Nitrifier abundance and diversity peak at deep redox transition zones

Rui Zhao1,2,4, Bjarte Hannisdal2, José M. Mogollon3 & Steffen L. Jørgensen2

More than half of the global ocean floor is draped by nutrient-starved sediments characterized by deep oxygen penetration and a prevalence of oxidized nitrogen. Despite low energy availability, this habitat hosts a vast microbial population, and geochemical characteristics suggest that nitrogen compounds are an energy source critical to sustaining this biomass. However, metabolic rates of nitrogen transformation and their link to microbial survival in this global-scale ecosystem remain virtually unknown. Here we provide quantitative constraints on microbial nitrogen cycling in open ocean oligotrophic sediments from seafloor to basement, spanning approximately 8 million years. We find active microbial nitrogen transformation throughout the sediment column but at very low rates. Local peaks in diversity and abundance of nitrifiers and denitrifiers occur at redox transition zones deep within the sediments, strongly indicating that these microbes are revived from their maintenance state and start growing again after millions of years of attrition.

Microbial communities in marine sediments impact all major global biogeochemical cycles, including those of oxygen, carbon, nitrogen, manganese, iron, and sulfur1–4. By mediating the flux of chemical compounds in and out of the sediment, this hidden biosphere influences the composition of the ocean and the atmosphere. Many of the processes constituting the global element cycles can occur without microbial catalyzation, albeit often at a considerably lower rate; the nitrogen cycle, however, is unique in that it is almost exclusively dependent on redox reactions regulated by microorganisms5. Hence, not only are the microbes essential for keeping the cycle turning6, but also for making nitrogen bioavailable and thereby a key controlling factor on primary production.

It is clear that microorganisms in marine sediments play a substantial part in balancing the global nitrogen cycle6, but despite recent progress in our understanding of nitrogen transformations in the marine environment6–11, our knowledge of key processes, fluxes, turnover rates, energy yields, spatial distributions, and population sizes in deep-sea sediments is strikingly limited12. One major reason for this limitation is the historical focus on organic-rich sediments on continental margins or in upwelling zones13. In such systems, the availability of energy, in the form of organic carbon, is accompanied by high microbial activity, restricting oxygen and nitrate penetration to a depth of centimeters or less14,15. Hence, the importance of nitrogen transformation in sediments with high organic load is dwarfed by other redox reactions such as sulfate reduction, for which the reaction zones can extend to depths of several hundred meters2,4. However, as researchers turn their attention to the vast oligotrophic regions on the ocean floor, it is becoming apparent that deep oxygen penetration and persistent nitrate throughout the sediment column represent a widespread geochemical scenario that differs markedly from the classic diagenetic redox sequence of organic-rich sediments16–21. Considering that more than half of the ocean floor is draped by oligotrophic sediments22, these new insights suggest that nitrogen transformation is a pervasive process in the deep sedimentary biosphere.

We present a comprehensive investigation of microbial nitrogen cycling in oligotrophic deep subseafloor sediments, using samples collected from North Pond, a small sediment filled basin, on the western flank of the Mid-Atlantic Ridge visited during the Integrated Ocean Drilling Program (IODP) Expedition 336. Two sediment cores from Holes U1383E and U1384A, hereafter referred to as site 3E and site 4A, respectively, provide access to the entire sediment column from seafloor to basement23. North Pond sediments are characterized as oligotrophic, with a total organic carbon content of <0.3 weight percent16,24. With deep oxygen penetration and nitrate present throughout the sediment19,25, these cores offer an ideal opportunity to study nitrogen transformation processes in deep oligotrophic subseafloor sediments. Our approach is to 1) study the nature and long term fate of the

1Department of Biology, University of Bergen, Thormøhlens gate 53A, Bergen, 5007, Norway. 2K.G. Jebsen Centre for Deep Sea Research, Department of Earth Science, University of Bergen, Allegaten 41, Bergen, 5007, Norway. 3Institute of Marine Sciences (CML), Leiden University, Leiden, Netherlands. 4Present address: School of Marine Science and Policy, University of Delaware, Lewes, DE, 19958, USA. Correspondence and requests for materials should be addressed to R.Z. (email: ruizhao@udel.edu) or S.L.J. (email: steffen.jorgensen@uib.no)
2) Combine in situ reaction rates predicted by reaction-transport models with quantitative microbial abundance data to derive the mean cell-specific metabolic rates of functional groups involved in nitrogen cycling. We find a predominance of nitrifiers and denitrifiers surviving in a maintenance state, with strong indication of revival and local growth driven by increased energy supply in transition zones between oxic and anoxic regimes deep within these oligotrophic sediments.

**Results**

**Geochemical profiles and fluxes.** At each site, the dissolved oxygen depth profile shows a characteristic “C” shape, with high concentrations at both the sediment-water interface and sediment-basalt interface, and decreasing concentration towards a central anoxic zone (Fig. 1). This pattern is partly caused by diffusion of dissolved oxygen into the sediment from the overlying seawater and from the underlying oxic basaltic aquifer,
resulting in two distinct redox transition zones at each site: an oxic-anoxic transition zone (OATZ) and an anoxic-oxic transition zone (AOTZ) (Fig. 1). Following the traditional oceanographic definition, we identify the OATZ as an interval over which the O$_2$ concentration drops below 10 $\mu$M down to the detection limit of $\leq$3 $\mu$M and, correspondingly, we define the AOTZ as the interval over which the concentration increases above detection limit and up to 10 $\mu$M. Consequently, the OATZs is found 21–24 meters below seafloor (mbsf) at site 3E and 26–30 mbsf at site 4A, while the AOTZs is located at 28–33 mbsf at 3E and 54–58 mbsf at 4A, respectively (Fig. 1, Table S1). The integrated O$_2$ consumption in the OATZ (i.e. the difference between O$_2$ fluxes in and out of the zone) accounts for 4% of total O$_2$ influx from seawater at 4A (low data resolution prevented estimates for the OATZ at 3E), while the AOTZs account for 29% and 44% of O$_2$ influx from the underlying basement at 4A and 3E, respectively (Table S1).

