MICROBIAL ECOLOGY IN THE SEDIMENT-COVERED OCEAN BASEMENT OF THE JUAN DE FUCA RIDGE

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We certify that we have read this dissertation and that, in our opinion, it is satisfactory in scope and quality as a dissertation for the degree of Doctor of Philosophy in Oceanography.

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DEDICATION

To the outstanding teachers and mentors that have inspired me, and to my family and friends for their love and encouragement.

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ABSTRACT

Investigations of microbial life inside of the deep seafloor and most reviews on the topic have focused on sediments and largely ignore the prospect of a biosphere inside the basaltic crust underlying the global system of ocean basins. This is despite the potential global importance of biogeochemical cycling that may be occurring *in situ* within the uppermost igneous ocean crust; a location that is predicted to be one of the most habitable subsurface environments due to its porosity, hydrothermal circulation, and expected chemical disequilibria. Sedimentation processes occuring over geologic time scales cause a majority of the global seafloor to be covered by thick and relatively impermeable blankets that prevent access to the underlying basaltic seafloor. As a result, studies of microorganisms inside the basaltic crust have traditionally been restricted to the exposed seafloor or to locations where hydrothermal fluids exiting the seafloor act as "windows" into the subsurface. However, these traditional methods for observing basaltic rock seafloor microorganisms are inadequate because ocean crust is hydrogeologically active until up to ~65 million years old and a majority of flow is likely to occur over long time scales and deep within the sediment-covered basement. Seafloor observatories that penetrate through sediments and into basement rock provide the infrastructure needed to collect samples from one of the planet's most remote environments. The broad goals of this study were to estimate the concentrations of microbial biomass and explore the microbial diversity in anoxic, deep subseafloor crustal fluids.

Building on the first characterizations of microbial life in the aging ocean basement, discrete fluid samples were collected and analysed here from new borehole observatories that are the first to incorporate dedicated stainless steel or Teflon-coated fluid delivery lines running along the exterior of the reactive iron casing. Biofouling-resistant materials used during the construction of the seafloor observatory fluid delivery lines permit collection of pristine samples that can be used for estimation of the *in situ* microbial biomass and reveal a range of cellular abundances that are, on average, roughly an order of magnitude lower that those found in bottom seawater. The cellular abundances reported here will help to constrain estimates of biomass inside the global seafloor and elucidate partitioning between the basaltic crustal and marine sediment communities, and furthermore, underscore the difficulties associated with collecting uncontaminated samples from the deep subsurface.

Sampling from a combination of older and newer borehole observatories has revealed novel microbial diversity and community structure from the seafloor that is distinct from overlying sediments and varies with the alteration state of the basement fluids. Microorganisms detected were largely from uncultivated groups, which means one can only speculate about the metabolic lifestyle for these organisms; however, comparisons to distant relatives indicate that a combination of autotrophic and heterotrophic lifestyles and active iron- and sulfur-cycling processes are present in the deep subseafloor. Temporal variation over annual timescales in

the types of microorganisms collected from a single borehole observatory was observed and is the first instance of microbial community turnover observed in the deep subseafloor. A distinct bacterial lineage of *Firmicutes* first retrieved in 1998 during the first study of this kind was detected here again from a neighboring borehole location, which is consistent with the inferred hydrogeologic connectivity of the system and implies that the subseafloor crust likely has some permanent and widespread residents. Phylogenetic groups of microorganisms identified here extend previous observations by identifying additional lineages that may truly exist in the deep subseafloor, and provide a foundation for future studies exploring important topics relating to the community metabolic potential and contribution to active global geochemical cycling.

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LIST OF ABBREVIATIONS

ACORK	Advanced Circulation Obviation Retrofit Kit
AT or ATL	Atlantis
AUV	Automated Underwater Vehicle
BHI80-139	Brain-Heat Infusion temperature 80°C culture clone 139 (aka: *Aerophobetes*)
BLAST	Basic Local Alignment Search Tool
bp	(Nucleic Acid) Base Pairs
CARD-FISH	Catalyzed Reporter Deposition Fluorescence *In Situ* Hybridization
CORK	Circulation Obviation Retrofit Kit
CTD	Conductivity, Temperature and Depth
Cy3	Cyanine dye number 3
DAPI	4',6-Diamidino-2-PhenylIndole
DHVEG	Deep Hydrothermal Vent Euryarchaeota Group
DNA	Deoxyribonucleic Acid
DOC	Dissolved Organic Carbon
DSDP	Deep Sea Drilling Project
D/V	Drilling Vessel
EM3	Environmental clone 3 (*Thermotoga*)
EM19	Environmental clone 19 (aka: KB1; *Calescamantes*)
ESCS	Extended Shoes Coring System
FACS	Fluorescence-Activated Cell Sorting
Fe_d	Dissolved iron
HOV	Human-Occupied Vehicle
IODP	Integrated Ocean Drilling Program
JdFR	Juan de Fuca Ridge
JS1	Japan Sea clone 1
JTB35	Japen Trench Bacteria clone 35 (*Gammaproteobacteria*)
KB1	Kebrit Deep Bacteria clone 1 (aka: EM19, *Calescamantes*)
L-CORK	Lateral CORK

LVBS	Large Volume Bag Sample/Sampler
Ma	Million years old
MBGB	Marine Benthic Group B
MBGE	Marine Benthic Group E
MBSF	Meters Below Seafloor
MBSL	Meters Below Sealevel
MCG	Miscellaneous Crenarchaeotic Group (aka: *Bathyarchaeota*)
MFW	Meters From Wellhead
MG-1	Marine Group-1 (*Thaumarchaeota*)
MG-2	Marine Group-2 (*Euryarchaea*)
MG-3	Marine Group-3 (*Euryarchaea*)
MPS	Mobile Pump Sampler
MSB	Meters Sub-Basement
MVBS	Medium Volume Bag Sample/Sampler
NCBI	National Center for Biotechnology Information
OCS116	Oregon Coast Surface clone 116 (*Alphaproteobacteria*)
ODP	Ocean Drilling Program
OM43	Ocean Margins clone 43 (*Betaproteobacteria*)
OM190	Ocean Margins clone 190 (*Planctomycetes*)
OPB41	Obsidian Pool Bacteria clone 41 (*Actinobacteria*)
OP8	Obsidian Pool clone 8 (aka: *Aminicenantes*)
OTU	Operational Taxonomic Unit
PBS	Phosphate Buffered Saline
PCoA	Principal Coordinates Analysis
PC	Principal Component
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PFC	Perfluorocarbon
PMCH	Perfluoromethylcyclohexane
PTFE	Polytetrafluoroethylene
QIIME	Quantitative Insights Into Microbial Ecology
RCB	Rotary Core Barrel

RF3	Rumen Fluid clone 3 (*Firmicutes*)
RNA	Ribonucleic Acid
ROV	Remote-Operated Vehicle
rRNA	Ribosomal Ribonucleic Acid
R/V	Research Vessel
SAGMEG	South African Gold Mine Euryarchaeota Group
SAR11	Sargasso Sea clone 11 (aka: *Pelagibacter*)
SAR86	Sargasso Sea clone 86 (*Gammaproteobacteria*)
SAR116	Sargasso Sea clone 116 (*Alphaproteobacteria*)
SAR324	Sargasso Sea clone 324 (aka: Marine Group B)
SAR406	Sargasso Sea clone 406 (aka: *Marinimicrobia*)
SCIMPI	Simple Cabled Instrument for Measuring Parameters In-Situ
SS	Stainless Steel
SSU	Small Subunit
TA06	Terephthalate-degrading consortium clone 06
TDN	Total Dissolved Nitrogen
THSCG	Terrestrial Hydrothermal Spring Crenarchaeotal Group
TMEG	Terrestrial Miscellaneous Euryarchaeotal Group
TN	Total Nitrogen
TOC	Total Organic Carbon
XCB	Extended Core Barrel

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CHAPTER 1

Introduction: microbial ecology in the sediment-covered ocean basement of the Juan de Fuca Ridge

By Sean Jungbluth

Jennifer F Biddle, Mark A Lever, and Michael S Rappé (co-authors)

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1.1 Background

Overview of Life in the Seafloor

The detection of life within the seafloor - sediment and the underlying basaltic ocean crustal basement[1] - is among the most exciting discoveries of the 20th century and, because of the enormous volume of this habitat, the discovery carries enormous implications for our understanding of global biogeochemical cycling. Investigation of microbial life in the dark seafloor began in the 1930s with the discovery of bacteria in surficial sediments (<1 meter depth) collected from the deep sea (ZoBell and Anderson, 1936). The subsequent discovery of deep-sea hydrothermal vent environments (Corliss *et al.*, 1979; Karl *et al.*, 1980) challenged the fundamental ecological tenet that all energy sustaining life is originally derived from the sun (Figure 1.1). In recent decades, tens to hundreds of microbiological surveys have been conducted within the deep sea at seafloor sediments and hydrothermal and/or chemically-reducing environmental settings, such as sediment gas hydrates (e.g. Briggs *et al.,* 2012), ultra-hot (> 350˚C) hydrothermal vents (e.g. McCliment *et al.,* 2006; Frank *et al.,* 2013), exposed ultra-mafic rock systems (e.g. Brazelton *et al.,* 2012), cold seeps (e.g. Inagaki *et al.,* 2002), hydrocarbon seeps (e.g. Kleindienst *et al.,* 2012), mud volcanoes (e.g. Niemann *et al.,* 2006), and whale falls (e.g. Tringe *et al.,* 2005; Treude *et al.,* 2009), expanding the cataloged diversity of life and, through the discovery of novel metabolisms (e.g. Strous and Jetten, 2004; Beal *et al.,* 2009), yielding paradigm-changing discoveries relevant to biogeochemical cycling.

At present, the depth to which microbial life penetrates and subsists within deep subseafloor sediments (>10 meters depth) and continues into the upper ocean basement (generally <500 meters depth) is still poorly known. Evidence for microbial life has been observed in deeply buried sediments (e.g., Parkes *et al.*, 1994; D'Hondt *et al.*, 2002; Inagaki *et al.*, 2006; Davis *et al.*, 2009; Røy *et al.*, 2012), deep trench environments (e.g. ZoBell, 1952; Kato *et al.*, 1997), and exposed basement rock (e.g. Rogers *et al.*, 2003; Santelli *et al.*, 2008; Mason *et al.*, 2009; Brazelton *et al.*, 2010); however, few direct tests have been carried out in buried basement rocks or fluids (Giovannoni *et al.*, 1996; Fisk *et al.*, 1998; Torsvik *et al.*, 1998; Furnes *et al.*, 2001; Bach and Edwards, 2003; Cowen *et al.*, 2003; Huber *et al.*, 2006; Orcutt *et al.*, 2011b). The most widely cited estimates of subseafloor biomass assert that subseafloor sediments contain 0.6 – 33% of Earth's total living biomass (Parkes *et al.*, 1994; Whitman *et al.*, 1998; Lipp *et al.*, 2008; Kallmeyer *et al.*, 2012) and contain 5 – 66% of the total microbial cells in the biosphere (Whitman *et al.*, 1998; Kallmeyer *et al.*, 2012). Notably, Kallmeyer's estimates are

[1] "Ocean crust" classically refers to sediments and basement rock; in this document, marine basement rock is called "crust" for ease of reading and is exclusive of sediments in all instances, or unless otherwise stated.

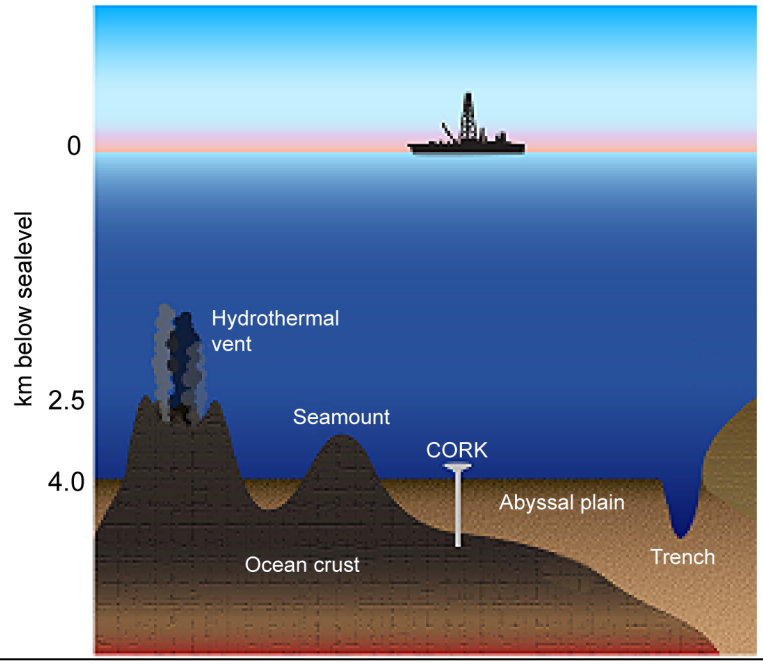

Figure 1.1 Vertical section of the aphotic ocean seafloor showing major features that have been accessed to varying degrees for microbiological study. (modified from Orcutt *et al.*, 2011a)

more accurate because they incorporate cell estimates from the subsurface of oligotrophic marine gyres and are not biased to the continental margins (Kallmeyer *et al.*, 2012). However, both the Whitman and Kallmeyer studies fail to account for the ocean crustal basement biosphere despite an estimated global rock volume of 2.3 x 10^{18} m^3, which is ca. 6-10x that of global marine sediments (Orcutt *et al.*, 2011a).

Overview of the Microbial Biosphere in Upper Ocean Basaltic Crust

The discovery of hyperthermophilic archaea and recurring geochemical evidence of crustal alteration led to the first hypotheses of a subsurface biosphere in the Earth's basaltic crust (Deming *et al.*, 1993). Initial suggestions of this subsurface biosphere came from experiments at seafloor hydrothermal vents, in which elevated DNA levels in hydrothermal plumes were measured that could not be explained by simple seawater entrainment; this implied that microbes were likely living in underlying sediments and rocks. Around this time, advances were also made with experiments showing that microbes likely grow on the Earth's crust, furthering the concept of a subsurface crustal biosphere (Thorseth *et al.*, 1995; Giovanonni *et al.*, 1996; Fisk *et al.*, 1998). Despite the difficulty of sample retrieval, and low and heterogeneous biomass on crust samples, experimental evidence has now confirmed the existence of crustal life, consisting of a vast microbial community including viruses (reviewed in Orcutt *et al.*, 2011a; Edwards *et al.*, 2011; Lever, 2013; Anderson *et al.*, 2013). The early evidence for a crustal biosphere inevitably led to lines of inquiry intended to address questions

concerning its extent and global biomass, transport, and distribution. The crustal biosphere extends over a wide temperature range, with evidence for biogenic alteration in the temperature range from 15–80 °C (Heberling *et al.*, 2010). Yet, most of the subseafloor oceanic crust is at least in theory habitable, based on measured and modeled isotherms, which show a conservative 120 °C isotherm to extend thousands of meters into the crust, which is below the predicted (150°C; Deming *et al.*, 1993) and experimentally measured temperature maxima of life (122°C; Takai *et al.*, 2008). The volume of this potentially habitable zone in ocean crust equals that of the modern ocean and exceeds that within continental crust by nearly a factor of two (Heberling *et al.*, 2010). Based on modeling crustal porosity, it has even been proposed that the oceanic crust might be the largest reservoir of microbial life on the planet (Heberling *et al.*, 2010).

The first evidence that life existed in the oceanic crust came from the observation of textural changes in basalts (Fisk *et al.*, 1998), as well as the measurement of DNA that came specifically from basaltic glass (Giovanonni *et al.*, 1996). Since then, there has been widely documented textural evidence observed in a variety of locations (e.g. Fisk *et al.*, 1998; Furnes *et al.* 1999; Fisk *et al.*, 2000; Furnes *et al.*, 2001; Thorseth *et al.*, 2001; Banerjee *et al.*, 2003; Edwards *et al.*, 2003; Fisk *et al.*, 2003; Einen *et al.*, 2006; Staudigel *et al.*, 2008; Walton, 2008); see Staudigel *et al.* (2008) for comprehensive review. Evidence of microbial habitation of deep crustal basalts has since even been found for eukaryotes, with fossilized fungi documented in 46 Ma old, deep basaltic rocks (Schumann *et al.*, 2004). Energy is predicted to be available in both shallow and deep basalts, with some limitation in metabolism coming from aged basalts. Here, flow may be severely restricted by mineral precipitates in fractures within, and thick overlying sediments with low permeability that block vertical advection. Support of this notion comes from a compilation of Fe(III)/ΣFe ratios measured in basalts ranging in age from several hundred thousand to several hundred million years. Here, increasing ratios were measured in the first 10–15 million years after formation, followed by constant ratios afterward (Bach and Edwards, 2003). In terms of microbial energy sources, this has been interpreted to mean that the potential for Fe(II) to serve as an energy source to life in subseafloor basalt may be restricted to the initial 10–15 million years after crust formation (Bach and Edwards, 2003). More recent data, however, indicate that a sufficient number of reductants, and thus possible indigenous, microbial fuel sources, remains 23 million years after formation in certain locations (Wheat and Fisher, 2008).

With the exception of mid-ocean ridge and seamount features, sediments cover the oceanic crust and conceal the largest aquifer system on the planet. With an upper ocean basement (<500 msb) porosity of roughly 10% (Alt and Bach, 2003; Nielsen and Fisk, 2010), this system is hypothesized to provide a site for attachment of up to 10^{34} microbial cells (Nielsen and Fisk, 2010). This porous and permeable environment, estimated to be about 2% of the total

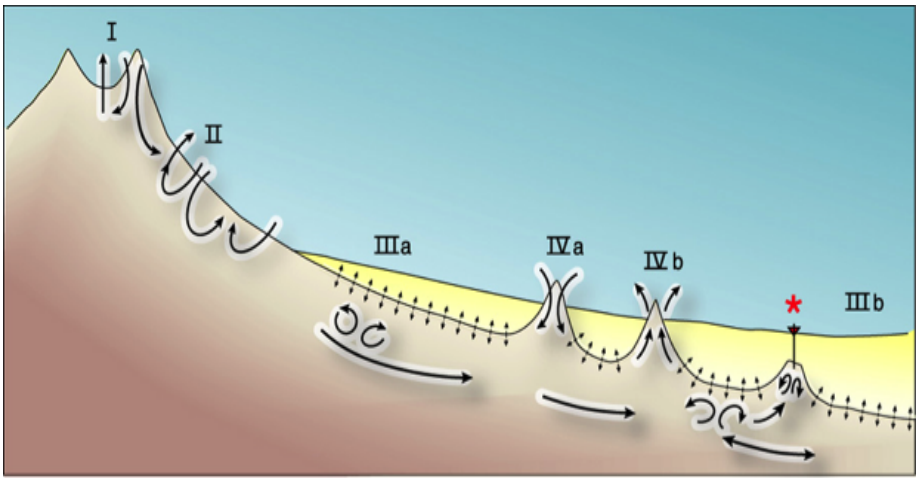

Figure 1.2 Diagram of mid-ocean ridge, ridge flank and ocean basin showing zones of relative fluid exchange between water column and basement crusts. (I) Ridge axis, (II) Unsedimented ridge flank, (III) Sedimented ridge flank, (IV) Active exchange via exposed outcrops, (*) Location of a CORK tapping into sediment-covered, sealed oceanic basement (modified from Cowen 2004).

ocean volume (Johnson and Pruis, 2003), permits the circulation of the entire volume of ocean every 10^5 – 10^6 years (Fisher and Becker, 2000), making the oceanic crust the locus of extensive hydrothermal circulation and geologically rapid chemical exchange between the crust and oceans. Consequently, low temperature hydrothermal fluids (<150 °C) circulating through much of the upper ocean basement are thought to be the most suitable for microbial life in the subseafloor (Baross *et al.*, 2004) by providing advantageous temperatures and useful chemical gradients (e.g. Cowen, 2004; Schrenk *et al.*, 2010; Orcutt *et al.*, 2011a; Figure 1.2). Despite interest in the ocean crustal biosphere, sampling access is exceptionally limited, being restricted to locations where venting fluids or formations are used as windows to the subsurface or through use of expensive, logistically difficult, and technically challenging seafloor drilling operations.

Efforts to understand the biosphere of basaltic ocean crust have largely been undertaken at surface exposed locations (i.e. hydrothermal vents and seamounts) through a combination of studies focused on both the surface- attached and fluid-hosted (planktonic) microbial communities. Surface-attached microbial communities found at seafloor-exposed basalts range between 6 x 10^5 and 1 x 10^9 cells g^{-1} in abundance, and are often dominated by *Alphaproteobacteria* and *Gammaproteobacteria* (Einen *et al.,* 2008; Santelli *et al.,* 2008; Mason *et al.,* 2009) that have been implicated in methane-, sulfide-, and iron-oxidation (Orcutt *et al.,* 2011a). Diffuse flow and high-temperature hydrothermal vent fluid microbial communities range in abundance from 1 x 10^4 to 1.95 x 10^6 cells ml^{-1} (Takai and Horikoshi, 1999; Kato *et al.,* 2009; Huber *et al.,* 2010) and harbor abundant archaeal lineages such as *Archaeoglobales*, *Thermococcales*, *Methanococcales* (Perner *et al.,* 2007a), and Marine Group I Thaumarchaeota

(Takai and Horikoshi, 1999; Perner *et al.,* 2007b), and bacterial lineages including *Gammaproteobacteria* and *Epsilonproteobacteria* (Sunamura *et al.,* 2004; Perner *et al.,* 2007a; Perner *et al.,* 2007b; Huber *et al.,* 2010). Microbial processes such as methanogenesis and sulfate-reduction via hydrogen-oxidation are suggested as a dominant anaerobic processes within high-temperature vent fluids, whereas sulfide-, metal-, and methane- oxidation appear to become increasingly important in diffuse flow fluids (Orcutt *et al.,* 2011a).

Direct access to the sediment-covered ocean basement requires expensive and logistically-involved ocean drilling expeditions. With respect to studying the deep biosphere, ocean drilling expeditions have typically resulted in the collection of rock and mineral specimens for biological analysis; however, direct cell counts are difficult to obtain from these materials due in part to the heterogenous colonization by microbial cells, resulting in data that are either qualitative (Fisk *et al.,* 1998; Thorseth *et al.,* 2001; Fisk *et al.,* 2003; Lysnes *et al.,* 2004), or below the limits of detection via microscopy ($<10^3$ cells cm^{-3} rock; Mason *et al.,* 2010). Surveys of microbial diversity within subsurface basaltic rocks have revealed several abundant groups, including the Thaumarchaeota lineage Marine Group I (Fisk *et al.,* 2003) and bacterial lineages *Gammaproteobacteria* (Lysnes *et al.,* 2004; Huber *et al.,* 2010), *Alphaproteobacteria* and *Betaproteobacteria* (Huber *et al.,* 2010). Overall, the microbial diversity and community composition in subsurface basalts is poorly known due to a lack of high-quality samples, often as a result of contamination issues (Smith *et al.,* 2000a; Smith *et al.*, 2000b; Lever *et al.,* 2006; Santelli *et al.,* 2010). The ability to explore rock and mineral material is likely to improve over time; however, a distinct disadvantage of rock retrieval is that it largely, if not completely, precludes the ability to conduct an *in situ* experiment (e.g. Smith *et al.,* 2011; Orcutt *et al.,* 2011b) or investigate temporal variability and dynamics.

1.2 Sampling Tools and Contamination Issues

Samples of ocean crust are exceptionally difficult to acquire due to the relatively impenetrable nature of the crustal environment. However, within the past decade, a suite of tools has been developed that permit the collection of subsurface rocks and fluids from depths ranging from shallow to deep in order to study life in the oceanic crust. Life in the surficial ocean crust has been explored by a variety of mechanisms, including the collection of surface bedrock fragments (e.g. Santelli *et al.*, 2008, Mason *et al.*, 2009) and hydrothermal vent fluid sampling (e.g. Karl *et al.*, 1988; Robidart *et al.*, 2013); however, these samples may not be reflective of the biosphere that is thought to reside deeper within the ocean crust (Gold *et al.*, 1992).

Tools for accessing the deep basement biosphere

The exploration of life in the oceanic crust has usually involved the use of ocean drilling expeditions conducted under the auspices of the Integrated Ocean Drilling Program (IODP) and its previous inceptions [i.e. the Deep Sea Drilling Program (DSDP) and Ocean Drilling Program (ODP)] (Table 1.1). In contrast to seafloor-based drilling operations, drillship-based systems are more frequently operated in the deep-sea environment and permit deeper drilling penetration depths. Wireline-retrievable coring tools are often used in association with the IODP's two principal drilling vessels, the *Joides Resolution* (USA) and *Chikyu* (Japan). The PETROBRAS corporation (Brazil) has also operated its own wireline-retrievable coring tools that have been used for deep biosphere research (Von Der Weld *et al.*, 2008). For a complete overview of IODP drilling and coring technology, consult Huey (2009). When combined with the low porosity of most basalts, the widespread use of circulation mud that typically contains seawater and other amendments such as organic polymers and mineral salts prohibits the collection of uncontaminated crustal fluids from within rock cores (Yanagawa *et al.*, 2013). Nonetheless, contamination tests performed on rock samples obtained by drilling indicate that, in most cases, the interior of rocks are free of significant microbial contamination (also see next section on "Contamination induced during drilling"). Thus, drilling provides a suitable, and in fact, the only, way by which the rock-associated indigenous microbial community within ocean crust can be sampled and studied. Downhole logging of physical parameters (temperature, pressure, resistivity, etc.) is possible using "logging-while-coring" or downhole devices such as the Simple Cabled Instrument for Measuring Parameters *in situ* (SCIMPI) (Moran *et al.*, 2006; Expedition 341S Scientists and Engineers, 2013). Existing instrumentation is largely set up for non-biological investigations but the Dark Energy Biosphere Investigative Tool (DEBI-T) downhole profiler has been adapted for use in deep subseafloor biosphere investigations (Bhartia *et al.*, 2010; Salas *et al.*, 2011). This *in situ* instrument utilizes deep ultraviolet excitation to induce organic molecules found in the membranes of microbial cells or spores to fluoresce differently from the opaque, organic-rock matrix material (Bhartia *et al.*, 2010; Salas *et al.*, 2011). While useful for the study of rock attached-microbes, "logging-while-coring" techniques are also not suited for investigations focused on crustal fluids. Subseafloor IODP drilling borehole observatories known as Circulation Obviation Retrofit Kits (CORKs) currently provide the best means to investigate life inside basalt-hosted, deep basement fluids (Wheat *et al.*, 2011; for review). CORKs facilitate downhole microbiological experimentation opportunities (Orcutt *et al.*, 2011b; Smith *et al.*, 2011) and, in some iterations, have been equipped with fluid delivery systems that permit collection of pristine fluids from a re-sealed basement environment (Cowen *et al.*, 2012). The past two decades have seen the launch of the original CORKs (Davis *et al.*, 1992) and the evolution of newer versions [CORK-II (Fisher *et al.*, 2005a); ACORK (Wheat *et al.*, 2011; Becker and Davis, 2005)]. The original CORK design was of great value to

hydrodynamic studies, but allowed fluids to interact with the interior of a reactive-iron casing, leading to contamination effects in chemical and microbiological samples. Newer CORK-II, A-CORK and CORK-Lite versions incorporate newer features such as sample intake ports positioned away from casing material and fluid delivery lines that are made of microbiologically-friendly polytetrafluoroethylene (PTFE) material and run external to the CORK casing. While there are clear advantages to conducting *in situ* experiments and obtaining relatively pristine samples from the newer CORK observatories (Orcutt *et al.*, 2012), old CORK installations can provide samples useful for comparative microbiological analysis (Jungbluth *et al.*, 2014). CORK observatories also facilitate the collection of samples at varying temporal resolution from the same basaltic crust environment (Jungbluth *et al.*, 2013), which is not possible with *in situ* coring methods.

