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Microbe-mineral biogeography from multi-year incubations in oceanic crust at North Pond, Mid-Atlantic Ridge

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Summary

Subseafloor oceanic crust is a vast yet poorly sampled habitat for life. Recent studies suggest that microbial composition in crustal habitats is variable in space and time, but biogeographic patterns are difficult to determine due to a paucity of data. To address this, we deployed hundreds of mineral colonization experiments at and below the seafloor for 4–6 years at North Pond, a borehole observatory network in cool (<10°C) and oxic oceanic crust on the western flank of the Mid-Atlantic Ridge. The overall community composition of mineral incubations reveals that colonization patterns are site dependent, with no correlation to mineral type. Only a few members of the *Thioalkalispiraceae* and *Thioprofundaceae* exhibited a mineral preference pattern, with generally higher abundance on metal sulphides compared to silicates, while taxa of the Gammaproteobacteria and Deltaproteobacteria were common in the colonization experiments. In comparison to datasets from other crustal habitats, broader biogeographic patterns of crustal communities emerge based on crustal habitat type (surface-attached communities versus fluid communities), redox environment and possibly crustal age. These comparisons suggest successional biogeography patterning that might be used as an indicator of how recently permeable pathways were established within oceanic crust.

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Introduction

One of the four themes of the decadal International Ocean Discovery Program (IODP) is to investigate the marine deep biosphere – the vast habitat for life below the seafloor – through scientific ocean drilling (IODP, 2011; D'Hondt *et al*., 2019b). While there have been major advances in understanding the abundance, diversity and patterns of microbial life in the sedimentary marine deep biosphere in the past decade (Edgcomb *et al*., 2011; Kallmeyer *et al*., 2012; Orsi *et al*., 2013; Inagaki *et al*., 2015; Wörmer *et al*., 2019; D'Hondt *et al*., 2019a), similar knowledge from the deep biosphere within oceanic lithospheric crust has lagged behind. Understanding the form and function of the crustal deep biosphere is important because microbial activity can influence fluid-rock reactions that can affect global chemical cycles including carbon cycling (Wheat *et al*., 2017; Shah Walter *et al*., 2018; McManus *et al*., 2019). As humanity considers subseafloor carbon capture and sequestration within oceanic crust as a negative emission technology (Goldberg *et al*., 2008; Matter *et al*., 2009; Goldberg *et al*., 2018), the need for understanding fluid-rock-microbe interactions in these environments gains in importance (Trias *et al*., 2017).

Some patterns in crustal deep biosphere biogeography are beginning to emerge based on IODP-related expeditions within the past decade. For example, an emergent pattern is the prevalence of Gammaproteobacteria and Alphaproteobacteria taxa in basaltic habitats exposed to cold (<10°C) and oxic seawater and the abundance of spore-forming sulphate reducing microbial groups under warm (~65°C) and anoxic conditions (Orcutt *et al*., 2020). Taking advantage of subseafloor borehole observatories called 'CORKs' (Becker and Davis, 2005), repeated sampling of crustal fluids post-drilling-disturbance reveals dynamic microbial communities in fluids over space and time, even though functional potential appears to be constant (Jungbluth *et al*., 2016; Meyer

et al., 2016; Tully *et al*., 2017). Some studies indicate that mineralogy has a structuring influence on crustal microbial communities (Toner *et al*., 2013; Smith *et al*., 2016), whereas other studies suggest that mineralogy may not be as strong of a governing factor when compared to temperature and/or redox conditions (Baquiran *et al*., 2016; Ramírez *et al*., 2019). Moreover, there appears to be microbial community structure differences between the subsurface rock-attached biofilms and the fluids that circulate through the rock habitat (Ramírez *et al*., 2019).

In an attempt to resolve biogeographic patterns of microbial communities in oceanic crust, we executed a multi-year mineral colonization experiment in the cool, oxic, ~8 Ma basaltic subseafloor at the North Pond site on the western flank of the Mid-Atlantic Ridge (Fig. 1, Fig. S1, Table S1). The study was designed to place known minerals within the ocean crust and at the seafloor to assess colonization patterns of microbial communities that settle on these materials. The study used a mineral colonization experiment approach with flow-through osmotic colonization systems (FLOCS) that have also been used in other subsurface crustal settings (Orcutt *et al*., 2010; Edwards *et al*., 2012b; Ramírez *et al*., 2019). The operational concept of FLOCS is that a plastic sleeve is filled with cassettes of mineral preparations, either crushed to sand-size particles or as polished coupons, through which environmental fluid is continuously drawn via an attached fluid sampling system called an OsmoSampler. The experiments were deployed in three different locations at North Pond: at Hole U1382A with a fibreglass CORK observatory in the southern region of the pond, at Hole U1383C with a similar CORK observatory in the eastern end of the pond, and at Hole U1382B with a mild-steel casing 'CORK-Lite' observatory. These boreholes access cool and oxic conditions within upper oceanic crust, and sampling has documented a recovery of crustal fluid conditions following drilling disturbance (Orcutt *et al*., 2013; Tully *et al*., 2017; Wheat *et al*., 2020). Details about the study site and the configuration of the observatories are provided in Appendix S1 and detailed elsewhere (Wheat *et al*., 2012; Edwards *et al*., 2012b; Wheat *et al*., 2020).

A detailed description of the experiment design is available in the Appendix S1. In brief, two types of mineral colonization experiments (i.e., FLOCS) were deployed:

