobaceriales,* and *Rhodospirialles* were in general the most abundant orders (**Figure 2C**). At both sites, *Burkholderiales* was by far the most prominent member of the *Betaproteobacteria* at the order level, with the genus *Variovorax* accounting for approximately half of this group and the family *Oxalobacteraceae* representing the other half (**Figure 2D** and Data sheet S1). The overall abundance of *Deltaproteobacteria* was relatively low and in Hole 1382A it was dominated primarily by *Myxococcales* and the *SAR324* clade whereas *Bdellovibrionales* and *Desulfobacterales* were the most abundant orders in Hole 1383C (**Figure 2E**). In addition

to the mentioned members of the *Proteobacteria* the following phyla were found in relatively high abundances; *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, and *Acidobacteria* along with a number of less abundant groups, but still representing more than 1% of all reads in one or more samples, such as *Planctomycetes* and *Chloroflexi* (see **Figure 2A**).

The archaeal community constitute only a minor fraction of the entire community (max. 5%, avg. 0.6% of all reads) and is represented by five phyla; *Ancient Archaeal Group* (AAG), *Crenarchaeota*, *Euryarchaeota*, *Thaumarchaeota,* and the newly proposed *Lokiarchaeota* (Spang et al., 2015; **Figure 2A** and Data sheet S1). Of these, Thaumarchaeal *Marine Group I* is by far the most abundant.

A comparison between the rank abundance of the 576 OTUs shared between the basalt and the overlying sediment was performed using the average abundances of the OTUs across all basalt and across all sediment samples, excluding the sedimentary breccia from Hole 1384A (**Figure 5**). The shared OTUs represented between 63 and 86% (average 80%) of all reads in the basalt and 44–71% (average 58%) in the sediments, but show a markedly different rank abundance distribution (**Table 2** and **Figure 5**).

Ordination and Clustering Analysis

The variation in the microbial community structure (relative abundance of OTUs) found in the basaltic samples beneath North Pond was compared to the composition in a number of sedimentary horizons in Hole 1383E directly overlying the basaltic crust by means of NMDS. The sedimentary community was investigated at 17 different depths, spanning from the top of the sediment to a few meters above the sediment basement interface and clearly shows a separation from those observed in the underlying upper crust (**Figure 3**). The microbial composition in the sedimentary breccia (lithological unit V) in Hole 1382A are markedly different from the rest of the samples from this sites and also from the basaltic rocks from Hole 1383C, causing these samples to cluster alone, with the exception of sample 8R_1B (the only basalt sample within the breccia unit). An NMDS analysis was also carried out on the full dataset, before removal of any of the OTUs found in the four controls, as described in the Materials and Methods Section. This result shows the same pattern, as with the "cleaned" dataset presented in **Figure 3**, and verifies that all control samples are markedly different from any of the indigenous samples (Figure S1).

In order to investigate any link between community composition and lithology a hierarchical clustering analysis based on the relative abundance of OTUs was executed. The results show several minor (group 1, 2, 3, 6) and two major (group 4 and 5) clusters separated by high bootstrap value (**Figure 4**). The major clusters largely distinguish the two sites from one another. However, three samples from Hole 1383C cluster within 1382A. The bootstrap values are generally low and clustering according to lithology or depth cannot be inferred. The clustering using the full dataset, before removal of potential contaminant reads in general shows the same clustering pattern, however a higher degree of mixing between the two sites is observed (Figure S2). The results from clustering based on Jaccard and Dice dissimilarity indexes, showed no clear difference in clustering pattern as compared to Bray-Curtis (data not shown). Using the geochemical data (major and trace elements) from the same core sections as those used for microbiology, in a hierarchical clustering, shows only very small variation in composition. No clear clustering pattern between sites or lithology could be observed and most branching points were unsupported (Figure S3).

Contamination Control

The presence of fluorescent microspheres in the sterile seawater used to wash the basalt rock surface was investigated on board the ship. After three washing rounds, microspheres were detected in 36% of the samples (**Figure 4**) and more frequently observed in Hole 1383C than 1382A.