Seafloor-based drilling and coring operations are a less expensive alternative to drillship operations; various tools have been developed and are maintained by different countries and organizations (Table 1.1). While access to cored sample is sometimes limited because of the precious nature of the material, boreholes can serve as convenient access points to basement fluids. Although access to seafloor-based drilling samples is limited and the penetration depth of these systems is considerable less than ship-based drilling, they provide a less expensive alternative that could complement drilling vessels for purposes of exploring the subseafloor biosphere (Freudenthal and Wefer, 2007). Many of the seafloor drills listed in Table 1.1 have not been used for subsurface biosphere investigations, but could, in principle, be used for this purpose. While most of the examples noted are relatively immobile seafloor-based systems, the Multiple-Barrel Rock Coring System can be used in conjunction with ROV and HOV platforms (Stakes *et al.*, 1997). Few microbiological studies have utilized seafloor-based basement penetration methodology (Table 1.1). The Benthic Multicoring System (BMS), operated by the Japan Oil, Gas and Metals National Corporation, has been used to generate and case a shallow borehole permitting fluid sampling and *in situ* microbiological experiments (Higashi *et al.*, 2004; Hara *et al.*, 2005; Kato *et al.*, 2009b). Another example is the 2000 kg coring apparatus designed by Johnson *et al.* (2003), which does not utilize drilling, but instead relies upon the weight of the instrument to drive metal casing into exposed rocky seamount. Inserted probes contained hollow elements that allowed crustal fluid sampling for microbiology (Huber *et al.*, 2006). Sampling of ocean crustal fluids requires only that boreholes are producing fluids and that measures are taken to maintain borehole integrity and ensure pristine sampling. These requirements theoretically permit a vast array of deep ocean and hydrothermal vent sampling equipment to be used for virtually all hydrothermal fluid sample access points (e.g. probes, CORKs, vents, seeps, etc.) (Table 1.2). Sampling tools for fluids come in several types, including syringe/plunger, passive flow and active flow (Table 1.2). Because of the low biomass expected in the subsurface ocean crust, the most successful tools used for subsurface fluid sampling to date are capable of

Table 1.1 Selected drillship- and seafloor-based basement penetration tools and sampling methods used or theoretically available for study of the ocean crust biosphere

	Ocean Crust Sample Type	*Tool(s)*	*Used for Subsurface Biosphere Investigation?*	*Max Penetration Depth (m)*	*Brief Overview*	*Example Citation(s)*
Wireline-based drillship operations	Rock	D/V *Joides Resolution* Rotary Core Barrel	yes	>2000	8200-m max operating depth	Huey, 2009; Mason *et al.*, 2009; Santelli *et al.*, 2010; Lever *et al.*, 2013
	Rock	D/V *Chikyu* Rotary Core Barrel	yes	~10000[a]	Accompanied by riser drilling capabilities, permitting deeper drilling penetration depths; 2500-m max operating depth when performing riser drilling operations	Huey, 2009; Expedition 331 Scientists, 2011
	Rock	Proprietary drill pipe w/ outer and inner barrel	yes	>2800	Type of rotary core barrel; operated by PETROBRAS corp. (Brazil)	Von Der Weld, 2008
	Fluid	Circulation Obviation Retrofit Kit (CORK)[b]	yes	NA	Long-term ODP/IODP borehole observatories; multiple design inceptions: CORK, CORK-II, A-CORK, CORK-Lite; accommodate *in situ* experimentation and basement fluid sampling	Davis *et al.*, 1992; Cowen *et al.*, 2003; Jannasch *et al.*, 2003; Orcutt *et al.*, 2011b; Wheat *et al.*, 2011; Wheat *et al.*, 2012; Jungbluth *et al.*, 2013)
	Rock	Benthic Multicoring	yes	20	6000-m max depth; multiple design inceptions: BMS-1, -2, -3;	Matsumoto and Sarata, 1996; Higashi *et al.*, 2004; Ishibashi

		Systems (BMS)			operated by JOGMEC (Japan)	*et al.*, 2007; Kato *et al.*, 2009b
Seafloor-based drilling operations	Rock	Portable Remotely Operated Drill (PROD)	no	125	3000-m max depth; multiple design inceptions: PROD-1, -2, -3; operated by Benthic Ltd	Sager *et al.*, 2003; Pallanich, 2010
	Rock	Multiple-Barrel Rock Coring System (MCS)	no	1	Used in conjunction with ROV and HOV platforms; operated by Debra Stakes & MBARI (USA)	Sager *et al.*, 2003; Stakes *et al.*, 1997
	Rock	Rockdrill (RD2)	no	50	4000-m max depth; operated by BGS (UK)	Wilson, 2006
	Rock	Rovdrill	no	80	3000-m max depth; multiple design inceptions: Rovdrill-1, -2, -3; operated by Nautilus Minerals Inc	Spencer *et al.*, 2011
	Rock	Autonomous coring system (ACS)	no	100	3000-m max depth; operated by NIOT (India)	Freudenthal and Wefer, 2013
	Rock	MeBo drill rig	no	80	4000-m max depth; operated by MARUM (Germany)	Freudenthal and Wefer, 2007; Krastel *et al.*, 2011; Freudenthal and Wefer, 2013
	Rock	Gregg's seafloor drill	no	150	Developed by Gregg Drilling & Testing Inc (USA)	Freudenthal and Wefer, 2013
	Fluid	Short-length Hollow Casing Pipe	yes	?	Inserted directly using 2000-kg coring apparatus or post-drilling; hollow stainless steel or titanium probes permit venting of fluids	Higashi *et al.*, 2004; Kato *et al.*, 2009b, Johnson *et al.*, 2003; Hara *et al.*, 2005; Huber *et al.*, 2006)

[a]Hypothetical max penetration depth based upon length of drill pipe available

[b]CORK-Lite designs do not require use of drill-ship operations

Table 1.2 Selected ocean incubation and crust sample collection tools used or relevant to deep biosphere investigations

Sample Type	*Tool Type*	*Tool(s)*	*Brief Description*	*Example Citation(s)*
Fluid				
	Syringe/ plunger sampling			
		Miniature Pore Water Sampler inside IODP Advanced Piston Corer (APC)	Never used really worked; not theoretically adapted for basalt rock	Huey, 2009
		Titanium gas-tight sampler	Used for high-temperature fluid sampling *in situ* sample pressure	Von Damn *et al.*, 1985; Butterfield *et al.*, 2004
		SIPPER acrylic sampler	Small volume fluid samples (1-10 ml) for discrete sampling	Di Meo *et al.*, 1999
	Passive fluid sampling			
		In situ vent devices	Used for *in situ* enrichment at hydrothermal vents; never used for deep subsurface biosphere research	Karl *et al.*, 1988; Reysenbach *et al.*, 2000; Nercessian *et al.*, 2003; Phillips *et al.*, 2003; Alain *et al.*, 2004
		BioColumn *in situ* filter	Multiple day *in situ* filtration of positively-pressured CORK borehole fluids	Cowen *et al.*, 2003
		Titanium bottle with hose	Connected to CORK wellhead L-port via flexible sampling hose	Nigro *et al.*, 2012
		Osmo Sampler w/ Flow through Osmo Colonization Systems (FLOCS)	Can be deployed in conjunction with CORK and SmartPlug/GeniusPlug observatory system; utilized largely for rock chip *in situ* incubations	Jannasch *et al.*, 2004; Orcutt *et al.*, 2010; Kopf *et al.*, 2011

	Osmo Sampler w/ Biological OsmoSampling System (BOSS)	Capable of *in situ* DNA and protein preservation; time-series sampling permits daily resolution for >1 year	Jannasch *et al.*, 2004; Robidart *et al.*, 2013
Active fluid sampling			
	Submersible-Coupled *In situ* Sensing and Sampling System (SIS)	Used for hydrothermal vent fluid sampling only; can be equipped with either Titanium syringes or gas-tight samplers as fluid collection devices	Massoth *et al.*, 1989
	Hydrothermal Fluid and Particle Sample (HFPS)	Features titanium intake nossels and multiple temperature probes	Butterfield *et al.* 2004; Huber *et al.*, 2006
	Hydrothermal Vent BioSampler (HVB)	Used for hydrothermal vent fluid sampling only; capable of *in situ* filtration and concentration of 10 l of fluids	Behar *et al.*, 2005
	Teflon tube and impellor	Teflon tube placed inside titanium pipe inserted into ocean crust	Hara *et al.*, 2005
	Autonomous Microbial Sampler (AMS)	Never used for deep subsurface biosphere research; feature titanium intake nossels; handles filtered and unfiltered samples	Taylor *et al.*, 2006
	KIPS fluid sampling system	Pump composed of Teflon parts; features multiple nossels, including titanium-based	Garbe-Schönberg *et al.*, 2006
	Deep-sea Environmental Sample Processer (D-ESP)	Never used for deep subsurface biosphere research; capable of fluid filtration, *in situ* quantitative PCR, and a variety of other functions	Scholin *et al.*, 2009; Ussler *et al.*, 2013
	Rotary clean seawater sampling system (ROCS)	Six 500 ml polycarbonate, Teflon, and silicon cylinders; sample performed using a peristal pump	Kato *et al.*, 2009b

		Large-scale fluid (LF) stainless steel sampling system	Used to collect up to 10 l of fluids; utilized ROPOS suction pump	Kato *et al.*, 2009b
		Large volume bag sampling system (BAG)	Polyethylene bag system capable of collecting up to 20 l; utilized ROPOS suction pump	Kato *et al.*, 2009b
		GeoMICROBE/Mobile Pumping System (MPS), Medium-Volume Bag System (MVBS), and Large Volume Bag System (LVBS)	Used to collect up to 70 l of fluids; handles filtered and unfiltered samples	Wommack *et al.*, 2004; Cowen *et al.*, 2012; Lin *et al.*, 2012; Jungbluth *et al.*, 2013; Jungbluth *et al.*, 2014
Rock				
	Basement coring			
		Rotary Core Barrel (RCB)	Standard basement core collection tool of *Joides Resolution* and *Chikyu*; used often with catchers to increase core retainment	Huey, 2009; Santelli *et al.*, 2010; Mason *et al.*, 2010; Expedition 336 Scientists, 2012; Lever *et al.*, 2013
		Extended Shoe Coring System (ESCS)	Variation of RCB employed on D/V *Chikyu*; used often with catchers to increase core retainment; relatively new tool in IODP arsenal	Huey, 2009; Expedition 331 Scientists, 2011; Yanagawa *et al.*, 2013
	In situ deployable experiments	Osmo Sampler w/ colonization systems and microbial flow cells	Rocks and minerals can be incubated *in situ* inside CORK borehole observatories	Orcutt *et al.*, 2010; Kopf *et al.*, 2011; Orcutt *et al.* 2011b; Smith *et al.*, 2011
		Autonomous *in situ* Instrument Colonization System (AISICS) and fluid sample probe	Rocks and minerals can be incubated *in situ*; developed specifically to study colonization processes; never used for deep biosphere research	Callac *et al.*, 2013

collecting large fluid volumes or concentrated the biomass from large volumes of crustal fluids (e.g. via *in situ* filtration). Passive fluid sampling using a "BioColumn" filter device (Cowen *et al.*, 2003) and downhole Osmo samplers connected in-line with flow through microbial colonization chambers have both been successful in association with CORK borehole observatories, yielding high-integrity samples for subsurface microbiology (Table 1.2). Active fluid sampling devices, including the Hydrothermal Fluid and Particle Samples (HFPS) (Butterfield *et al.*, 2004; Huber *et al.*, 2006), the trio of sampling devices used by Kato and colleagues (Kato *et al.*, 2009b), and the Mobile Pumping System (MPS) combined with both medium- and large-volume bag systems (Cowen *et al.*, 2012; Lin *et al.*, 2012) have proven highly successful in sampling subsurface fluids. Active fluid sampling systems such as the GeoMICROBE sled (Cowen *et al.*, 2012) are actively deployed for microbiological analysis; systems such as the GeoMICROBE provide the means to collect crustal fluid samples autonomously in time series fashion (Table 1.2). The retrieval of rock samples from the ocean crust provides the opportunity to study microorganisms that are living in direct association with rocks, and thus likely to be involved in the physical and chemical alteration of ocean crust. Drilling conducted by the IODP vessels for the purpose of rock sample collection typically utilizes Rotary Core Barrel (RCB) technology. However, both the *Joides Resolution* and the *Chikyu* utilize slight variations of this tool called the extended core barrel (XCB), and the Extended Shoe Coring System (ESCS), respectively. Both the XCB and the ESCS incorporate features that enhance penetration and recovery of basement rock; however, the ESCS contains upgrades that make it superior to the XCB (Huey, 2009). Huey (2009) provides a complete overview of IODP basement coring technology.

Contamination induced during drilling

Monitoring of sample contamination during drilling and subsequent operations is essential to downstream microbiology and molecular biological studies due to the large potential for cell and nucleic acid contamination of subseafloor samples with cells and nucleic acids from seawater and chemical compounds pumped into boreholes during drilling operations (Masui *et al.*, 2008; Yanagawa *et al.*, 2013). Two methods have traditionally been used to monitor contamination in sediment and rock cores during ocean drilling expeditions: fluorescent microbeads (microspheres) and chemical [perfluorocarbon (PFC)] tracers (Smith *et al.*, 2000a; Smith *et al.*, 2000b; House *et al.*, 2003). Both methods have their strengths and shortcomings. Fluorescent microbeads are suspended in distilled water and placed in a sealed plastic bag that is subsequently mounted to the core-catcher sleeve prior to advancement into the borehole. This bag breaks open during drilling operations, thereby releasing microbeads into drilling fluids. The advantage of this method is that appropriately sized microbeads (0.5–1.0 µm diameter) are likely to be good analogs for the transport of (contaminant) microbial cells in fluids during drilling operations. Detection and quantification is, moreover, methodologically

straightforward and can be performed by epifluorescence microscopy – even months after drilling expeditions, on frozen or fixed samples. A pitfall is that homogeneous concentrations of microbeads cannot be achieved by bag breakage and microsphere spillage into drilling fluids. As a result, certain parts of drilling fluids may contain very high concentrations of microbeads, whereas other parts only have low concentrations or may even be devoid of microbeads (House *et al.*, 2003). As a result, although visual observation of microbeads is evidence for drilling fluid contamination, the failure to detect microbeads is not reliable evidence for its absence. A more quantitative and sensitive method is the use of chemical tracers. During past ocean drilling expeditions, the PFC compound perfluoromethylcyclohexane (PMCH; C_7F_{14}) has been used. This tracer is injected into the drilling mud stream on board, to a constant concentration of 1 mg l^{-1}. From there, this mud stream is pumped directly into the borehole to the drill bit. Because the detection limit is so low (i.e., parts per trillion level), PFC tracers offer a highly sensitive method by which drilling fluid intrusion in the nanoliter range per cm^3 of sample can be detected (Lever *et al.*, 2006). PMCH is also less volatile than initially thought, indicating that loss from volatilization during drilling operations, sampling, or short-term sample storage on board will not result in significant PFC loss from samples, and thus underestimation of contamination in samples (Lever *et al.*, 2006; Inagaki *et al.*, 2012). Potential shortcomings of the PFC method are, however, that samples need to be taken within three hours after core sampling to avoid diffusion of PFC from contaminated core exteriors to clean interiors. Otherwise, false positives might occur, since microbial cells diffuse at a vastly lower rate than PFC. Moreover, recent data from IODP Expedition 337 indicate that PFC can sorb to drilling mud components, sediment and/or rock material (Inagaki *et al.*, 2012). If confirmed, drilling fluid contamination estimated by PFC measurements might have resulted in underestimation of drilling fluid contamination in some cases. This problem of incomplete PFC release during headspace incubation has been reduced through modifications of the PFC incubation protocol (Inagaki *et al.*, 2012). Both microspheres and PFC have been used to quantify drilling fluid contamination during drilling operations into ocean basement (Smith *et al.*, 2000a; Shipboard Scientific Party, 2003; Lever *et al.*, 2006). Both tracers were quantified on exteriors and in interiors of gabbro cores in the Middle America Trench (Shipboard Scientific Party, 2003) and indicated high contamination of core exteriors and vastly lower, though still significant, contamination of the innermost core interiors. These findings were partially confirmed by the studies of Smith *et al.* (2000a) and Lever *et al.* (2006) on basalt cores, which also demonstrated high contamination of outer rock surfaces. Contrary to the study on gabbro cores, however, these studies found the PFC content in the interiors of intact rock samples to be very low or even below detection. There was no significant difference (i.e. drilling fluid contamination within basalt cores was not higher) compared to sediment cores obtained by the advanced piston coring technique. A key difference between the study by Morris *et al.* (Shipboard Scientific Party, 2003) and the studies by Smith *et al.* (2000a) and Lever *et al.* (2006) might be

that, in the latter two studies, additional treatments besides washing with water (i.e. washing with methanol or flaming) were used to remove tracer from core exteriors. After 2x washing with artificial seawater, Lever *et al.* (2006) found PFT on rock exteriors to only have decreased by ~80%, compared to decreases of ~99% after additional flame treatment. Without this additional treatment, which was equivalent to flame-sterilizing rock exteriors, the risk of cross-contaminating rock interiors through contact with still highly contaminated rock exteriors during rock crushing was higher. Thus, taking together the published data on drilling fluid contamination, it appears that the interiors of intact basalt rocks are only minutely contaminated during the drilling process and thus suitable for microbiological analyses.

Contamination during fluid sampling

The sampling of subsurface crustal fluids carries contamination challenges independent of those that can occur during drilling because of the low amount of biomass contained in fluids: fluids emanating from CORK boreholes appear to contain microbial cells that are roughly an order of magnitude less abundant than those in bottom seawater (Jungbluth *et al.*, 2013). Thus, from a microbiological perspective, even a small percentage of bottom seawater entrainment can result in significant contamination. Real-time temperature measurements of fluids being sampled is somewhat sufficient to assess chemical integrity; however, the addition of *in situ* electrochemical measurements of redox species is the best way to assess potential for leaks in the CORK and instrument fittings (Cowen *et al.*, 2012).

1.3 Deep Subsurface Biosphere Investigations

Sediment-Buried Crustal Fluid Sampling

Since the basement rock of most mid-ocean ridge flanks and ocean basins is buried under thick, impermeable layers of sediment (i.e., sediments thick enough to act as a barrier to rapid exchange of fluids) (Embley *et al.*, 1983), the fluids circulating within the underlying ocean basement are difficult to access for direct studies. Although retrieval of ocean basement rocks is only possible through the use of drilling operations, fluids circulating with the sediment-covered basement can be collected using three methods: (1) retrieval of fluids suspected of having a deep subsurface origin via access through an exposed seamount (Huber *et al.,* 2006), (2) retrieval of fluids suspected of having a deep subsurface origin from a drilled and cased, open borehole drilled through shallow (<5m) sediment covering a seamount (Hara *et al.,* 2005; Kato *et al.,* 2009b), or (3) use of deep sediment- and basement-penetrating sampling platforms (CORKs; explained below) in order to collect discrete fluid samples directly from the basement aquifer (Cowen *et al.,* 2003; Huber *et al.,* 2006; Nigro *et al.,* 2012). The most prevalent

technology for collecting deep subsurface crustal fluids is through the use of Circulation Obviation Retrofit Kit (CORK) observatories (Davis *et al.*, 1992) placed inside of Ocean Drilling Program (ODP) and Integrated Ocean Drilling Program (IODP) boreholes. CORK sampling and instrumentation platforms have permitted the collection of pristine samples suitable for probing the microbial ecology of the deep subsurface biosphere (Figure 1.3) through the implementation of fluid delivery lines capable of channeling subseafloor fluids up to the seafloor for sampling access. Investigations into the deep biosphere have utilized CORK observatories within a variety of different field programs including: (1) the Costa Rica subduction zone (Jannasch *et al.,* 2003; Nigro *et al.,* 2012), (2) the Mid-Atlantic ridge flank sediment pond ("North Pond") (Expedition 336 Scientists, 2012; Wheat *et al.,* 2012), and (3) the Juan de Fuca ridge eastern flank (Shipboard Scientific Party, 1997; Cowen *et al.,* 2003; Expedition 301 Scientists, 2005; Fisher *et al.,* 2005b; Huber *et al.,* 2006; Expedition 327 Scientists, 2011; Figure 1.4).

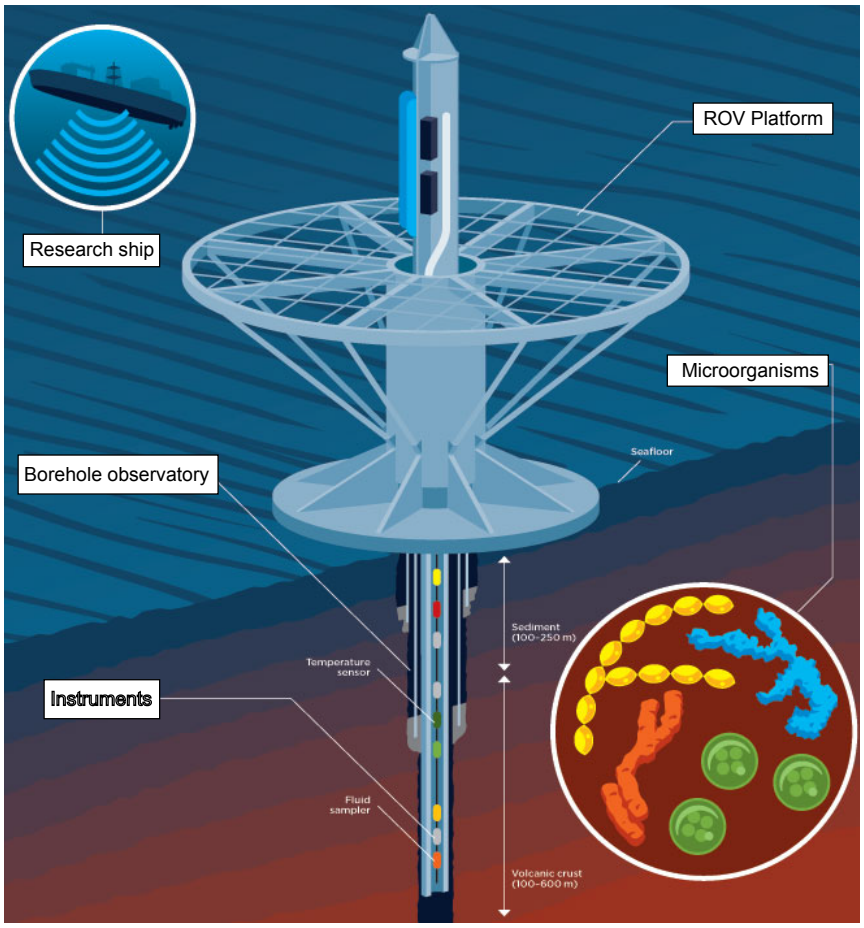

Figure 1.3 Illustration of Circulation Obviation Retrofit Kit (CORK) observatory used for deep subsurface monitoring and fluid sampling. (Biba, 2011; Illustration by James Provost, 2011; *Wired Magazine*)

Juan de Fuca Ridge Crustal Fluid Sampling

Fluid sampling for deep subseafloor microbial ecology surveys on the Juan de Fuca Ridge (JdFR) flank have been conducted within 3.5 million-year old ocean crust via stainless steel probes stuck into an exposed seamount (Huber *et al.,* 2006) or directly from the deep basement aquifer using CORK observatories (Cowen *et al.,* 2003; Huber *et al.,* 2006). In this location, basement aquifer fluids are thought to be moving through the basement rock due to the high basement permeability, which combines with nearby rocky seamounts protruding through the sediments to provide hydrogeological exchange between hydrothermal recharge and discharge points over distances >50 km (Fisher *et al.*, 2003; Figure 1.5). These geological features allow deep seawater to be entrained into the ocean basement and to evolve during its passage through the relatively warm and chemically reducing basement rock environment (Cowen, 2004). A subset of ODP and IODP boreholes that are drilled through ~250 meters of sediments and penetrate the flowing basement aquifer have been fitted with CORK platforms (1026B, U1301A, U1362A, U1362B; Table 1.3 and Figure 1.5). The biological and chemical characteristics of crustal fluids collected via CORK observatories tapping into basement fluid recharge zones likely reflect the response of the *in situ* basement microbial community to an influx of terminal electron acceptors (Wheat *et al.*, 2010; Lin *et al.*, 2012). Seamount fluids sampled by Huber and colleagues (2006) were collected from Baby Bare seamount, which is venting fluids thought to

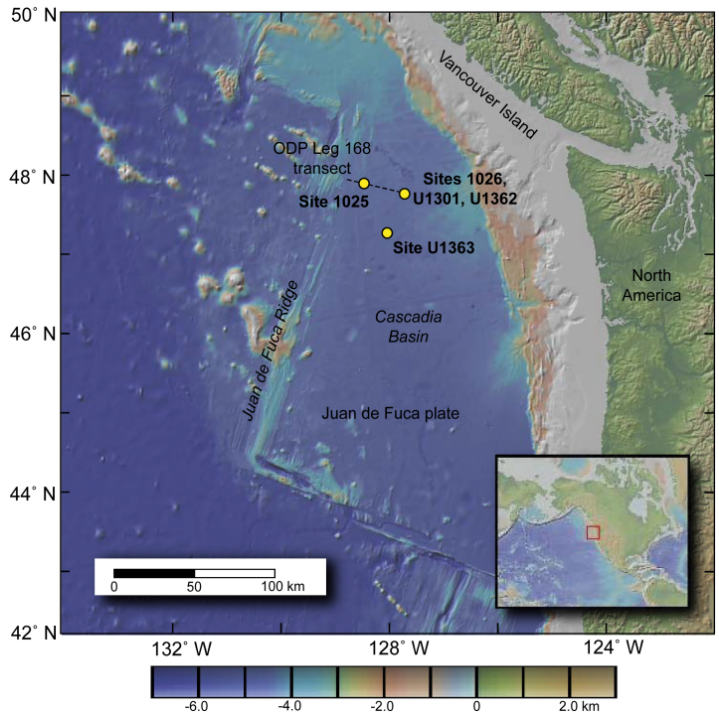

Figure 1.4 Map of the eastern flank of the Juan de Fuca Ridge (JdFR) showing the location of the ODP Leg 168 and CORK-enabled fluid sampling. (modified from Expedition 327 Scientists, 2011)

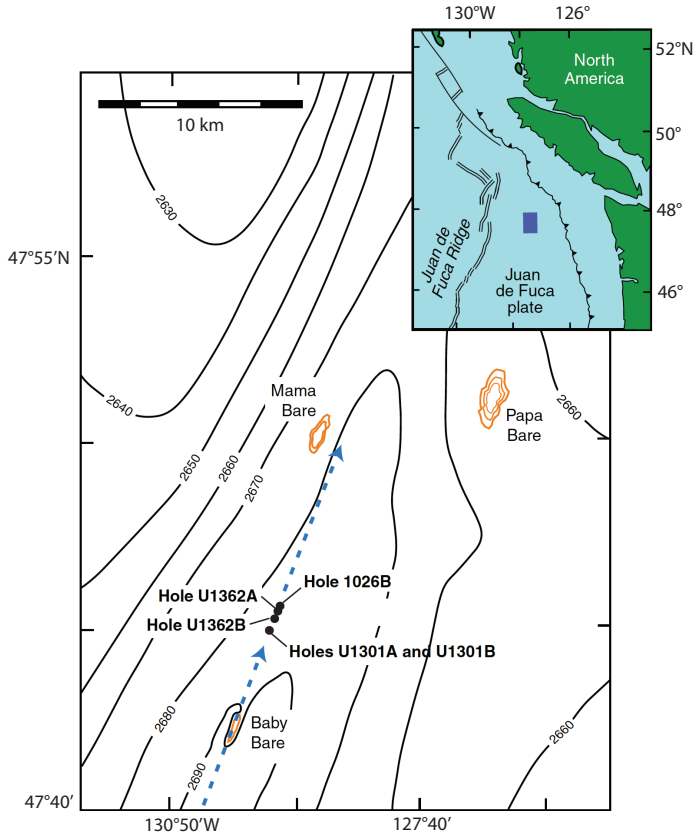

Figure 1.5 Bathymetric map showing locations of borehole observatories placed within 3.5 million year old crust of the Juan de Fuca Ridge eastern flank. Gold contours indicate basement outcrops. Blue dashed arrows indicate general direction of inferred fluid flow in basement. (Fisher *et al.,* 2011)

Table 1.3 Summary of JdFR flank CORK-enabled borehole fluid sampling locations.

	1025C	*1026B*	*U1301A*	*U1362A*	*U1362B*
Crustal Age (million-years old)	1.2	3.5			
Sediment-Basement Interface Temperature (°C)	39	65			
Sampling Depth (meters) (water column depth at sampling spigot + sediment + permeated basement rock)	2606 + 101 + 46	2658 + 247 + 48	2667 + 262 + 108	2672 + 236 + 292	2672 + 242 + 117
CORK Generation	1st	2nd (CORK-II)		3rd (ACORK)	
Fluid Delivery Line Type	none	1/8” SS	1/8” SS	½” PTFE	½” PTFE

SS: Stainless Steel; PTFE: Polytetrafluoroethylene

represent hydrothermally-altered end-member basement fluids. The studies conducted by Cowen and colleagues (2003) and Huber and colleagues (2006) represent the sole attempts to date that have assessed the microbial community diversity and structure of ridge flank crustal fluids. Cowen and colleagues (2003) used a first-generation CORK observatory to show that fluids escaping from the top of the ODP borehole 1026B, a member of the 1301 borehole suite, are derived from the oceanic basement and that the chemical characteristics and temperature (65°C) of these fluids are conducive to microbial growth (Cowen *et al.*, 2003). Using standard molecular microbial ecology techniques, small-subunit ribosomal RNA (SSU rRNA) gene sequence data indicated the presence of diverse *Bacteria* and *Archaea*, including gene clones of varying degrees of relatedness to thermophilic sulfate reducers and thermophilic fermentative heterotrophs (Cowen *et al.*, 2003). The most abundant lineages identified by Cowen and colleagues include a phylotype closely related to *Candidatus* Desulforudis audaxviator, a lineage that has been discovered within subsurface terrestrial goldmine fluids and implicated in sulfate reduction (Chivian *et al.,* 2008), and the family *Desulfohalobiaceae* within the phylum *Deltaproteobacteria*, which is an uncultivated group that has only been discovered in this initial study and another CORK-focused study where a black rust biofilm was sampled (Nakagawa *et al.,* 2006). The abundance of microbial cells as determined from the 'Biocolumn'- collected sample was 8.5 x 10^4 cells ml^{-1}, though this was suspected to be an underestimate due to the presence of extensive polymers that obscured cells (Cowen *et al.,* 2003).

Sampling fluids from borehole 1026B and a nearby exposed seamount (Baby Bare Seamount), Huber *et al.*, (2006) similarly found fluids to host microbial lineages with metabolisms that are consistent with the physical and geochemical characteristics of the system (i.e. anaerobic thermophiles). A variety of groups were detected within seamount fluids that range from being likely subsurface fluid residents (e.g. *Deltaproteobacteria, Epsilonproteobacteria, Thermotoga, Aquifex,* Candidate division OP8, Miscellaneous Crenarchaeota Group), to likely seawater inhabitants (e.g. SAR86, SAR116, OM43, *Cyanobacteria*, Marine Groups I & II). Huber and colleague's attempts to sample the CORK fluids from borehole 1026B were less successful, with the samples largely yielding either seawater-associated (e.g. *Cyanobacteria, Flavobacteria, Verrucomicrobia*) or microorganisms likely originating from contamination (e.g. *Betaproteobacteria – Burkholderia, Ralstonia, Gammaproteobacteria – Oceanospirillales, Actinobacteria – Micrococcus*). However, a few lineages were identified that tend to be associated with deep subsurface systems (e.g. *Thiomicrospira, Thermotoga*). These results underscore the difficulties associated with contamination-free sampling. Microbial cell abundances reported for two seamount fluid samples (9.19 x 10^4 – 1.30 x 10^5) were slightly elevated compared to the CORK borehole 1026B fluid sample (3.81 x 10^4), and the enumeration of *Bacteria* and *Archaea* by fluorescence *in situ* hybridization (FISH) revealed the presence of a relatively low numbers of active microbial cells, which were dominated by *Bacteria* (Huber *et al.*, 2006).

Contemporary microbial ecology studies of deep subsurface fluids are poised to take advantage of expanded spatial and temporal opportunities and state-of-the-art molecular biology techniques. Borehole fluid-sampling capabilities evolved over two decades have most recently been implemented within a subset of CORKs on the Juan de Fuca Ridge flank (U1301A, U1362A, U1362B), permitting collection of the most pristine, unaltered basement fluids yet through the use of non-contaminating CORK parts and relatively inert fluid delivery lines (Wheat *et al.*, 2011). Dedicated microbiological sampling ports composed of biofouling-resistant materials (i.e. stainless steel, Tefzel "BioLine") were employed on CORKs installed during IODP Expedition 301 (Fisher *et al.*, 2005a), where several of the original CORKs emplaced within the boreholes during the initial drilling leg were replaced with second- and third-generation advanced CORKs (Fisher *et al.*, 2005b). During IODP Expedition 327 (Expedition 327 Scientists, 2011), similar third- generation advanced CORK were installed within several of the freshly drilled boreholes. This latest borehole fluid sampling technology upgrade fortuitously coincides with the era of next-generation DNA sequencing techniques, which permit molecular microbial ecology surveys orders of magnitude more deeply than those that were conducted previously (Cowen *et al.,* 2003; Huber *et al.,* 2006). Here, I exploit the unprecedented opportunity to apply the latest molecular techniques upon samples provided by the new array of CORK installations situated within the JdFR flank to study the microbial ecology of basement fluids.

1.4 Rationale

The analyses performed can generally be grouped into two areas: (i) microbial biomass and (ii) microbial diversity and community structure.

(i) Microbial Biomass

Measures of microbial biomass within the sediment-covered ocean basement are restricted to the studies by Cowen *et al.,* (2003) and Huber *et al.,* (2006), which were both one-time, limited-sampling events. In both studies, either particulate biofilms (Cowen *et al.,* 2003) or marine sediments (Huber *et al.,* 2006) - both potential contaminants - were tentatively identified using early-generation deep biosphere sampling methods; however, these are still the best estimates of microbial cell abundance in ridge flank basement fluids. Currently, microbial biomass within borehole fluids retrieved from newer CORK observatories (i.e. 1301A, 1362A, 1362B) has not been assessed (outside of my work presented below), even though these sampling platforms utilize the cleanest fluid sampling lines to be installed in any CORK observatories (i.e. stainless steel, Teflon-like). Crustal fluid sampling described here was performed annually for multiple years from the new CORKs; this helps to resolve spatial and

temporal variability in the magnitude of ridge flank basement biomass. Fluorescence *in situ* hybridization experiments are described and were used to enumerate specific microbial lineages, such as total *Bacteria* and *Archaea.* Because this study utilized the cleanest sampling technology to date, microbial cell numbers reported here provide robust estimates that can be used to constrain the magnitude of the microbial biomass within the sediment-covered ocean basement.

(ii) Microbial Diversity and Community Structure

Knowledge of microbial diversity and community structure in the anoxic, sediment-covered ocean basement fluids is restricted to the studies by Cowen *et al.,* (2003) and Huber *et al.,* (2006), which were both one-time, limited-sampling events. Excluding the research I have performed and presented here, the microbial diversity and community structure of borehole fluids has not been assessed using the latest CORK observatories to be installed in either new holes (U1301A, U1362A, U1362B) or old holes (1025C, 1026B). Annual and other opportunistic sampling from a variety of boreholes described here has helped resolve spatial and temporal variability in the ridge flank basement microbial community. This work focused on the use of SSU rRNA gene sequence comparisons.

1.5 Research Objectives

The overall goals of the proposed research are to enhance our understanding of how environmental conditions such as ocean basement age, temperature, and redox activity influence the abundance, diversity, and community structure of microbial life in the Juan de Fuca Ridge sediment-covered ocean basement.

1. Enumerate total microbial cells and selected lineages (e.g. *Bacteria* and *Archaea*) in basalt-hosted borehole fluids to constrain marine subsurface microbial cell numbers and biomass estimates.

2. Investigate the ecology, diversity, and community structure of microbial cells in relation to spatial and temporal changes in sedimented, young ridge flank crustal fluids.