Nitrate concentrations in the pore water are higher than values measured in the bottom seawater and in the crustal fluids [~21.1 $\mu$M] throughout both sediment cores. Depth profiles of nitrate are mirror images of the oxygen pattern at both sites, with nitrate increasing downcore all the way into the AOTZ, reaching a maximum of 40–50 $\mu$M (Fig. 1). Below these maxima, nitrate concentrations decline and approach the bottom seawater values at the sediment-basalt interface. High sedimentary nitrate concentrations result in an efflux of nitrate from the sediment into both the overlying seawater (0.031–0.047 mmol m$^{-2}$ yr$^{-1}$) and the underlying oceanic crust (0.010–0.011 mmol m$^{-2}$ yr$^{-1}$), with the latter accounting for 19–24% of total sedimentary nitrate efflux (Table S1). Pore water nitrite and manganese concentrations were below the detection limit throughout both cores. The same was true for ammonium at site 4A, while low values ranging between 5–25 $\mu$M were found at site 3E (Supplementary Fig. S6).

Quantification of functional genes and microbial population size. At both sites, the estimated total abundance of microbes (the sum of archaeal and bacterial 16S rRNA genes) was highest near the surface, with $\sim$10$^8$ copies g$^{-1}$ wet sediment, and then decreased with depth to a relatively stable level of $\sim$10$^6$ at site 3E and $\sim$10$^5$ at site 4A (Fig. 2). However, distinct abundance peaks were observed for both Bacteria and Archaea in the OATZs at both sites and less pronounced, in the AOTZ at 3E (Fig. 2).

The abundance of functional groups involved in nitrogen transformation was quantified by targeting their diagnostic functional genes (Fig. 2). Out of 12 target genes, we successfully detected six genes that encode key enzymes involved in nitrification and denitrification processes (Fig. 2, Table S4 show a complete list of targeted genes). In the uppermost sediments, the functional gene abundances are similar across cores, but decrease more rapidly with depth at 4A, resulting in lower overall abundances at this site (Fig. 2). Ammonia-oxidizing archaea (AOA), detected via the archael amoA gene, account for almost the entire archaeal population in the surface sediments, with the latter accounting for 19–24% of total sedimentary nitrate efflux (Table S1). Pore water nitrite and manganese concentrations were below the detection limit throughout both cores. The same was true for ammonium at site 4A, while low values ranging between 5–25$\mu$M were found at site 3E (Supplementary Fig. S6).
abundances approximately two orders of magnitude lower than archaeal amoA throughout both cores (Fig. 2). Nitrite-oxidizing bacteria (NOB), which catalyze the second step in the nitrification process (oxidizing nitrite to nitrate), were detected via the nxrB gene, with a vertical distribution and abundance similar to that of the bacterial amoA (Fig. 2), including the local abundance increases in the OATZs at both sites. Additionally, we successfully detected genes encoding nitrate reductase (narG) and nitrite reductase (nirK and nirS), which are key enzymes that catalyze the first two steps of denitrification, respectively (NO\textsubscript{3}→NO\textsubscript{2}→NO). The abundance of the narG gene in 3E shows an initial decrease of two orders of magnitude, after which it stabilizes around 10\textsuperscript{4} copies per gram sediment from 5 mbsf and until the AOTZ where a further decline is observed (Fig. 2a). The narG distribution in 4A is more variable with apparent elevations at or near the two redox transition zones (Fig. 2b). The nitrite-reductase gene nirK is generally more abundant than nirS in the uppermost sediments, but has a more pronounced decrease with depth (Fig. 2), resulting in nirS-dominated denitrifier communities in deeper sediments. Slight increases of nirK occur in the OATZs, whereas nirS gene copy numbers are more evenly distributed (Fig. 2). The following functional genes were not detectable by qPCR in any of the analyzed samples: anammox bacteria (hzsA and hzo genes), DNRA bacteria (nrfA genes) and nitrogen-fixing bacteria (nifH genes), suggesting that these microbes play a negligible role in nitrogen cycling at the investigated depths.

Figure 3. Functional group abundances. Values are based on taxonomic affiliation of partial 16S rRNA genes in core 3E (A) and 4A (B) and given as percent relative abundances of the total community population. Left to Right: Oxygen characteristics, putative ammonium oxidizing archaea (AOA), ammonium oxidizing bacteria (AOB), nitrite oxidizing bacteria (NOB) and denitrifiers (see material and methods for taxa included in each functional group). The oxic/anoxic transition zones (OATZ) and the anoxic/oxic transition zones (AOTZ) are marked with grey shaded area. Note the difference in range of x-axis values of AOA between the two cores.

Prokaryotic community composition. A substantial fraction of the total community are putative nitrifiers (AOA, AOB, and NOB) and denitrifiers, based on the 16S rRNA gene amplicons, and their vertical distribution is largely consistent with the abundance variation of the corresponding functional genes (Figs 2 and 3). Putative AOA belonging to the order Nitrosopumilales (formerly known as Marine Group I/MG-I/marine group 1.1a\textsuperscript{20}), within the phylum Thaumarchaeota occur in most samples and account for 21% and 66% of total richness in near-surface sediments at 3E and 4A, respectively (Fig. 3A,B). Their relative abundance decreases downcore but distinct peaks occur at the OATZs at both sites and in the AOTZ at site 4A. Occurrences of the AOB genera Nitrosospira and Nitrosococcus (Figs 3 and S5a) are consistent with the distribution of the bacterial amoA gene detected by qPCR (Fig. 2). Putative NOB affiliated with the genera Nitrospina and Nitrospira are also detected at most depths (Figs 3, S5b). Although the relative abundances of AOB and NOB (<3% of the total community) are much lower than AOA, their vertical distributions display similar distinct peaks in the OATZs (Fig. 3). Potential
Denitrifiers are also detected in our amplicon sequences and include members from *Aeromonas*, *Arcobacter*, *Pseudomonas* and Woeseiaceae (formerly known as JTB255-MBG 29), all of which show elevated abundances in the OATZs (Fig. 3, and Tables S5 and S6). However, we note that functional prediction based solely on partial 16S rRNA gene information is challenging, especially of denitrifiers due to their high phylogenetic and metabolic diversity. Consistent with the qPCR results, we found little or no evidence for taxa suspected to be involved in anammox, DNRA, or nitrogen fixation.