Drill mud and the microsphere bag yielded a total of ∼47,500 high-quality reads comprising a number of different bacterial taxa (Data sheet S1). After removal of extraction blanks, 12 OTUs were obtained from the drill mud and 46 from the microsphere bag, most of these were affiliated with *Streptococcus*, a group that are often associated with human pathogens. A larger number of OTUs were obtained from the microsphere plastic bag, many of which were affiliated with different SAR clades. However, also here a number of reads were associated with bacterial groups often associated with humans (e.g., *Streptococcus* and *Dermabacter*; Data sheet S1). Standard drilling protocols inevitably introduce contaminants to drilling components and we propose this as an explanation for the detection of human associated taxa.

Extraction blanks were represented by ∼16,000 reads whereof the vast majority could be assigned to the following three taxonomic groups: *Ralstonia*, *Enterobacteriaceae,* and *Methylobacterium*. On average 38% of all reads were removed during the cleaning procedure (total of 158 OTUs) highlighting the importance of performing operation controls and analysing blank extractions, especially when working with low biomass sample material (Data sheet S1).

DISCUSSION

Microbial Abundances in Subsurface Basaltic Crust

Based on our qPCR results of prokaryotic 16S rRNA gene abundances, we estimate that the samples contain $\sim 10^4$ cells per gram of rock sample, with *Bacteria* outnumbering *Archaea* in all samples (**Table 2**). However, as with all cell estimates based on a primer-based approach it is prone to bias and the numbers should be evaluated with this in mind. It is difficult to compare our estimates with earlier reports since, to the best of our knowledge, only one previous study has been conducted in which direct cell abundances were estimated from native cold subsurface basalt (Fisk et al., 2003). Based on amino acid concentration they suggest $\sim 10^5$ cells per gram sample. A recent study estimated cell numbers in the basaltic fluids from both investigated sites, to be between 1.4 and 2.2×10^4 per ml fluid, based on direct cell counts (Meyer et al., 2016). Considering the average basaltic porosity of 4% and assuming a density of 3, implies that our quantification