1.6 Guiding Questions

(i) Microbial Biomass

1. *What is the particle load in pristine borehole fluids as assessed using microscopy, and how do samples collected from CORKs employing old and new sampling technologies compare?*
2. *How similar are microbial cell abundances within crustal fluids from borehole U1301A collected one year apart?*
3. *How variable is the concentration of microbes within crustal fluids derived from different boreholes in crust having the same temperature (65˚C) and age (3.5 million-years old)?*
4. *What is the spatial and temporal variability of absolute abundance within domain-level microbial groups (Bacteria, Archaea) and specific lineages that will be identified throughout the duration of the study?*

(ii) Microbial Diversity and Community Structure

1. *Is the microbial diversity and community assemblage within crustal fluids accessible through the CORK observatory at borehole U1301A the same as that inhabiting crustal fluids sampled previously from borehole 1026B* (Cowen *et al.*, 2003)*?*
2. *How similar are microorganisms inhabiting crustal fluids to previously characterized microbial isolates known to utilize redox active compounds that are enriched in the subsurface relative to the seafloor?*
3. *What is the variability in diversity and community structure of microorganisms inhabiting crustal fluids from borehole U1301A in samples collected one year apart?*
4. *Is the diversity and community structure of microorganisms inhabiting crustal fluids found within younger (1.2 million-years old) and cooler (39˚C) borehole 1025C different from those found in older (3.5 million-years old) and warmer (65˚C) boreholes 1026B, U1301A, U1362A, and U1362B?*
5. *Is the diversity and community structure of microorganisms inhabiting crustal fluids having the same temperature (65˚C) and crustal age (3.5 million-years old) identical (boreholes 1026B, U1301A, U1362A, and U1362B)?*
6. *What is the variability in diversity and community structure of microorganisms inhabiting crustal fluids within borehole U1362A and U1362B sampled on fine temporal (i.e. hourly) time scales?*

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CHAPTER 2

Microbial diversity within basement fluids of the sediment-buried Juan de Fuca Ridge flank

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2.1 Abstract

Despite its immense size, logistical and methodological constraints have largely limited microbiological investigations of the subseafloor basement biosphere. In this study, a unique sampling system was used to collect fluids from the subseafloor basaltic crust via a Circulation Obviation Retrofit Kit (CORK) observatory at Integrated Ocean Drilling Program borehole U1301A, located at a depth of 2667 m in the Pacific Ocean on the eastern flank of the Juan de Fuca Ridge. Here, a fluid delivery line directly accesses a 3.5 million years old basalt-hosted basement aquifer, overlaid by 262 m of sediment, which serves as a barrier to direct exchange with bottom seawater. At an average of 1.2×10^4 cells ml^{-1}, microorganisms in borehole fluids were nearly an order of magnitude less abundant than in surrounding bottom seawater. Ribosomal RNA genes were characterized from basement fluids, providing the first snapshots of microbial community structure using a high-integrity fluid delivery line. Interestingly, microbial communities retrieved from different CORKs (1026B and U1301A) nearly a decade apart shared major community members, consistent with hydrogeological connectivity. However, over three sampling years, the dominant gene clone lineage changed from relatives of *Candidatus* Desulforudis audaxviator within the bacterial phylum *Firmicutes* in 2008 to the Miscellaneous Crenarchaeotic Group in 2009 and a lineage within the JTB35 group of *Gammaproteobacteria* in 2010, and statistically significant variation in microbial community structure was observed. The enumeration of different phylogenetic groups of cells within borehole U1301A fluids supported our observation that the deep subsurface microbial community was temporally dynamic.

2.2 Introduction

It is now generally accepted that a subseafloor biosphere extends throughout the enormous volume of sediments and basement basalt that make up the global system of mid-ocean ridge spreading centers, flanks and ocean basins (e.g., Gold, 1992; Parkes *et al.*, 1994; Fisk *et al.*, 1998; Bach and Edwards, 2003; Cowen *et al.*, 2003; Schrenk *et al.*, 2010; Orcutt *et al.*, 2011a). Of the different oceanic subsurface environments, the porous, uppermost igneous crust originating from the mid-ocean ridge spreading centers is likely to be the most suitable for microbial life, because it is the locus of extensive hydrothermal circulation (Baross *et al.*, 2004). Vigorous seawater entrainment into young, <10 million years old (Ma) ridge flanks actively injects a chemical disequilibrium (generally in the form of terminal electron acceptors) into the deep ocean crust, fueling redox-active processes involving iron and sulfur cycling. This disequilibrium is thought to sustain a substantial subseafloor microbial ecosystem (Bach and Edwards, 2003).

Despite the presumed pervasiveness of the basalt-hosted deep subsurface biosphere, a thick layer of sediment that significantly restricts sampling opportunities covers much of the

oceanic basement of the mid-ocean ridge flanks and ocean basins. However, seafloor instrumentation platforms known as Circulation Obviation Retrofit Kit (CORK) observatories (Davis *et al.*, 1992; Edwards *et al.*, 2011) affixed to the Ocean Drilling Program (ODP) and Integrated Ocean Drilling Program (IODP) boreholes (Davis and Becker, 2001; Fisher *et al.*, 2005a) provide a rare opportunity to conduct *in situ* geophysical studies and sample fluids from the sediment-covered basement rock. By channeling fluids through dedicated microbiological and geochemical sampling lines, natural crustal fluids originating from the deep subsurface can be retrieved at the seafloor for subsequent interrogation.

Several boreholes drilled in the seafloor on the eastern flank of the Juan de Fuca Ridge (JdFR) in the northeastern Pacific Ocean are equipped with CORK observatories, providing access to 1.2 to 3.5 Ma crust that is covered by up to several hundred meters (m) of sediment. This sediment results in an impermeable seal between bottom seawater and the basement rock (Embley *et al.*, 1983). In this particular region of seafloor, nearby rocky seamounts protruding through the sediments combine with high basement permeability to provide hydrogeological exchange between hydrothermal recharge and discharge points over distances greater than 50 km (Fisher *et al.*, 2003). These geological features allow deep seawater to be entrained into the ocean basement and to evolve during its passage through the relatively warm and chemically reducing basement rock environment (Cowen, 2004). The biological and chemical characteristics of crustal fluids collected via CORK observatories tapping into basement fluid recharge zones likely reflect the response of the *in situ* basement microbial community to an influx of terminal electron acceptors (Wheat *et al.*, 2010; Lin *et al.*, 2012).

In 2003, Cowen *et al.* used an early-generation CORK observatory to collect basement fluids originating deep within the JdFR borehole 1026B, to conduct one of the first microbiological studies of young ridge flank crustal fluids. A passive flow 'BioColumn' device was connected in series to the CORK spigot to channel discharging fluids through a filter membrane. Subsequently, Huber *et al.* (2006) sampled fluids emanating from borehole 1026B, as well as those exiting Baby Bare Seamount (an exposed basaltic seamount nearby), using an *in situ* filtration system to collect microbial biomass. Subsurface fluids exiting the seamount were sampled by Huber *et al.* (2006) using stainless steel probes inserted through thin sediment and into the basement rock. Although young ridge flank fluid microbial diversity has been assessed using CORK-enabled *in situ* enrichment techniques (Smith *et al.*, 2011; Orcutt *et al.*, 2011b) and biofilm sampling of a retrieved CORK exposed to ridge flank crustal fluids (Nakagawa *et al.*, 2006), the two samples collected from borehole 1026B and samples collected from Baby Bare seamount have provided the lone assessments of planktonic microbial community structure within young ridge flank fluids.

Recent technological advances in CORK-assisted basement fluid sampling permit unprecedented access to basement fluids. Relatively inert stainless steel fluid delivery lines running exterior to the CORK casing are featured on a new generation of CORK observatories

deployed on both pre-existing (1026B) and new (U1301A) boreholes (Fisher *et al.*, 2005b) in an effort to sample deeply buried basement fluids while controlling contamination and minimizing biofouling effects noted in previous studies (Cowen *et al.*, 2003). In this study, a pump with titanium- and Teflon-wetted parts was used to draw basement fluids up the fluid delivery line and through the CORK spigot, permitting thorough flushing of the fluid delivery lines before the collection of basement fluids (Cowen *et al.*, 2012; Lin *et al.*, 2012). The upgraded materials and extensive flushing of the fluid delivery line facilitate high-integrity sampling of the crustal fluids, providing for an accurate assessment of *in situ* microbial community characteristics from the sediment-covered basement rock of the ridge flanks. Here, we describe the use of these improvements to retrieve crustal fluids from CORK U1301A in the summers of 2008–2010. Small subunit ribosomal RNA (SSU rRNA) gene cloning and sequencing and epifluorescence microscopy were used to provide insight into the diversity of microbial communities inhabiting the igneous crustal biosphere.

2.3 Materials and Methods

Sample collection

During R/V *Atlantis* cruises ATL15_35 (28 July 2008–13 August 2008), ATL15_51 (20 August 2009–6 September 2009) and ATL15_66 (13 June 2010–1 July 2010), basement crustal fluids were collected from a CORK-II borehole observatory at IODP borehole U1301A located 101 km east of the JdFR spreading center (47°145.2090'N, 127°145.8330'W; Figure 2.1). Fluids were sampled from the microbiological and geochemical sampling lines associated with the CORK-II installation (Fisher *et al.*, 2005a). Borehole U1301A penetrates 262 m of sediments and another 108 m into ~3.5 Ma basement rock (Table 2.1). A deep-sea pumping system was used that was improved significantly over the course of the study, becoming more powerful and incorporating a non-contaminating titanium and Teflon pump head and complementary in-line sensors (Lin *et al.*, 2012; Figure 2.1c). Fluid samples were ultimately collected in custom-made 60-l acid-washed Tedlar bags (MiDan Co., Chino, CA, USA) protected by a high-density polyethylene box (Figure 2.1). To minimize the introduction of chemical and microbial contaminants during sampling, acid-washed tubing and sampling bags were employed. In 2008, sample bags were filled after flushing the fluid delivery line with three times their volume of borehole fluids, and elevated fluid temperatures were observed. In 2009 and 2010, fluids were collected into sample bags after the fluid temperature had risen and stabilized at ~20 °C for 1 h. Subsurface samples described herein were collected during human-occupied vehicle *Alvin* dives 4434 (2008) and 4532 (2009), and remote-operated vehicle *Jason II* dive 497 (2010).

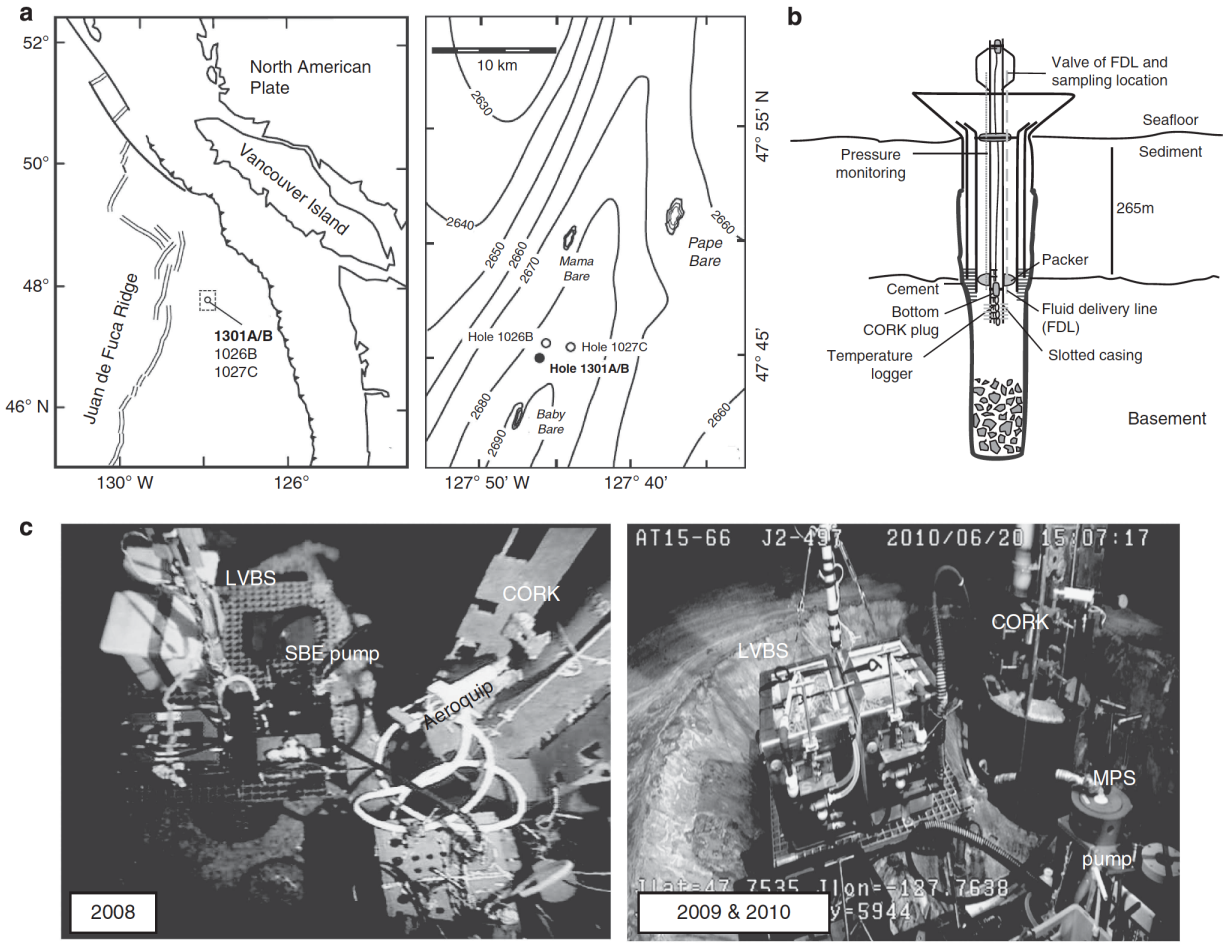

Figure 2.1 (a) Location of CORK observatory sampling sites on the Juan de Fuca Ridge flank, Pacific Ocean. (b) Schematic diagram of CORK located at borehole U1301A. Borehole crustal fluids were sampled from the exit valve of the fluid delivery line. (c) Photos of the fluid sampling device used in 2008, when a SeaBird pump (SBE) was used to divert fluids into the large volume bag sampler (LVBS), and 2009 and 2010, when a mobile pump sampler (MPS) was used to draw fluids and delivery into the LVBS. Figure modified from Lin et al. (2012).

Sample preparation for DNA extraction

In 2008, approximately 2 l of fluids were pumped from the bag sampler through a 25 mm-diameter, 0.1 µm pore-sized polyethersulfone membrane filter (Pall Corporation, Port Washington, NY), which was subsequently stored in 0.5 ml of DNA lysis buffer [20 mM Tris-HCl, 2 mM EDTA, 1.2% Triton X-100, 2% lysozyme (w/v), pH 8] at -80°C until further processing. In 2009 and 2010, 2.6 l and 11.2 l of fluids, respectively, were pumped from the bag sampler through a 0.22-µm-pore-sized Sterivex-GP filter cartridge (Millipore Corporation, Billerica, MA),

Table 2.1. General characteristics of samples analyzed for this study

	Borehole U1301A	*Bottom seawater*	
Year sampled	2008-2010	2008-2010	2010
Sampling apparatus	pump/bag	Niskin on rosette	Niskin on AUV
Temperature (°C)	65	~2	~2
Sampling depth (m)	2667+262+108[a]	2644-2660	~2670

Abbreviation: AUV, autonomous underwater vehicle; CORK, Circulation Obviation Retrofit Kit.
[a]Water column depth at CORK sampling spigot **+** sediment **+** permeated basement rock (Wheat *et al.*, 2010).

and subsequently stored in 2.0 ml of DNA lysis buffer at -80°C until further processing.

Seawater samples were collected in the vicinity of borehole U1301A in order to provide background controls for the borehole fluid samples (Table 2.1). In 2008, seawater from within the nepheloid layer at 2644 m depth was collected in a 10 l Niskin bottle using a conductivity-temperature-depth (CTD) guided rosette. A total of 2.6 l of fluids were filtered through a 25 mm-diameter, 0.1 µm pore-sized polyethersulfone membrane filter (Pall Corporation), and subsequently stored in 0.5 ml of DNA lysis buffer at -80°C until further processing. Also via CTD rosette, 3.8 l of seawater were collected from a depth of 2660 m in 2009, and 5.0 l of seawater were collected from a depth of 2648 m in 2010. The fluids were filtered through 0.22-µm-pore-sized Sterivex-GP filter cartridges (Millipore Corporation), filled with 2.0 ml of DNA lysis buffer, and stored at -80°C until further processing. In 2010, a 5 l Niskin bottle fitted to the ROV Jason II was used to collect 4.9 l of seawater from a depth of approximately 2660 m in the vicinity of CORK U1301A (dive 505) and processed in the same manner as other fluid samples collected in 2010.

DNA extraction

Membrane filters were thawed to room temperature prior to nucleic acid extraction. For the 2008 borehole fluid sample, environmental DNA was extracted using the PowerWater DNA isolation kit (MO BIO Laboratories, Carlsbad, CA) following the manufacturer's protocol. Environmental DNA was extracted from all other samples (2008-2010 seawater and 2009-2010 borehole fluid) via the PowerSoil DNA isolation kit (MO BIO Laboratories) following the manufacturer's protocol. Quantification of resulting genomic DNA was performed using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

SSU rRNA gene cloning and sequencing

Small subunit ribosomal RNA (SSU rRNA) gene fragments were amplified via the polymerase chain reaction (PCR) using the universal oligonucleotide forward and reverse primers 519F (5'-CAGCMGCCGCGGTAATWC-3') and 1406R (5'- ACGGGCGGTGTGTRC-3'), respectively. Each 20 µl PCR reaction contained 0.25 U of PicoMaxx high fidelity DNA polymerase (Stratagene, La Jolla, CA), 1x PicoMaxx reaction buffer, 200 µM of each of the four deoxynucleoside triphosphates (dNTPs), 200 nM of both forward and reverse primer, and ~3-4 ng of environmental DNA template. PCR cycling conditions consisted of an initial denaturation step at 95°C for 4 minutes, followed by 35 to 38 cycles of 95°C denaturation for 30 sec, 55°C annealing for 1 min, 72°C extension for 2 min, and a final extension step at 72°C for 20 min. For the 2008 borehole fluid sample, a 3-cycle reconditioning PCR was performed in order to help eliminate heteroduplexes (Thompson *et al.*, 2002). Amplification products of the anticipated length were excised from an agarose gel and subsequently purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA). Products were cloned using either the pGEM-T Easy kit (Promega, Madison, WI) or the TOPO TA Cloning kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Clones were sequenced unidirectionally on an ABI 3730XL DNA Analyzer (Applied Biosystems, Carlsbad, CA).

Phylogenetic analysis

DNA sequences were trimmed of vector sequence and manually curated using Sequencher version 4.9 software (GeneCodes, Ann Arbor, MI) and subsequently checked for chimera formation via Bellerophon (Huber *et al.*, 2004) and CHECK_CHIMERA, available from the Ribosomal Database Project (Cole *et al.*, 2005). Using the ARB software package (Ludwig *et al.*, 2004), curated clone sequences were aligned with version SSURef_106 of the SILVA ARB database (Pruesse *et al.*, 2007) modified to include short (<1200 nucleotides) environmental gene clones that were highly similar to the clone sequences obtained in this study. Phylogenetic analyses were performed with the RAxML maximum likelihood method using a gamma distributed rate variation model for nucleotide substitution (Stamatakis, 2006). Sequences of short length were added to the maximum likelihood-derived phylogenies using the parsimony insertion tool in ARB. Bootstrap analyses were determined by RAxML (Stamatakis *et al.*, 2008) via the CIPRES Portal V 3.1 (Miller *et al.*, 2010). All sequences generated in this study have been deposited in GenBank under accession numbers JX194240 - JX194738 and JX255729 - JX255730.

Microbial community analysis

The UniFrac significance test (Lozupone and Knight, 2005) was used to evaluate whether the microbial community structure was different between the three sample years. A maximum likelihood-based phylogenetic tree containing all borehole U1301A fluid environmental sequences derived in this study was evaluated using an online implementation of the hypothesis testing approach (Lozupone *et al.*, 2006). Significant differences in community structure within pairs of samples were evaluated using 100 permutations of both the weighted and unweighted implementations of the UniFrac algorithm. All p-values were corrected for multiple comparisons using the Bonferroni correction. Microbial community α-diversity estimators, rarefaction curves, and community relatedness were generated or assessed using lane-masked (α-diversity estimators, rarefaction curves) or unmasked (community relatedness) clone sequences grouped into operational taxonomic units (OTUs) defined at 99% and 97% SSU rRNA gene sequence similarity cut-off values using the nearest neighbor clustering method as implemented by the mothur software package (Schloss *et al.*, 2009). Microbial richness, evenness, and diversity were assessed by the Chao1 richness estimator (S_{chao1}) (Chao, 1984), Simpson evenness index ($E_{simpson}$) (Simpson, 1949), and the non-parametric Shannon diversity index ($\hat{H}_{shannon}$) (Shannon, 1948), respectively, as implemented in mothur (Schloss *et al.*, 2009).

Sample preparation for microscopy

In 2008, 40 ml seawater sub-samples were fixed with a final concentration of 3% formaldehyde (0.2 µm-filtered) for 2 to 4 hours at 4°C, and subsequently filtered through 0.2 µm pore-sized polycarbonate membranes (Whatman, Maidstone, United Kingdom). After air-drying, membranes were stored desiccated at -80°C until microscopic analysis. In 2009, 40 ml seawater and borehole fluid sub-samples were fixed as above, but subsequently stored at -80°C until thawed and filtered on 0.2 µm pore-sized polycarbonate membranes (Whatman). Borehole and seawater sub-samples collected in 2010 were fixed, filtered and stored as in 2008.

Probe design and optimization

Using version 106 of the SILVA ARB database (Pruesse *et al.*, 2007) and the 'probe design' function of the ARB sequence analysis software (Ludwig *et al.*, 2004), taxon-specific oligonucleotide probes were designed to target borehole fluid environmental gene clones within the *Candidatus* Desulforudis clade of the bacterial phylum *Firmicutes* (1026B3_590; 5'-TTCACCCGCGACTTCTCGG-3'), and borehole fluid clones related to the uncultivated gammaproteobacterial lineage JTB35 (1301A10_105_986; 5'-TTCTGATGATGTCAAGGGAT-3') (Table 2.2). Specificity of the probes was validated using the 'probe match' tool within the ARB

software package. Because no cultivated strains with SSU rRNA genes matching the probe target sequences were available to serve as positive controls, hybridization conditions were optimized using environmental samples: probe melting curves were constructed via whole-cell hybridizations using a range of formamide concentrations (20-60%) on high-integrity fluid samples collected from borehole U1301A in 2009 (probe 1026B3_590) and 2010 (probe 1301A10_105_986).

CARD-FISH

Catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH) was carried out according to the protocol of Grote *et al.*, (2007), modified from Sekar *et al.*, (2003) and Pernthaler *et al.*, (2002). For enumeration of cells of the domain *Bacteria*, a mix of horseradish peroxidase (HRP)-labeled oligonucleotide probes EUB338, EUB338-II, and EUB338-III were employed, while the HRP-labeled oligonucleotide probe Arch_915 was used to enumerate *Archaea* (Table 2.2). Hybridization specificity was evaluated using the HRP-labeled negative control probe NonEUB (Table 2.2). All HRP-labeled probes were obtained from Biomers.net (Ulm, Germany).

The 25 mm-diameter polycarbonate membrane filters were sectioned into one-eighth pieces and embedded in agarose as described by Pernthaler *et al.*, (2002). For hybridization reactions employing the EUB338 bacterial probe suite and probe 1026B3_590, cell permeabilization was achieved by incubating filter sections in a solution containing 10 mg ml^{-1} lysozyme, 0.05 M EDTA (pH 7.6), and 0.1 M Tris-HCl (pH 8.3) for 1 hr at 37°C. After washing once in ultrapure water, the filter sections were incubated in an achromopeptidase solution [12 U achromopeptidase, 0.01 M NaCl, and 0.01 M Tris-HCl (pH 8.3)] for 30 min at 37°C, followed by a final ultrapure water wash. Hybridization reactions employing HRP-labeled probe 1301A09_077_986 were only treated with the lysozyme solution and subsequent ultrapure water wash, while those employing HRP-labeled probe Arch_915 were permeabilized with a proteinase K solution [>120 µAU ml^{-1} proteinase K, 0.05 M EDTA (pH 7.6), 0.1 M Tris-HCl (pH 8.3)] and washed three times in ultrapure water. Following permeabilization, all filter sections were incubated for 20 min in 0.01 M HCl at room temperature in order to inactivate endogenous peroxidases, washed twice with ultrapure water, and rinsed briefly in 95% ethanol. Hybridization reactions with probes Arch_915, 1026B3_590, and 1301A10_105_986 contained a final probe concentration of 90 µM in hybridization buffer [900 mM NaCl, 20 mM Tris-HCl (pH 8.3), 20-55% formamide (Table 2.2), 0.01% SDS, 0.1 g ml^{-1} dextran sulfate, 1% blocking reagent (Roche, Basle, Switzerland)], while the EUB338 probe suite was used at a final concentration of 30 µM for each of the three probes. Hybridization reactions were performed for ca. 12 hr at 35°C in the dark on a rotary shaker. Following hybridization, the filter sections were blotted on tissue paper and transferred into pre-warmed (37°C) wash buffer [20 mM Tris-HCl (pH 8.3), 5

mM EDTA (pH 7.6), 0.01% sodium dodecyl sulfate, 13-74 mM NaCl (Table 2.2)] for 15 min. After removing excess wash buffer, filter sections were transferred to a substrate mix containing 1 part cyanine 3 (Cy3) labeled tyramides to 50 parts amplification buffer (TSA Plus Cyanine 3 System, Perkin Elmer, Waltham, MA) and incubated for 10 min at room temperature in the dark. Filter sections were subsequently dabbed on blotting paper to remove excess substrate mix and then washed once in phosphate-buffered saline (PBS) solution (23 mM NaH_2PO_4, 100 mM Na_2HPO_4, 1.3 M NaCl, pH to 7.7) for 15 min at room temperature in the dark. The filter sections were subsequently washed with ultrapure water, rinsed with 95% ethanol, and air-dried.

Fluorescence Microscopy

All filter sections were counterstained with a mounting solution [5.5 parts of Citifluor (Citifluor Ltd., London, United Kingdom), 1 part of VectaShield (Vector Laboratories, Burlingame, CA), and 0.5 parts of 1x PBS supplemented with 4',6- diamidino-2-phenylindole (DAPI) to a final concentration of 1 µg ml^{-1}] (Pernthaler *et al.*, 2002) and inspected with a Leica DM5000B epifluorescence microscope (Leica Microsystems, Wetzlar, Germany) equipped with a 100x objective (HCX PL APO CS, Leica Microsystems), filter sets appropriate for Cy3 and DAPI fluorescence, an ORCAII-ER cooled CCD camera (Hamamatsu, Hamamatsu City, Japan) and IPLab imaging software suite version 3.9.5 (Scanalytics, Rockville, MA). Between 500 and 1000 DAPI-positive objects were counted for each sample. Probe-hybridized cells were enumerated directly by overlaying Cy3 images onto corresponding DAPI images and identifying Cy3 coincident DAPI signals. Micrograph images were taken with a mounted camera setup using a uniform exposure time of 50 ms for both DAPI and Cy3 images. The detection limit of cell counts determined by DAPI staining was evaluated via enumeration of 40 ml of 0.2 µm filtered ultrapure water (Kallmeyer *et al.*, 2008). The detection limit of CARD-FISH enumeration was determined by the hybridization of a subsurface sample with the non-EUB oligonucleotide probe.

2.4 Results and Discussion

Physical and chemical characteristics of borehole U1301A fluid

The chemical characteristics of crustal fluids from borehole U1301A retrieved concomitantly with the samples analyzed in this study have been described previously (Lin *et al.*, 2012). High-integrity fluid samples were of near neutral pH (7.4–7.5), depleted in dissolved organic carbon, carbon dioxide, nitrate, sulfate and phosphate, and enriched in ammonium, silicate, calcium, and ferric and ferrous iron (Table 2.3). Magnesium concentrations serve as an

indicator of hydrothermally altered subsurface fluids (Mottl and Wheat, 1994); the high-integrity borehole fluid samples analyzed in this study were selected on the basis of depleted magnesium concentrations relative to bottom seawater (Table 2.3).

Microbial community structure

Universal oligonucleotide PCR primers were used to clone and sequence SSU rRNA genes from one high-integrity crustal fluid sample collected from borehole U1301A in each of three consecutive field seasons (2008–2010). A total of 238 (2008), 353 (2009) and 191 (2010) environmental gene clones were sequenced (Table 2.4). In addition, 72 (2008), 363 (2009) and 176 (2010) gene clones were sequenced from libraries generated from bottom seawater collected in the vicinity of borehole U1301A via conductivity, temperature and depth (CTD) rosette, and 95 environmental gene clones were sequenced from a sample of bottom seawater collected in the vicinity of borehole U1301A using a Niskin bottle attached to the remote-operated vehicle *Jason II* in 2010 (Table 2.5). Borehole fluid sample microbial communities were analyzed using a variety of α-diversity calculators and operational taxonomic units defined at 99% and 97% SSU rRNA gene sequence similarity (Table 2.6). The Shannon diversity index showed an increasing trend over the 3-year sampling period (Table 2.6), indicating an increase in community diversity over time. Rarefaction curves generated using the same operational taxonomic unit definitions were for the most part steeply sloping, indicating that the clone libraries are undersampled (Figure 2.2).

In each of the 3 years, the microbial community structure within borehole U1301A fluid was significantly different than that of its corresponding bottom seawater control(s), based on the UniFrac weighted and unweighted tests (Table 2.7). In general, borehole fluid microbial communities were dominated by clones related to microorganisms harboring physiological attributes consistent with the physical and chemical conditions of this environment (e.g., thermophiles, anaerobes, sulfate-reducers, etc.), or related to SSU rRNA gene sequences previously recovered from related environments (Table 2.4). In contrast, seawater samples were generally dominated by environmental clones related to bacterial and archaeal lineages previously recovered from seawater (Table 2.5). On the basis of the operational taxonomic unit clustering, bottom seawater and borehole fluid microbial communities shared a minor component of their respective diversity (Figure 2.3). The three borehole fluid clone libraries were also significantly different between years (Table 2.7), and were each dominated by different SSU rRNA gene lineages (Figure 2.4).