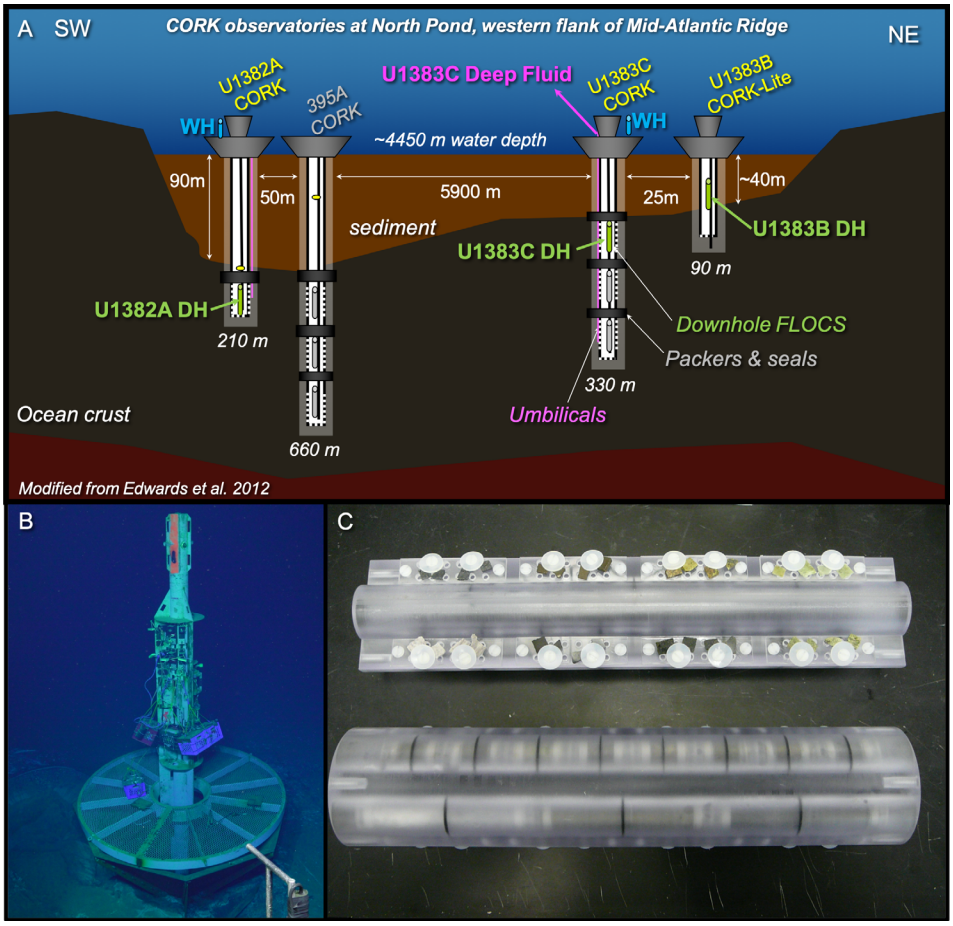

Fig 1. Overview of the North Pond site (A) with borehole observatories in oceanic crust (B) and the FLOCS colonization experiments in this study (C). A: In 2011 during IODP Expedition 336, FLOCS experiments (example in photograph in C) were deployed downhole (DH, green and grey cylinder symbols) in the Holes U1382A and U1383C observatories. In 2012 during cruise MSM20-5, DH FLOCS experiments were deployed at Hole U1383B. Finally, in 2014 during cruise MSM37, wellhead (WH) FLOCS experiments were deployed at Holes U1382A and U1383C (blue cylinder symbols; example in photograph in B); these experiments sampled bottom seawater. In A, green and blue coloured FLOCS experiments were recovered in 2017; FLOCS coloured in grey not recovered due to instrument failures. Figure modified with permission from (Edwards *et al*., 2012b). See Figs S1 and S2 for more details.

(i) downhole within the borehole observatory casing on instrument strings, exposed to subsurface crustal fluids and conditions and (ii) at the wellhead, exposed to bottom seawater (Fig. 1, Fig. S2). The FLOCS contained cassettes with various igneous silicates (basalts, fayalite, olivine), metal sulphides (pyrite, pyrrhotite, sphaelerite, chalcopyrite), metal oxides (haematite, goethite), metal carbonate (siderite) and less-reactive surfaces including glass beads, glass wool, and plastic. Some of the FLOCS contained an additional enrichment solution that added nitrate to the inoculating fluids at 150 μM final concentration compared to ambient conditions closer to 20 μM (Wheat *et al*., 2020). The enrichment and non-enrichment FLOCS experiments were deployed in 2011 and 2012 within the three boreholes, and the FLOCS experiments that were incubated in bottom seawater were deployed in 2014 (Table S1). Microorganisms within the environmental fluid (i.e., crustal fluid or bottom seawater) could colonize the mineral preparations in the FLOCS over the course of the deployments. Effluents from FLOCS colonization chambers were continuously collected in OsmoSamplers (Jannasch *et al*., 2004) to determine solute concentration changes over time and confirm fluid origin and reaction progression. All experiments were recovered in October 2017 and processed as detailed in the Appendix S1 following protocols of recent studies (Ramírez *et al*., 2019; Jones *et al*., 2020). After verifying DNA extraction protocols on test samples (Table S2, Figs S3 and S4), the microbial communities on the colonized materials were compared to each other, and also to prior community data from rock and fluid samples from this and other study sites, to determine the degree that inoculation origin, redox and nutrient conditions, mineral type, length of exposure or other factors contribute to biogeographic patterning.

Results and discussion

Documentation of FLOCS performance and microbial abundance estimates

Analysis of solutes from the OsmoSamplers document that crustal fluids were pulled through the downhole FLOCS and that bottom seawater was pulled through the wellhead FLOCS experiments (Fig. S5, Table S3). Scanning electron microscopy analysis of polished colonization coupons of different composition confirmed the presence of cells and secondary alteration products on the coupon surfaces, as well as mineral precipitates and possible organic films on plastic materials that were part of the colonization experiment (Fig. S6). Secondary alteration products appeared thicker on the metal sulphides compared to the silicates (see (Orcutt and D'Angelo, 2019) for more detail), as has been observed in other studies (Orcutt *et al*., 2011).

Quantification of the 16S rRNA gene via quantitative PCR revealed a biomass density of the microbial biofilms on the downhole and wellheads FLOCS samples of approximately $1.2 \pm 0.5 \times 10^4$ and $1.2 \pm 1.0 \times 10^4$ 16S rRNA gene copies per gram of material, respectively (Table S4). None of the no-template-control samples resulted in an amplification product (data not shown). If we assume that every cell in this low energy environment has only one 16S rRNA gene copy per cell, these values translate to a cell density of approximately 1×10^4 cells g^{-1}, which is similar to 16S rRNA gene-based density analyses measured in basalt cores that were collected during drilling operations (Jørgensen and Zhao, 2016; Zhang *et al*., 2016). There were no trends in 16S rRNA gene copy density across FLOCS location, mineral type, or nitrogen enrichment.

16S rRNA gene amplicon sequencing resulted in 2697 unique ASVs that passed quality control (Fig. 2; Appendix S2). The average number of ASVs per sample was 134 ± 74, which is consistent with the estimated number of operational taxonomic units described at 97% or greater sequence similarity in basalts from this site (Jørgensen and Zhao, 2016). There were no patterns in alpha diversity metrics (e.g., observed ASVs, Shannon Diversity Index) in relation to site, mineral type or nitrate enrichment, although the wellhead samples were less diverse than the downhole samples. The colonization experiment samples were significantly less diverse in both richness (fewer observed ASVs) and evenness (lower Shannon Diversity Index) than the communities living on the 8-Ma basalt samples from this site, the crustal fluids within this system and the seafloor basalts from the Dorado Outcrop (*t*-test *p*-values <0.05).