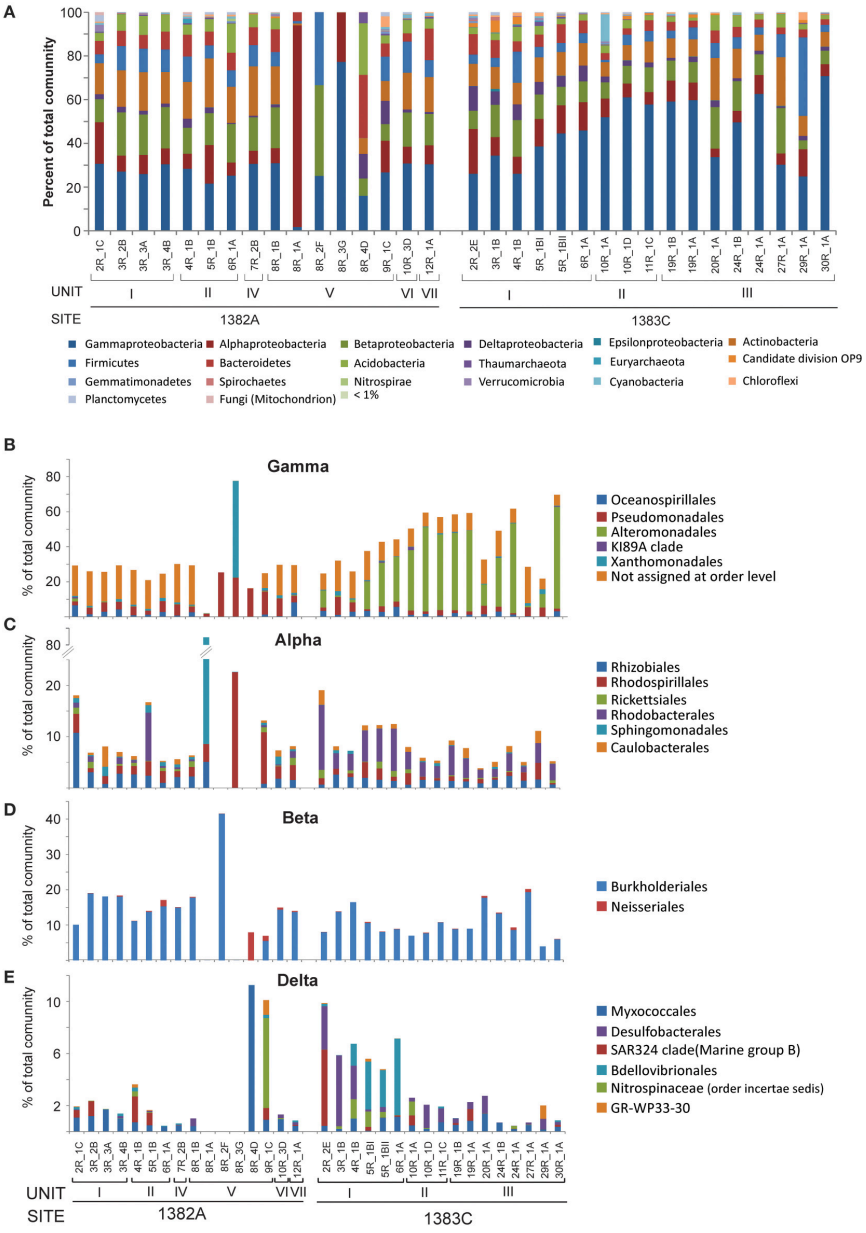

FIGURE 2 | Relative abundance of taxonomic groups. (A) Class level abundances of *Proteobacteria* and phylum level for all other groups comprising more than 1% of total community in one or more samples. Abundances for the taxonomic orders representing more than 1% of total community of **(B)** *Gammaproteobacteria*, **(C)** *Alphaproteobacteria*, **(D)** *Betaproteobacteria*, and **(E)** *Deltaproteobacteria*.

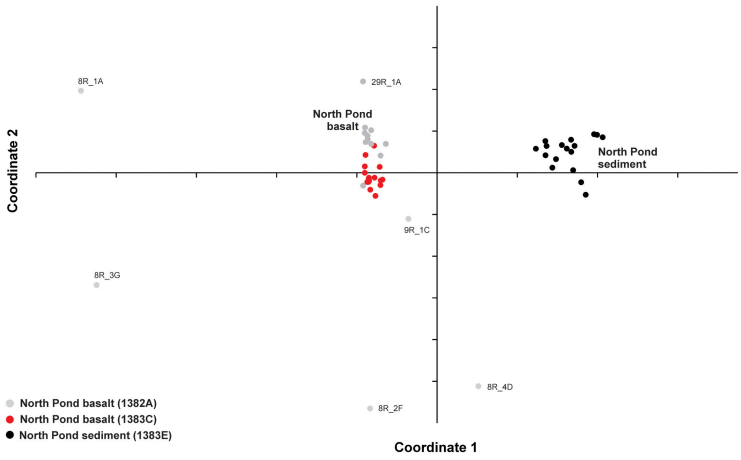

FIGURE 3 | Comparison between community structures. Relative abundance of OTUs in each sample was used to compare the variation between the basalt-hosted communities and the sediment-hosted communities by means of non-metric multidimensional scaling (NMDS). Samples are color coded gray: Hole 1382A, red: 1383C, black: North Pond sediment from Hole 1383E.

is not merely representing cells in the fluid, but that the majority must be attached to the rock surfaces.

Due to the restricted number of sites in our study we have restrained ourselves from the tantalizing prospect of extrapolating the cell abundance to global biomass. However, we note that a previous estimate based on thermodynamic and bioenergetic models was suggested to translate into $\sim 10^7$–10^9 cells per gram rock (Santelli et al., 2008). In other words, 3–5 orders of magnitudes off our estimations. In support of a lower cell abundance is the relatively low oxygen consumption (<1 nmol O_2 cm^{-3} rock d^{-1}) estimated beneath North Pond (Orcutt et al., 2013). Another interesting observation related to the cell numbers is the relative consistency across all samples, which suggest that the cells are limited by a common vital anabolic or catabolic resource.