When examining the OTU-level community structure of AOA as a function of sediment depth/age (Fig. 4B,D), we find that most OTUs located in the deeper sediments also occur in the surface layers, albeit at a much lower relative abundance. In addition, a marked increase in AOA diversity (richness) is found in the OATZs, with a taxonomic composition similar to the surface layer (Fig. 4B,D). Phylogenetic analysis revealed that members of the Nitrosopumilales Eta (η) cluster dominate within the AOA community in deeper horizons, whereas the surface layers and the OATZ contain a much higher diversity (Fig. 4C,D). Similar to the Nitrosopumilales, the overall microbial richness is highest in the surface sediment layer (280 at 3E and 169 at 4A) and decreases sharply downcore, but is interrupted by a marked richness increase in the OATZs at both sites, resembling that of the surface layer (Fig. 4A,C).

**Reaction rates.** We used a reaction-transport model to simulate the profiles of oxygen and nitrate, and to model the reaction rates of nitrification, denitrification, and oxygen respiration simultaneously. The simulated profiles of oxygen and nitrate match well with those observed, and the predicted TOC is within the range of previous reports (0.02–0.3% (20); Fig. S1). Nitrification rates vary two to three orders of magnitude (4.1 × 10⁻⁷–2.5 × 10⁻⁴ in 3E and 1.3 × 10⁻⁷–3.4 × 10⁻⁵ mol m⁻³ yr⁻¹ in 4A) and are higher in the upper and lower oxic zones than in the anoxic zone, while the opposite trend was predicted for denitrification rates (Fig. 1). Oxygen consumption rates show relatively high initial surface respiration followed by a slow but steady decrease with depth (Fig. 1). To evaluate whether or not denitrification is active in the oxygenated sediments, we performed a sensitivity test by varying the inhibition concentration of O₂ (h₁) over a wide range (0.01–50 µM) and found a best-fit h₁ value of 10 µM (Fig. S2, Table S4), suggesting that the denitrifiers detected in the oxygenated parts of the sediments are actively performing denitrification.

Calculated cell-specific oxygen consumption rates vary between 10⁻⁵–10⁻² fmol e⁻ cell⁻¹ d⁻¹ similar to the cell-specific rates of nitrifiers and denitrifiers (Figs 1 and S3). We translated the cell specific rates of nitrifiers into carbon metabolic rates assuming one mole carbon is fixed at the expense of 10 moles of ammonium oxidized and 14 fg of weight for each cell. These metabolic rates range between ~10⁻⁷–10⁻⁴ g C (g C cell)⁻¹ hour⁻¹ and...

**Figure 4.** Microbial diversity and distribution of nitrifiers. Microbial richness as a function of depth measured by OTU abundance in core 3E (A) and 4A (C). The oxic/anoxic (OATZ) and the anoxic/oxic (AOTZ) transition zones are marked with grey. Heatmap of the relative abundances of different Nitrosopumilales species (OTUs) is represented by each individual column in core 3E (B) and 4A (D). Distinct phylogenetic clusters are marked with Greek letters.

---

**Scientific Reports**  (2019) 9:8633 | https://doi.org/10.1038/s41598-019-44585-6
characterize the nitrifiers as being in maintenance state with cell turnover times spanning approximately 3 orders of magnitude, from months to several hundred years (Fig. 5).

Discussion

Microbial ecology of the N cycling population. The near absence of detectable ammonium and the prevalence of nitrate in concentrations exceeding that of the overlying seawater (Fig. 1) are clear indicators of active nitrogen cycling within the sediments beneath North Pond. Moreover, a substantial fraction of the microbial population is inferred to be involved in nitrogen transformation processes, primarily nitrification. Our reaction rate estimates and the abundance of ammonia-oxidizers (Figs 1–3) point to nitrification as the overall most prominent nitrogen cycling process above and below the anoxic zone. Among the ammonia-oxidizing microbes, which mediate the first step of nitrification, Archaea (AOA) are highly dominant relative to their bacterial counterpart in all but one sample (Figs 2, 3). Specifically, the relative abundance of AOA belonging to the Nitrosopumilales is at least one order of magnitude higher than AOB (Nitrosospira and Nitrosococcus) (Fig. 3). This result might be attributed to a higher oxygen affinity of AOA having extremely low $K_m$ values and a highly energy efficient CO$_2$ fixation pathway, which allows them to outcompete AOB in oxygen- and ammonium-limited regions where energy is low. With respect to nitrification rates, however, some AOB are known to have higher metabolic rates than AOA, implying that the relative contribution of AOB to overall nitrification might be higher than inferred from their abundance. Our community profiling suggests that the second step of nitrification, nitrite oxidation, is catalyzed by NOB affiliated with Nitrospira and Nitrospina. A tight coupling of ammonium oxidation and nitrite is suggested by dual isotopic analysis of pore-water nitrate at this site. Hence, the absence of nitrite in the pore water could suggest that the catabolic activity of the NOB population is as high as the ammonia-oxidizers, irrespective of the significantly lower abundance of NOB, throughout both cores (Figs 2, 3). This inference would be in agreement with recent findings from the dark ocean indicating that NOB have a higher metabolic efficiency than the numerically dominant AOA. However, we cannot exclude partial nitrite removal by denitrifiers, which would lead to overestimations of the NOB activity level.