Microbial community composition and beta diversity

Diverse bacterial and archaeal phyla, including candidate phyla radiation/Patescibacteria groups (Hug *et al*., 2016), were present in the FLOCS biofilms (Fig. 3; Fig. S7). Overall, most phyla observed in the downhole FLOCS were also observed on FLOCS incubated in bottom seawater, and there was also overlap with phyla detected in the crustal fluids. Similar to previous studies at North Pond and other cold (<10°C) and oxic oceanic crust environments (Santelli *et al*., 2008; Lee *et al*., 2015; Jørgensen and Zhao, 2016; Tully *et al*., 2017), the majority of the documented taxa belong to Bacteria, with typically less than 1% of sequences grouping within Archaea. While there was high phyla-level overlap between the FLOCS and the crustal fluids, there were some taxa that were only observed in the fluids or only observed in the FLOCS biofilms. The highest relative

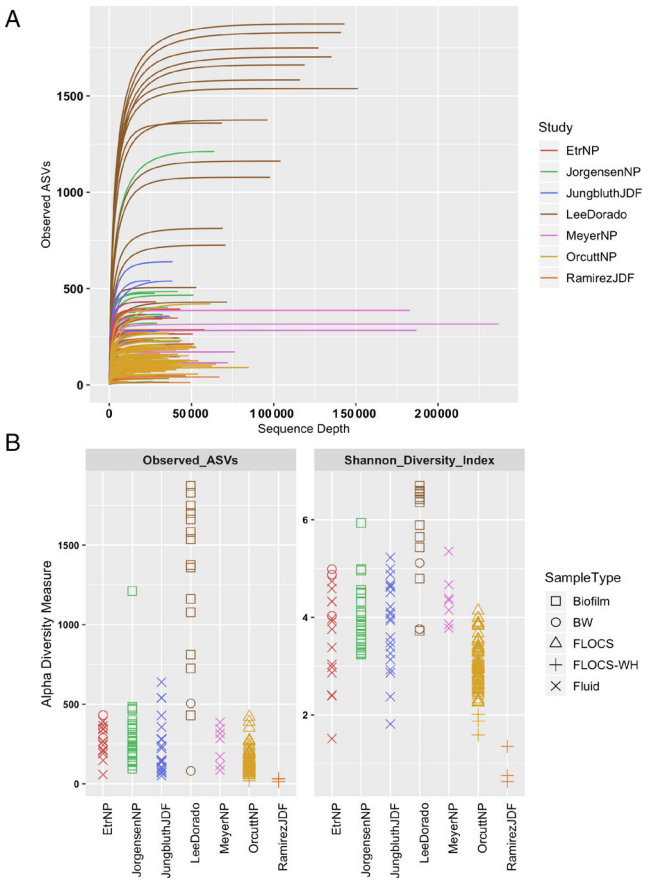

Fig 2. Summary of sample species richness and diversity in samples from this study ('OrcuttNP') in comparison to previous published studies (North Pond crustal fluids 'EtrNP' (NCBI Sequence Read Archive project PRJNA603242) and 'MeyerNP' (Meyer *et al*., 2016); North Pond basalts 'JorgsensenNP' (Jørgensen and Zhao, 2016); seafloor basalts from the Dorado Outcrop 'LeeDorado' (Lee *et al*., 2015); crustal fluids from the warm and anoxic Juan de Fuca Ridge flank 'JungbluthJDF' (Jungbluth *et al*., 2016); and colonization experiments from the Juan de Fuca subsurface 'RamirezJDF' (Ramírez *et al*., 2019)).
A. Rarefaction curves to predict species richness for sample sequence libraries documents that most were sampled to saturation, and thus, the application of alpha and beta diversity metrices is valid.
B. Left: Summary of the predicted number of species grouped by sample type, with symbol colour indicating sample type (Biofilm, communities from rock surfaces; BW, bottom seawater communities; FLOCS, biofilm communities from downhole FLOCS experiments; FLOCS-BW, biofilm communities from seafloor FLOCS experiments; Fluid, crustal fluid communities). Right: Estimate of the Shannon Diversity Index for each sample, which considers species richness and evenness (higher numbers indicate higher species richness and/or more unevenness).

sequence abundances across all FLOCS incubations grouped within the phyla of Proteobacteria, Bacteriodota, and Planctomyceota. Sequences of Campilobacterota (formerly in Epsilonbacterota), Desulfobacterota, Firmicutes, Spirochaetota and Zixibacteria were also abundant in the Hole U1383B downhole FLOCS, as well as in the crustal fluid samples, but rare or absent within the other downhole samples. Although in low abundance, sequences grouping within Nanoarchaeota and Crenarchaeota (specifically, *Nitrosopumilaceae*-related taxa, which were recently reclassified from the Thaumarchaeota in the latest Silva v138 database release), were also commonly observed in the downhole FLOCS and crustal fluids. Within the *Nitrosopumilaceae*, the ASVs grouped in the Alpha and Gamma 16S rRNA gene clades (data not shown); these taxa are presumed to be aerobic ammonia oxidizers and are common in low abundance on seafloor and subseafloor basalts (Zhao *et al*., 2020).

Within the Proteobacteria phylum (Fig. 3), almost all samples had an abundance of sequences grouped within the Alphaproteobacteria (clades *Hyphomonadaceae*, *Magnetospiraceae*, *Parvibaculaceae*, the PS1 clade and *Rhodobacteraceae*), the Gammaproteobacteria (clades *Colwelliaceae*, *Methylophagaceae*, and *Thiotrichaceae*), and the Zetaproteobacteria (clade *Mariprofundaceae*). Sequences of six Deltaproteobacteria taxa (*Desulfobacteraceae*, *Desulfobulbaceae*, *Desulfocapsaceae*, *Desulfovibrionaceae*, *Desulfuromonadaceae* and *Geopsychrobacteraceae*) were abundant in the Hole U1383B FLOCS but were rare or absent in the other samples, with the exception of *Desulfocapsaceae* sequences also being abundant in crustal fluids and *Geopsychrobacteraceae* sequences also being prevalent in bottom water FLOCS. FLOCS from Holes U1383C and U1382A had an abundance of sequences grouping within *Thioalkalispiraceae* and *Thioprofundaceae* of the Gammaproteobacteria, as did the U1383B FLOCS with nitrate enrichment. By contrast, sequence groups observed in crustal fluids and/or bottom seawater, but rare/absent on the FLOCS, included the *Alcanivoraceae*, *Alteromonadaceae*, Clades I and II of the SAR11 Alphaproteobacteria, *Gallionellaceae, Idiomarinaceae, Marinobacteraceae, Pseudoalteromonadaceae* and *Pseudomonadaceae*. Within the Bacteriodota phylum, sequences from all samples dominantly grouped within the *Flavobacteriaceae* family, with other families similarly abundant among all sample types samples (Fig. S8).