Microbial Community Composition in Subsurface Basaltic Crust

Based on the 16S rRNA amplicon libraries the microbial communities in the subsurface basalt in North Pond are all dominated by Bacteria, in general leaving the archaeal domain represented by <1%. Although, our qPCR estimations vary slightly from this, both analyses confirm the bacterial dominance (**Table 2**). By far the most abundant phylum was *Proteobacteria*, with *Gamma-*, *Beta-,* and *Alphaproteobacteria* constituting the majority within this phylum. In addition *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, and *Acidobacteria* were all represented by relatively high abundances. To the best of our knowledge, only one previous published study has successfully amplified DNA from native subsurface material and determined the community composition (Lysnes et al., 2004). By means of DGGE that study reported sequences related to *Gammaproteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi,* and *Firmicutes*, of which *Gammaproteobacteria* was the most abundant. A more recent study compiled taxonomic data from a number of studies regarding surface-exposed basalts and identified a set of commonly found abundant microbial groups, including *Gamma-*, *Alpha-*, and *Deltaproteobacteria*, as well as *Actinobacteria*, *Bacteroidetes*, *Acidobacteria*, *Planctomycetes*, *Gemmatimonadetes,* and *Nitrospirae* (Lee et al., 2015). Finally, the microbial community composition in the fluids underneath North Pond has revealed a similar dominance of *Proteobacteria,* also with *Gammaproteobacteria* being most abundant, then followed by *Alpha-*, *Epsilon-*, *Beta-*, and *Deltaproteobacteria* (Meyer et al., 2016). Further, relative high abundances of *Actinobacteria*, *Bacteroidetes*, *planctomycetes, Gemmatimonadetes,* and *Chloroflexi* were observed. Based on this it is tempting to suggest that a basalt-hosted taxonomic core group exists, including surface, subsurface fluids, and hard rocks. However, it is important to stress that the similarity of microbial groups in all cases is based on high taxonomic rank and future in-depth phylogenetic comparison, founded on full-length 16S rRNA sequences, are needed to resolve this issue.

The origin of the subsurface oceanic crustal community is an open question and different scenarios have been debated, one of them being that dispersion may occur via the above sediment column (Huber et al., 2006; Schrenk et al., 2010). When we compared the relative community composition in the basaltic rocks with that in overlying sediment striking differences were observed (**Figure 3**). However, this is by no means indicative of community isolation between the crust and sediment. On the contrary, a high degree of overlap was revealed from the OTUs distribution pattern. Of the 1,802 OTUs

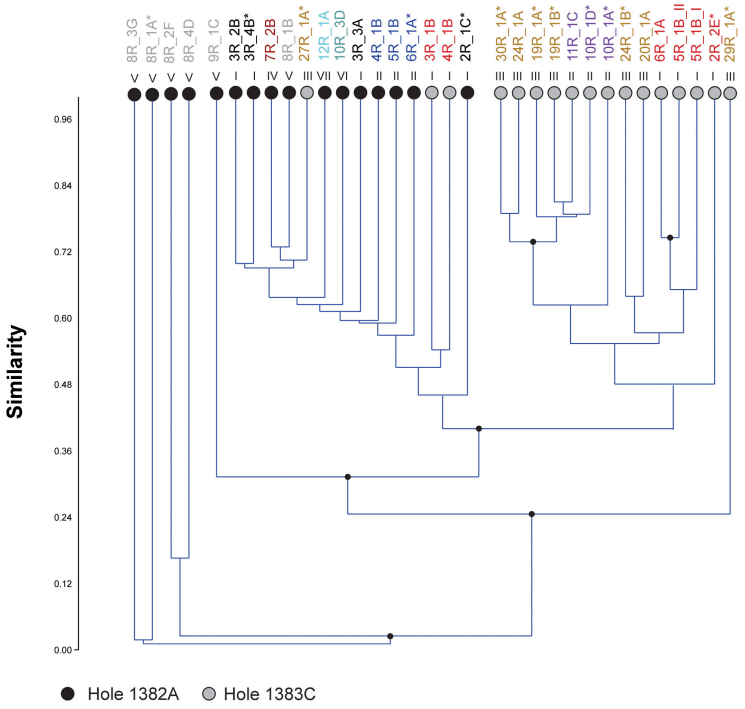

FIGURE 4 | Microbial community clustering. Relative abundance of OTUs in each sample was used to cluster the different communities within the crustal samples from North Pond by means of Bray-Curtis distance calculations. Circles at the end of each branch indicates sampling site; gray: Hole 1383C, black; Hole 1382A. Roman numerals above the branches indicate the lithological unit, also indicated by colors in the sample name above. Asterisk indicates presence of microspheres in the wash fluids after last washing. Numbers above sample name indicates clusters supported by high bootstrap values (> 90) and are identical to numbers used in **Table 2**.