Despite the difficulties of taxonomically identifying denitrifiers, both the reaction-transport model results (Fig. 1) and the distribution of functional genes (Fig. 2) indicate that denitrifiers are present and active even in theoxic zones. While this result seemingly contradicts the traditional view that denitrification is limited to anoxic/hypoxic environments, it supports mounting evidence of denitrification in oxic marine sediments. Whether this finding is best explained by the presence of anaerobic micro-niches or by oxygen tolerance is still unclear.

From surface to basement, the microbes involved in nitrogen transformation are taxonomically congruent with those found in surface sediments from other open ocean sites, suggesting that a canonical assemblage of higher-rank microbial taxa regulate nitrogen cycling in the oligotrophic sedimentary realm. Beyond the community composition, however, the vertical distribution and abundances of these taxa shed light on processes and activity at depth. The occurrence of Nitrosopumilales 16S rRNA and AOA amoA genes at depths without detectable oxygen (Figs 2, 3) supports the inferred presence of this group in oxygen-deprived marine sediments, and raises questions about their metabolic capabilities. To this end, our calculated nitrification rates in zones without detectable oxygen require only nanomolar levels of O$_2$ (well below the analytical detection limit). Furthermore, with our estimated oxygen consumption rates, which are always higher than the nitrification rates (Fig. 1), the O$_2$ flux should be sufficient to accommodate the nitrification process. Recent studies also show that pelagic AOA in oxygen minimum zones have extremely low $K_m$ values of 333 nM O$_2$ and that oxygen concentrations less
than 10 µM are sufficient to support the growth of specific AOA strains. Hence, our data provide no evidence to suggest that AOA cells found in oxygen-deprived zones are dead, inactive, or involved in any other metabolism than aerobic ammonium oxidation.

**Cell-specific reaction rates, physiological status and turnover time.** When considering the sedimentary community as a whole (excluding nitrifiers), and assuming it to be involved in aerobic heterotrophic metabolism, the cell-specific oxygen consumption rates, at North Pond fall within those estimated for communities at similar depths from oligotrophic and oxygenated sediments beneath the North and South Pacific gyres (Fig. S3). Our estimates help constrain the basal energy requirements for the community as a whole, indicating a lower energy limit around $10^{-5}$ fmol electrons cell$^{-1}$ day$^{-1}$, equivalent to a daily electron transfer on the order of 10$^{3}$. However, such estimates assume equal activity and function for all members of the community and are thus unable to distinguish between different metabolic strategies, which could mask significant variation in the level of activity among microbial groups and individual cells. Although mounting evidence points toward a deep sedimentary community in which the majority of cells are metabolically active, the specific minimum energy required to sustain a microbial cell is likely to depend on environmental conditions as well as differences in metabolic strategy e.g. autotrophy vs heterotrophy.

Our data enable us to go beyond bulk respiration estimates, where all cells are treated as equal, and to resolve cell-specific rates of nitrogen transformation, specifically nitrification rates (Fig. 1). In contrast to the cell-specific oxygen consumption, which is relatively stable (roughly within one order of magnitude in each core), cell-specific nitrification rates are highly variable, spanning several orders of magnitude between sampled horizons. Despite very low metabolic activity levels, cell-specific nitrification rates at >10 mbsf depth are notably higher than the cell-specific oxygen respiration rates (Fig. 1C), and this pattern also holds true when cell-specific oxygen rates are corrected for cell variation in 16S rRNA gene copy numbers (Fig. S3). It is unclear if this relatively higher energy output is related to their autotrophic lifestyle and a potentially higher anabolic energy requirement. Nevertheless, the nitrification rates are many orders of magnitude lower than *in situ* estimates in surface environments, e.g. freshwater surface sediments [29–65 fmol cell$^{-1}$ d$^{-1}$; Könneke, et al.4] as well as rates obtained from cultured representatives of AOB [24–550 fmol cell$^{-1}$ d$^{-1}$; Prosper50] and AOA [Nitrosopumilus maritimus, 4 fmol cell$^{-1}$ d$^{-1}$; Könneke, et al.4].

Our analysis also provides reliable estimates of total denitrification rates and predicts active denitrification throughout both cores (Figs 1–3). However, these inferred cell-specific rates (Fig. S3) have to be qualified, since denitrifiers are metabolically versatile and can switch electron acceptors, e.g. from oxidized nitrogen compounds to oxygen.

Transformation of cell-specific nitrification rates into carbon metabolic rate estimates categorize the nitrifiers as being in maintenance state (Fig. 5), in agreement with growing evidence that the majority of microorganisms to oxygen. denitrifiers are metabolically versatile and can switch electron acceptors, e.g. from oxidized nitrogen compounds to oxygen.

**Local growth in redox transition zones.** At depth greater than 20 mbsf the OATZs and the AOTZs have been isolated from fresh organic carbon input and surface community recruitment for several million years. Nonetheless, local peaks in archaeal and bacterial cell abundance at both the OATZs and the AOTZs (Fig. 2) indicate that these zones are able to sustain a greater biomass than adjacent horizons, in agreement with diaconetic model predictions of enhanced biological activity in sedimentary OATZs. The motility of microbial cells in subsurface sediments is considered to be limited by energy and space. If so, and assuming steady surface input, then the observed abundance peaks can only be explained as a result of *in situ* growth. Local abundance peaks have previously been found in a sulfate-methane transition zone (SMTZ) in organic-rich sediments. Taken together, these findings suggest that the increased energy available at redox transition zones where reduced and oxidized chemical species meet, such as the OATZ, AOTZ and SMTZ, allows starved cells to grow and divide *in situ*.