Ordination of the FLOCS microbial community composition data revealed grouping of samples based on fluid inoculum source (bottom seawater versus crustal fluid) and nitrate enrichment and also indicated that mineral-type did not influence community composition grouping (Fig. 4). Different approaches for ordinating amplicon-based sequence data were compared, but they did not result in statistically significant differences in ordination (Fig. S9). The FLOCS experiments incubated in bottom seawater at two sites (Holes U1382A and U1383C) grouped most closely to each other as compared to the samples incubated downhole at those two sites (Fig. 4A). Likewise, the Holes U1382A and U1383C downhole

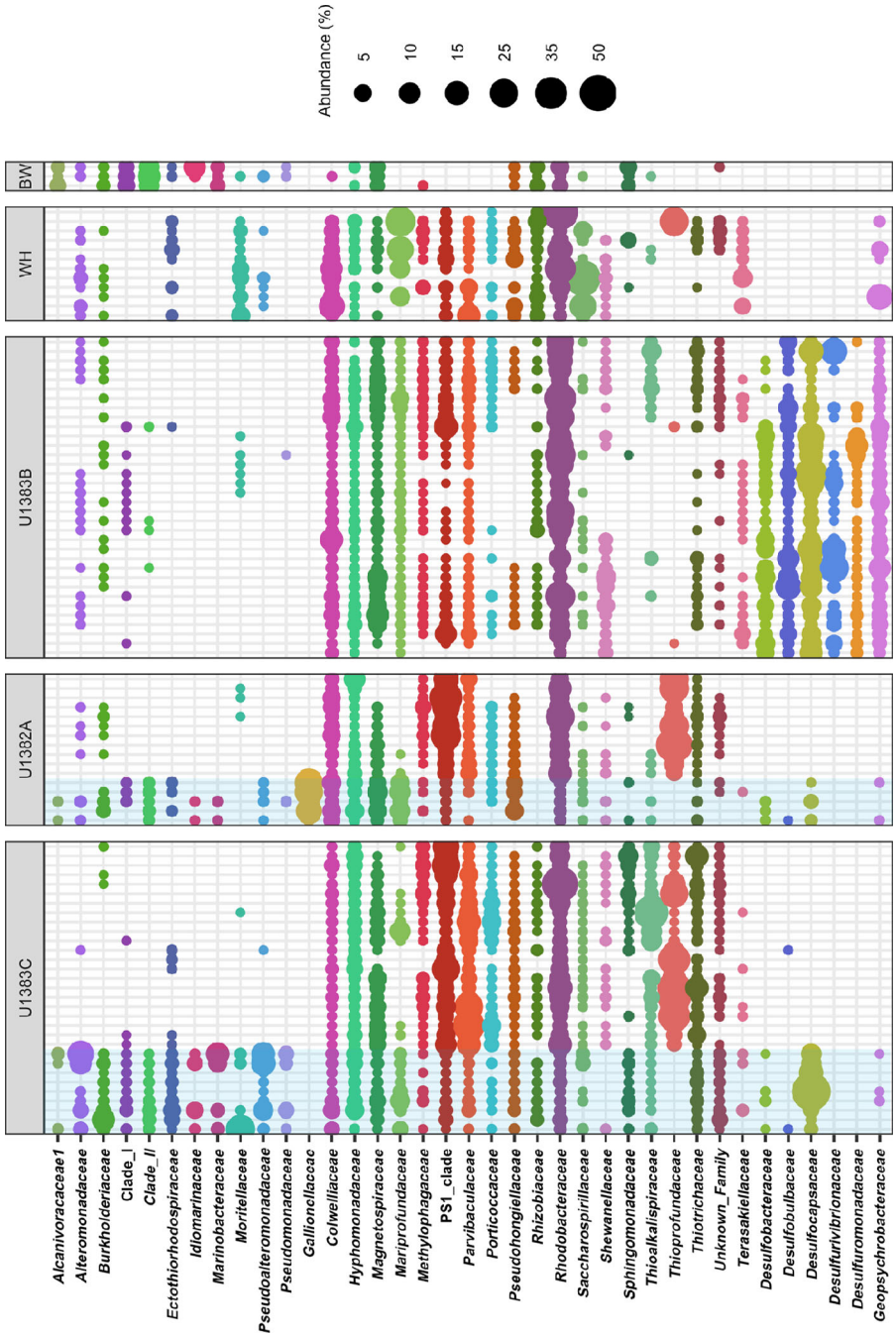

Fig 3. Relative abundance of 16S rRNA gene sequences of Proteobacteria families in FLOCS (this study) and crustal fluid (in blue boxes; (NCBI Sequence Read Archive project PRJNA603242) samples recovered in 2017 from subsurface oceanic crust at North Pond, Mid-Atlantic Ridge. Samples are grouped according to location and sample type (facets: U1383C downhole fluids and FLOCS, U1382A downhole fluid and FLOCS, U1383B downhole FLOCS, wellhead (WH) bottom water FLOCS, and bottom water (BW)). The order of samples along the x-axis can be cross referenced with Fig. S7, Appendix S2 and Table S4. The y-axis order grouped top to bottom by taxa found predominantly in fluids and not the FLOCS, those found in both fluids and FLOCS, and those found predominantly in FLOCS but not fluids.