Relatives of *Candidatus* Desulforudis audaxviator, an uncultivated bacterium of the phylum *Firmicutes* (Chivian *et al.*, 2008), formed the most abundant lineage of clones recovered from borehole U1301A fluids collected in 2008 (36% of clones; Figure 2.4). This lineage was

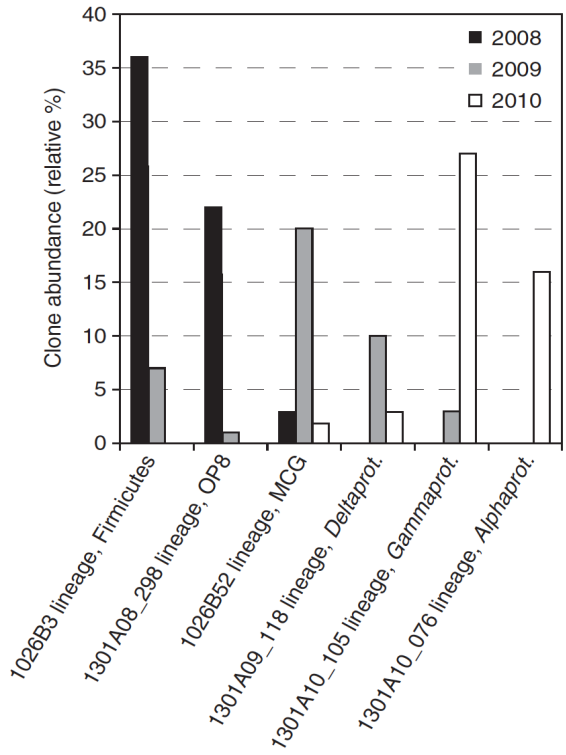

Figure 2.4 Change in abundance of the two most common SSU rRNA gene clone types recovered from borehole U1301A fluids in each year of sampling.

nearly identical (>99% similarity) to environmental gene clone sequences recovered previously from rock chips incubated within borehole U1301A (Orcutt *et al.*, 2011b) and fluids collected from borehole 1026B (Cowen *et al.*, 2003; Figure 2.5a). In addition, this lineage was also recovered from borehole U1301A fluids collected in 2009 (7% of clones; Figure 2.5a), but not in 2010 (Figure 2.4). Also abundant in the 2008 borehole U1301A clone library was a lineage within the candidate bacterial phylum OP8 (22% of clones; Figure 2.5b). This phylum was initially identified from a hydrothermal spring within Yellowstone National Park (Hugenholtz *et al.*, 1998) and has since been detected in the marine subsurface (e.g., Dhillon *et al.*, 2003; Huber *et al.*, 2006). Borehole U1301A gene clones related to the candidate phylum OP8 identified in this study formed a monophyletic lineage with clones derived from rock chips incubated within borehole U1301A (Orcutt *et al.*, 2011b) and hydrothermal sediments of Guaymas Basin (Teske *et al.*, 2002; Figure 2.5b). This lineage was also recovered from borehole U1301A fluids collected in 2009 (1% of clones; Figure 2.5b), but not 2010 (Figure 2.4). Other less abundant clone groups recovered from borehole U1301A fluid collected in 2008 include members of both the archaeal phyla and the bacterial phyla *Actinobacteria*, *Planctomycetes*, *Synergistetes*, *Proteobacteria*, *Cyanobacteria*, and the candidate phyla EM19 and SAR406/marine Group A (Table 2.4 and Figures 2.6 and 2.7). In contrast to the 2008 sample, a specific lineage within the archaeal Miscellaneous Crenarchaeotic Group (MCG; Takai *et al.*,

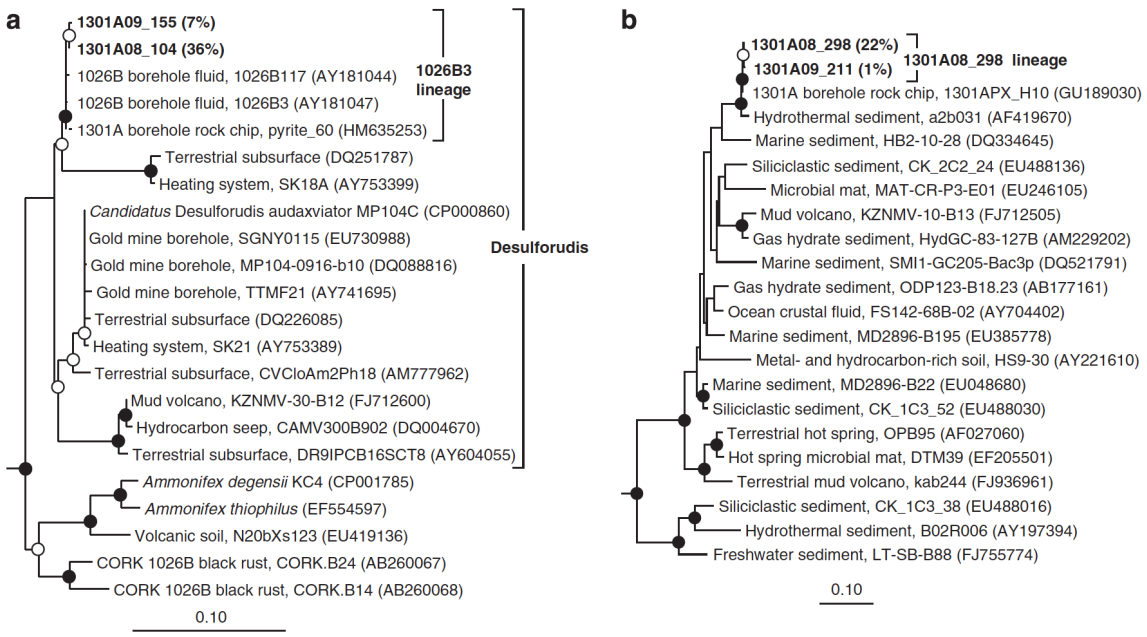

Figure 2.5 Phylogenetic relationships of borehole U1301A fluid-derived SSU rRNA gene clones related to (a) *Candidatus* Desulforudis audaxviator of the phylum *Firmicutes* and (b) candidate phylum OP8. Open circles indicate nodes with bootstrap support between 50–80%, whereas closed circles indicate bootstrap support >80%, from 1000 replicates. Gene clones recovered in this study are highlighted in bold font; the relative abundance of identical clones recovered from the same sampling year is listed in parentheses. Gene clones that were targeted for fluorescence *in situ* hybridization using oligonucleotide probe 1026B3_590 are included within the '1026B3 lineage' bracket. Cultivated *Firmicutes* (a) and *Thermoanaerobacter* (b) were used as outgroups (data not shown). The scale bars correspond to 0.1 substitutions per nucleotide position. Relevant gene clones of short length were added after tree construction and bootstrapping, and are indicated by dashed lines.

2001; Inagaki *et al.*, 2003a), referred to here as the 1026B52 lineage, was the most abundant group of clones recovered from borehole U1301A fluids collected in 2009 (20% of clones; Figure 2.4). The 1026B52 lineage included a group of clones recovered previously from borehole 1026B fluids (Cowen *et al.*, 2003), as well as clones recovered from borehole U1301A fluids collected in 2008 and 2010 (Figure 2.8a). In addition to the highly abundant 1026B52 lineage, several less abundant lineages within the MCG were recovered in all three sampling years (Figure 2.8a). Also abundant in the 2009 borehole U1301A clone library was a lineage related to the genus *Desulfocapsa* within the *Deltaproteobacteria* (10% of clones; Figure 2.4). Closely related clones were also recovered from borehole U1301A fluids collected in 2010, as well as gene clones recovered from such environments as an anaerobic methane-oxidizing sulfate-reducing enrichment (Jagersma *et al.*, 2009), marine sediments of a hydrocarbon seep (Lloyd *et al.*, 2010) and a mud volcano (Pachiadaki *et al.*, 2010) (Figure 2.8b). Other less abundant clone groups recovered from borehole U1301A fluid collected in 2009 include members of both the archaeal phyla and the bacterial phyla *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Fusibacter*,

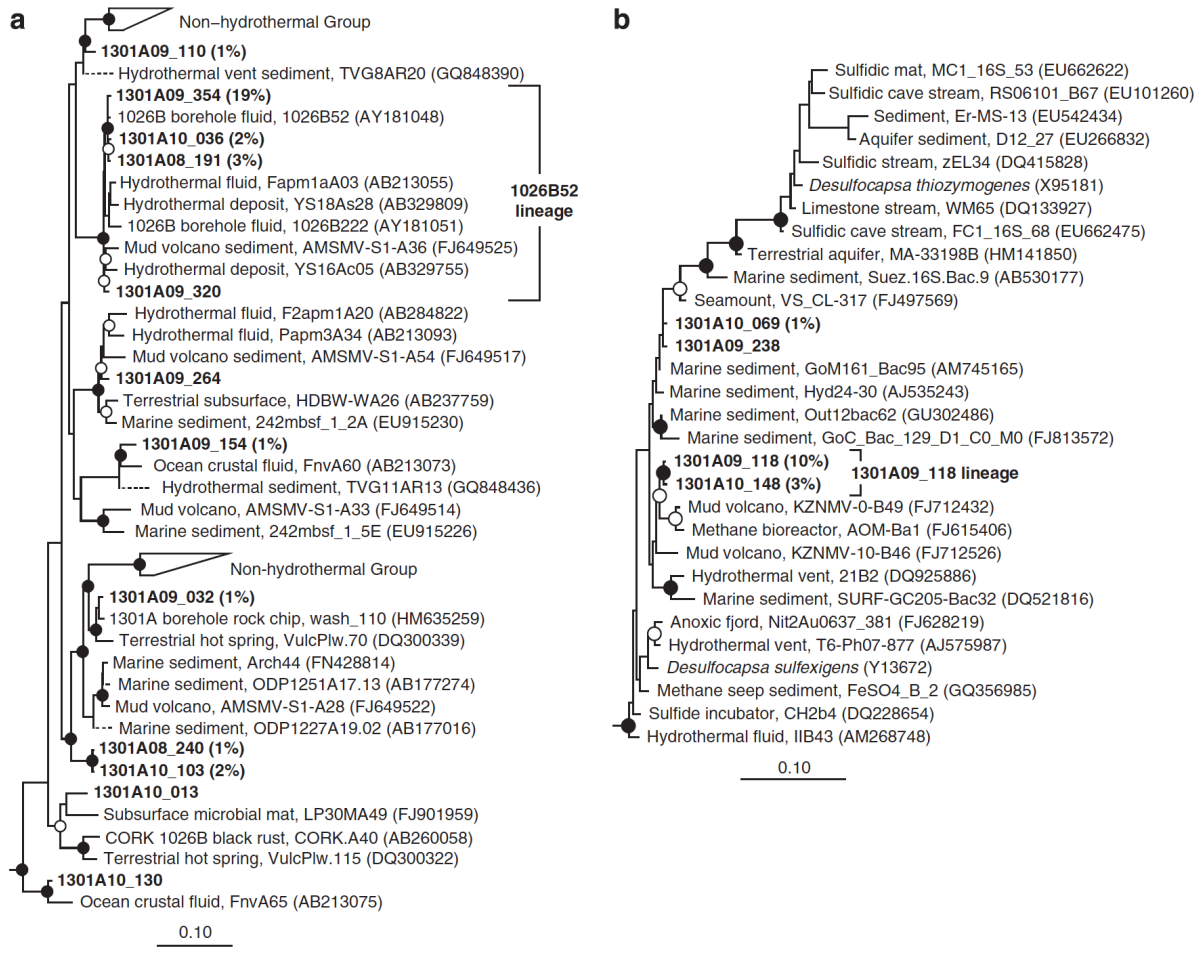

Figure 2.8 Phylogenetic relationships of borehole U1301A fluid-derived SSU rRNA gene clones related to (a) the Miscellaneous Crenarchaeotic Group and (b) *Desulfocapsa* of the *Deltaproteobacteria*. Cultivated *Thermoprotei* (a) and *Desulfobulbus* (b) were used as outgroups (data not shown). Other information as in Figure 2.5.

Verrucomicrobia, *Proteobacteria*, *Cyanobacteria*, and the candidate phyla RF3 and OP8 (Table 2.4 and Figures 2.6 and 2.7).

The most abundant gene clone group identified from borehole U1301A fluids collected in 2010 was phylogenetically affiliated with a lineage of uncultivated *Gammaproteobacteria* (27% of clones; Figure 2.4) and were most closely related to environmental gene clones recovered from hydrothermal sediments (Orcutt *et al.*, 2010; Pachiadaki *et al.*, 2010) and basaltic ocean crust (Santelli *et al.*, 2008; Figure 2.9a). Closely related clones were also recovered from borehole U1301A fluids collected in 2009 (Figures 2.4 and 2.9a). Also abundant in the 2010 borehole U1301A clone library was a lineage related to the family *Hyphomicrobiaceae* of the *Alphaproteobacteria* (16% of clones; Figure 2.4). Close relatives of this gene clone lineage have been recovered from hydrothermal vent chimneys (Suzuki *et al.*, 2004; Voordeckers *et al.*, 2008; Figure 2.9b). Other less abundant clone groups recovered from

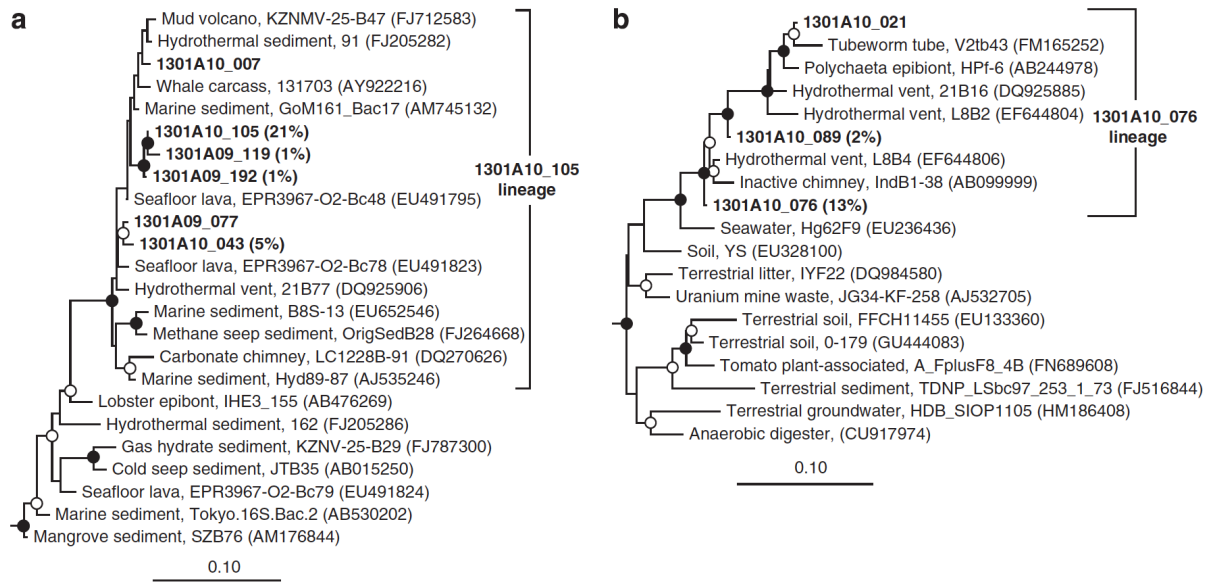

Figure 2.9 Phylogenetic relationships of borehole U1301A fluid-derived SSU rRNA gene clones related to (a) the JTB35 lineage of *Gammaproteobacteria* and (b) the *Hyphomicrobiaceae* lineage of *Alphaproteobacteria*. Cultivated *Methylobacter* sp. (a) and distantly related *Hyphomicrobiaceae* (b) were used as outgroups (data not shown). Gene clones that were targeted for fluorescence *in situ* hybridization using oligonucleotide probe 1301A10_105_986 are included within the '1301A10_105 lineage' bracket. Other information as in Figure 2.5.

borehole U1301A fluid collected in 2010 include members of the archaeal phylum *Crenarchaeota* and the bacterial phyla *Bacteroidetes*, *Fusibacter*, *Planctomycetes*, *Verrucomicrobia*, *Proteobacteria*, and the candidate phylum RF3 (Table 2.4 and Figures 2.6 and 2.7).

The three borehole U1301A fluid environmental SSU rRNA gene clone libraries shared four clone groups in common (Table 2.4). Three of these groups were most closely related to microorganisms harboring physiological attributes consistent with the physical and chemical conditions of this environment or related to SSU rRNA gene sequences previously recovered from similar environments, and included the archaeal MCG lineage, the genus *Sulfurimonas* within the *Epsilonproteobacteria*, and the genus *Thiomicrospira* within the *Gammaproteobacteria*. Within the genus *Sulfurimonas*, environmental gene clones derived from borehole U1301A fluids did not form a monophyletic lineage, but were all closely related to environmental gene clones derived from a variety of sediment, cold seep and oceanic crustal environments (e.g., Li *et al.*, 1999; Huber *et al.*, 2006; Sudek *et al.*, 2009; Figure 2.10a). Within the genus *Thiomicrospira*, environmental gene clones derived from borehole U1301A fluids formed a monophyletic lineage that was closely related to environmental gene clones originating from hydrothermal fluids (Huber *et al.*, 2006; Kato *et al.*, 2009; Figure 2.10b). The fourth clone group shared by all the three borehole U1301A fluid clone libraries was phylogenetically affiliated with the ubiquitous SAR11 clade of planktonic marine

Alphaproteobacteria (Morris *et al.*, 2002). Environmental SSU rRNA gene clone libraries from borehole U1301A fluids collected in 2008 and 2010 did not share any clone groups in common that were also not shared with the 2009 sample.

Direct cell counts and CARD-FISH

After staining with the DNA-specific dye DAPI (4',6- diamidino-2-phenylindole), microscopic examination of borehole U1301A fluid samples revealed average microbial cell abundances of 8.98 x 10^3 (2009) and 1.53 x 10^4 (2010) cells ml^{-1} (Table 2.8). These values ranged from 9.5 (2009) to 20.1% (2010) of the microbial cell abundance measured in identically processed bottom seawater samples (Table 2.8). The majority of cells within borehole U1301A fluids appeared as single, free-living cells that were not clumped or otherwise associated with particles (Figure 2.11 and data not shown). However, dense clusters of cells that appeared attached to small (5–10 µm in diameter) particles were occasionally observed (data not shown). Taxon-specific enumeration via catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH) revealed *Bacteria* and *Archaea* to each make up ca. 30% of the total microbial cells (as determined by DAPI) in borehole U1301A fluids collected in 2009 (Table 2.8). In 2010, bacterial cells made up nearly 41% of the total counts, whereas archaeal cells accounted for 12% (Table 2.8). Archaeal cells were predominantly ca. 1 µm diameter cocci, whereas bacterial cells were of diverse morphology (Figure 2.11).

Two clone groups found to be abundant in borehole U1301A fluid libraries were specifically targeted for enumeration via CARD-FISH (Table 2.2). The gene clone lineage related to *Candidatus* Desulforudis audaxviator of the bacterial phylum *Firmicutes* made up 32% of the bacterial cells in borehole U1301A fluids collected in 2009. Consistent with the SSU rRNA gene clone library findings, this lineage was below the limit of detection (276 ± 79 cells ml^{-1}) in borehole U1301A fluid collected in 2010. Cells hybridized with the *Candidatus* Desulforudis audaxviator-specific oligonucleotide probe were ca. 1-5 µm rods (Figure 2.11). The gene clone lineage related to an uncultivated group, JTB35, of the *Gammaproteobacteria* made up 12% of the bacterial cells in borehole U1301A fluids collected in 2009, and 9% of the bacterial cells in fluids collected in 2010 (Table 2.8). Cells hybridized with the JTB35-related oligonucleotide probe specific to the borehole clones retrieved in this study were ca. 1–2 µm long and ovoid in shape (Figure 2.11). The *Candidatus* Desulforudis audaxviator and JTB35 gene clone lineages were not detected in bottom seawater samples collected in either 2009 or 2010.

Discussion

Few direct measurements are available to constrain the magnitude of microbial biomass residing within basalt-hosted deep subseafloor basement aquifers (Cowen *et al.*, 2003; Huber *et*

al., 2006). This study is the first to utilize a second-generation CORK-II equipped with a stainless steel fluid delivery line that, after flushing, yielded uncontaminated ocean crustal fluid samples derived directly from the crustal aquifer. The chemical signature of borehole U1301A fluids (Lin *et al.*, 2012) is consistent with previous results from basement fluids collected from borehole 1026B (Wheat *et al.*, 2000; Cowen *et al.*, 2003) and the Baby Bare ridge flank outcrops (Mottl *et al.*, 1998; Sansone *et al.*, 1998; Wheat *et al.*, 2000). In this case, efforts to ensure sampling of pristine crustal fluids produced cellular abundances from borehole U1301A that are 83–93% less than previous reports from borehole 1026B (Cowen *et al.*, 2003; Huber *et al.*, 2006) and 59–67% less than Baby Bare Seamount-derived crustal fluid (Huber *et al.*, 2006). In addition to the lower cell counts, flocs and clumps of cells noted previously in borehole 1026B fluids (Cowen *et al.*, 2003; Huber *et al.*, 2006) were rarely observed in the borehole U1301A fluid samples. Differences in cellular abundance measurements between the two boreholes are most readily explained by differences in CORK design: the CORK affixed to borehole U1301A greatly reduces the possibility of fluid interaction with casing cement and steel liners (Fisher *et al.*, 2005a; Wheat *et al.*, 2011), and the potential for sampling biofilms attached to these materials. However, because the borehole U1301A and Baby Bare Seamount samples used for direct cell counts yielded floc-free fluid with chemical signatures, indicating that they were high-integrity crustal fluids, the differences in cellular abundance may reflect *bona fide* differences between the two sites. This is further supported by differences in microbial community structure, as microorganisms prevalent in seawater made up a large fraction of the Baby Bare fluid-derived microbial community (Huber *et al.*, 2006), but were rare in fluids derived from borehole U1301A. Thus, this study provides robust data that constrains the magnitude of microbial communities residing in the hydrothermal fluids of the deep-ocean crustal biosphere, albeit at a lower quantity than previous estimates.

Unexpectedly, significant differences in microbial community structure were observed over three consecutive years of sampling crustal fluids from borehole U1301A. Between years, overall community structure was statistically different, the most abundant microbial lineages changed, and absolute cellular abundances of both *Bacteria* and *Archaea* varied. This suggests a temporally dynamic microbial community. Borehole U1301A was drilled, cased and equipped with a CORK during the summer of 2004. From the time of drilling until September 2007, bottom seawater flowed into the borehole, resulting in fluids that possessed chemical characteristics identical to the surrounding seawater environment (Wheat *et al.*, 2010). Abruptly (i.e., within 1 week), fluid flow reversed, resulting in the production of warm fluids of a chemical composition consistent with the high-integrity basement fluids (Wheat *et al.*, 2010). It is feasible, perhaps even likely, that successional changes in basement fluid microbial community structure in response to such an abrupt change in chemistry are much more gradual, resulting in the inter-annual differences observed here. The observed temporal variation is, perhaps, not unlike the temporal succession of microbial communities thought to

occur during the active-to-inactive transition of hydrothermal chimneys and associated venting fluids (e.g., Hentscher and Bach, 2012; Sylvan *et al.*, 2012). However, it is also feasible that the variability in community structure is unrelated to the specific turnaround event experienced in September 2007. Instead, it may reflect not well-understood patchiness in the physical and/or chemical characteristics of this system, or result from changes in our methodological approach from year to year (Lin *et al.*, 2012). Continued temporal sampling should help to identify and differentiate stochastic and deterministic processes influencing the structure of microbial communities inhabiting borehole U1301A fluids.

Despite inter-annual variability, the microorganisms found to be abundant in borehole U1301A SSU rRNA gene surveys bared close phylogenetic relationships with cultivated anaerobic thermophiles and/or environmental gene clones previously recovered from related environments, indicating that they are plausible inhabitants of the deep subsurface environment. Environmental gene clones baring close phylogenetic relationships with clones or isolates of apparent seawater origin were infrequent and made up only a small fraction of the libraries originating from the borehole U1301A fluids. One abundant lineage in the borehole U1301A fluid samples from 2008 and 2009 (though curiously not detected by SSU rRNA gene clone library analysis or CARD-FISH in 2010) was nearly identical to the most abundant clone lineage recovered in fluid samples retrieved from borehole 1026B in 1998 (Cowen *et al.*, 2003). This marine crustal fluid lineage forms a monophyletic clade with *Candidatus* Desulforudis audaxviator and a variety of other environmental gene clones of terrestrial (Moser *et al.*, 2005; Lin *et al.*, 2006; Chivian *et al.*, 2008) and marine (Gihring *et al.*, 2006; Nakagawa *et al.*, 2006; Niemann *et al.*, 2006; Pachiadaki *et al.*, 2010; Orcutt *et al.*, 2011b) deep subsurface origin. *Candidatus* Desulforudis audaxviator is the tentative name given to the uncultivated bacterium possessing a composite genome constructed from metagenomic DNA sequence data of South African gold mine fracture water origin (Chivian *et al.*, 2008). The 2.35 Mbp genome revealed a motile, sporulating thermophilic chemolithoautotroph capable of sulfate reduction, nitrogen fixation and carbon fixation via the acetyl coenzyme-A synthesis pathway (Chivian *et al.*, 2008), which makes it well suited for an independent lifestyle within the deep subsurface crustal environment. In future studies, it will be valuable to determine the extent to which these metabolic features are shared between abundant members of these distinct but globally related environments, using whole-genome sequence data from the subseafloor-dwelling relatives of *Candidatus* Desulforudis audaxviator.

The relative percentage of archaeal cells detected within the borehole U1301A fluids was significantly higher than had been measured previously from borehole 1026B and Baby Bear Seamount (12–30% vs <2%; Huber *et al.*, 2006). Although this may partially be explained by improvements in methodology (i.e., FISH vs CARD-FISH), analysis via SSU rRNA gene clone libraries supports the conclusion that the differences were not simply methodological. Gene clones related to a major lineage of uncultivated *Crenarchaeota* known as the MCG (Takai *et al.*,

2001; Inagaki *et al.*, 2003a) were recovered from borehole U1301A fluids in all three sampling years, as well as from borehole 1026B fluids sampled in 2000 (Cowen *et al.*, 2003) and other ocean crustal environments (Huber *et al.*, 2006; Nakagawa *et al.*, 2006; Kato *et al.*, 2009; Orcutt *et al.*, 2011b). The MCG is a diverse microbial assemblage that has been recovered from a wide variety of terrestrial and marine habitats (Teske and Sørensen, 2008), and some evidence indicates that it may be heterotrophic (Biddle *et al.*, 2006; Webster *et al.*, 2010). Consistent with this phylogenetic diversity, recent evidence from fosmid gene clones recovered from marine sediments indicated little gene-order consistency with known archaeal genomes and genomic fragments (Li *et al.*, 2012).

In addition to the abundant *Candidatus* Desulforudis audaxviator and MCG lineages, several other interesting clone groups worthy of subsequent investigation have emerged from this study. Gene clones related to the candidate bacterial phylum OP8 were detected in consecutive years from borehole U1301A fluids, and have previously been detected in crustal fluids from Baby Bare Seamount (Huber *et al.*, 2006) and rock chips incubated within borehole U1301A (Orcutt *et al.*, 2011b). Gene clones related to the genus *Sulfurimonas* were recovered in all the three borehole U1301A fluid samples characterized here, which is consistent with their recovery from other chemically reducing environments, such as mid-ocean ridge spreading centers (Santelli *et al.*, 2008), crustal fluids (Huber *et al.*, 2006), whale falls (Tringe *et al.*, 2005) and pelagic redoxclines (Grote *et al.*, 2008). Although evolutionary relatedness between the gene clones identified here and the cultivated representatives of the genus *Sulfurimonas* is distant, characterized strains are capable of chemolithoautotrophic processes, such as sulfur oxidation and thiosulfate oxidation (Inagaki *et al.*, 2003b; Takai *et al.*, 2006). Members of the *Thiomicrospira* lineage of *Gammaproteobacteria* were also recovered in all the three borehole U1301A fluid samples, and characterized isolates of this group are also chemolithoautotrophic sulfur oxidizers (e.g., Jannasch *et al.*, 1985; Knittel *et al.*, 2005).

Although we identified a number of microbial lineages that appear to be common inhabitants of the subseafloor crustal fluid environment, none appear to fit within the Ocean Crustal Clade lineages defined previously (Mason *et al.*, 2007, 2009). As the nature of these studies differed (basalt surface-attached vs basalt-hosted fluids), it is possible that, like the marine water column (e.g., DeLong *et al.*, 1993), distinct surface-attached and planktonic microbial communities exist within the basaltic ocean crust.

In this study, we focused on cloning and sequencing of PCR-amplified SSU rRNA genes rather than next-generation, deep sequencing of rRNA gene amplicons (e.g., Sogin *et al.*, 2006) because of the increase in sequence length afforded by the more traditional approach. Although a massively parallel sequencing strategy would enable much deeper sampling of individual microbial communities than could routinely be achieved by cloning and sequencing, and thus a more comprehensive assessment of the diversity, evenness and community structure contained within our samples (thousands vs hundreds of sequences; Sogin *et al.*,

2006), limitations to the sequencing technology currently restrict it to a short fragment of the 16S rRNA gene. As little is known regarding the diversity of microorganisms inhabiting the deep subsurface basement biosphere, we chose to maximize the phylogenetic signal we could obtain from representative environmental sequences, so that they could serve as quality reference points for future, potentially high-throughput, studies. In addition, longer representative sequences are useful for the identification of taxon-specific regions of variability that can be targeted by oligonucleotide probes in whole-cell *in situ* assays, such as CARD-FISH, or via quantitative PCR assays targeting extracted nucleic acids. In all 3 years, estimations of richness and rarefaction analysis reveal that there is more microbial diversity to be discovered if deeper sequencing were performed.

The study presented here results from continually evolving sampling equipment and methodology, which serve as a source of variability that may impact our findings or their interpretation. Over the course of the study, the pumping system became better sealed from intruding seawater, permitting a steady increase in the borehole fluid temperature at which sampling began, and an increase in the volume of high-integrity borehole fluid samples. Instead of using a multi-day *in situ* filtration (Cowen *et al.*, 2003), a clean pump was used here to fill large-volume sampling bags over the course of hours; this relatively rapid sampling method appears to have minimized the contaminating effects of surrounding seawater as evident by the collection of samples that appear to be of pristine ocean basement crustal fluid (Cowen *et al.*, 2012; Lin *et al.*, 2012). The evolution of cleaner sampling equipment and methodology are positive developments for the field of research and represent the best window available into the deep subsurface oceanic crust.

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2.6 Appendix

Table 2.2 Characteristics of the oligonucleotide probes used for fluorescence *in situ* hybridization

Name	*Probe sequence (5'-3')*	*Target region*[a]	*Hyb. FA (%)*[b]	*Wash NaCl (mM)*[c]	*Specificity*	*Reference*
EUB_338 EUB_338 II EUB_338 III	GCTGCCTCCCGTAGGAGT GCAGCCACCCGTAGGTGT GCTGCCACCCGTAGGTGT	338-355	55	13	Domain *Bacteria*	Amann *et al.,* 1990; Daims *et al.,* 1999
Arch_915	GTGCTCCCCCGCCAATTCCT	915-934	55	13	Domain *Archaea*	Stahl and Amann, 1991
NonEUB	ACTCCTACGGGAGGCAGC	n.a.	55	13	Negative control	Wallner *et al.,* 1993
1026B3_590	TTCACCCGCGACTTCTCGG	590-608	50	19	1026B117 lineage	this study
1301A09_077_986	TTCTGATGATGTCAAGGGAT	986-1005	30	74	1301A09_077 lineage	this study

[a]*Escherichia coli* 16S rRNA positions
[b]Percent formamide (v/v) in hybridization reaction
[c]Concentration of NaCl in wash buffer
n.a., not applicable

Table 2.3 Chemical characteristics of fluid samples collected from borehole U1301A and background seawater

	Borehole U1301A			*Bottom Seawater*
	2008	*2009*	*2010*	*2008-2010*
pH	7.5	7.4	7.4	7.7
Ca^{2+} (mM)	51.7	54.8	53.0	10.4
Mg^{2+} (mM)	6.9	2.0	3.4	53.7
K^{+} (mM)	6.4	6.0	6.4	10.2
CH_4 (µM)	1.95[a]	n.d.	1.83	n.d.
H_2 (µM)	0.65[a]	n.d.	0.48	n.d.
NH_4^{+} (µM)	98	102	100	<0.05
PO_4^{3-} (µM)	0.45	0.10	0.14	2.89
NO_3^{-2} (µM)	4.7	0.6	1.5	40.8
SO_4^{2-} (mM)	18.7	17.2	18.3	28.4
Fe_{aq} (µM)	0.8	1.1	0.63	<0.1
DOC (µM)	20.6	12.9	12.0	39
Alkalinity (meq/L)	0.73	0.42	0.52	2.48

Abbreviations: DOC, dissolved organic carbon; n.d., not determined.

[a]Determined from gas sample collected at CORK borehole 1026B.