Recent advances in our basic understanding of microbial evolution and community assembly support the notion that microbes currently populating the deep sedimentary biome are direct descendants of a persistent sub-seafloor population. The recent finding that genomic evolution in subseafloor sediments is negligible suggests that turnover times could be significantly lower than previous assumed for the deep sedimentary population. We note that the slowest turnover rates for nitrifiers are within the anoxic zones of our investigated cores.
Nitrogen fluxes. Oligotrophic conditions at North Pond cause an imbalance in the nitrification-denitrification rates, which we observe as nitrate accumulation in the sediment pore water. This in turn leads to an efflux of nitrate both into the overlying seawater and into the underlying oceanic basement (Table S1). The upward nitrate efflux (0.03–0.05 mmol m$^{-2}$ y$^{-1}$) is in agreement with the general understanding of deep-sea sediments acting as a source of dissolved nitrogen for the overlying water masses. We note that although our upward nitrate efflux estimates for the topmost active layer are probably biased due to the relatively low sampling effort in the surface sediments, they are comparable to those reported from the oligotrophic South Pacific Gyre. Moreover, we estimate that 19–24% of total sedimentary nitrate efflux diffuses into the underlying oceanic crust (Table S1), which matches exactly the range estimated from the oligotrophic sediments at the Clarion-Clipperton fraction zone. This downward supply of sedimentary nitrate could be important for sustaining microbial life in the crustal habitat as indicated by incubation experiments, and by the vast nitrogen cycling potential of microbes in both the crustal fluids and hard rocks.

To summarize, a conceptual model of the nitrogen cycling processes and the associated community dynamics in the oligotrophic sediments of North Pond is provided in Fig. 6. The imbalance of metabolic activities of nitrifiers and denitrifiers maintain high nitrate concentrations throughout the sediment column, and is likely caused by limited carbon availability for denitrifiers. The result is a nitrate efflux into the overlying seawater and the underlying basaltic aquifer, both habitats where nitrate may act as a limiting nutrient. Our calculated cell-specific rates of nitrification and denitrification in the oxygenated sediments are comparable to previously obtained aerobic bulk respiration rates from similar environments in the North and South Pacific gyres. However, we note that our nitrification rates are generally higher than the estimated aerobic respiration rates, potentially as a consequence of the nitrifiers’ autotrophic lifestyle. Overall, nitrifiers are in a maintenance state, with turnover times varying between months to hundreds of years. We highlight the OATZ as a microbial hotspot of subsurface nitrogen transformation, where reduced nitrogen (ammonia) diffuses up from deeper anoxic layers and provides additional substrate, which otherwise is limited. This redox transition zone hosts a higher abundance of cells and
a greater microbial diversity, particularly of nitrifiers, relative to adjacent horizons. We argue that these isolated peaks serve as indicators of local microbial growth, even though these cells have been sequestered from fresh organic matter input for millions of years. The microbial community composition in the OATZ suggests that the original surface nitrifying community has been resurrected at depth after a protracted period of dormancy. Despite a suggestive increase in AOA cell abundance at the AOTZ, the absence of a comparable diversity peak implies that the majority of the nitrifying community cannot rebound after passing through the anoxic zone.

Materials and Methods

Sample location and collection. The two cores used in this study were obtained during the Integrated Ocean Drilling Program (IODP) Expedition 336 to North Pond, an isolated small sediment pond (8 km × 14 km) above basaltic ocean crust located on the western flank of the Mid-Atlantic Ridge at 22°49′N; 46°05′E. The water depths of the two coring sites were 4425 (U1383E) and 4475 (U1384A) meters below sea level. Temperature of the bottom seawater is assumed to be 1.5 °C. The cores were cut into 1.5 meter-long sections on deck and immediately sampled for microbiological and sediment pore-water analyses, as described elsewhere. For this study, sediment from the interior part of intact whole cores was sub-sampled using autoclaved cut-end 10 ml plastic syringes and stored in sterile Whirlpaks® bags at −80 °C until further analysis. We note that a few horizons at site 3E showed signs of weak to moderate coring disturbance.

Flux calculations. Based on the pore-water profiles of oxygen reported in Orcutt, et al. and nitrate profiles reported in, the diffusive fluxes of nitrate between sediments, the overlying seawater and the underlying base-water were calculated using Fick’s first law of diffusion (details can be found in SI). Due to relatively low spatial resolution of data points in the surface sediments where the most radical curvature of geochemical profiles occur, the values reported should be regarded as minimum estimates.

Reaction-transport modeling. Geochemical interactions for the entire sedimentary sequence at both sites were simulated using a one-dimensional reaction-transport model, which discretizes the advection-diffusion-reaction equation. The model used in this study considers three primary reactions: aerobic respiration (R_a), heterotrophic denitrification (R_d); and two secondary reactions: nitri-fication (R_n) and Mn oxidation (R_o), between six chemical species (organic matter, O_2, NO_3^−, NH_4^+, Mn^{2+}, and MnO_2). The model simulations assume that the geochemical profiles, including all implicit reactive intermediates, are near steady state. For details see SI.

DNA extraction. A total of 43 sediment horizons were selected for DNA extraction (~3 meter intervals) from sites 3E (19 horizons) and 4A (25 horizons). Genomic DNA was extracted using PowerLyse® DNA Isolation Kit (MOBIQ Laboratories, Inc.) following the manufacturer’s instruction with two modifications. First, the special bead coating G2 DNA/RNA enhancer (Ampliqon A/S, Odense, Denmark) was used as described in Bælum et al. Second, 200 µg of sterile filtered polyadenylic acid (Sigma) was added to each lysis mixture prior to bead beating (Hugenholtz et al. 1998). Bead beating was performed using the MP-Biomedical FastPrep®-24 for 45 seconds (speed setting 6). To track potential contaminants introduced during the drilling and experimental processes, DNA from the drill mud, the plastic bag carrying the fluorescent microspheres, and the kit reagents were also extracted as described elsewhere. DNA extracts from each sample was finally eluted into 100 µl PCR-grade double-distilled water (ddH2O), and preserved at −20 °C until further analysis.