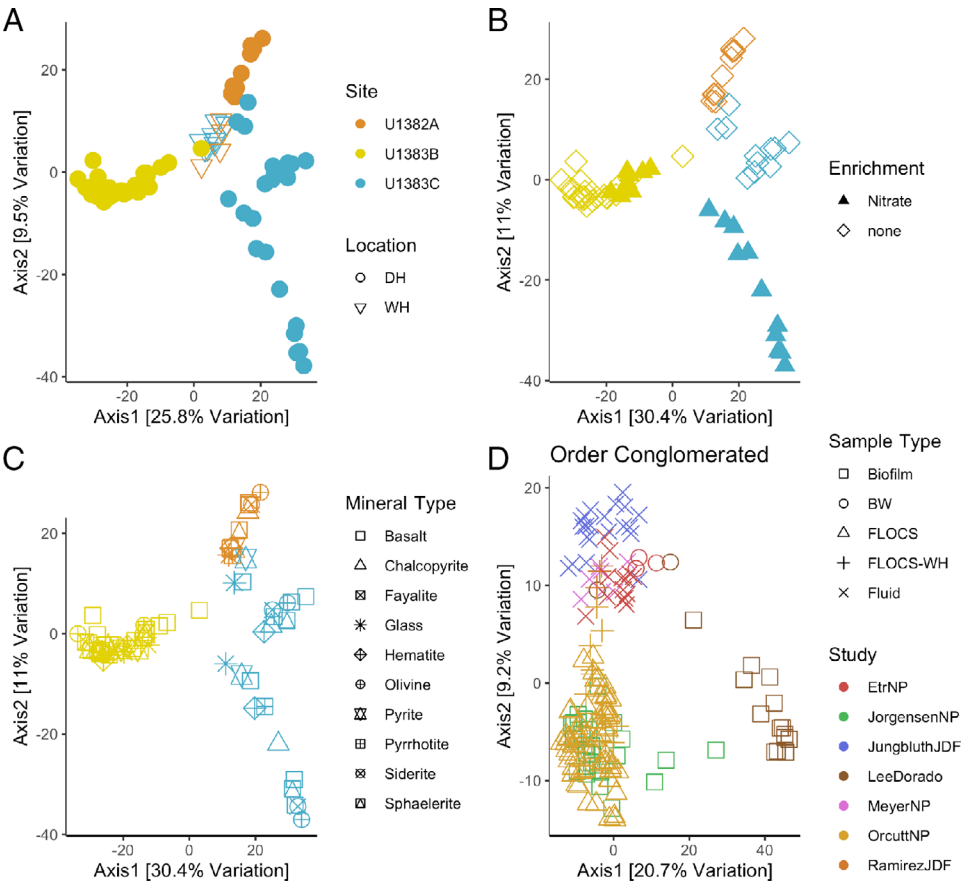

Fig 4. Similarity comparisons of 16S rRNA gene sequence libraries between various sample sets, with symbols clustering closer together having more similar community composition. Principle Coordination Analysis (PCoA) ordination plots of the sequence Aitchison distance matrix of Centre Log Ratio transformed data for all downhole (DH) and wellhead (WH) differentiated FLOCS (A), downhole-only FLOCS differentiated by nitrate-enriched or non-enriched samples (B) and downhole-only differentiated by FLOCS substrate type (C); symbol colour in plots reflects borehole observatory location. In (D), an ordination plot of sequence comparisons of the FLOCS data from this study with previous published studies from comparable locations, with symbol shape and colour representing the Sample Type and Study, as coded in Fig. 2. More details on the approaches described in Appendices S1 and S2.

FLOCS samples grouped on one side of the ordination axis versus the downhole samples from Hole U1383B. Samples enriched in nitrate at Holes U1383B and U1383C formed distinct clusters as compared to the non-enriched samples from those same locations (Fig. 4B). There was no community-level clustering trend of the FLOCS communities based on mineral type of the downhole FLOCS experiments (i.e., no clustering of silicate or metal sulphide samples together; Fig. 4C). A similar analysis comparing the FLOCS microbial community composition data to sequence datasets from comparable locations (see Methods; Fig. 4D; Fig. S10) revealed clustering of samples based on habitat type, with the strongest separation of community structure between surface and subsurface samples (i.e. axis 1), and with the second strongest delineation being between fluid communities and biofilm communities (i.e. axis 2). A similar ordination grouped by primer set used for amplicon sequencing in these various studies did not reveal clustering of data based on primer set (Fig. S11).

Since ordination analysis revealed clustering of the FLOCS samples based on inoculum source (bottom seawater versus crustal fluid), site, and solute conditions (nitrate amendment), we performed differential abundance analysis to determine which ASVs vary between these categories. The strongest separation of the samples was based on site type (i.e. axis 1 of the ordinations separating the downhole samples from Holes U1382A and U1383C from the Hole U1383B samples; Fig. 4A-C, Fig. S10). The taxonomy of the ASVs that were differentially abundant between this separation (Fig. 5) may indicate an oxygen sensitivity preference driving this pattern, based on the documented phenotypic traits of isolates from these same taxa. Namely, many taxonomic groups that were differentially abundant in Hole U1383B samples are commonly associated with anaerobic, microaerophilic and redox transition environments. For example, several ASVs related to *Deltaproteobacteria* families with known anaerobic sulphate- or sulphur-cycling capabilities (i.e., *Desulfobacteria*, *Desulfobulbia* and *Desulfuromonadia* ASVs) were differentially abundant in Hole U1383B. Likewise, strictly or facultatively anaerobic heterotrophic families of the Bacteroidia (Huang *et al*., 2014; Iino *et al*., 2014; Sun *et al*., 2016) were also differentially abundant in the Hole U1383B FLOCS, as were several other microaerophilic taxa with diverse redox cycling capabilities (e.g., *Mariprofundus*, Zixibacteria, and *Melioribacteraceae* (Emerson