Table 2.4 Summary of SSU rRNA gene clones recovered from borehole U1301A fluids

Phylogenetic affiliation[a]	*2008 (n=238)*	*2009 (n=353)*	*2010 (n=191)*	*Representative clones*
Archaea				
Crenarchaeota				
20a_6 (THSCG)	<1	0	0	08_170
Group C3	0	0	<1	10_110
Marine Group I	0	<1	0	09_234, 09_286, 09_227
Misc. Crenarchaeotic Group	5	23	4	08_191, 08_316, 08_109; 09_110, 09_264, 09_354, 09_162, 09_059, 09_254; 10_036, 10_107, 10_126, 10_013, 10_130
Euryarchaeota				
Candidatus *Paravarchaeum*	0	<1	0	09_151, 09_098
Miscellaneous Euryarcheotic Group	<1	<1	0	08_341; 09_024
Bacteria				
Actinobacteria				
Propionibacterium	6	0	0	08_096, 08_283, 08_199, 08_133
OCS155	0	<1	0	09_082
Bacteroidetes				
AL-5	0	0	<1	10_121
B-68	0	0	2	10_48
Crocinitomix	0	0	2	10_112
CS_B045	0	<1	2	09_114; 10_160
Elizabethkingia	0	0	<1	10_172
F4C34	0	0	2	10_100, 10_094

Lutibacter	0	0	1	10_169, 10_161
Marinoscillum	0	<1	0	09_206
NB1-m	0	0	2	10_142
NS5	0	<1	0	09_172
Owenweeksia	0	0	1	10_168, 10_039
Reichenbachiella	0	0	3	10_129
Sediminibacterium	0	<1	0	09_170
Cyanobacteria				
Synechococcus	<1	<1	0	08_347; 09_152
Deferribacteres				
SAR406	<1	0	0	08_150
EM19	<1	0	0	08_228
Firmicutes				
Candidatus *Desulforudis*	36	7	0	08_104; 09_155
RF3	0	2	2	09_001; 10_033
Fusibacter	0	2	1	09_115, 09_271, 09_058, 09_230; 10_079, 10_113
Nitrospirae				
Candidate Division OP8	22	1	0	08_298, 09_211
Planctomycetes				
BD7-11	3	0	0	08_346
OM190	0	0	<1	10_186
Planctomyces	0	0	1	10_018, 10_159
SM1A02	0	0	3	10_032, 10_149
Proteobacteria				
Alphaproteobacteria				

DB1-14	0	0	<1	10_162
Hyphomicrobiaceae	0	0	16	10_021, 10_089, 10_076
IndB1-2	0	0	<1	10_027
Larval intestine clone D	1	0	0	08_142
Pelagicola	0	0	2	10_163
Rhizobium	0	<1	0	09_276
Rhodobacteraceae	0	0	<1	10_193
Robiginitomaculum	0	0	3	10_120
SAR11	4	5	1	08_220, 08_332, 08_167, 08_299; 09_340, 09_147, 09_089, 09_040, 09_078, 09_052, 09_133, 09_131, 09_095, 09_050, 09_148, 09_010, 09_054; 10_098
SB1-18	0	0	<1	10_132
Sphingomonadaceae	0	<1	0	09_278
Sphingopyxis	0	0	<1	10_116
Betaproteobacteria				
Achromobacter	0	<1	0	09_028
Azospira	0	<1	0	09_346
Cupriavidus	0	4	0	09_220, 09_223, 09_139
Herbaspirillum	0	8	0	09_101, 09_0127
Herminiimonas	0	<1	0	09_352
OM43	0	<1	0	09_200
Ralstonia	0	<1	0	09_045
Undibacterium	4	0	0	08_318
Deltaproteobacteria				
CWTST01_F02	0	<1	0	09_261

Desulfobacula	0	0	<1	10_185
Desulfocapsa	0	10	4	09_238, 09_118; 10_069, 10_148
Desulfonatronaceae	<1	0	0	08_215
Desulfovibrionaceae	2	0	0	08_212
Desulfuromusa	0	0	<1	10_134
Desulfurivibrio	0	<1	1	09_159, 10_131
Nannocystineae	0	0	<1	10_122
Nitrospinaceae	0	<1	0	09_307
SAR324	0	<1	0	09_031
Smithella	0	<1	0	09_255
Epsilonproteobacteria				
Campylobacteraceae	0	<1	0	09_076
Sulfurimonas	<1	7	<1	08_324; 09_042, 09_196, 09_163, 09_362; 10_057
Sulfurovum	0	<1	0	09_125
Gammaproteobacteria				
9NBGBact_8	0	<1	0	09_090
Aeromonadaceae	0	<1	0	09_329, 09_301, 09-229
Alcanivorax	3	0	0	08_152
Alteromonadaceae	<1	0	0	08_213
Arctic96BD-19	0	2	0	09_053, 09_250, 09_363, 09_327
Colwelliaceae	0	1	<1	09_026, 09_079, 09_005, 09_258; 10_165
Halomonadaceae	3	0	0	08_291, 08_190
HTCC2089	0	<1	0	09_181
Hyd24-012	0	0	<1	10_054
Idiomarinaceae	0	0	<1	10_157

IndB3-17	0	<1	0	09_292
JTB35	0	3	27	09_192, 09_119, 09_077; 10_007, 10_105, 10_043
JTB255	0	0	<1	10_179
Marinobacter	2	0	0	08_227
Methylophaga	0	0	1	10_144
Moraxellaceae	<1	0	0	08_231, 08_246
OM182	0	<1	3	09_360, 09_235; 10_010, 10_001
Pseudoalteromonadaceae	0	3	0	09_165, 09_193, 09_275, 09_361, 09_081, 09_259
Pseudomonas	0	<1	0	09_056, 09_083
SAR156	0	<1	0	09_157
Sedimenticola	0	<1	0	09_137
Shewanellaceae	0	<1	0	09_002, 09_017, 09_283
Thiomicrospira	2	<1	2	08_309; 09_087; 10_004
Xanthomonadaceae	2	6	0	08_180 08_168; 09_349, 09_191, 09_179, 09_222, 09_274, 09_365
ZA2202c	0	0	5	10_051
ZD0417	0	<1	0	09_184
Synergistetes				
KCL50b_15_16	<1	0	0	08_256
Verrucomicrobia				
DEV007	0	0	1	10_174
Roseibacillus	0	<1	0	09_022
Eukaryotes				
Metazoa				
Rhizaria				

Radiolaria	0	<1	<1	09_249, 09_325; 10_046
Polycystinea	0	<1	0	09_070

[a]Phylogenetic affiliations were derived from SILVA SSU database release 106. In cases where taxonomic affiliations were obviously incorrect (i.e. polyphyletic), groups were named after the first gene clone derived from a particular lineage

Table 2.5 Summary of SSU rRNA gene clones recovered from background seawater near borehole U1301A

	Rosette (i.e. shipboard)			*ROV*	
Phylogenetic affiliation[a]	*2008 (n=72)*	*2009 (n=363)*	*2010 (n=176)*	*2010 (n=95)*	*Representative clones*
Archaea					
Crenarchaea					
Marine Group I	4	<1	11	4	08sw_037, 08sw_005, 08sw_070; 09sw_283, 09sw_284; 10sw_024, 10sw_132, 10sw_122, 10sw_131, 10sw_086, 10sw_071; 10jsw_048, 10jsw_089
Euryarchaeota					
Marine Group II	0	0	<1	2	10sw_011; 10jsw_061
Bacteria					
Acidobacteria					
DA023	0	0	1	0	10sw_060, 10sw_144
Actinobacteria					
Mycobacterium	0	0	<1	0	10sw_111
Nocardioides	0	0	1	0	10sw_043, 10sw_173
Pseudonocardia	0	0	<1	0	10sw_142
Sva0996	1	0	0	0	08sw_056
Bacteroidetes					
Brumimimicrobium	0	0	0	1	10jsw_005
Crocinitomix	0	0	<1	0	10sw_085
Elizabethkingia	0	0	0	1	10jsw_004
Flavobacteriaceae	1	0	0	0	08sw_009
Marinoscillum	1	0	0	0	08sw_088

NS2b	1	0	0	0	08sw_079
NS5	0	<1	0	0	09sw_013
NS9	4	0	<1	0	08sw_080, 08sw_087; 10sw_169
Candidate Division BRC1	0	0	2	0	10sw_007, 10sw_091
Chloroflexi					
SAR202	1	0	6	2	08sw_022; 10sw_050, 10sw_067, 10sw_005, 10sw_093; 10jsw_066
TK10	0	0	<1	1	10sw_137
Deferribacteres					
SAR406	10	2	2	6	08sw_035, 08sw_013, 08sw_043, 08sw_065, 08sw_032; 09sw_079, 09sw_126, 09sw_281
Gemmatimonadetes	0	0	<1	1	10sw_146; 10jsw_055
Planctomycetes					
028H05-P-BN-P5	1	0	0	0	08sw_027
Blastopirellula	1	0	0	0	08sw_029
CL500-3	1	0	0	0	08sw_034
OM190	0	<1	0	0	09sw_050, 09sw_154
Planctomyces	1	0	<1	0	08sw_015; 10sw_018
Proteobacteria					
Alphaproteobacteria					
AEGEAN-169	1	0	0	0	08sw_050
Rhodobium	0	0	<1	0	10sw_042
Rhodospirillaceae	0	0	5	0	10sw_152, 10sw_109, 10sw_140, 10sw_013, 10sw_159, 10sw_124

SAR11	26	2	1	13	08sw_066, 08sw_002, 08sw_004, 08sw_072, 08sw_028, 08sw_031, 08sw_019, 08sw_024, 08sw_014, 08sw_089, 08sw_052, 08sw_061; 09sw_002, 09sw_165, 09sw_240, 09sw_198, 09sw_343, 09sw_070; 10jsw_051, 10jsw_071, 10jsw_016, 10jsw_068, 10jsw_053, 10jsw_002, 10jsw_082
Sneathiella	0	0	<1	0	10sw_180
Betaproteobacteria					
OM43	1	31	0	0	08sw_053; 09sw_211, 09sw_053, 09sw_202, 09sw_214, 09sw_356, 09sw_317, 09sw_302
Deltaproteobacteria					
JTB38	1	0	2	0	08sw_068; 10sw_039, 10sw_143
Nannocystaceae	0	0	1	0	10sw_076, 10sw_156
Nitrospinaceae	4	<1	4	0	08sw_083, 08sw_051; 10sw_027, 10sw_155, 10sw_083, 10sw_047
OM27	0	0	1	0	10sw_066, 10sw_121
SAR324	6	1	37	6	08sw_010, 08sw_062; 09sw_175; 10sw_062, 10sw_026, 10sw_033
Gammaproteobacteria					
AGG47	3	0	4	1	08sw_018, 08sw_055; 10sw_171, 10sw_056
Alcanivorax	1	0	1	0	08sw_023; 10sw_065
ALO40	0	0	<1	0	10sw_078
Arctic96BD-19	4	<1	1	4	08sw_012, 08sw_084
CF-26	0	0	0	2	10jsw_026
Colwelliaceae	0	<1	0	0	09sw_042
Granulosicoccus	0	0	<1	0	10sw_181

H2_10.4_2	0	0	<1	0	10sw_107
JTB23	1	0	0	0	08sw_058
NB-1d	0	0	<1	0	09sw_090
OM60	0	0	<1	0	10sw_135
OM182	1	0	1	5	08sw_040; 10sw_129, 10sw_031; 10jsw_006
Pseudoalteromonadaceae	0	<1	<1	19	09sw_176; 10sw_138; 10jsw_086, 10jsw_041, 10jsw_024, 10jsw_088, 10jsw_096, 10jsw_077, 10jsw_012, 10jsw_063, 10jsw_029, 10jsw_015
SAR156	3	<1	0	3	08sw_045; 09sw_289
Sinobacteraceae	0	0	<1	0	10sw_104
SUP05	0	0	0	4	10jsw_057
ZA2202c	0	<1	0	0	09sw_047
ZD0417	0	<1	<1	2	09sw_039; 10sw_069; 10jsw_025
Verrucomicrobia					
Arctic97B-4	1	0	5	3	08sw_073; 10sw_118, 10sw_080; 10jsw_058
MB11C04	0	0	<1	0	10sw_025
Roseibacillus	0	0	2	0	10sw_168
Eukaryotes					
Alveolata	3	19	<1	2	08sw_039, 08sw_047; 09sw_231, 09sw_162, 09sw_016, 09sw_267, 09sw_119, 09sw_156, 09sw_040, 09sw_288, 09sw_146, 09sw_141, 09sw_145, 09sw_152, 09sw_067, 09sw_066, 09sw_054, 09sw_099, 09sw_206, 09sw_233, 09sw_239, 09sw_103, 09sw_243, 09sw_171, 09sw_127, 09sw_250, 09sw_100, 09sw_024, 09sw_089, 09sw_037, 09sw_062
Excavata					

Discoba	0	<1	0	0	09sw_265
Fungi					
Hypocreales	0	0	0	14	10sw_004
Hacrobia					
Picobiliphyta	0	<1	0	0	09sw_017
Metazoa					
Annelida	0	<1	<1	0	09sw_011; 10sw_017
Arthropoda	0	<1	0	1	09sw_095; 10jsw_060
Cnidaria	0	3	0	0	09sw_043, 09sw_273, 09sw_256
Ctenophora	0	<1	0	0	09sw_134
Rhabdocoela	1	0	0	0	08sw_078
Rhizaria					
Cercozoa	0	<1	0	0	09sw_184
Radiolaria	3	<1	0	0	08sw_064; 09sw_049
Acanthrea	0	1	0	0	09sw_218, 09sw_101, 09sw_094
Polycystinea	6	30	1	0	08sw_011, 09sw_244, 09sw_263, 09sw_072, 09sw_003, 09sw_057, 09sw_001; 10sw_016
RAD B	1	4	0	0	08sw_042; 09sw_080, 09sw_087, 09sw_205
RAD C	0	<1	0	0	09sw_111
Stramenopiles					
Chrysophyceae	0	0	0	1	10jsw_078

[a]Phylogenetic affiliations were derived from SILVA SSU database release 106. In cases where taxonomic affiliations were obviously incorrect (i.e. polyphyletic), groups were named after the first gene clone derived from a particular lineage

Table 2.6 Summary of OTU-based approaches to borehole fluid α-diversity

	Genetic distance used for OTU clustering	*2008*	*2009*	*2010*
Observed richness (OTUs)	0.01	56	106	86
	0.03	32	74	65
Chao1 richness estimator (S_{chao1})	0.01	108	345	330
	0.03	43	152	583
Simpson evenness index ($E_{simpson}$)	0.01	0.12	0.16	0.21
	0.03	0.17	0.19	0.17
Non-parametric Shannon diversity index ($\hat{H}_{shannon}$)	0.01	3.11	4.00	4.24
	0.03	2.52	3.53	3.65

Table 2.7 Summary of microbial community structure comparisons

Environmental pair		*UniFrac Significance (p-value)*	
#1	*#2*	*Weighted*	*Unweighted*
2008 subsurface	2009 subsurface	≤ 0.03	≤ 0.03
2008 subsurface	2010 subsurface	≤ 0.03	≤ 0.03
2009 subsurface	2010 subsurface	≤ 0.03	≤ 0.03
2008 subsurface	2008 seawater	≤ 0.01	≤ 0.01
2009 subsurface	2009 seawater	≤ 0.01	≤ 0.01
2010 subsurface	2010 seawater (CTD)	≤ 0.03	≤ 0.03
2010 subsurface	2010 seawater (AUV)	≤ 0.03	≤ 0.03
2010 seawater (CTD)	2010 seawater (AUV)	≤ 0.03	≤ 0.03

Table 2.8 Group-specific CARD-FISH and microbial cell counts (DAPI) from borehole U1301A fluids and background seawater

	Borehole U1301A			*Seawater from Rosette*			*Seawater from ROV*
	2008	*2009*	*2010*	*2008*	*2009*	*2010*	*2010*
Total cell abundance ($x10^3$ cells ml^{-1})	n.d.[b]	8.98 ± 0.55	15.3 ± 0.66	87.8 ± 2.85	95.0 ± 3.45	76.0 ± 2.66	88.6 ± 3.97
Total *Bacteria* ($x10^3$ cells ml^{-1})	n.d.[b]	2.94 ± 0.14	6.23 ± 0.47	47.6 ± 1.13	47.3 ± 1.61	24.6 ± 1.68	30.7 ± 1.48
Total *Archaea* ($x10^3$ cells ml^{-1})	n.d.[b]	2.72 ± 0.18	1.84 ± 0.14	6.55 ± 0.30	30.3 ± 1.92	4.14 ± 0.57	5.73 ± 0.48
1026B117 lineage ($x10^3$ cells ml^{-1})	n.d.[b]	0.95 ± 0.11	b.d.[a]	n.d.[b]	b.d.[a]	b.d.[a]	b.d.[a]
1301A09_077 lineage ($x10^3$ cells ml^{-1})	n.d.[b]	0.36 ± 0.061	0.56 ± 0.081	n.d.[b]	b.d.[a]	b.d.[a]	b.d.[a]

[a]b.d., below detection; DAPI limit is 751 ± 83 cells ml^{-1}; CARD-FISH detection limit is 276 ± 79 cells ml^{-1}

[b]n.d., not determined

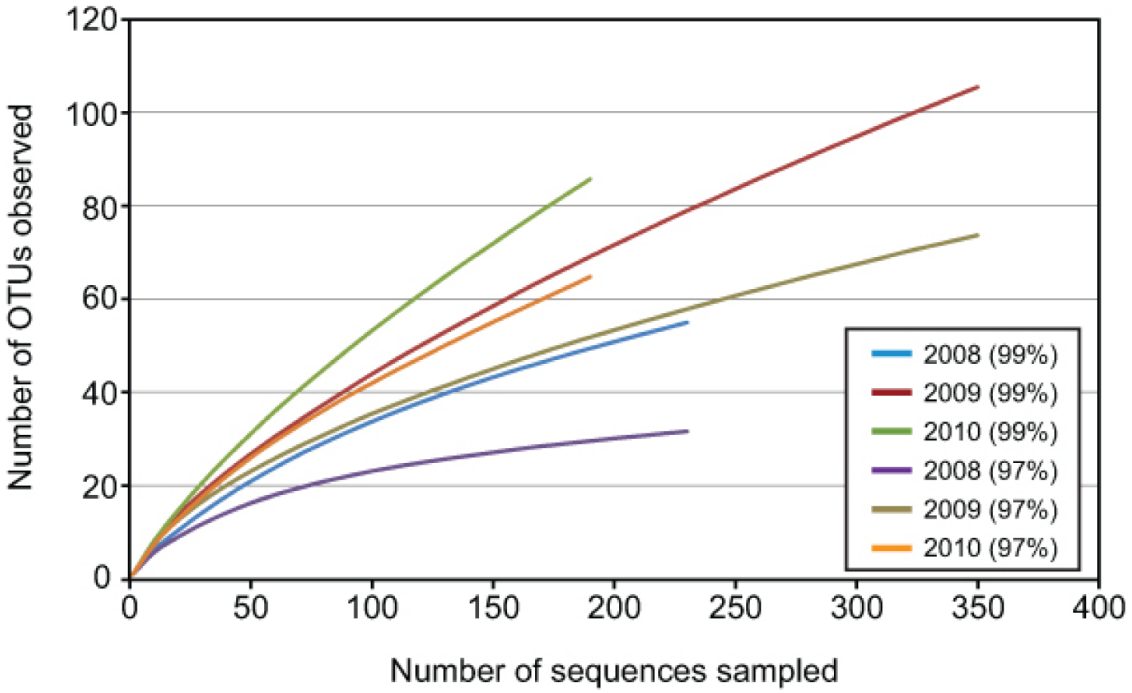

Figure 2.2 Rarefaction curves displaying the number of operational taxonomic units (OTUs) observed given the sequencing effort in each year of sampling, clustered at either 99% or 97% similarity cut-off values.

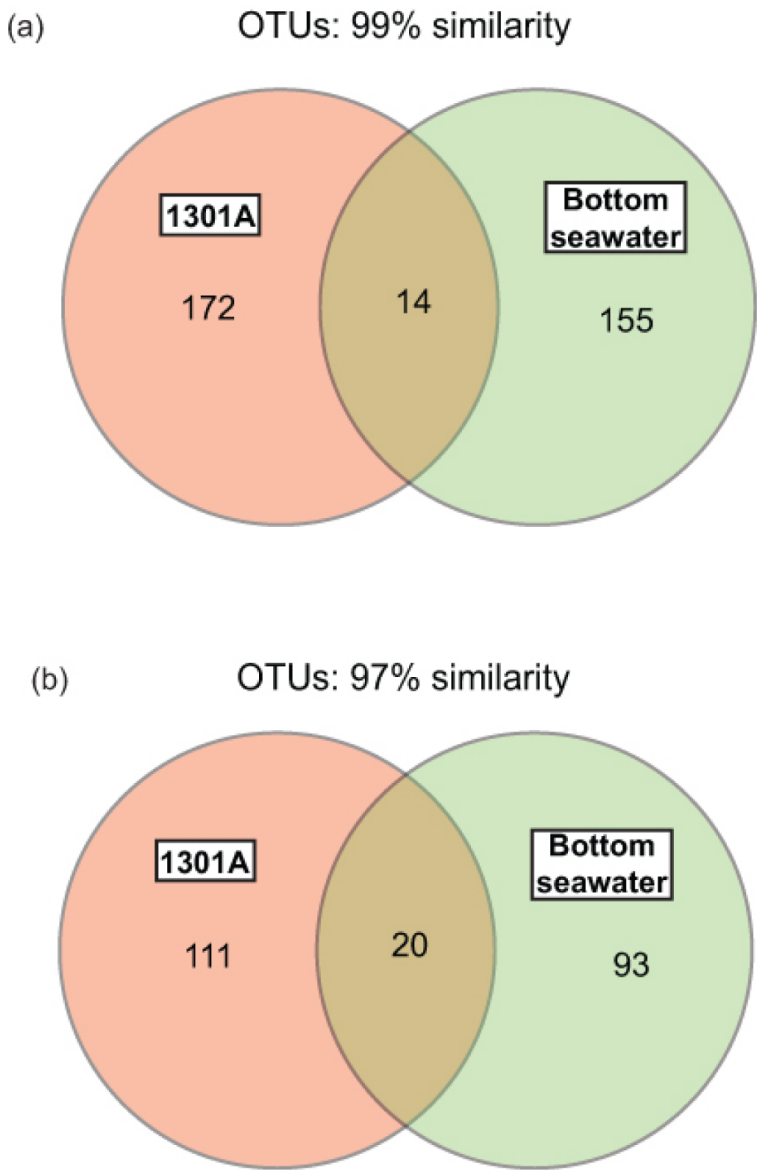

Figure 2.3 Venn diagrams showing microbial community relatedness between borehole U1301A fluid-derived and bottom seawater-derived SSU rRNA gene clones from each individual borehole fluid sample and all seawater samples clustered at (A) 99% or (B) 97% similarity cut-off values.

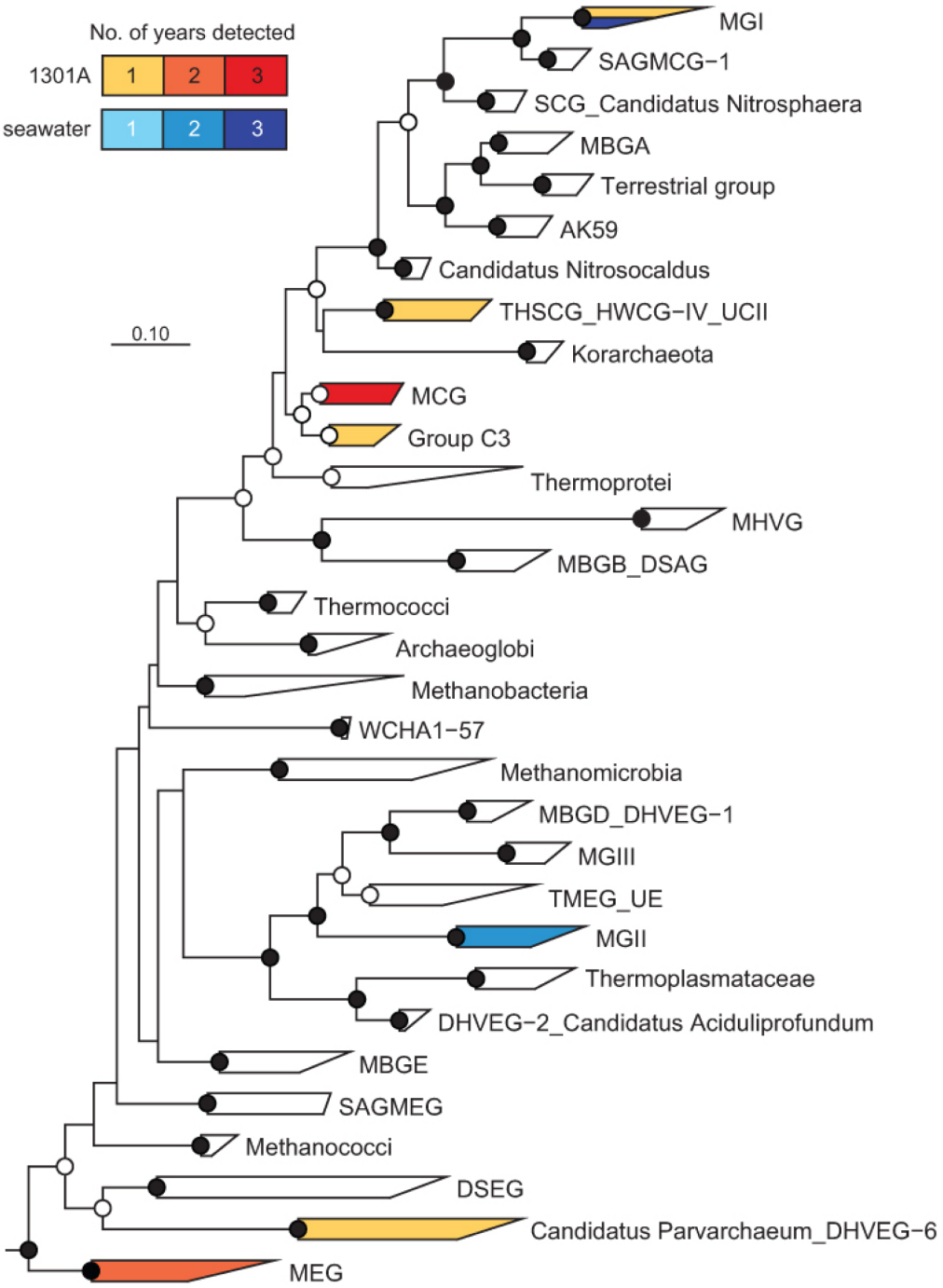

Figure 2.6 Phylogenetic relationships of SSU rRNA gene clones related to the domain *Archaea*, colored according to source (borehole U1301A fluids vs. rosette-derived surrounding bottom seawater) and total number of years recovered (out of three sampling years). Open circles indicate nodes with bootstrap support between 50-80%, while closed circles indicate bootstrap support >80%, from 1000 replicates. Cultivated *Bacteria* were used as outgroups (not shown). The scale bar corresponds to 0.10 substitutions per nucleotide position.

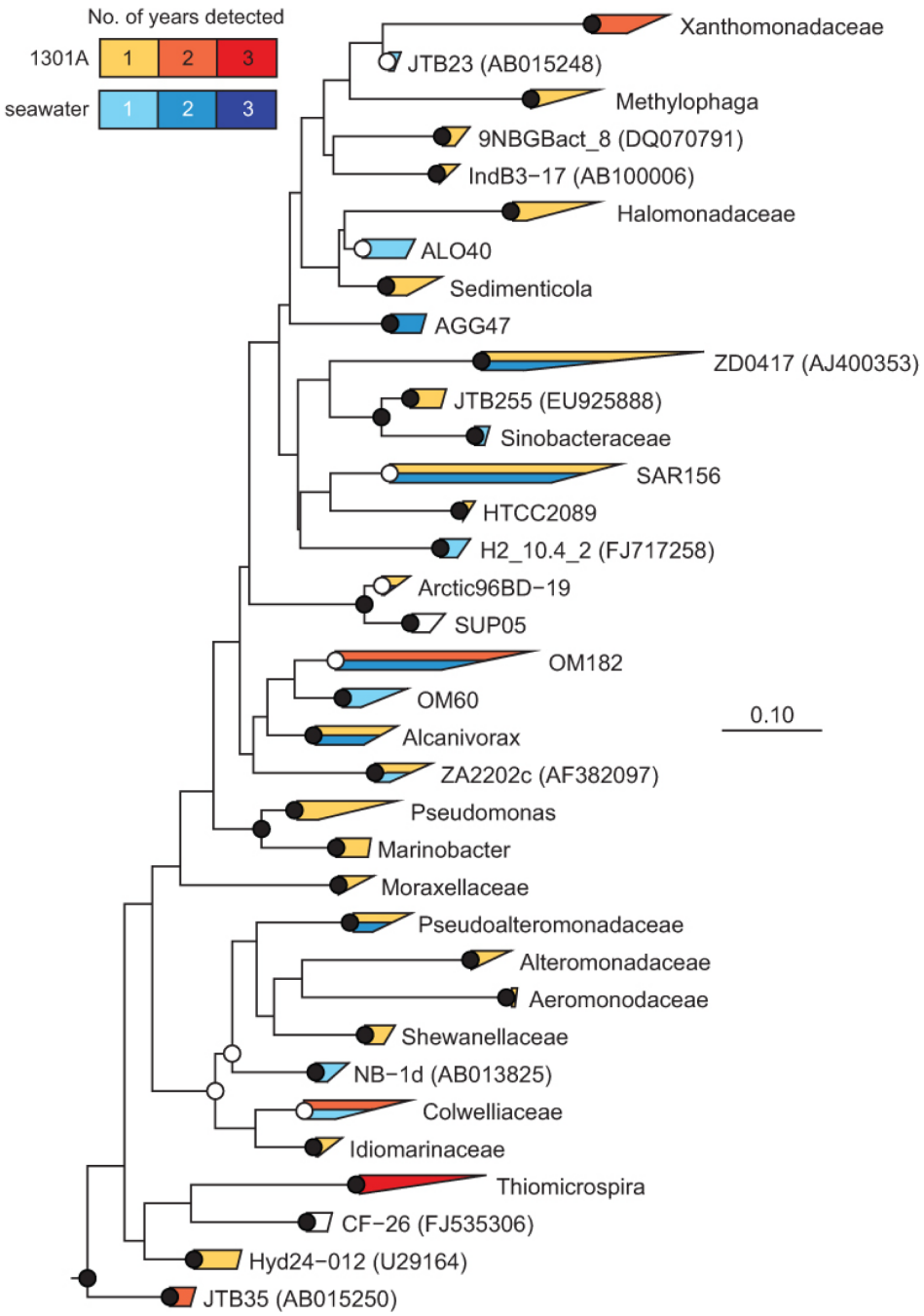

Figure 2.7 Phylogenetic relationships of SSU rRNA gene clones related to the *Gammaproteobacteria*, colored according to source (borehole U1301A fluids vs. rosette-derived surrounding bottom seawater) and total number of years recovered (out of three sampling years). Open circles indicate nodes with bootstrap support between 50-80%. while closed circles indicate bootstrap support >80%, from 1000 replicates. Cultivated *Betaproteobacteria* were used as outgroups (not shown). The scale bar corresponds to 0.10 substitutions per nucleotide position. When applicable, groups are named according to family; however, in some instances a genus name is provided. For clades with no cultivated representatives, the name of the original gene clone representing the lineage is used.