PCR screening of functional genes. The functional genes encoding the key enzymes of the following nitrogen transformation processes were screened using conventional PCR in all 43 horizons: archael and bacterial ammonia oxidation (AOA amoA and AOB amoA, respectively), nitrite oxidation (nxrB), nitrate reduction (narG and napA), nitrite reduction (nirS and nirK), nitrous oxide reduction (nosZ), anaerobic ammonium oxidation (hzaA and hzo), dissimilative nitrate reduction to ammonium (nrfA), and nitrogen fixation (nifH). In addition we screened for the presence of sulfate reducers, by targeting the dsrB marker gene. A complete list of primers and specific PCR conditions can be found in Table S5. Each reaction (25 µl total volume) contained the following: 1× HotStar Taq® Master Mix (Qiagen, Hilden, Germany), 1.2 µM of each primer and 1 µl template DNA. PCR amplification for each gene was performed for 40 cycles, and products evaluated by visual inspection on 1% agarose gels.

Enumeration of gene abundance by quantitative PCR. Based on initial PCR screening, the six functional genes successfully amplified in at least one of the 43 sediment horizons (i.e. AOA amoA, AOB amoA, NOB nxrB, nitrate reducer narG, as well as denitrifier nirK and nirS) were subsequently enumerated by quantitative PCR (qPCR). In addition, archael and bacterial 16S rRNA gene abundances were quantified as described elsewhere. All standards were quantified using BIO-analyzer (DNA 1000 chips, Aglient Technologies) and 10-fold serial diluted to 10−10⁶ copies µl−1. Details can be found in SI, including a complete list of primers and thermal conditions Table S5.

Cell specific reaction rates and metabolic activity. Mean cell-specific rates of nitrifiers and denitrifiers were calculated by dividing the total volumetric reaction rates predicted from the reaction-transport model by the total abundance of the respective functional genes measured by qPCR (details in SI). Due to the possible presence of multiple copies of a given functional gene in one genome, the calculated cell-specific rates should be considered minimum values. The cell-specific rates were normalized to femtomoles of electrons (e−) transferred per cell per day, under a number of assumptions, which can be found in the SI. For comparison with other relevant studies, cell specific reaction rates were recalculated into carbon metabolic rates as described in the SI.
16S rRNA gene libraries and sequencing. DNA extracted from site 3E and 4A (43 horizons in total) was amplified in duplicate reactions using a previously described two-step amplification strategy, minimizing amplification bias\(^\text{4-5}\). 16S rRNA genes were PCR amplified using primers Uni519F (5\'−CAGCMGGCGGGCTA−3\') and 1392R (5\'−ACGGGCGTGWGTRC−3\') for samples from 4A, and Uni519F and 806R (5\'−GACTACHVGGGTATCCTR−3\') for 3E, to create the initial amplicon libraries. More details can be found in the SI section.

Taxonomic evaluation and functional classification. After quality control (details in SI) the filtered reads were clustered into Operational Taxonomic Units (OTUs) at 97% nucleotide similarity cutoff using UPARSE\(^\text{73}\). Taxonomy of the representative sequences of each OTU were assigned using the software packages CREST against the SilvaMod reference database\(^\text{74}\) using a common ancestor algorithm. OTUs with taxonomic affiliation relevant to nitrogen transformation were manually assigned into functional groups as follows: the order Nitrospumilales was assigned into ammonium oxidizing archaea (AOA), the genus Nitrososphaera, Nitrospina and Nitrosococcus were considered ammonium oxidizing bacteria, AOB, the genus Nitrosira and Nitrospina were treated as nitrite oxidizing bacteria (NOB), and denitrifying bacteria i encompass the taxa of Pseudoana, Arcobacter, Aeromonas, and Woesieliae (Tables S6, S7). OTUs of Nitrospumilales were extracted from the decontaminated OTU table, and the relative abundance of each OTU in the total AOA communities in each core was normalized by total-sum-scaling before displayed in the heatmap (Fig. 4) prepared using the R package ggplot2\(^\text{75}\). For diversity measures (richness) each sample was randomly subsampled to 1,000 reads in USEARCH v10\(^\text{8}\), prior to any comparison between samples.

Data Availability Raw read files generated in this study have been deposited in the NCBI Sequence Read Archive under accession number PRJNA489438. Geochemical data related to the two investigated cores can be found at IODP Expedition 336 webpages (http://publications.iodp.org/proceedings/336/336toc.htm).