found across all 33 crustal samples, 576 OTUs were shared with the sedimentary habitat above, comprising as much as 63–86% (average 80%) of all reads in the basaltic samples. However, the relative distributions of the shared OTUs in the two environments are markedly different, which might reflect environment-specific taxon recruitment based on availability of electron donors and acceptors (**Figure 5**). Based on these results the two components of the deep biosphere do indeed share many of their microbial inhabitants. Although, the dynamics and mechanisms of taxon dispersal between the crustal and sedimentary subseafloor regimes is beyond the scope of this study, we note that a recent study support the dispersal of sedimentary bacteria via the ocean (Walsh et al., 2016).

Regarding the heterogeneity of crust-associated microbial communities beneath North Pond, we note that although the communities from the two different sites to a large degree cluster together, (**Figure 3**), there are also clear differences (**Figure 4**). Two major clusters, supported by high bootstrap values (> 90) were found, largely separating the two investigated sites from one another. Notable differences are the presence of a few highly abundant OTUs affiliating with an uncultured *Alteromonas* lineage (*Gammaproteobacteria*) in Hole 1383C and a much higher occurrence of *Rhodobacterales* (*Betaproteobacteria*) in Hole 1383C than in Hole 1382A. The significance of this is not known, but it is possible that the physico-chemical nature, and associated redox coupling, of advective crustal fluids, drive community differentiation.

Potential Metabolic Traits and Dominant Groups

The vast majority of lineages reported here do not group within taxonomic clades with known metabolism, and therefore their potential role in the ecosystem is unresolved. However, a number of less abundant groups with relatively constrained metabolic potential are present, thereby allowing us to assign their function with some degree of certainty. For example, as putative sulfate reducers in other environments a number of different genera were observed, including, *Desulfotomaculum*, *Desulfurispora*, *Desulfosporosinus*, *Desulfobaca*, *Desulfobulbus*, *Desulforomonas*, *Desulfovibrio,* and *Desulfobacula*, suggesting that at least the potential for active sulfate reduction is present. The classical sulfur oxidizers (mainly within *Epsilonproteobacteria*), on the

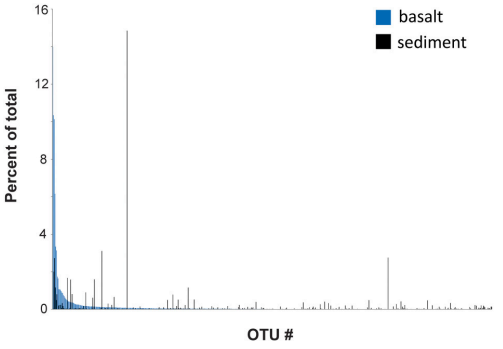

FIGURE 5 | OTU rank abundance. Comparing the rank abundance of OTUs shared between the basalt-hosted community (Holes 1382A and 1383C) and the sediment-hosted (1383E). The abundance of each OTU across all basalt-hosted samples and across all sediment samples was compiled and the average abundance used (excluding the sedimentary breccia in Hole 1382A).

other hand, were only sporadically observed. This observation is worth mentioning in the context of the relatively high abundances of sulfur oxidizers found in the fluids by Meyer et al. (2016) and in the overlying sediments, suggesting different functions between the free-living and the surface-attached communities.

Iron and hydrogen (beside sulfur) has been proposed to be important electron donors in this type of habitat (Bach and Edwards, 2003; Edwards et al., 2005). However, known metal reducers such as, *Marinobacter, Shewanella*, *Geobacter,* and *Ferruginibacter* made up only a minor fraction of the entire community (*Marinobacter* up to 0.3%). Hydrogen utilization is another widespread trait that is difficult to pinpoint based on taxonomy alone, hence we could only assign this to *Hydrogenophilus*, *Hydrogenophaga,* and *Paracoccus,* all represented in the dataset, but in low abundances.