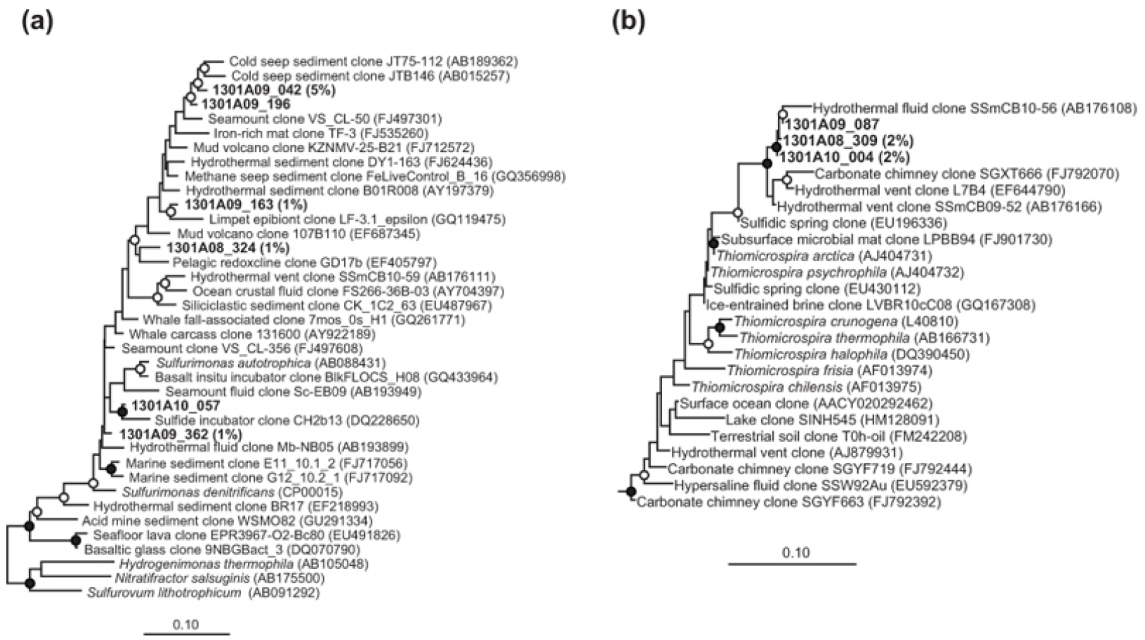

Figure 2.10 Phylogenetic relationships of borehole U1301A fluid-derived SSU rRNA gene clones related to (A) the *Sulfurimonas* lineage of the *Epsilonproteobacteria* and (B) the *Thiomicrospira* lineage of the *Gammaproteobacteria*. Open circles indicate nodes with bootstrap support between 50-80%, while closed circles indicate bootstrap support >80%, from 1000 replicates. Gene clones recovered in this study are highlighted in bold font; the relative abundance of identical clones recovered from the same sampling year is listed in parentheses. Cultivated *Epsilonproteobacteria* (A) and *Thioalkalimicrobium* (B) were used as outgroups (not shown). The scale bars correspond to 0.1 substitutions per nucleotide position. Relevant gene clones of short length were added after tree construction and bootstrapping, and are indicated by dashed lines.

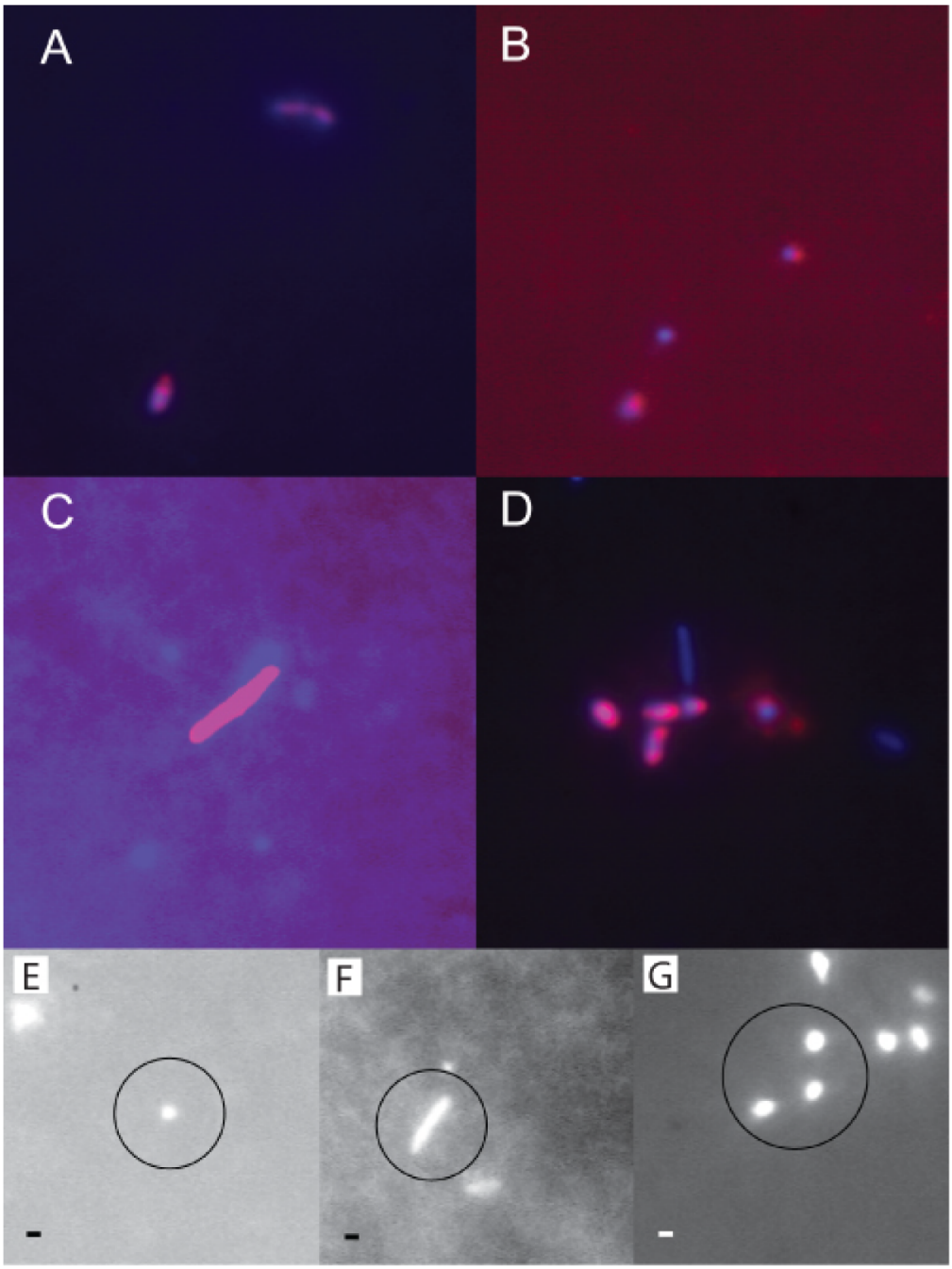

Figure 2.11 Representative photomicrographs of *Bacteria* (A), *Archaea* (B), 1026B3 lineage of the *Firmicutes* (C) and 1301A10_105 lineage of *Gammaproteobacteria* (D) in fluids from borehole U1301A illuminated via CARD-FISH. Images are false-colored dual image overlays of DNA-containing cells stained with DAPI (blue) and probe-conferred Cy3 fluorescence (red). Panels E-G are DAPI-stained images corresponding to typical oligonucleotide probe-positive cells of *Archaea* (E), 1026B3 lineage of the *Firmicutes* (F) and 1301A10_105 lineage of *Gammaproteobacteria* (G). Scale bar, 1 µm.

CHAPTER 3

Phylogenetic diversity of microorganisms in subseafloor crustal fluids from Holes 1025C and 1026B along the Juan de Fuca Ridge flank

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3.1 Abstract

To expand investigations into the phylogenetic diversity of microorganisms inhabiting the subseafloor biosphere, basalt-hosted crustal fluids were sampled from Circulation Obviation Retrofit Kits (CORKs) affixed to Holes 1025C and 1026B along the Juan de Fuca Ridge (JdFR) flank using a clean fluid pumping system. These boreholes penetrate the crustal aquifer of young ocean crust (1.24 and 3.51 million years old, respectively), but differ with respect to borehole depth and temperature at the sediment-basement interface (147 m and 39°C vs. 295 m and 64°C, respectively). Cloning and sequencing of PCR-amplified small subunit ribosomal RNA genes revealed that fluids retrieved from Hole 1025C were dominated by relatives of the genus *Desulfobulbus* of the *Deltaproteobacteria* (56% of clones) and *Candidatus* Desulforudis of the *Firmicutes* (17%). Fluids sampled from Hole 1026B also contained plausible deep subseafloor inhabitants amongst the most abundant clone lineages; however, both geochemical analysis and microbial community structure reveal the borehole to be compromised by bottom seawater intrusion. Regardless, this study provides independent support for previous observations seeking to identify phylogenetic groups of microorganisms common to the deep ocean crustal biosphere, and extends previous observations by identifying additional lineages that may be prevalent in this unique environment.

3.2 Introduction

Several studies now support the notion that the enormous volume of sediments and basement basalt that compose the global system of mid-ocean ridge spreading centers, flank and ocean basins harbors microbial life (e.g., Gold, 1992; Parkes *et al.*, 1994; Fisk *et al.*, 1998; Bach and Edwards, 2003; Cowen, 2004; D'Hondt *et al.*, 2004; Schrenk *et al.*, 2010; Orcutt *et al.*, 2011a; Jungbluth *et al.*, 2013). Fueled by cooling of basement rock, fluid circulation occurring within porous and permeable young ridge flanks (<10 million years) introduces fresh terminal electron acceptors into the ocean crust (i.e. oxygen, nitrate, sulfate); the electron acceptors mix with warm, chemically-reducing fluids to make the uppermost igneous basement a suitable marine subsurface environment for microorganisms (Baross *et al.*, 2004). Although the uppermost (40–500 m) basement aquifer is estimated to contain ~2% of the world ocean volume (Johnson and Pruis, 2003), information regarding the evolutionary history, community structure, functional properties, and metabolic activity of microorganisms inhabiting this unique system is scarce (Schrenk *et al.*, 2010; Orcutt *et al.*, 2011a).

Most mid-ocean ridge flank and ocean basin basement is buried under thick, impermeable layers of sediment that significantly restrict sampling opportunities. However, Circulation Obviation Retrofit Kit (CORK) observatories (Davis *et al.*, 1992; Edwards *et al.*, 2011) affixed to Ocean Drilling Program (ODP) and Integrated Ocean Drilling Program (IODP)

boreholes (Davis and Becker, 2001; Fisher *et al.*, 2005) offer access to perform measurements and experiments *in situ* or collect crustal fluids. Fluids within the basement rock can be channeled up through the sediment horizon via fluid delivery lines and collected from sampling ports at the seafloor via submersible (Cowen *et al.*, 2003; Huber *et al.*, 2006; Cowen *et al.*, 2012; Edwards *et al.*, 2012; Lin *et al.*, 2012; Nigro *et al.*, 2012; Jungbluth *et al.*, 2013).

During ODP Leg 168, an array of boreholes was drilled into ocean basement of increasing age along a transect perpendicular to the Juan de Fuca Ridge (JdFR) axis on its eastern flank (Figures 3.1a, 3.1b, and 3.2) (Shipboard Scientific Party, 1997). Two of these, ODP Holes 1025C and 1026B, penetrate over-pressured basaltic crust and were sealed with CORK sampling platforms. The sediment cover at Hole 1026B is sufficiently thick to act as an impermeable seal (Embley *et al.*, 1983), preventing circulating basement fluids from directly mixing with deep ocean seawater, while Hole 1025C lies within a transition zone between sediment-free regions that may allow for open hydrothermal circulation and sediment-covered, hydrologically sealed igneous crust (Shipboard Scientific Party, 1997). While both boreholes were originally equipped with early-generation CORKs that delivered crustal fluids directly through a potentially reactive iron casing (Davis *et al.*, 1992; Shipboard Scientific Party, 1997), in 2004 the CORK at Hole 1026B was replaced with an upgraded CORK-II, which is more amenable to microbiological sampling due to dedicated stainless steel fluid delivery lines that circumvent fluid passage through the casing itself (Becker and Davis, 2005). Also in 2004, Hole U1301A was drilled in close proximity to Hole 1026B and affixed with a CORK-II and stainless steel fluid delivery lines (Expedition 301 Scientists, 2005).

Several studies have used the CORK observatories along the JdFR flank to investigate the coupled microbiology and chemistry of basalt-hosted crustal fluids in this region (Cowen *et al.*, 2003; Huber *et al.*, 2006; Nakagawa *et al.*, 2006; Steinsbu *et al.*, 2010; Orcutt *et al.*, 2011b; Jungbluth *et al.*, 2013). From these and other studies (e.g., Wheat *et al.*, 2004; Lin *et al.*, 2012) it is now known that basaltic crustal fluids are enriched in several compounds that are highly likely to impact biological processes in this system, including methane, hydrogen, ammonium, and iron, and are depleted in others, including magnesium, phosphate, nitrate, sulfate, and dissolved organic carbon (DOC), relative to bottom seawater. In addition to mineral weathering and serpentinization, the chemical composition of these fluids also suggests that microbially-mediated processes including biogenic methane cycling, iron metabolism, sulfate reduction and fermentation could also be occurring, with microorganisms drawing down DOC, nitrate, phosphate, and sulfate stocks in the process. Consistent with some of these processes, several microbial lineages identified from Holes 1026B or U1301A fluid samples (Cowen *et al.*, 2003; Huber *et al.*, 2006; Jungbluth *et al.*, 2013) and solid substrates (Nakagawa *et al.*, 2006; Steinsbu *et al.*, 2010; Orcutt *et al.*, 2011b; Lever *et al.*, 2013) via both culture-based and cultivation independent studies are related to *Bacteria* and *Archaea* known to transform sulfur compounds, including the bacterial lineages Candidatus *Desulforudis*, *Desulfocapsa*,

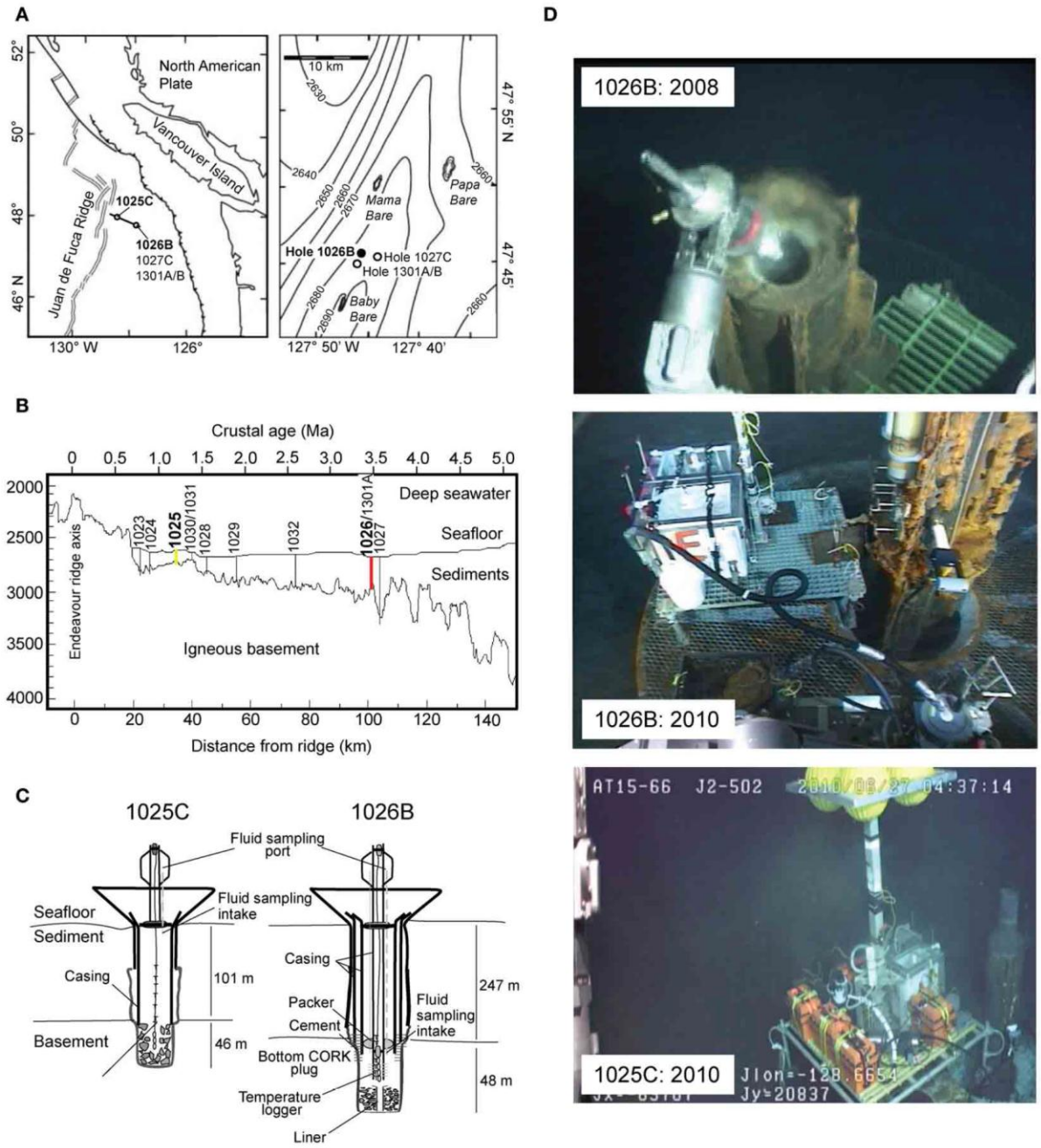

Figure 3.1 (A) Location of CORK observatory sampling sites on the Juan de Fuca Ridge flank, Pacific Ocean. **(B)** Cross-sectional diagram of ODP Leg 168 showing depth of basement crust and sediment thickness, basement age and associated distance from ridge axis, and locations of Holes 1025 (yellow) and 1026 (red) in boldface font (modified from Wheat et al., 2004). **(C)** Schematic diagram of CORKs located at Holes 1025C and 1026B (not drawn to scale). Fluids were sampled from the exit valve of the fluid delivery line (modified from Lin et al., 2012). **(D)** Photo of squeeze sample taken for biogeochemical analysis from top of CORK 1026B in 2008, fluid sampling device used in 2010 at Hole 1026B, and Borehole Flushing Unit and fluid sampling device used at Hole 1025C in 2010.

Thiomicrospira, *Sulfurimonas*, and the archaeal lineage *Archaeoglobus*. Some of these may grow chemolithoautotrophically via the coupling of hydrogen oxidation to sulfate reduction. However, many of the microbial lineages identified via phylogenetic marker-based cultivation independent methods within basalt-hosted deep subsurface fluids of the JdFR flank are not closely related to any known cultivated strains, and possess unknown functional roles in this system (Jungbluth *et al.*, 2013).

In this study, we sought to use a phylogenetic marker-based approach in order to compare the phylogenetic identity of microorganisms inhabiting fluid samples retrieved from three JdFR flank CORK observatories sampled within the span of 1 week: 1025C, 1026B, and U1301A, and to compare these samples to similar data derived previously from fluids retrieved from Hole 1026B. Our goals were to understand how CORK characteristics may influence both the microbial community structure and underlying chemistry of fluid samples from CORKS in close proximity but of different design and construction, as well as to understand how changes to the CORK at a single borehole (1026B), and the loss of CORK integrity at this location over time, impact the resulting chemical and microbial properties of the resultant fluids.

3.3 Materials and Methods

Sample collection

During R/V *Atlantis* cruise ATL15-66 (13 June–1 July 2010), fluids were collected from CORK observatories at ODP Holes 1025C (47°53.247'N, 128°38.919'W) and 1026B (47°45.759'N, 127°45.552'W), located 34 and 101 km east of the JdFR spreading center, respectively (Figures 3.1 and 3.2). Fluids were sampled from the microbiological and geochemical sampling lines associated with the original 1025C CORK (Davis *et al.*, 1992) and 1026B CORK-II (Fisher *et al.*, 2005) installations. Holes 1025C and 1026B penetrate 101 and 247 m of sediments and another 46 and 48 m into ~1.2 and ~3.5 Ma basement rock, respectively (Table 3.1 and Figure 3.1c). In 2008, a small volume (~150 mL) fluid sample was collected from Hole 1026B on HOV *Alvin* dive 4432 during R/V *Atlantis* cruise ATL15–35 using a squeeze sampler while the CORK head was opened for redeployment of downhole instrument strings (Figure 3.1d); the volume of this sample was only sufficient for geochemical analysis.

In 2010, a deep-sea pumping system incorporating a non-contaminating titanium and Teflon pump head and complementary in-line sensors was used to draw and collect fluids from Holes 1025C and 1026B into custom-made 60 l acid-washed Tedlar bags (MiDan Co., Chino, CA, USA) protected by a high-density polyethylene box (Large Volume Bag Sampler, LVBS, Wommack *et al.*, 2004; Cowen *et al.*, 2012; Lin *et al.*, 2012; Jungbluth *et al.*, 2013) (Figure 3.1d). The fluid flowpath contained a custom flowcell with an *in situ* oxygen optode (Aanderaa Data Systems, Bergen, Norway), temperature sensor (Sea-Bird Electronics Inc., Bellingham, WA,

USA), and a custom fluid flow sensor to allow for real-time assessment of the integrity of fluid connections and plumbing flowpath (e.g., Cowen *et al.*, 2012). All tubing and sampling bags along the fluid delivery path were acid washed prior to deployment on the submersible. Borehole fluids were flushed through the CORK observatory fluid delivery lines prior to sampling into the LVBS. Hole 1025C was flushed overnight using a Borehole Flushing Unit pump to clear out the section of 10.75" iron casing while less time (~30 min) was used to flush Hole 1026B due to the much smaller void volume of the 1/4" fluid delivery line (Figure 3.1d). Samples for microbiological analysis were collected on ROV *Jason II* dives J2–502 (1025C) and J2–500 (1026B).

Subsamples for geochemistry were taken immediately after shipboard retrieval of the LVBS samplers by transferring fluids directly to gastight syringes (Hamilton Co., Reno, NV, USA) or via acid-washed tubing and a peristaltic pump into acid-cleaned high-density polyethylene (HDPE) bottles. All geochemical sub-sampling was conducted inside a laminar flow hood with a high-performance air filter (HEPA) to ensure a clean sampling environment. Samples for major ion analysis were stored refrigerated, while samples for inorganic nutrients (nitrate, nitrite and phosphate), DOC and total organic carbon (TOC) were stored frozen until further processing. Subsequently, 1.95 l (1025C) and 1.50 l (Hole 1026B) of sample fluids were passed through 0.22 µm pore-sized Sterivex-GP filter cartridges (Millipore Corporation, Billerica, MA, USA) for the collection of microbial biomass. Filters were subsequently stored in 2.0 ml of DNA lysis buffer [20 mM Tris-HCl, 2 mM EDTA, 1.2% Triton X-100, 2% lysozyme (w/v), pH 8] at −80°C until further processing. All samples were stored frozen within 2 h of shipboard retrieval of the bag samplers.

As described previously, seawater samples for comparison were collected in the vicinity of Hole 1026B on cruises in 2008, 2009 and 2010 (Lin *et al.*, 2012; Jungbluth *et al.*, 2013).

Genomic DNA extraction and rRNA gene sequencing

In a shore-based laboratory, Sterivex membrane filters were subsequently thawed to room temperature and extracted for DNA using the PowerSoil DNA isolation kit (MO BIO Laboratories, Carlsbad, CA, USA) following the manufacturer's protocol. Quantification of the resulting genomic DNA was performed using a NanoDrop DN-100 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Small subunit ribosomal RNA (SSU rRNA) gene fragments were amplified via the polymerase chain reaction (PCR) using the universal oligonucleotide forward and reverse primers 519F (5'-CAGCMGCCGCGGTAATWC-3') and 1406R (5'-ACGGGCGGTGTGTRC-3'), respectively (Lane *et al.*, 1985). Each 20 µl PCR reaction contained 0.25 U of PicoMaxx high fidelity DNA polymerase (Stratagene, La Jolla, CA, USA), 1x PicoMaxx reaction buffer, 200 µM of each deoxynucleoside triphosphate (dNTPs), 200 nM of both forward and reverse primer, and ~4–8 ng of environment DNA template. Appropriate positive

and negative control reactions were also included. PCR cycling conditions consisted of a denaturation step at 95°C for 4 min, followed by 30 cycles of 95°C denaturation for 30 s, 55°C annealing for 1 min, 72°C extension for 2 min, and a final extension step at 72°C for 20 min. Amplification products were size-selected and cleaned using the QIAquick gel extraction kit (Qiagen, Valencia, CA, USA) and subsequently cloned with the TOPO TA Cloning kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Clones were sequenced using an ABI 3730XL DNA Analyzer (Applied Biosystems, Carlsbad, CA, USA).

Phylogenetic analysis

DNA sequences were trimmed of vector sequence and manually curated using Sequencher version 4.9 software (GeneCodes, Ann Arbor, MI, USA) and subsequently checked for chimera formation via Bellerophon (Huber *et al.*, 2004) and CHECK_CHIMERA, available from the Ribosomal Database Project (Cole *et al.*, 2005). Using the ARB software package (Ludwig *et al.*, 2004), hand-curated clone sequences were aligned with version SSURef_111 of the SILVA ARB database (Pruesse *et al.*, 2007) modified to include short (<1200 nucleotides) environmental gene clones that were highly similar to clone sequences obtained in this study. Phylogenetic analyses were performed with the RAxML maximum likelihood method using the general time-reversible model with a gamma distributed rate variation for nucleotide substitution (Stamatakis, 2006) and selection of the tree with the highest likelihood value based on 100 simulations. Sequences of short length were added to the maximum-likelihood-derived phylogenies using the parsimony insertion tool in ARB. Bootstrap analyses employed RAxML (Stamatakis *et al.*, 2008) via the CIPRES Portal V 3.1 (Miller *et al.*, 2010). All non-redundant sequences generated in this study have been deposited in GenBank under accession numbers KF574286-KF574384.

Microbial community analysis

The UniFrac significance test (Lozupone and Knight, 2005) was used to evaluate whether the microbial community structure was different between the three sample years. A maximum likelihood-based phylogenetic tree containing all borehole U1301A fluid environmental sequences derived in this study was evaluated using an online implementation of the hypothesis testing approach (Lozupone *et al.*, 2006). Significant differences in community structure within pairs of samples were evaluated using 100 permutations of both the weighted and unweighted implementations of the UniFrac algorithm. All p-values were corrected for multiple comparisons using the Bonferroni correction. Microbial community α-diversity estimators, rarefaction curves, and community relatedness were generated or assessed using lane-masked (community relatedness) or unmasked (α-diversity estimators, rarefaction curves)

clone sequences grouped into operational taxonomic units (OTUs) defined at 99 and 97% SSU rRNA gene sequence similarity cut-off values using the average neighbor clustering method as implemented by the mothur software package (Schloss *et al.*, 2009). Microbial richness, evenness, and diversity were assessed by the Chao1 richness estimator (S_{chao1}) (Chao, 1984), Simpson evenness index ($E_{simpson}$) (Simpson, 1949), and the non-parametric Shannon diversity index ($\hat{H}_{shannon}$) (Shannon, 1948), respectively, as implemented in mothur (Schloss *et al.*, 2009).

Analytical methods for geochemistry

Subsamples for shipboard colorimetric measurement of iron species by UV-vis spectrophotometer using the ferrozine method (modified after Stookey, 1970) were split into three aliquots. For Fe(II), samples from a gastight syringe were 0.2-µm filtered in an N2 atmosphere and immediately reacted with a ferrozine solution consisting of 50% of a 2.5 M ammonium acetate buffer and 50% of 0.01 M ferrozine. Samples were allowed to stand for ~30 min for color development, followed by absorbance measurement at 562 nm. Total dissolved iron (Fed) was derived by reacting a separate 0.2-µm filtered aliquot for 24 h with 0.2 M hydroxylamine in 0.1 M HCl prior to ferrozine analysis. Total iron (FeT) was determined by reacting an unfiltered aliquot for 24 h with 0.2 M hydroxylamine in 0.1 M HCl prior to ferrozine analysis. Major ions were analyzed with a Dionex ICS-1100s ion chromatograph (Thermo Fisher Scientific, Sunnyvale, CA, USA). In addition, magnesium and calcium were analyzed shipboard with ethylene diamine tetraacetic acid (EDTA) and ethylene glycol tetraacetic acid (EGTA) titration (Grasshoff *et al.*, 1999). Nitrate, nitrite and phosphate were analyzed by spectrophotometric methods (Grasshoff *et al.*, 1999). Ammonium was measured by a modified flow-injection fluorescence method (Jones, 1991). Methane and hydrogen were measured by gas chromatography (Lin *et al.*, 2012).

End-member correction

Bottom seawater entrainment at the time of sampling (e.g., via leaks in sampling equipment or compromised integrity of the CORK seal) was estimated using a two end-member mixing model (Libes, 2009) based on nitrate concentration (Mottl *et al.*, 1998; Wheat and Mottl, 2000). In anoxic basement fluids, our working assumption is that end-member nitrate concentration is zero because nitrate is exhausted (G. Wheat, personal communication), while bottom seawater contains ~40 µM nitrate in this region (Wheat *et al.*, 2010; Lin *et al.*, 2012).

3.4 Results and Discussion

Biogeochemical characteristics of borehole fluid samples

Dissolved oxygen concentrations in fluids collected from CORK observatories immediately revealed that 1026B (O_2 = 88 µM) showed signs of bottom seawater intrusion, while fluids from 1025C and U1301A were deplete in O_2 (18 µM and 10 µM, respectively) relative to bottom seawater (110 µM). After end-member correction to a nitrate concentration of 0 µM, the 6.4 µM of nitrate measured in the Hole 1025C fluid sample suggests that it consisted of ~84% basement fluid and ~16% seawater (Table 3.1). The concentration of nitrate in the Hole 1026B fluid sample characterized here was nearly identical to that in bottom seawater (40.6 vs. 40.8 µM), while the concentration of dissolved iron was far higher than that in a sample from Hole 1026B collected from the top of the open CORK in 2008 (1000 µM vs. ~0.5 µM, respectively). The lower magnesium and sulfate and higher calcium and ammonium relative to bottom seawater suggests that end-member basement fluid represented only ~3–10% of the 1026B fluid sample characterized here. It is not known at what time between 2008 and 2010 sampling periods that 1026B became catastrophically compromised to bottom seawater intrusion; however, end-member chemical concentrations calculated for crustal fluids accessed via 1026B in 2008 were 77% end-member basement fluid. Overall, the end-member basement fluid from Hole 1026B is more similar to that from Hole U1301A than Hole 1025C, consistent with previous observations (Elderfield *et al.*, 1999; Wheat *et al.*, 2004).

Microbial community structure

A total of 734 and 754 ng of DNA was extracted from Holes 1025C and 1026B fluid samples, respectively. After amplification with universal oligonucleotide primers and cloning, 70 (1025C) and 87 (1026B) SSU rRNA gene clones were sequenced (Table 3.2). Microbial communities were analyzed using a variety of α-diversity calculators and OTUs defined at 99% and 97% SSU rRNA gene sequence similarity, resulting in 22 (99%) and 16 (97%) OTUs from 1025C and 53 (99%) and 42 (97%) OTUs from 1026B. The Shannon diversity index was depressed in the Hole 1025C sample, indicating that this sample possessed lower community diversity, while rarefaction curves and Chao1 richness estimators generated using the same OTU definitions indicated that the clone libraries were under sampled (data not shown and Table 3.3).

Phylogenetic analyses indicated that the borehole fluid samples described here contained a majority (~80%, 1025C) or significant fraction (~40%, 1026B) of clones related to microorganisms that harbor physiological attributes consistent with the physical and chemical conditions of life within the crustal subsurface environment (e.g., meso- and thermophiles,

anaerobes, sulfate-reducers, etc.), or are related to SSU rRNA gene sequences previously recovered from related environments (Table 3.2 and Figure 3.3). The 1025C and 1026B fluid samples characterized here showed little overlap in the taxonomic identity of OTUs, as well as little overlap with fluids sampled from Holes 1026B and U1301A previously (Figure 3.4) (Cowen *et al.*, 2003; Huber *et al.*, 2006).