References

1. Colwell, F. S. & D'Hondt, S. Nature and extent of the deep biosphere. Rev Mineral Geochem 75, 547–574 (2013).
2. D'Hondt, S. et al. Distributions of microbial activities in deep subseafloor sediments. Science 306, 2216–2221, https://doi.org/10.1126/science.1101155 (2004).
3. Falkowski, P. G., Fenchel, T. & Delong, E. F. The microbial engines that drive Earth’s biogeochemical cycles. Science 320, 1034–1039, https://doi.org/10.1126/science.1153213 (2008).
4. Parkes, R. J. et al. Deep sub-seaﬂoor prokaryotes stimulated at interfaces over geological time. Nature 436, 390–394, https://doi.org/10.1038/nature03796 (2005).
5. Canfield, D. E., Glazer, A. N. & Falkowski, P. G. The evolution and future of Earth’s nitrogen cycle. Science 330, 192–196, https://doi.org/10.1126/science.1186120 (2010).
6. Thamdrup, B. New pathways and processes in the global nitrogen cycle. Annual Review of Ecology, Evolution, and Systematics 43, 407–428, https://doi.org/10.1146/annurev-ecolsys-102710-145048 (2012).
7. Strous, M. et al. Missing lithotroph identified as new planctomycete. Nature 400, 446–449 (1999).
8. Könneke, M. et al. Isolation of an autotrophic ammonia-oxidizing marine archaeon. Nature 437, 543–546, https://doi.org/10.1038/nature03911 (2005).
9. Ettwig, K. F. et al. Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. Nature 464, 543–548, https://doi.org/10.1038/nature08883 (2010).
10. Lam, P. et al. Revising the nitrogen cycle in the Peruvian oxygen minimum zone. Proceedings of the National Academy of Sciences of the United States of America 106, 4752−4757, https://doi.org/10.1073/pnas.0812444106 (2009).
11. Bristow, L. A. et al. Ammonium and nitrate oxidation at nanomolar oxygen concentrations in oxygen minimum zone waters. Proceedings of the National Academy of Sciences of the United States of America 113, 10601–10606, https://doi.org/10.1073/pnas.1600359113 (2016).
12. Wankel, S. D., Buchwald, C., Ziebis, W., Wenk, C. B. & Lehmann, M. F. Nitrogen cycling in the deep sedimentary biosphere: nitrate isotopes in porewaters underlying the oligotrophic North Atlantic. Biogeoosciences 12, 7483−7502, https://doi.org/10.5194/bg-12-7483-2015 (2015).
13. Durbin, A. M. & Teske, A. Microbial diversity and stratification of South Pacific abyssal marine sediments. Environmental Microbiology 13, 3219−3234, https://doi.org/10.1111/j.1462-2920.2011.02544.x (2011).
14. Revsbech, N. P., Jorgensen, B. B. & Blackburn, T. H. Oxygen in the sea bottom measured with a microelectrode. Science 207, 1355–1356 (1980).
15. Sørensen, J., Jorgensen, B. B. & Revsbech, N. P. A comparison of oxygen, nitrate, and sulfate respiration in coastal marine sediments. Microbial Ecology 5, 105−115, https://doi.org/10.1007/bf02010501 (1979).
16. Ziebis, W. et al. Interstitial fluid chemistry of sediments underlying the North Atlantic gyre and the influence of subsurface fluid flow. Earth and Planetary Science Letters 323, 79–91, https://doi.org/10.1016/j.epsl.2012.01.018 (2012).
17. D’Hondt, S. et al. Presence of oxygen and aerobic communities from sea floor to basement in deep-sea sediments. Nature Geoscience 8, 139−142, https://doi.org/10.1038/ngeo2387 (2015).
18. D’Hondt, S. et al. Subseaﬂoor sedimentary life in the South Pacific Gyre. Proceedings of the National Academy of Sciences of the United States of America 106, 11651−11656, https://doi.org/10.1073/pnas.0811793106 (2009).
19. Expedition; 336; Scientists. Sediment and basement contact coring. In Edwards, K. J., Bach, W., Klaus, A., and the Expedition 336 Scientists, Proc. IODP, 336: Tokyo (Integrated Ocean Drilling Program Management International, Inc.), https://doi.org/10.2204/iodp.proc.336.106.2012 (2012).
20. Mewes, K. et al. Diffusive transfer of oxygen from sea mount basaltic crust into overlying sediments: An example from the Clarion–Clipperton Fracture Zone. Earth and Planetary Science Letters 433, 215−225 (2016).
21. Kuhn, T. et al. Widespread seawater circulation in 18-22 Ma oceanic crust: Impact on heat flow and sediment geochemistry. Geology 45, 799−802, https://doi.org/10.1130/g39911.1 (2017).
22. Claustre, H. et al. Gross community production and metabolic balance in the South Paciﬁc Gyre, using a non intrusive bio-optical method. Biogeosciences 5, 463−474 (2008).
23. Expedition; 336; Scientists. Methods. In Edwards, K. J., Bach, W., Klaus, A., and the Expedition 336 Scientists, Proc. IODP 336: Tokyo (Integrated Ocean Drilling Program Management International, Inc.), https://doi.org/10.2204/iodp.proc.336.106.2012 (2012).
24. Reeve, B. K. et al. Nitrogen Cycling of Active Bacteria within Oligotrophic Sediment of the Mid-Atlantic Ridge Flank. Geomicrobiology Journal, 1–18 (2018).
61. Hensen, C., Landenberger, H., Zabel, M. & Schulz, H. D. Quantification of diffusive benthic fluxes of nitrate, phosphate, and silicate

57. Hoehler, T. M. & Jørgensen, B. B. Microbial life under extreme energy limitation.

59. Walsh, E. A.

46. Røy, H.

47. Jørgensen, B. B. & Marshall, I. P. G. In

50. Prosser, J. Autotrophic nitrification in bacteria.

52. Morono, Y.

37. Prosser, J. I. & Nicol, G. W. Archaeal and bacterial ammonia-oxidisers in soil: the quest for niche specialisation and differentiation.

44. Inagaki, F.

42. Roussel, E. G.

34. Martens-Habbena, W., Berube, P. M., Urakawa, H., de la Torre, J. R. & Stahl, D. A. Ammonia oxidation kinetics determine niche separation of nitrifying Archaea and Bacteria.

29. Billen, G. Evaluation of nitrifying activity in sediments by dark 14C-bicarbonate incorporation. Water Research 10, 51–57, https://doi.org/10.1016/0043-1354(76)90157-3 (1976).

41. Tully, B. J. & Heidelberg, J. F. Potential mechanisms for microbial energy acquisition in oxic deep-sea sediments.

30. Billen, G. Evaluation of nitrifying activity in sediments by dark 14C-bicarbonate incorporation. Water Research 10, 51–57, https://doi.org/10.1016/0043-1354(76)90157-3 (1976).

49. Bai, Y. & Jørgensen, B. B. Microbial nitrifier abundance in deep-sea sediments: implications for nitrification rates in anoxic marine sediments.

53. Braun, S.

54. Lomstein, B. A., Langerhuus, A. T., D’Hondt, S., Jørgensen, B. B. & Spivack, A. J. Endospore abundance, microbial growth and necromass turnover in deep sub-seafloor sediment. Proceedings of the National Academy of Sciences of the United States of America 109, 16213–16216, https://doi.org/10.1073/pnas.1203849109 (2012).