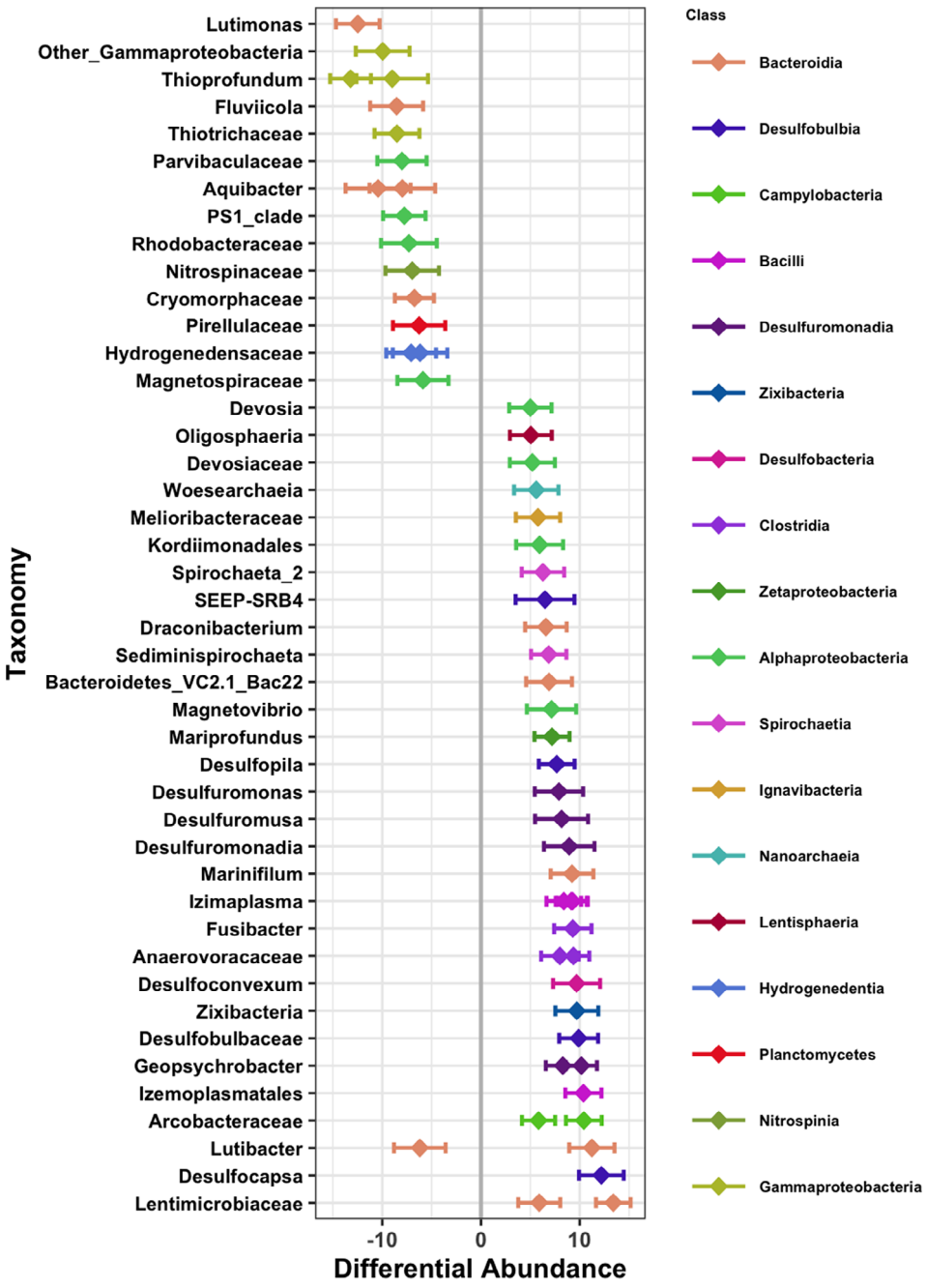

Fig 5. Differential abundance of taxa groups (at family level) between the Hole U1383B FLOCS samples (right side) versus the Holes U1382A and U1383C FLOCS samples (left side). Each diamond symbol represents a different ASV, with error bars representing the range of abundance of that taxa across all sample types in that category, and symbol colour indicates the class that the family groups in per the legend. X-axis shows median differential abundance on a log scale.

et al., 2007; Castelle *et al*., 2013; Podosokorskaya *et al*., 2013). In contrast, differentially abundant taxa in the Holes U1382A and U1383C sites are associated with aerobic oxygen-utilizing metabolisms, such as autotrophic sulphur-oxidizing Gammaproteobacteria in the families *Thiotrichaceae* and *Thioprofundaceae* (Garrity *et al*., 2007;Sorokin *et al*., 2007; Mori *et al*., 2011) and aerobic autotrophic nitrite oxidizers in the *Nitrospinaceae* that have been shown to be responsible for large fractions of dark-ocean carbon fixation (Pachiadaki *et al*., 2017). Some of these taxa were similarly abundant between the Hole U1382A and U1383C samples, with just a few of these being differentially more abundant at one or the other site (Fig. S12A). ASVs grouping with two taxa with known nitrate/nitrite-utilization phenotypes – the *Nitrospinaceae* and the *Thiotrichaceae* – were more abundant under nitrate enrichment at both site types (i.e. presumably more oxic at Holes U1382A and U1383C and less oxic at Hole U1383B; Fig. S12B). By contrast, ASVs in the *Thioalkalispiraceae* and

Flavobacteriaceae were more abundant under nitrate enrichment at the presumably less oxic site (Hole U1383B; Fig. S12C). At both site types, there were some ASVs within the *Magnetospiraceae* family, which has known nitrate reduction phenotypes (Li *et al*., 2012; Bazylinski *et al*., 2013), that were stimulated by nitrate enrichment, and others that correlated with lower nitrate. Finally, taxa that were differentially abundant in non-nitrate enriched FLOCS exposed to crustal fluids as compared to exposure to bottom seawater included groups with known association to nitrogen and sulphur cycling, including *Thioprofundaceae*, *Thioalkalispireae, Thiotrichaceae*, *Nitrospinaceae* and *Magnetospiraceae* (Fig. S12D). A taxonomic group with known iron-oxidation potential – the *Mariprofundaceae* – was stimulated under nitrate enrichment in the presumably lower oxygen conditions with higher iron contents (i.e., in Hole U1383B, where the observatory hardware was constructed of exposed mild steel instead of epoxy coated steel and fibreglass).

Consistent with the ordination indicating that mineralogy did not differentiate community structure (Fig. 4C), differential abundance analysis performed to identify taxa that preferentially colonized silicates over metal oxides or metal sulphides did not have any significant results (p-value threshold of 0.05; data not shown). Despite the insignificant results, a few ASVs in these tests had abundance patterns that indicated taxa with a mineral preference. For example, three Gammaproteobacteria groups within the families *Thioalkalispiraceae* and *Thioprofundaceae*, with cultivated isolates known to perform sulphur oxidation (Sorokin *et al*., 2007; Mori *et al*., 2011), were commonly enriched on metal sulphides and non-basalt samples (Fig. S13). The lack of statistical significance is due to high within-group variation for ASVs enriched in a particular category, but the trend is consistent with functional prediction.

Factors determining development of rock-attached biofilms in oceanic crust

Overall, the microbial community composition data from replicate mineral colonization experiments deployed within subsurface oceanic crust in this study reveal that the structure of microbial communities that form biofilms on rocks follows unique trajectories reflecting stochastic seeding from crustal fluids that have evolved from bottom seawater entrained into basaltic crust (Fig. 4A). Although the rock-attached biofilm communities exposed to bottom seawater and crustal fluids formed distinct clusters (Fig. 4A), there was nevertheless a high degree of taxa overlap between them and bottom seawater (Fig. 3; Figs S7 and S8). This observation of taxa overlap between rock-attached biofilms and bottom seawater was previously observed in subsurface basalts from the cold (<10°C) and oxic North Pond site (Jørgensen and Zhao, 2016) but differs from the trend observed at a warm (~64°C) and anoxic subsurface crustal site that had more distinct taxa separation between subsurface rock-attached biofilms and bottom seawater (Ramírez *et al*., 2019). This indicates that temperature and/or oxygen content is a major driver of community difference in crustal settings, as previously described (Ramírez *et al*., 2019). As temperature and oxygen content also reflects the average connectivity of the crustal fluids to the overlying ocean (i.e. more connected fluids are colder and more oxic), this may indicate that the average age of the crustal fluid plays a role in the structure of the microbial community that develops in rock-attached biofilms.