Hole 1025C fluid community structure

Members of the genus *Desulfobulbus*, a group of cultivated *Deltaproteobacteria* that are able to grow by dissimilatory sulfate reduction (e.g. Sass *et al.*, 2002; Suzuki *et al.*, 2007),

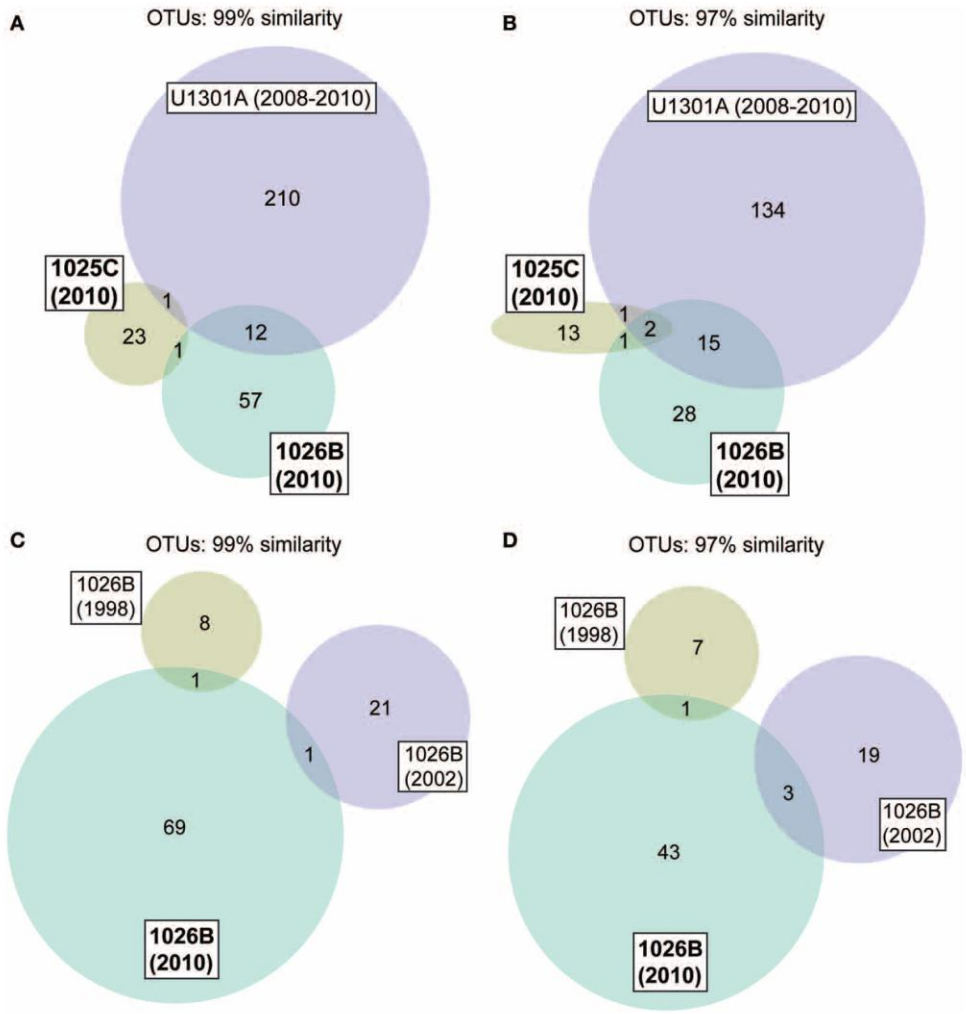

Figure 3.4 Venn diagrams showing the overlap in microbial communities between Holes 1025C, 1026B, and U1301A fluids (A,B), and between Hole 1026B fluids collected in different years (C,D). Data for "U1301A 2008–2010" appears in Jungbluth et al. (2013), "1026B 1998" in Cowen et al. (2003), and "1026B 2002" in Huber et al. (2006).

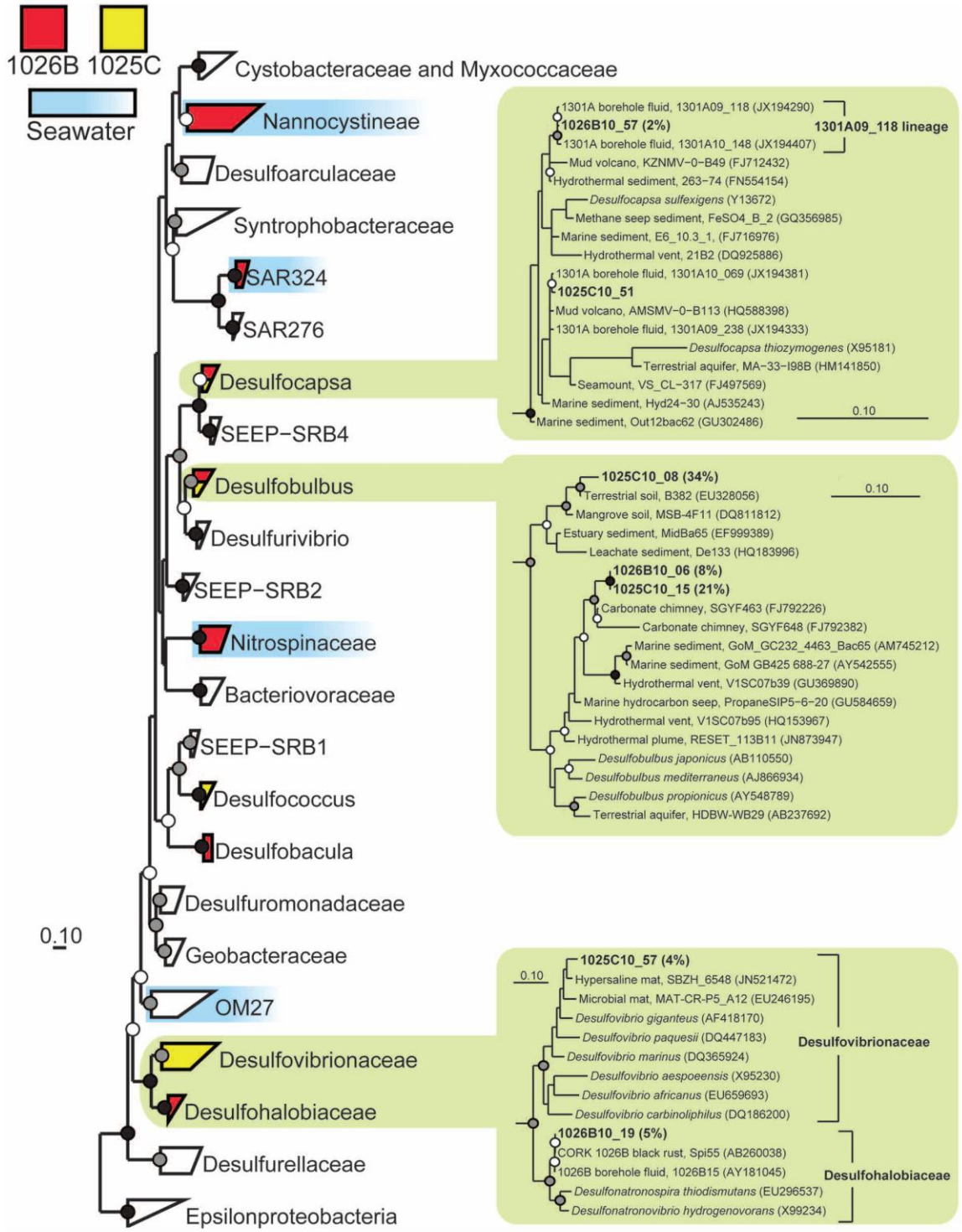

Figure 3.5 Phylogenetic relationships of SSU rRNA gene clones related to the phylum *Deltaproteobacteria*, colored according to borehole of origin. Clones derived from nearby bottom seawater are shown in blue (Jungbluth et al., 2013). Cultivated *Epsilonproteobacteria* were used as an outgroup (not shown). Detailed phylogenies are shown for selected lineages. Black (100%), gray (>80%), and white (>50%) circles indicate nodes with bootstrap support, from 1000 replicates. Gene clones recovered in this study are highlighted in bold font; the relative abundance of identical clones is listed in parentheses.

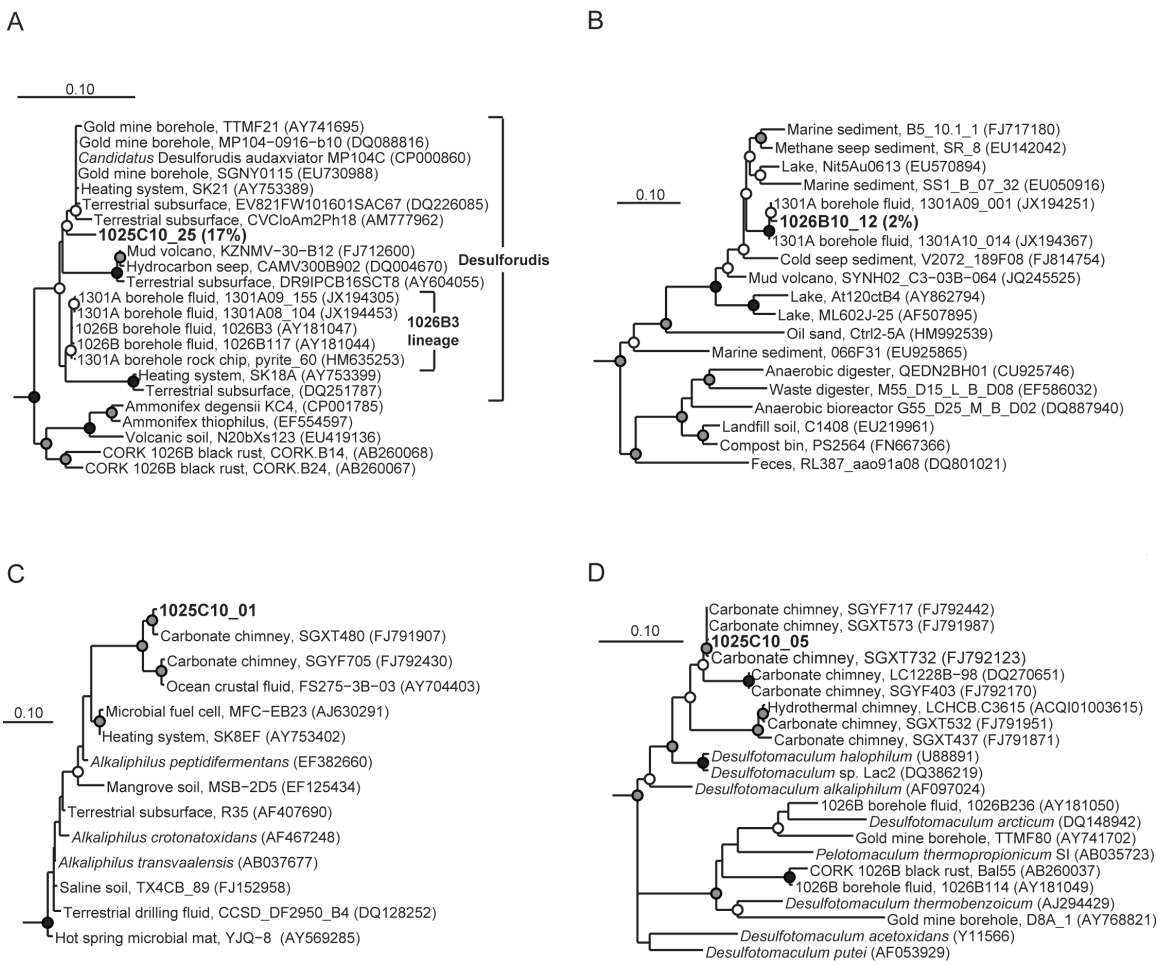

Figure 3.6 Phylogenetic relationships of SSU rRNA gene clones from Holes 1025C and 1026B fluids within the phylum *Firmicutes*, related to (A) *Candidatus* Desulforudis audaxviator, (B) RF3, (C) *Alkaliphilus*, and (D) *Desulfotomaculum* of the bacterial phylum *Firmicutes*. Short length gene clone pyrite_60 was added to the *Candidatus* Desulforudis audaxviator tree after tree construction and bootstrapping and is indicated by a dashed line. Other information as in Figure 3.5.

dominated the clones recovered from Hole 1025C fluid (56% of clones; Table 3.2). Two monophyletic lineages were detected; one was nearly identical (> 99% similarity) to environmental gene clones recovered from Hole 1026B fluids in this study, while the other was most closely related to environmental gene clones from terrestrial and mangrove soil (e.g. Berlendis *et al.*, 2010; Figure 3.5). The second most abundant group of environmental gene clones from Hole 1025C fluid was related to *Candidatus* Desulforudis audaxviator (17% of clones; Table 3.2), an uncultivated lineage within the phylum *Firmicutes* that has been detected previously in marine subsurface borehole fluids (e.g. Cowen *et al.*, 2003; Jungbluth *et al.*, 2013) and the terrestrial subsurface (e.g. Moser *et al.*, 2005, Chivian *et al.*, 2008). Interestingly, the environmental gene clones recovered from 1025C form a monophyletic lineage that is distinct from those previously recovered from Holes 1026B and U1301A fluids (Figure 3.6a). Several other less abundant bacterial lineages were recovered from Hole 1025C, and accounted for

27% of the microbial community (Table 3.2, Figure 3.5 and 3.7b). No *Archaea* were recovered from 1025C.

Hole 1026B fluid community structure

The two most abundant environmental gene clone groups recovered from Hole 1026B fluid were most closely related to the seawater-associated lineages SAR11 (17% of clones) and the *Pseudoalteromonadaceae* (9% of clones), including SAR11 and *Pseudoalteromonadaceae* gene clones recovered previously from Hole U1301A fluids and surrounding bottom seawater (Table 3.2) (Jungbluth *et al.*, 2013). However, several lineages were recovered from 1026B that, based on the physiology of the most closely related cultivated strains, may rely on inorganic sulfur-containing compounds for growth. These include *Desulfobulbus* (8% of clones) and *Desulfobacula* (6% of clones) of the *Deltaproteobacteria* and *Thiomicrospira* of the *Gammaproteobacteria* (6% of clones) (e.g., Jannasch *et al.*, 1985; Kuever *et al.*, 2001; Pagani *et al.*, 2011) (Table 3.2). These lineages are also closely related to environmental gene clones from the hydrothermally-influenced marine subsurface (Figures 3.5 and 3.8) (e.g., Brazelton *et al.*, 2010; Nigro *et al.*, 2012; Jungbluth *et al.*, 2013). Several other low-abundance environmental gene clone lineages recovered from Hole 1026B fluid were most closely related to cultured microorganisms or environmental gene clones that originated from the marine subsurface (Table 3.2, Figures 3.7a, 3.7c, 3.7d, 3.7e, and 3.7f), and accounted for 17% of the microbial community.

Overlap with previous oceanic crustal fluid studies

Several gene clones recovered from the two CORK fluid samples were closely related to environmental gene clones previously characterized from oceanic ridge flank crustal fluids. Hole 1025C fluids contained gene clones that were highly related to those recovered from Hole 1026B fluids in this study (*Desulfobulbus*; Figure 3.5), Hole U1301A fluids sampled across multiple years (*Desulfocapsa*; Figure 3.5) (Jungbluth *et al.*, 2013), and crustal fluids from Baby Bare seamount (*Alkaliphilus*) (Huber *et al.*, 2006). These lineages contain cultured representatives known to be involved in sulfur cycling (Finster *et al.*, 1998; Takai *et al.*, 2001). A lineage of gene clones recovered from Hole 1026B fluids here was discovered to form a monophyletic clade that has been previously detected from fluids (Cowen *et al.*, 2003) and a rusty biofilm (Nakagawa *et al.*, 2006) from CORK 1026B (Figure 3.5); closest cultivated relatives within the family *Desulfohalobiaceae* of the phylum *Deltaproteobacteria* have also been implicated in sulfur cycling (Zhilina *et al.*, 1997; Sorokin *et al.*, 2008). Several clones recovered from Hole 1026B had close phylogenetic relation to Hole U1301A fluid clones collected during previous years (Jungbluth *et al.*, 2013), including *Gammaproteobacteria* lineages

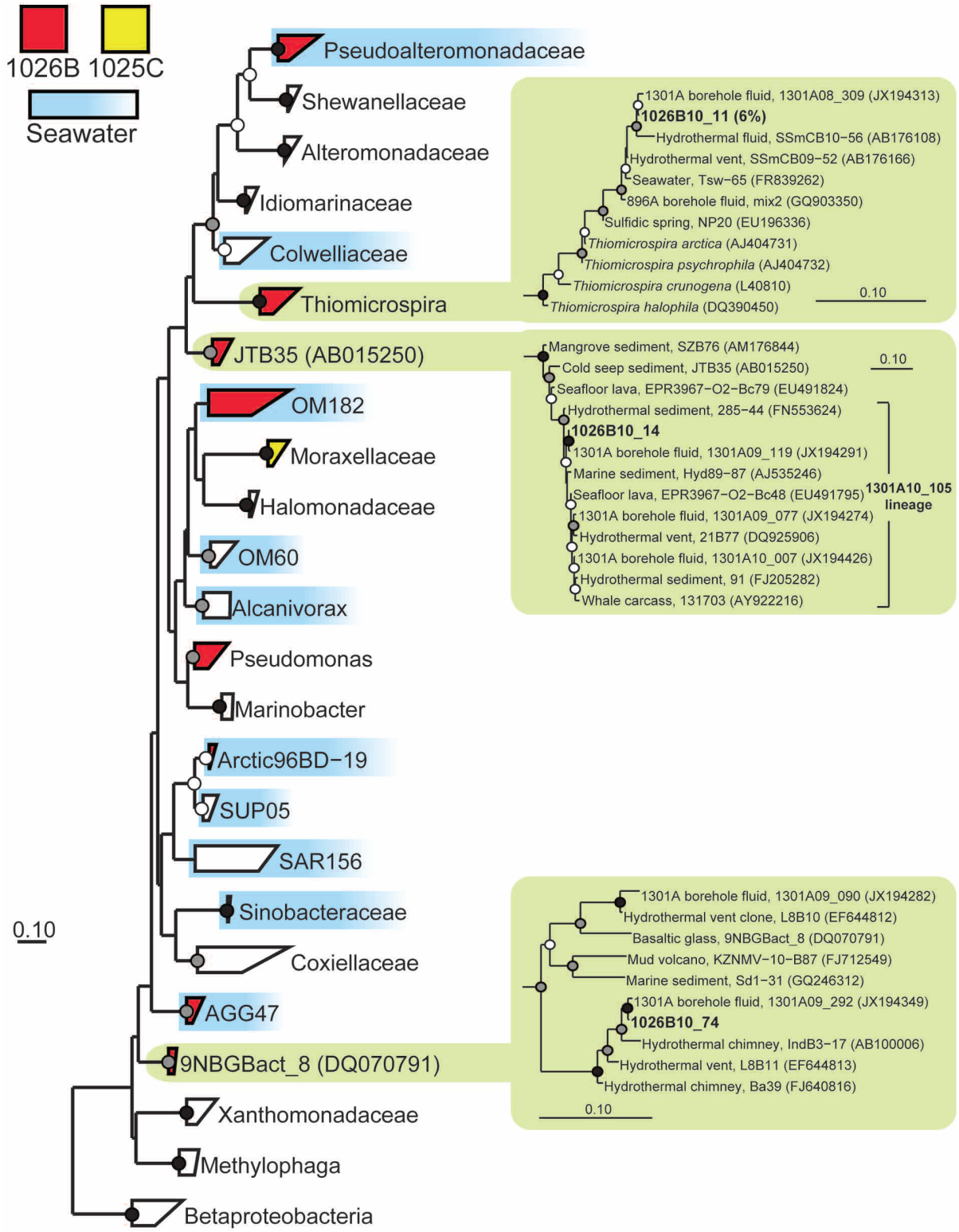

Figure 3.8 Phylogenetic relationships of SSU rRNA gene clones related to the phylum *Gammaproteobacteria*, colored as in Figure 3.5. A variety of *Betaproteobacteria* were used as outgroups (not shown). Detailed phylogenies are shown for selected lineages. Other information as in Figure 3.5.

Thiomicrospira, JTB35, 9NGBBact_8 (Figure 3.8); *Firmicutes* lineage RF3 (Figure 3.6b); *Epsilonproteobacteria* lineage *Sulfurimonas* (Figure 3.7c); *Planctomycetes* lineage OM190 (Figure 3.7e); *Fusibacter* (Figure 3.7f); and archaeal lineage DHVEG-6 (Figure 3.7a). UniFrac analysis suggests similar community structure in fluids from U1301A and 1026B (Table 3.4), although seawater contamination confounds this result.

Clustered at 97% similarity, two OTUs were common to Hole 1025C, 1026B, and U1301A fluids (SAR11 and *Desulfocapsa*), while an OTU within the genus *Desulfobulbus* was shared between Holes 1025C and 1026B crustal fluids to the exclusion of U1301A. Excluding OTUs that were also detected in bottom seawater, the Hole 1026B fluid sample characterized here contained a single lineage in common with Hole 1026B fluid samples characterized by Cowen *et al.* (2003) (*Desulfohalobiaceae*) or Huber *et al.* (2006) (*Sulfurimonas*) (Figure 3.9).

Overlap with other marine subsurface habitats

In addition to the clones described above, six gene clone lineages from 1025C and 1026B were closely related to sequences previously recovered from marine subsurface habitats typically associated with hydrothermal activity. A group of gene clones related to the mesophilic, sulfate-reducing group *Desulfovibrionaceae* (e.g., Abildgaard *et al.*, 2006; Thabet *et al.*, 2007) was detected in Hole 1025C fluids (Figure 3.5). Two groups within the *Alphaproteobacteria* were detected that fell within phylogenetic clades predominantly derived from seawater environments (OCS116 and *Rhodobacteraceae*; Suzuki *et al.*, 1997; Wagner-Döbler and Biebl, 2006; Morris *et al.*, 2012); however, phylogenetic inference that included gene clones detected within Hole 1026B fluids revealed that these clades may contain lineages specific to hydrothermally-active subsurface environments (Figure 3.10). Two additional lineages from 1025C that were related to environmental gene clones previously described from hydrothermal chimneys were detected in marine subsurface fluids for the first time, including *Bacteroidetes* lineage SB-1 (Figure 3.7b) and an uncultivated *Firmicutes* lineage related to known *Desulfotomaculum* discovered at Lost City carbonate vents (e.g., Brazelton *et al.*, 2010) (Figure 3.6d). However, this lineage was distinct from terrestrial (e.g., Moser *et al.*, 2005) and marine (Cowen *et al.*, 2003; Nakagawa *et al.*, 2006) environmental gene clones previously described from this group. A single gene clone related to environmental gene clones isolated from hydrothermal vents (Kato *et al.*, 2009) and sediments (Davis *et al.*, 2009) within the iron-oxidizing phylum *Zetaproteobacteria* (Emerson *et al.*, 2007; Singer *et al.*, 2011) was also detected in Hole 1026B fluids (Figure 3.7d).

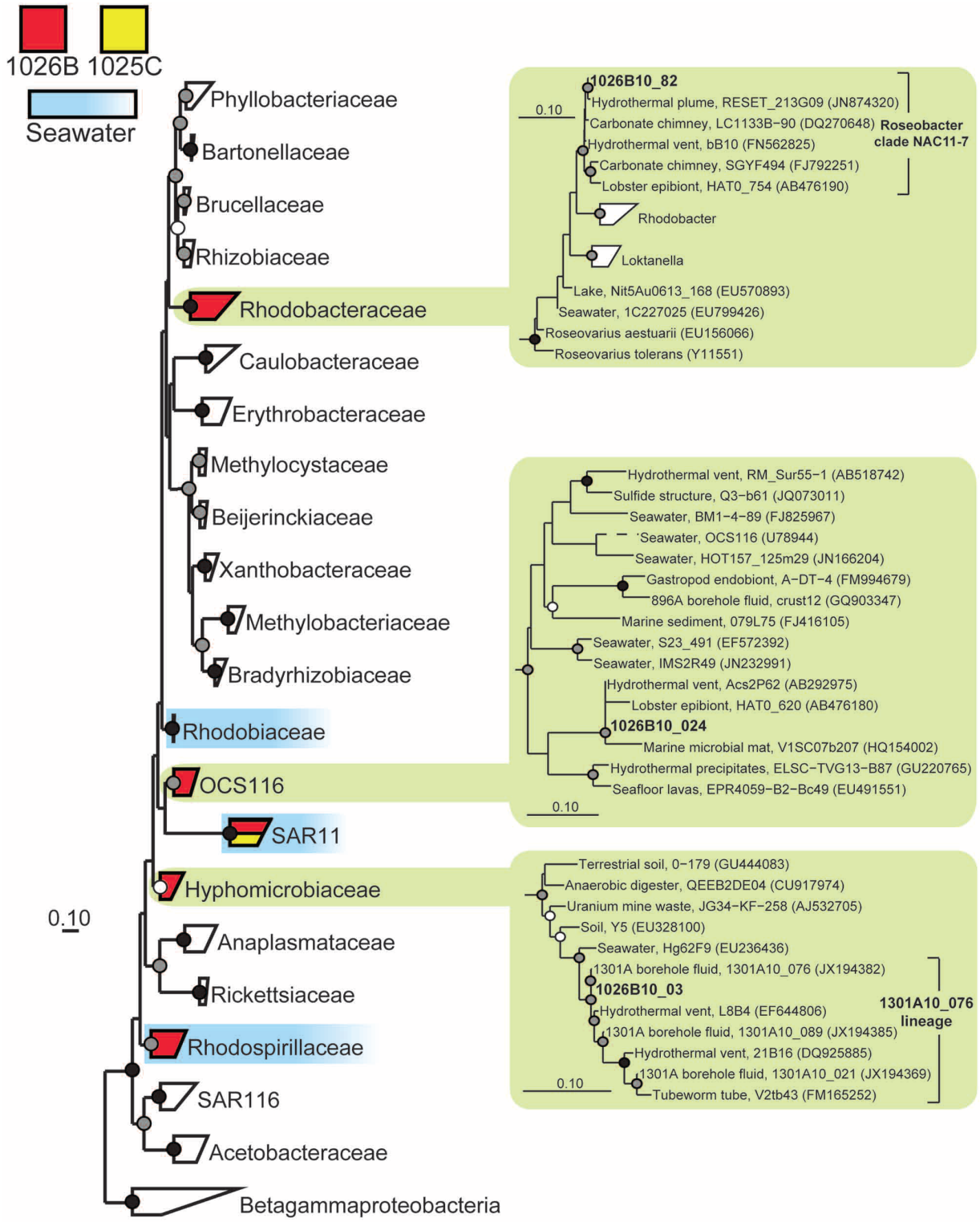

Figure 3.10 Phylogenetic relationships of SSU rRNA gene clones related to the phylum *Alphaproteobacteria,* colored as in Figure 3.5. A variety of *Betaproteobacteria* and *Gammaproteobacteria* were used as outgroups (not shown). Detailed phylogenies are shown for selected lineages. Short length gene clone OCS116 was added after tree construction and bootstrapping and is indicated by a dashed line. Other information as in Figure 3.5.

Discussion

While the CORKs affixed to Holes 1026B and 1025C were not designed with the intent to be exemplar platforms through which to investigate the microbiology of deep subsurface crustal fluids, they nonetheless offer independent points of access that can serve to complement and verify observations made from other CORK platforms that were designed specifically to maintain the biological integrity of retrieved borehole fluids, such as U1362A and U1362B on the JdFR flank (Fisher *et al.*, 2012). As priority for geochemical and microbiological sampling of older boreholes diminishes due to the antiquation of early-generation CORK materials and suboptimal fluid delivery systems, the likelihood of prioritizing research and thus submersible dive objectives in an effort to obtain fluid samples from them also continues to decrease. However, this study adds to previous research (e.g., Cowen *et al.*, 2003; Nigro *et al.*, 2012) that demonstrates that early generation CORKs are capable of yielding useful crustal fluid samples for comparative analysis, particularly when significant flushing measures are employed.

The biogeochemical composition of the Hole 1025C fluid sample investigated here was more similar to predicted end-member deep subsurface crustal fluid values than that of the Hole 1026B fluid sample. Efforts taken to minimize the sampling of contaminating seawater were predominantly successful at 1025C, as indicated by depleted O_2 concentration during sampling and other fluid chemistry suggesting only ~16% bottom water composition, and reflected in low detection of seawater environmental gene clones in the clone library (~10%). In contrast, the 1026B sample yielded at least 50% of typical seawater related environmental gene clones, in agreement with the near-background O_2 concentration of the collected sample, and further supported by the seawater-like chemical composition of the fluid sample. Unfortunately, it is not possible to estimate if the microbial community followed a conservative mixing model of two end-member fluids consisting of bottom seawater and crustal fluid because fluids collected from both 1026B and 1025C contained a high particulate matter load that prohibited the enumeration of microbial cells via microscopy (Figure 3.11). It is also not feasible to pinpoint the exact mechanism by which significantly more seawater was introduced into the sample from 1026B as opposed to 1025C; the two boreholes possess vast differences in a variety of factors that includes both CORK characteristics and host environment. However, one potential explanation lies in the observation that the seal of the CORK affixed to Hole 1026B has lost integrity and allows fluid to circumvent the fluid delivery lines of the CORK itself (Fisher *et al.*, 2011). Consistent with this explanation is that gastight samples collected in 2008 were comprised of 99% end-member basement fluids (Lin *et al.*, 2012), but only comprised 3–10% of the gastight sample collected in 2010. In addition, the low pH, extremely high iron concentration of the 2010 1026B sample, and ratios of dissolved Fe(II), total dissolved Fe, and

total Fe also indicate significant iron corrosion has taken place within fluid delivery lines and borehole casing.

Concurrent to sampling from Holes 1025C and 1026B, fluid samples were also collected from Hole U1301A for biogeochemical (Lin *et al.*, 2012) and microbial analysis (Jungbluth *et al.*, 2013). Hole U1301A was drilled in 2004 in close proximity to Hole 1026B and affixed with a CORK-II observatory possessing stainless steel fluid delivery lines (Expedition 301 Scientists, 2005), providing an independent access point to 3.5 million years old (Ma) crust and a means to support sample quality assessment from Hole 1026B. The physical proximity of the two boreholes suggests a similar fluid alteration history (Wheat *et al.*, 2010). Despite evidence from O_2, iron concentrations, and other parameters that the 1026B CORK seal has lost integrity and is allowing seawater intrusion, several environmental gene clone lineages recovered from this sample were closely related to environmental gene clones previously characterized from Hole 1026B fluids collected nearly 12 years ago (Cowen *et al.*, 2003) as well as from three successive years (2008–2010) of Hole U1301A fluids characterized previously (Jungbluth *et al.*, 2013). Thus, although contaminated with microorganisms of likely seawater origin, the Hole 1026B sample analyzed here provides independent support for the presence of certain microbial lineages in deep subsurface crustal fluids.

Despite being of higher integrity with regard to seawater intrusion, the microbial community within the Hole 1025C sample analyzed here overlapped little with either the JdFR flank fluid communities characterized previously or the Hole 1026B sample analyzed as part of this study. Two plausible explanations are that the old style CORK system in place at this borehole exerts and influence on the structure of the microbial community, or that the borehole taps an environment that hosts a distinct autochthonous microbial community. While neither scenario can be excluded, it is worth pointing out that the chemical characteristics of 1025C-derived crustal fluids are unique compared to that of the other boreholes investigated here in that they fall between those of bottom seawater and Hole U1301A, which is consistent with its classification within a Hydrothermal Transition Zone (Shipboard Scientific Party, 1997).

When considering the combined dataset from the two new fluid samples analyzed here, lineages within the bacterial phyla *Deltaproteobacteria* (*Desulfocapsa*, *Desulfohalobiaceae*), *Gammaproteobacteria* (JTB35, *Thiomicrospira*, 9NBGBact_8), *Alphaproteobacteria* (*Hyphomicrobiaceae*), *Epsilonproteobacteria* (*Sulfurimonas*), *Firmicutes* (*Desulforudis*, RF3), *Planctomycetes* (OM190), *Fusibacter*, and one within the archaeal domain (DHVEG-6) have all been previously found in JdFR flank crustal fluid samples (Cowen *et al.*, 2003; Huber *et al.*, 2006; Jungbluth *et al.*, 2013). In the deep terrestrial subsurface, the *Desulforudis* lineage can make up an extremely high proportion of microorganisms *in situ*, and has been associated with the potential for sulfate reduction, inorganic carbon fixation, and nitrogen fixation via genomic analysis (Chivian *et al.*, 2008). In the marine deep subsurface, this lineage has now been recovered in relatively high proportion in fluids retrieved from 1025C (17%), 1026B (39%;

Cowen *et al.*, 2003), and U1301A (36% of 2008 sample; Jungbluth *et al.*, 2013). Interestingly, *Desulforudis*-related gene sequences from Hole 1025C fluids form a monophyletic lineage with sequences detected from the terrestrial subsurface rather than other marine lineages recovered previously. Thus, within this lineage it appears that there may have been multiple transitions between the terrestrial and marine deep subsurface environments. The functional and evolutionary characteristics that are shared amongst the different deep subsurface *Desulforudis*-related lineages, and what differences may account for the pattern of evolutionary descent observed here, remain to be discovered.