Finally, we observe a number of groups with the ability to transform nitrogen compounds. This includes members of the putative ammonium-oxidizing archaeal Marine Group I (<5% of total community) and nitrifiers (*Nitrospira)*, which was detected in discrete samples up to 0.5%. Despite, the relative low abundances, their presence suggest an active nitrogen cycle, which is in congruence with the low concentration of organic carbon measured in this environment (Orcutt et al., 2015; Sakata et al., 2015).

Contamination Control

A great concern related to investigations of subsurface crustal material (and the deeply buried biosphere in general) has been the challenge of overcoming issues related to contamination constrains (Lever et al., 2006; Santelli et al., 2010). In order to delineate potential sources of contamination (acquired during drilling or subsequent sample processing) we analyzed two sample processing controls (extraction blanks) and two drilling operation controls (drill mud and a recovered empty microsphere bag). Designation of potential contaminants facilitates the tracking of their source in addition to their downstream removal from the bioinformatic pipeline as described before. As more reads were removed from the native samples due to their overlap with extraction blanks rather than with the drill mud and microsphere bag, we conclude that more contamination was introduced during DNA extraction and amplification than from the actual drilling procedure. However, we acknowledge that contamination could have originated from drilling or downstream procedures not accounted for by our contamination controls.

Despite, the observed contamination, several lines of evidence suggest that the final community structure is not corrupted: (i) the bifurcation of community structure of the two crustal sites, (ii) drastic differences between the composition and structure of sedimentary and crustal samples, despite equally low biomass, (iii) congruence between our results and taxonomic identity of enriched organisms from independent studies at this site, including *Pseudomonas, Burkholderia, Bacillus, Salinibacterium, Sphingomonas, Moraxella,* and *Methylobacterium* (Hirayama et al., 2015), (iv) agreement with core-taxa hitherto identified in basalt hosted environments (Lysnes et al., 2004; Lee et al., 2015) and in the fluids under North Pond (Meyer et al., 2016).

In sum, the data shows that contamination in low abundance habitats is a concern, and we encourage that both extraction and operation controls are performed. If such measures are taken the influence of contamination in data analysis seems manageable.

We also note that the presence of microspheres, a general measure of contamination deployed during IODP drilling operations, does not seem to be reflected in the magnitude of contamination in the 16S rRNA gene libraries (measured as number of reads removed in the filtering due to drill mud contamination).

CONCLUSIONS

Our study gives some of the first insights into the microbial inventory of the subsurface oceanic crust in a young cool ridge flank system and show a community dominated by *Proteobacteria* (*Gammaproteobacteria*, *Alphaproteobacteria,* and *Betaproteobacteria*), followed by *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, and *Acidobacteria*. In general, the same phyla are present in high abundances on seafloor-exposed basalts and in the crustal fluids, suggesting the possibility for a common basalt-hosted microbial biome. However, more data is needed to establish if a core group also exist at deeper taxonomic levels. Within the crustal communities we find microbial representatives that are likely to be involved in iron, sulfur, hydrogen, and nitrogen cycling but all in relative low abundances. However, the activity levels might be considerably higher than what the relative abundance implies.

Our estimated cell abundances are on average 0.7×10^4 cells per gram of igneous rock, several orders of magnitude lower than what has been found on seafloor-exposed basalt (e.g., Einen et al., 2008; Santelli et al., 2008). The abundance is relatively consistent across all samples and we therefor speculate that the available

energy is equal across the different samples and/or cell numbers are limited by a common nutrient factor.

Further, the variations in community structure between the samples do, to a large extent, separate the two investigated sites (1382A and 1383C) into two major clusters with no apparent link to differences in lithology. Comparing the basalt-hosted community structure to the overlying sediment using the relative distribution of individual OTUs clearly separates the two environments. However, ∼1/3 of all OTUs in the basalt, representing an average of 80% of all sequences, were also present in the overlying sediment, indicating coupling between the two compartments of the deep biosphere. This observation is interesting with respect to the origin of the microorganisms inhabiting the crustal aquifer, however, deciphering the direction of cell dispersal, the source and the nature and underlying mechanisms of such links needs further investigation.

AUTHOR CONTRIBUTIONS

SJ designed the research; SJ and RZ performed the research; SJ and RZ analyzed the data; and SJ and RZ wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00820

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