Differentiation of crustal biosphere microbial communities also reflected habitat type and some local environmental conditions. For example, there were some taxa unique to the crustal fluids and not found on the colonization biofilms, and vice versa (Fig. 3). This is consistent with a prior interpretation of 'biofilm' and 'planktonic' fractions of the crustal deep biosphere reported previously (Ramírez *et al*., 2019). This habitat distinction is also evident in the grouping of samples from this and prior studies (Fig. 4D). As another example, higher nitrate concentrations in the fluids inoculating the colonization experiment resulted in community shifts (i.e. Figure 4B) that reflected differential abundance of taxa with known nitrate/nitrite-utilization phenotypes, such as higher relative sequence abundance of *Nitrospinaceae* and *Thiotrichaceae* (Fig. 3).

By contrast, mineral-specific patterns in overall biofilm microbial biogeography were not observed on the time scale of the colonization experiments at North Pond (Fig. 4C). Only a few taxa displayed substrate-driven distribution patterns, such as *Thioprofundum* related sequences that are generally more abundant on metal sulphides than on non-sulphides (Fig. S13). This overall lack of mineral-driven community biogeography is consistent with a previous observation from similar colonization experiments deployed at the Juan de Fuca (Ramírez *et al*., 2019), but it differs from inferences of mineral-driven structuring in a comparable colonization experiment at that same site (Smith *et al*., 2016) as well as to broad microbial biogeography patterns observed on different seafloor rock types (Toner *et al*., 2013).

Although there was variability in mineral composition between the different studies, this is unlikely to explain the observation of limited mineral pressure on microbial community structure. For example, the substrate variability was higher in this study (silicates, metal sulphides, metal oxides, etc.) and the Ramírez *et al*. (2019) study (i.e., silicates and metal sulphides) than in the Smith *et al*. (2016) study, which only focused on silicates.

Recent comparative analysis among a broad collection of seafloor and subseafloor samples reveals that the clustering of the Smith *et al*. (2016) samples is very tight compared to the variability in other datasets (Orcutt *et al*., 2020), suggesting that mineral-driven community differences inferred in the Smith *et al*. (2016) study were relatively small scale in comparison to other studies. Likewise, the Toner *et al*. (2013) study compared seafloor rock specimens separated by considerable physical distance (m to km), whereas the scale of separation in the colonization experiments was smaller (mm to cm) and may have allowed for greater community cross-over between sample types. It is possible that autoclaving of the colonization materials allowed the release of some dissolved substrates from the samples prior to deployment, which could have blurred the variability between sample types in the colonization experiment studies compared to the Toner *et al*., 2013 study; however, the ample flushing of the colonization experiments with sterile distilled- and sea-water solutions prior to deployment should have minimized this issue.

Additional evidence against mineral-driven microbial community structuring in this experiment comes from the similarity of the biofilms formed on inert borosilicate glass in comparison to the mineral substrates, as well as similarity to communities that colonize marine plastic. The FLOCS samples containing glass grouped within each FLOCS sample set by site (Fig. 4C), with no differentiation between the inert substrates and the minerals. While known plastic-associated taxa (Kirstein *et al*., 2019) were observed in the FLOCS sequences – including Planctomycete classes OM190 and BD7-11, Bacteriodetes in the genus *Aquibacter* and Alphaproteobacteria in the families *Hyphomonadaceae* and *Rhodobacteraceae* (Appendix S2, Fig. 3, Figs S7 and S8), the closest environmental sequence relatives to many of these ASVs in the FLOCS data come from similar seafloor and subsurface locations (see additional discussion in Appendices S1 and S2). Overall, these observations suggest that pioneer biofilm-forming taxa generally colonize any surface, further supporting the observation that mineral type does not drive community structure in this setting. This observation is consistent with an earlier study where rock biofilm formers did not source energy from the rock itself but rather from the overlying fluids (Sudek *et al*., 2017).

While mineral type does not drive microbial community structure on the roughly decadal time scale of colonization experiments, we observe that the overall diversity of crustal biofilms may increase with time (Fig. 2B). Namely, the youngest (<4-years-old) wellhead-incubated samples have the lowest diversity, with increasing species richness and unevenness observed in the slightly older (~6 years old) downhole colonization followed by the 8-Ma basalts and crustal fluids from North Pond, and finally the 23-Ma basalts from Dorado Outcrop. Similar ecological succession patterns of increasing diversity with age have been reported in a wide diversity of other microbial ecosystems (Ortiz-Álvarez *et al*., 2018). We note that this is not a perfect comparison, however, as the trend compares artificial colonization experiments with both subsurface and seafloor rocks; these habitat differences could also contribute to the observed trend.

Following a logic outlined elsewhere (Edwards *et al*., 2005), we present the following possible explanation for the suggested successional pattern. First, fresher basalts are initially colonized by a pioneer community of surface generalists, with a few taxa such as *Thioprofundum* that exhibit preference for colonizing areas of the rock matrix richer in sulphides. Over time, as the biofilm develops and secondary alteration products form (i.e., sulphur or metal oxides) and carbonate precipitates, new niche spaces are created that allow for a higher diversity of microbial taxa. Hence, we posit that the relative abundance of taxa like *Thioprofundum*, and the relative diversity of biofilm communities, may be indicators of the relative age of colonization within the crust. If true, this indicates that microbial parameters could be another indication of fluid flow condition within upper oceanic crust (i.e. how recently fractures opened up to flow). Analysis of additional samples across a range of age and alteration spectra are needed to further resolve factors that drive crustal biogeography (Edwards *et al*., 2012a) and the linkages of this biogeography to crustal fluid flow.