31. Wuchter, C.

32. Kallmeyer, J., Pockalny, R., Adhikari, R. R., Smith, D. C. & D’Hondt, S. Global distribution of microbial abundance and biomass in subseafloor sediment. Proceedings of the National Academy of Sciences of the United States of America 109, 2846–2855, https://doi.org/10.1073/pnas.1207574109 (2012).

48. Majzub, A. & Jørgensen, B. B. Microbial nitrifier abundance in deep-sea sediments: implications for nitrification rates in anoxic marine sediments.

33. Billen, G. Evaluation of nitrifying activity in sediments by dark 14C-bicarbonate incorporation. Water Research 10, 51–57, https://doi.org/10.1016/0043-1354(76)90157-3 (1976).

36. Jørgensen, B. B. & Marshall, I. P. G. In

40. Ovchinnikova, I., Kallmeyer, J., Pockalny, R., Adhikari, R. R., Smith, D. C. & D’Hondt, S. Microbial nitrifier abundance in deep-sea sediments: implications for nitrification rates in anoxic marine sediments.

35. Leys, E. & Prosser, J. I. Distribution of ammonia-oxidising bacteria in coastal and deep-sea sediments of the North Sea and the eastern equatorial Atlantic Ocean. Applied Environmental Microbiology 70, 2802–2809, https://doi.org/10.1128/AEM.70.5.2802-2809.2004 (2004).

38. Martens-Habbena, W., Berube, P. M., Urakawa, H., de la Torre, J. R. & Stahl, D. A. Ammonia oxidation kinetics determine niche separation of nitrifying Archaea and Bacteria.

39. Martens-Habbena, W., Berube, P. M., Urakawa, H., de la Torre, J. R. & Stahl, D. A. Ammonia oxidation kinetics determine niche separation of nitrifying Archaea and Bacteria. Nature 461, 976–979, https://doi.org/10.1038/nature08465 (2009).

58. Starnawski, P.

59. Walsh, E. A.
63. Cowen, J. P. et al. Fluids from aging ocean crust that support microbial life. *Science* **299**, 120–123, https://doi.org/10.1126/science.1075653 (2003).
64. Bourbonnais, A. et al. Activity and abundance of denitrifying bacteria in the subsurface biosphere of diffuse hydrothermal vents of the Juan de Fuca Ridge. *Biogeochemistry* **9**, 4661–4678, https://doi.org/10.1016/j.biorto.2012.06.007 (2012).
65. Tully, B. J., Wheat, C. G., Glazer, B. T. & Huber, J. A. A dynamic microbial community with high functional redundancy inhabits the cold,oxic subsurface ocean. *ISME Journal* **12**, 1–16, https://doi.org/10.1038/ismej.2017.187 (2018).
66. Tyrrell, T. The relative influences of nitrogen and phosphorus on oceanic primary production. *Nature* **400**, 525–531, https://doi.org/10.1038/103822941 (1999).
67. Moore, C. M. et al. Processes and patterns of oceanic nutrient limitation. *Nature Geoscience* **6**, 701–710, https://doi.org/10.1038/ngeo1765 (2013).
68. Mogollón, J. M., Mewes, K. & Kasten, S. Quantifying manganese and nitrogen cycle coupling in manganese-rich, organic carbon-starved marine sediments: Examples from the Clarion-Clipperton fracture zone. *Geophysical Research Letters* **43**, 7114–7123 (2016).
69. Bælum, J. et al. A conceptual model linking functional gene expression and reductive dechlorination rates of chlorinated ethenes in clay rich groundwater sediment. *Water research* **47**, 2467–2478 (2013).
70. Zhang, Y. C. et al. Isotopic and microbiological signatures of pyrite-driven denitrification in a sandy aquifer. *Chemical Geology* **300**, 123–132, https://doi.org/10.1016/j.chemgeo.2012.01.024 (2012).
71. Jørgensen, S. L. & Zhao, R. Microbial inventory of deeply buried oceanic crust from a young ridge flank. *Frontiers in Microbiology* **7**, 820, https://doi.org/10.3389/fmicb.2016.00820 (2016).
72. Berry, D., Ben Mahfoudh, K., Wagner, M. & Loy, A. Barcoded primers used in multiplex amplicon pyrosequencing bias amplification. *Appl. Environ. Microbiol.* **77**, 7846–7849, https://doi.org/10.1128/aem.05220-11 (2011).
73. Edgar, R. C. UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nature methods* **10**, 996–998 (2013).
74. Lanzen, A. et al. CREST - Classification Resources for Environmental Sequence Tags. *PLoS One* **7**, e49334, https://doi.org/10.1371/journal.pone.0049334 (2012).
75. Wickham, H. ggplot2: elegant graphics for data analysis. (Springer 2016).
76. Edgar, R. C. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **26**, 2460–2461, https://doi.org/10.1093/bioinformatics/btq461 (2010).

**Acknowledgements**

We express our gratitude to Wolfgang Bach and the late Katrina Edwards for having the vision and ability to realize IODP expedition 336. We thank the entire scientific party and all crewmembers onboard for their help and expertise, especially Beth Orcutt for generating the extensive oxygen profiles and Geoffrey Wheat for making the pore water data available. Beth Orcutt and Wolfgang Bach are thanked for many valuable comments and suggestions. This study used samples and data provided by the IODP and was funded by the Norwegian Research Council through the Centre for Geobiology, University of Bergen.

**Author Contributions**

R.Z. and S.L.J. designed research; R.Z. and S.L.J. performed research; R.Z., S.L.J., J.M.M., and B.H. analyzed data; and R.Z., B.H., and S.L.J. wrote the paper.

**Additional Information**

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-019-44585-6.

**Competing Interests:** The authors declare no competing interests.

**Publisher’s note:** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2019