Biomass patterns in the oceanic crust deep biosphere

Biofilms that formed on crustal surfaces within the decade timeframe in this experiment are similar in density (~10^3–10^4 cells g^{-1}; Table S4) to rock-attached biofilms (or trapped cells in rock voids) observed in ~8 Ma subsurface basalt samples from this same location (Jørgensen and Zhao, 2016; Zhang *et al*., 2016). By comparison, cell biomass in subsurface gabbroic and ultramafic crustal materials is often lower (Früh-Green *et al*., 2018; Li *et al*., 2020), which may reflect lower permeabilities compared to extrusive basalts. Colonization experiments conducted under warm (~64°C) and anoxic conditions have yielded somewhat higher densities (Smith *et al*., 2011; Smith *et al*., 2016; Ramírez *et al*., 2019). Likewise, crustal boreholes that have been revisited have high cell densities and visible flocculent material (Becker *et al*., 2004; Nigro *et al*., 2012; Salas *et al*., 2015), indicating that high cell densities are possible with high fluid-rock ratios. Recently, very high cell densities have been suggested in veins of very old basaltic crust (Suzuki *et al*., 2020), although the fluid-rock ratio

in this system is unclear. These observations indicate that microbial biomass in oceanic crust could be a function of permeability and/or fluid: rock ratios. We caution that these trends should be viewed qualitatively, however, as different methods were used to determine cell densities across the studies.

In our experiments, while nitrate addition stimulated a shift in microbial community structure (Fig. 4B, Fig. S12), there was not a statistically significant increase in biomass associated with this change. This suggests that nitrate is not a limiting nutrient for biomass in this system, although nitrate did stimulate growth under *ex situ*, atmospheric pressure incubations with basalt samples from this site (Zhang *et al*., 2016). Basaltic crust has previously been identified as a phosphorous sink and a net heterotrophic system (Wheat *et al*., 2003; Shah Walter *et al*., 2018; Lin *et al*., 2019), and previous cell density measurements in North Pond basalt cores from Hole U1383C noted a positive correlation between cell counts and phosphate content (Zhang *et al*., 2016). These observations together suggest that carbon and/or phosphorous, but not nitrogen availability, may determine the carrying capacity for the North Pond hydrologic system.

Given that prior studies estimate that there are $2–4 \times 10^{29}$ total cells in subsurface oceanic crust (Bar-On *et al*., 2018; Magnabosco *et al*., 2018; Flemming and Wuertz, 2019) and that biofilms hold 20%–80% of these cells (Flemming and Wuertz, 2019), we estimate that the abundance of some key crustal biofilm taxa may rival the abundance of SAR11/Pelagibacterales – heralded as the most abundant plankton in the ocean at 2.4×10^{28} total cells (Giovannoni, 2017). For example, we estimate that the *Rhodobacteraceae* – a ubiquitous group of Alphaproteobacteria observed in the colonization experiments (median 2% relative abundance; mean $6 \pm 8\%$ standard deviation; Fig. 3) and also in older basalts at 1%–2% average abundance (Orcutt *et al*., 2020) – could comprise $0.4–6.4 \times 10^{27}$ total cells in biofilms in oceanic crust. A similar range of total cells is estimated for the PS1 clade of Alphaproteobacteria, which has similar abundance patterns to *Rhodobacteraceae* (Fig. 3 and Orcutt *et al*., 2020). While these estimates are quite crude and require further datasets to ground-truth, they nevertheless highlight that microbial groups that exist in the subsurface together may rival the abundances of well-studied surface-ocean taxa, underscoring the importance of resolving the distribution of these groups to understand their function and biogeochemical impact. Furthermore, there is little genomic information for some of the most prevalent and abundant taxa (such as the PS1 and unclassified clades of Alphaproteobacteria). This demonstrates a knowledge gap in our understanding of the physiology and ecology of these microbial groups that are prevalent in oceanic crustal subsurface systems and draws attention to the need for studies to resolve their physiologies.

Experimental procedures

Bulk community DNA extraction and 16S rRNA gene quantitative PCR and sequencing

DNA from biofilms on the colonization experiments was extracted with the MP Biomedicals FastDNA SpinKit for Soil with a modification that included the addition of polyadenylic acid (Appendix S1). This protocol was tested on excess basalt samples from wellhead FLOCS prior to processing study samples to confirm suitability of the method with appropriate sample types (Figs S3 and S4; Table S2). For all sample extraction batches, a no-template control sample was included to account for possible sequence contamination in low biomass samples (Sheik *et al*., 2018; Ramírez *et al*., 2019). Following protocols detailed in the Appendix S1, the abundance of the 16S ribosomal RNA (rRNA) gene in the DNA extracts was measured with quantitative polymerase chain reaction (qPCR) followed by amplicon sequencing of the V4-V5 region of 16S rRNA gene. Unique amplicon sequence variants (ASVs) were determined using the standard DADA2 pipeline Version 1.12 (Callahan *et al*., 2016). ASV sequences were assigned taxonomy by the naïve Bayesian classifier built into the DADA2 package using the Silva v138 database (Quast *et al*., 2013). Beta-diversity patterns were assessed by ordination after normalization by centre-log-transformation and calculation of the Aitchison distance metric via Principle Coordinate Analysis (PCoA), and differential-abundance tests were performed for pairwise comparisons between multiple combinations of sample categories. The sequence data from this study were compared to these prior studies to examine biogeographic patterns: North Pond crustal fluids (NCBI Sequence Read Archive project PRJNA603242; Tully *et al*., 2017); Juan de Fuca crustal fluids (Jungbluth *et al*., 2016); North Pond subsurface basalts (Jørgensen and Zhao, 2016); FLOCS experiments from the Juan de Fuca (Ramírez *et al*., 2019) and seafloor basalts from the Dorado Outcrop (Lee *et al*., 2015). Considering the difference in sequencing approaches and primers used between these various studies, we narrowed our 16S rRNA gene phylogenetic comparisons and diversity metrics to Family and broader taxonomic ranks as described in the Appendices S1 and S2.

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Author contributions

BNO and CGW designed and deployed the experiment; all authors participated in the experiment recovery. CGW analysed fluid chemistry data, and TD and ETR performed DNA and bioinformatic analyses. BNO and TD wrote the manuscript with input from CGW.

Data availability statement

16S rRNA gene amplicon data are available at the NCBI Sequence Read Archive BioProject PRJNA564565 samples SAMN12723399-489; Table S3 lists accession numbers for each sample individually. Bioinformatics processing steps are available at https://github.com/orcuttlab/northpond, https://github.com/orcuttlab/ocean-crust-micro and in Appendix S2. Scanning electron microscopy images from select mineral colonization experiments are available at the Biological and Chemical Oceanography Database Management Office (BCO-DMO) dataset ID 756152 (Orcutt and D'Angelo, 2019). OsmoSampler chemical data are available via BCO-DMO Project 707762.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1 Supporting Information.

Appendix S2 Supporting Information.