The recovery of multiple shared lineages provides evidence linking microbial communities from several boreholes along the JdFR flank. This is not altogether surprising, as the CORK-fitted boreholes ostensibly tap in to basalt-hosted deep subsurface crustal fluids in relatively close proximity to one another and experience broad similarities in physical and chemical conditions. While these linkages provide an initial framework for investigating the genetic and evolutionary characteristics of microbial populations in the marine deep subsurface, it would benefit greatly from a genomic approach that extends beyond a relatively conserved, single-gene phylogenetic marker such as the 16S rRNA. In addition, environmental genomics approaches—whether based on single cell whole genome amplification and sequencing or metagenomics—would also help to illuminate the functional attributes of these lineages, as nearly all contain no close phylogenetic relatives in laboratory culture.

A subset of clones that bear reasonably close phylogenetic relationships to known isolates allow for some speculation regarding the functional characteristics of a few microbial lineages detected in Holes 1025C and 1026B. A common theme of the *Deltaproteobacteria* lineages related to clones described in this study is the ability to utilize low-molecular weight organic compounds (e.g., acetate, formate, pyruvate) and intermediate redox-state sulfur compounds as electron donors (e.g., Suzuki *et al.*, 1997; Abildgaard *et al.*, 2006); in some cases hydrogen can be used directly (e.g., Kuever *et al.*, 2001; Alazard *et al.*, 2003). Clone lineages from Hole 1025C and Hole 1026B fluids related to anaerobic, heterotropic *Deltaproteobacteria* groups (e.g., *Desulfobulbus*, *Desulfovibrio*) were in relatively high abundance, while clones related to potentially autotrophic lineages of *Gammaproteobacteria* that perform the oxidation of intermediate sulfur compounds (e.g., *Sulfurimonas*, *Thiomicrospira*, and Arctic96BD-19; Jannasch *et al.*, 1985; Inagaki *et al.*, 2003; Marshall and Morris, 2013), were also present.

Microbial lineages related to mesophiles that perform anaerobic sulfur-cycling processes such as the disproportion and/or reduction of sulfate (e.g., *Desulfobulbus*, *Desulfovibrio*, *Desulfocapsa*) were detected in high abundance from Hole 1025C. However, lineages related anaerobic mesophiles were also members of the Hole 1026B fluid sample, which is inconsistent with the expected borehole fluid temperature (~64°C) within Hole 1026B. It is possible that the suspected seawater intrusion in this location has cooled the immediate surroundings of the permeated basement aquifer and is selecting for a mesophilic microbial

community. Consistent with this idea is the notable absence of relatives of the thermophilic sulfate-reducing lineage *Archaeoglobus*, which is a lineage that has been consistently detected from borehole 1026B in previous studies (Cowen *et al.*, 2003; Nakagawa *et al.*, 2006; Steinsbu *et al.*, 2010). Microbial lineages related to known methanogens were absent, and only a single gene clone related to a known iron oxidizer was recovered, from Hole 1026B (i.e., *Zetaproteobacteria*). This suggests that either iron oxidation is not a common metabolic trait, or is being performed by as-yet unidentified iron oxidizers in this environment.

In summary, the basement fluid samples investigated here leverage the spatial array of borehole observatories located on the JdFR flank to reveal some aspects of deep subseafloor microbial community biogeography, particularly with regard to the overlap in microbial community members between boreholes located in close proximity, 1026B and U1301A. While 1026B and 1025C are not ideal targets for assessing the quantitative characteristics of microorganisms residing within the deep subseafloor, here they yield important, independent evidence for the presence of a number of phylogenetic lineages within the basalt-hosted deep subseafloor biosphere.

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3.6 Appendix

Table 3.1 General and chemical characteristics of fluid samples

	1025C uncorrected[a]	*1026B uncorrected*[a]	*U1301A uncorrected*[b]	*Bottom seawater*[b]
Clone prefix	1025C10	1026B10	1301AXX[c]	1301AXXsw[c]
Sampling depth (m)	2606+101+46.2[d]	2658+247+48.2[d]	2667+262+108[d]	
pH	7.9	6.8 (7.6[e])	7.4	7.7
Ca^{2+} (mM)	30.4 (34.1)	12.0 (60.0)	53.0	10.4
Mg^{+2} (mM)	29.7 (25.2)	48.5 (2.20)	3.40	53.7
K^{+} (mM)	9.4 (9.2)	9.6 (6.2)	6.4	10.2
CH_4 (µM)	5.3 (6.3)	n.d. (2.0)[b]	1.5	0.0002[f]
H_2 (µM)	n.d. (n.d.)	n.d. (0.7)[b]	0.3-2	0.0004[f]
NH_4^{+} (µM)	43 (51)	2.5 (122)	100	<0.05
PO_4^{3-} (µM)	0.10 (0)	<0.01 (0.16)	0.14	2.89
NO_2^{-1}+ NO_3^{-2} (µM)	6.4 (0)	40.6 (0)	1.5	40.8
SO_4^{2-} (mM)	26.2 (25.9)	27.2 (18.5)	18.3	28.4
Dissolved Fe (µM)	0.07 (0.08)	450 (0.5)	0.60	<0.10
Total Fe (µM)	43.3 (51.3)	1360 (n.d.)	0.8	<0.1
DOC (µM)	22 (19)	43 (8-17[g])	12	39
Alkalinity (meq/L)	0.88 (0.58)	0.36 (0.53[e])	0.52	2.48

[a]Corrected values provided in parentheses represent end-member fluids (nitrate=0)
[b]Chemical data from Lin *et al.*, 2012
[c]Jungbluth *et al.*, 2013
[d]Water column depth at CORK sampling spigot + sediment + permeated basement rock
[e]Cowen *et al.*, 2003
[f]Kelley *et al.*, 1998
[g]Lang *et al.*, 2006
n.d., not determined

Table 3.2 Relative abundance of SSU rRNA gene clones recovered from borehole 1025C & 1026B fluids

Phylogenetic affiliation[a]	*1025C (n=70)*	*1026B (n=87)*	*Representative clones*
Archaea			
Crenarchaeota (Thaumarchaeota)			
Marine Benthic Group A	0	1	1026B_51
Marine Group I	0	2	1026B_30, 1026B_73
Euryarchaeota			
DHVEG-6 (Figure 3.7a)	0	1	1026B_15
Bacteria			
Bacteroidetes			
Marinilabiaceae	1	0	1025C_63
NS9	0	2	1026B_18
SB-1 (Figure 3.7b)	4	0	1025C_22, 1025C_61
Cand. phylum SAR406	3	3	1025C_18, 1026B_53, 1026B_13
Chloroflexi			
Anaerolineaceae	1	0	1025C_30
Firmicutes			
Acholeplasmataceae	1	0	1025C_27
Clostridiaceae			
Alkaliphilus (Figure 3.6c)	1	0	1025C_01
Ca. *Desulforudis* (Figure 3.6a)	17	0	1025C_45
Peptococcaceae			
Desulfotomaculum (Figure 3.6d)	1	0	1025C_05
RF3 (Figure 3.6b)	0	2	1026B_12
Fusibacter (Figure 3.7f)	0	1	1026B_60
Planctomycetes			
OM190 (Figure 3.7e)	0	2	1026B_72
Proteobacteria			
Alphaproteobacteria (Figure 3.10)			
Hyphomicrobiaceae			
1301A10_076 lineage (Figure 3.10)	0	1	1026B_03

OCS116 (Figure 3.10)	0	1	1026B_24
Rhodobacteraceae			
Roseobacter clade NAC11-7 (Figure 3.10)	0	1	1026B_82
Rhodospirillaceae			
Defluviicoccus	0	1	1026B_23
SAR11	3	17	1025C_67, 1026B_29, 1026B_01, 1026B_20, 1026B_52, 1026B_80, 1026B_58, 1026B_41, 1026B_47, 1026B_87, 1026B_69
Betaproteobacteria			
Burkholderiaceae			
Cupriavidus	0	1	1026B_67
Deltaproteobacteria (Figure 3.5)			
Desulfobacteraceae			
Desulfobacula	0	6	1026B_07, 1026B_70
Desulfococcus	3	0	1025C_53
Desulfobulbaceae			
Desulfobulbus (Figure 3.5)	56	8	1025C_08, 1025C_15, 1026B_06, 1026B_21
Desulfocapsa (Figure 3.5)	1	0	1025C_51
1301A09_118 lineage	0	2	1026B_57
Desulfohalobiaceae (Figure 3.5)	0	5	1026B_19
Desulfovibrionaceae (Figure 3.5)	4	0	1025C_57
Nannocystineae	0	1	1026B_76
Nitrospinaceae	0	2	1026B_42
SAR324	0	1	1026B_55
Epsilonproteobacteria			
Helicobacteraceae			
Sulfurimonas (Figure 3.7c)	0	3	1026B_05, 1026B_25, 1026B_62
Gammaproteobacteria (Figure 3.8)			
9NBGBact_8 (Figure 3.8)	0	1	1026B_74
AGG47	0	2	1026B_34, 1026B_36

Arctic96BD-19	0	5	1026B_64, 1026B_46, 1026B_17
JTB35			
1301A10_105 lineage (Figure 3.8)	0	1	1026B_14
Moraxellaceae	1	0	1025C_31
OM182	0	1	1026B_83
Pseudoalteromonadaceae	0	9	1026B_66, 1026B_79, 1026B_77, 1026B_56, 1026B_35
Pseudomonas	0	5	1026B_40, 1026B_86
Thiomicrospira (Figure 3.8)	0	6	1026B_11
Zetaproteobacteria (Fiureg 3.7d)	0	1	1026B_31
Verrucomicrobia			
Arctic97B-4	0	1	1026B_59

[a]Phylogenetic affiliations were determined using SILVA SSU database release 111. However, in some cases manual phylogenetic assignment was required because the SILVA taxonomy was inconsistent. In these instances, lineages were named after the first gene clone derived from the group

Table 3.3 Microbial richness and evenness in borehole 1025C & 1026B fluids

	Similarity cutoff	*1025C*	*1026B*
No. of sequenced clones		70	87
Observed OTUs	99%	22	53
	97%	16	42
Estimated OTUs (Chao1)	99%	67	183
	97%	22	86
Simpson's evenness index	99%	0.26	0.83
	97%	0.33	0.63
Shannon diversity index	99%	2.66	4.32
	97%	2.29	3.82

Table 3.4 Summary of microbial community structure comparisons

	Significance of UniFrac metric (p-value)	
Comparison	*Weighted*	*Unweighted*
1025C x 1026B	≤ 0.03	≤ 0.03
1025C x U1301A	≤ 0.03	≤ 0.03
1026B x U1301A	≤ 0.03	0.06
1025C x sw[a]	≤ 0.03	≤ 0.03
1026B x sw[a]	≤ 0.03	≤ 0.03

[a]sw, Juan de Fuca Ridge bottom seawater (Jungbluth *et al.*, 2013)

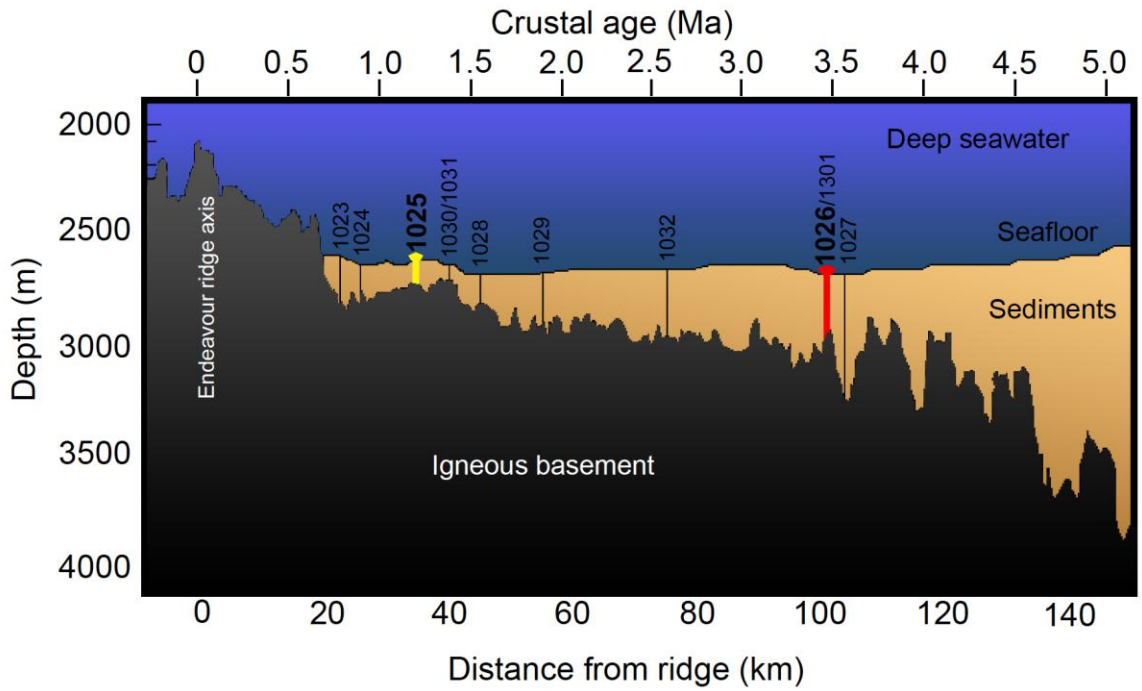
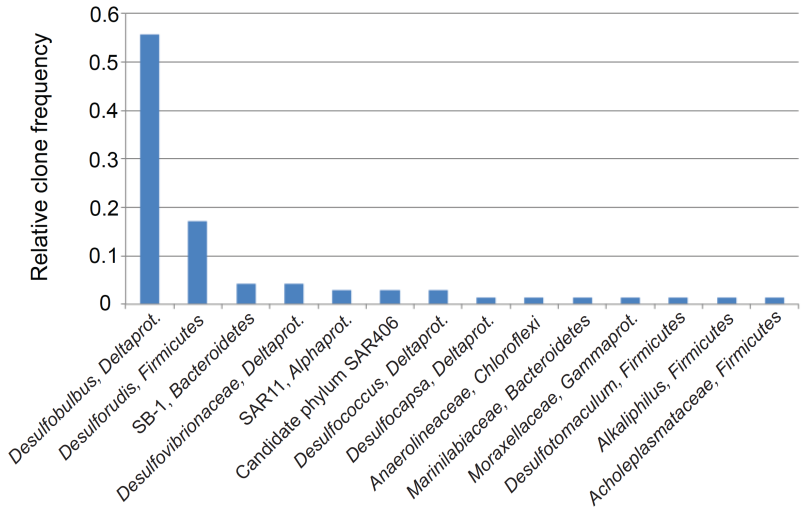


Figure 3.2 Colorized cross-sectional diagram of ODP Leg 168 showing depth of basement crust and sediment thickness, basement age and associated distance from ridge axis, and locations of Holes 1025 (yellow) and 1026 (red) in boldface font (modified from Wheat et al., 2004).

A



B

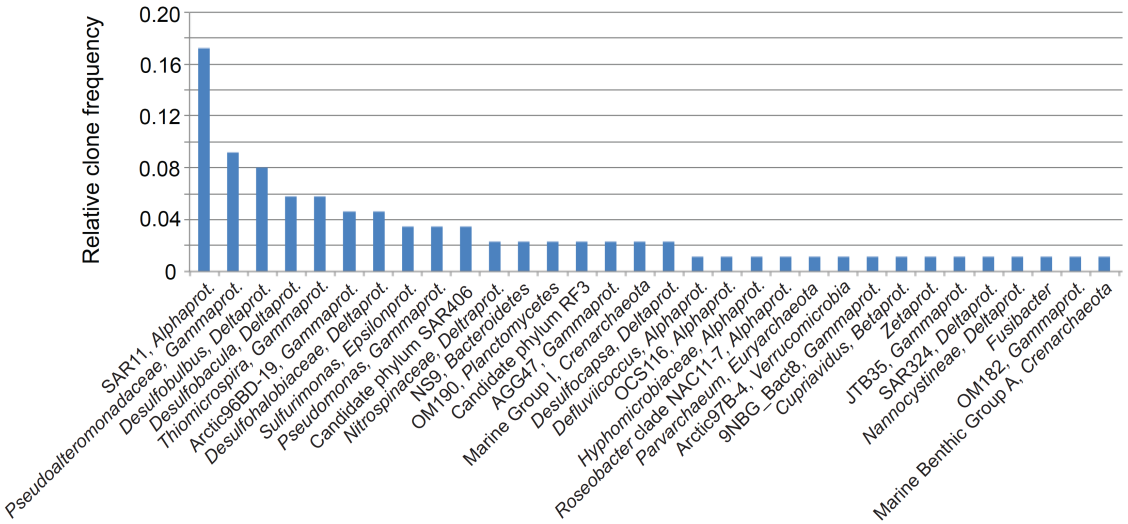


Figure 3.3 Rank order histograms of SSU rRNA gene clone sequences from (A) borehole 1025C and (B) borehole 1026B fluids sampled in the summer of 2010. The relative abundance of all gene clone sequences are shown, from a total of 70 (1025C) and 87 (1026B) sequences.

Figure 3.7 Phylogenetic relationships of borehole 1025C and borehole 1026B fluid-derived SSU rRNA gene clones within the domain *Archaea* related to (A) DHVEG-6, and the domain *Bacteria* related to (B) lineage SB-1 of phylum *Bacteroidetes*, (C) *Sulfurimonas* of phylum *Epsilonproteobacteria*, (D) phylum *Zetaproteobacteria*, (E) lineage OM190 of phylum *Planctomycetes,* and (F) phylum *Fusibacter*. Cultivated *Thermoplasmatales* (A), *Bacteroidetes* (B), *Campylobacteracreae* (C), *Proteobacteria* (D) *Planctomycetes* (E), and *Firmicutes* (F) were used as outgroups (data not shown). Other information as in Figure 3.5.

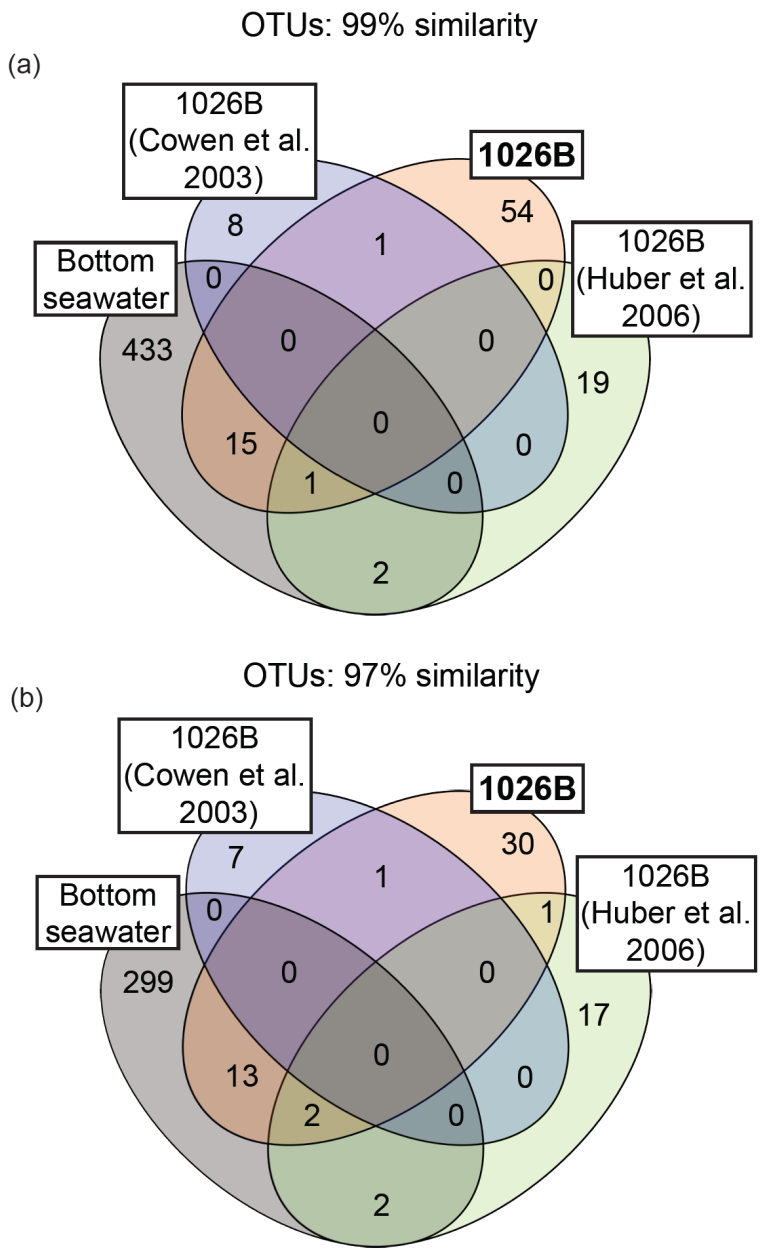


Figure 3.9 Venn diagrams showing microbial community relatedness fluid-derived SSU rRNA gene clones from all fluid samples collected to date from borehole 1026B (Cowen et al., 2003; Huber et al., 2006, this study) and locally-collected bottom seawater (Huber et al., 2006; Jungbluth et al., 2013) clustered at (A) 99% or (B) 97% similarity cut-off values.

Figure 3.11 Photograph of fixed and preserved samples of borehole 1026B fluids filtered onto polycarbonate membranes for microscopy. Note the iron particulates that prevented cellular enumeration.

CHAPTER 4

Microbial diversity in sediment near Grizzly Bare Seamount in Holes U1363B and U1363G

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4.1 Abstract

Microorganisms inhabiting sediment in close proximity to recharging basement outcrops are of interest because of the enhanced advective fluid flow in these locations, which is expected to exert unique selective pressures on the resident microbial communities. Here, Integrated Ocean Drilling Program (IODP) boreholes were used to access sediment microbial communities near Grizzly Bare recharge seamount on the Juan de Fuca Ridge eastern flank. The two locations examined in this study, Holes U1363G and U1363B, are 50 and 177 m away from the center of the outcrop, respectively. In general, small subunit ribosomal RNA gene clones from all three domains of life were detected; these groups were predominantly related to microorganisms known to reside in marine sediment. A large fraction of environmental gene clones recovered from Hole U1363B and U1363G sediment were related to uncultivated, candidate phyla of *Bacteria* such as BHI80-139, BRC1, JS1, OPB41, and TA06. Hole U1363B and U1363G sediment clone libraries were generally dominated by the domain *Bacteria* and particularly the phylum *Chloroflexi*, which comprised approximately one-quarter of the total gene clones identified. However, borehole sediment also contained several archaeal lineages that were phylogenetically affiliated with the Miscellanenous Crenarchaeotal Group. Eukaryotic *Fungi* were only detected within the interstitial water sample from Hole U1363B. Finally, a minor portion of clones recovered from sediment in this study were also recovered from basement fluid samples previously characterized from Baby Bare discharge seamount and Ocean Drilling Program and IODP borehole Circulation Obviation Retrofit Kit Observatories (CORKs) in Holes 1026B and U1301A, which are ~50 km to the north-northeast.

4.2 Introduction

For more than two decades, Ocean Drilling Program (ODP) and Integrated Ocean Drilling Program (IODP) expeditions to the eastern flank of the Juan de Fuca Ridge have been conducted for the purpose of describing the hydrogeological, geochemical, and microbiological processes and properties within a young ridge flank environment (e.g., Shipboard Scientific Party, 1992, 1997; Expedition 301 Scientists, 2005; Expedition 327 Scientists, 2011a; Wheat *et al.*, 2013). Driven by lithospheric cooling of the ridge flank, the porous and permeable igneous ocean basement acts as a subsurface conduit for hydrothermal fluid flow between exposed rocky outcrops (Wheat *et al.*, 2013). Although the nature of microbial life in the sediment-covered basement is the focus of much active research on the Juan de Fuca Ridge, the microbial diversity within marine sediment is still poorly cataloged, due in large part to the restriction of most marine sediment diversity surveys to shallow depths (<1 m) and continental margins (Orcutt *et al.*, 2011a).

Microbial diversity surveys on the Juan de Fuca Ridge have focused at diffuse flow hydrothermal vents at Axial Seamount (e.g., Lynch, 2000; Alain *et al.*, 2002; Pagé *et al.*, 2004; Huber *et al.*, 2006a, 2009; Kaye *et al.*, 2011; Meyer *et al.*, 2013), high-temperature hydrothermal vents at Endeavor Segment (e.g., Wang *et al.*, 2009a, 2009b; Zhou *et al.*, 2009; Anderson *et al.*, 2013), a hydrothermal mound located at off-axis Cleft Segment (Davis *et al.*, 2009), seafloor basalt (Mason *et al.*, 2007, 2009), sediment-buried basement fluid (Cowen *et al.*, 2003; Huber *et al.*, 2006b; Nakagawa *et al.*, 2006; Jungbluth *et al.*, 2013), and sediment-buried rock (Orcutt *et al.*, 2011b; Smith *et al.*, 2011; Lever *et al.*, 2013). The limited number of microbiological studies focused on sediment of the Juan de Fuca Ridge have typically not incorporated phylogenetic marker-based surveys of community diversity such as small subunit ribosomal RNA (SSU rRNA) gene sequencing (e.g., Cragg *et al.*, 2000; Mather and Parkes, 2000; Engelen *et al.*, 2008); only studies focused on Cascadia margin methane hydrate–bearing sediment have incorporated such an approach (e.g., Bidle *et al.*, 1999; Marchesi *et al.*, 2001; Knittel *et al.*, 2003; Inagaki *et al.*, 2006; Nunoura *et al.*, 2008; Briggs *et al.*, 2012). Descriptions of the phylogenetic and metabolic composition of microbial communities from other Juan de Fuca Ridge sediment environments are important for comprehensively investigating patterns in microbial distribution and community evolution with respect to gradients in the physical and chemical properties of the system.

During IODP Expedition 327, five boreholes were cored at Site U1363 (Figure 4.1) adjacent to an exposed rocky seamount, Grizzly Bare outcrop (Figure 4.2). Grizzly Bare serves as a point of seawater recharge into the igneous basement that is superimposed over basal basement fluid flow likely originating predominantly from the unsedimented ridge flanks. Sediment in this area is composed of turbidites interspersed with hemipelagic clay, consistent with core recovery from ODP and IODP boreholes at Sites 1026 and U1301 located >50 km to the north-northeast. Three sample types were collected from Holes U1363B and U1363G for the purpose of investigating microbial community structure:

1. Raw uncompressed sediment samples,
2. Squeezed sediment (i.e., squeeze cakes), and
3. Interstitial water derived from the squeeze cakes.

It is possible that the three sample types differ with respect to their resident microbial community structure based on strength of attachment of individual microbial lineages to sediment substrate. For example, squeeze cakes may represent communities that might be more tightly attached to the sediment, whereas interstitial water derived from the squeeze cakes may reflect a more loosely associated microbial community. In this investigation, the variety of samples from Holes U1363B and U1363G (Figure 4.2) were investigated using SSU rRNA gene cloning and sequencing.

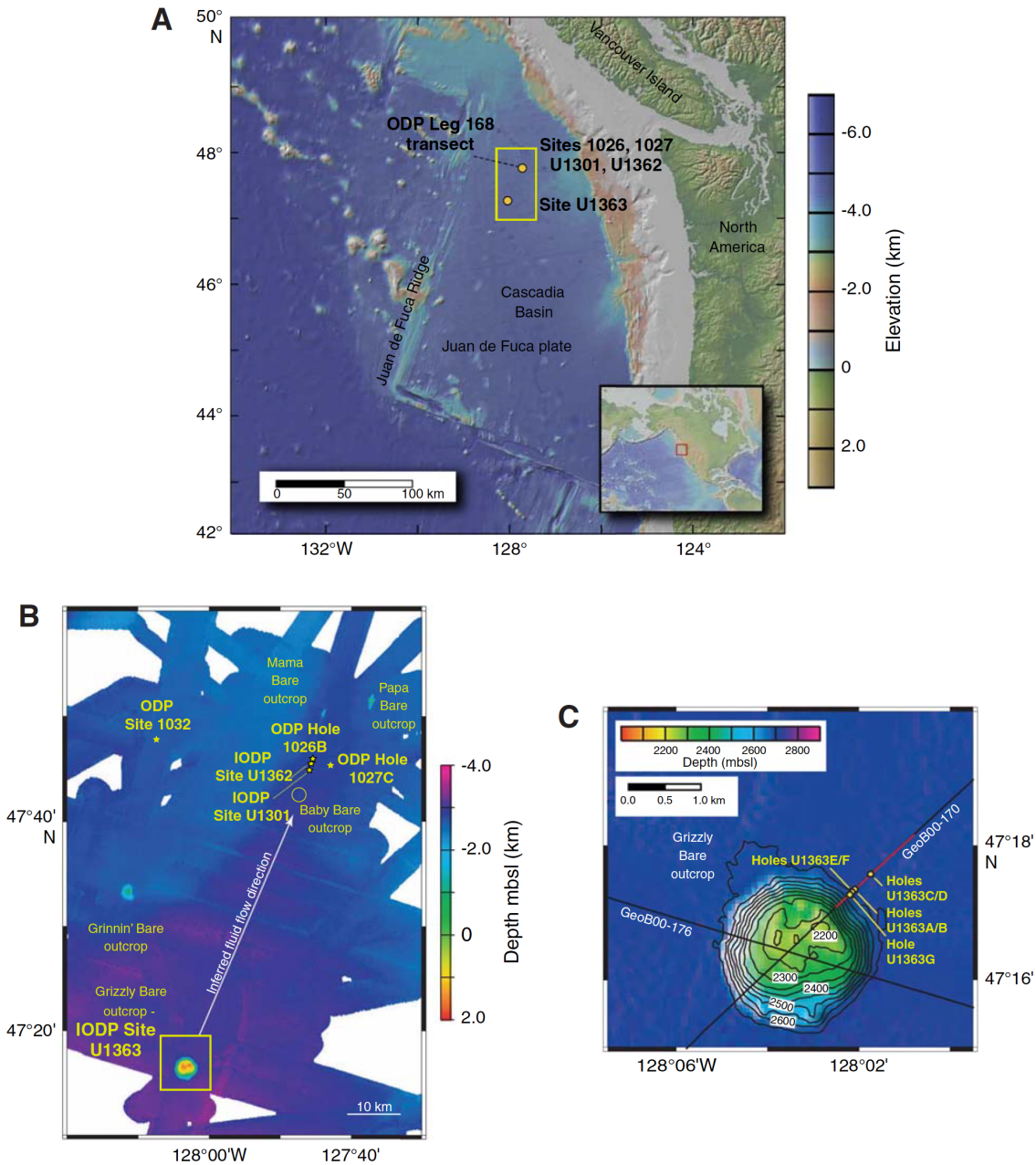

Figure 4.1 A. Site locations, Integrated Ocean Drilling Program (IODP) Expedition 327. Site U1363 is adjacent to a large basement outcrop ~52 km south-southwest of the dashed line that denotes Ocean Drilling Program (ODP) Leg 168 (Shipboard Scientific Party, 1997). The yellow box indicates the location of B (modified from Expedition 327 Scientists, 2011a). B. Regional bathymetry of Expedition 327 and previous expedition sites and basement outcrops. Thermal and chemical data suggest hydrothermal fluids recharge at Grizzly Bare outcrop and flow north-northeast (white arrow). The yellow box indicates the location of C (modified from Expedition 327 Scientists, 2011a). C. Bathymetry around Grizzly Bare outcrop and Site U1363. Black lines indicate tracks of reflection seismic lines around Hole U1363B collected during the 2000 ImageFlux expedition (Zühlsdorff et al., 2005; Hutnak et al., 2006). Part of Line GeoB00-170 (red line) is shown in Figure 4.2A (modified from Expedition 327 Scientists, 2011c).

4.3 Materials and Methods

Sample collection and processing

Samples were cored and collected from Holes U1363B and U1363G as described previously (Expedition 327 Scientists, 2011c); the subset of samples analyzed in this study is summarized in Table 4.1. Within the sample name, H and X indicate the two different coring methods that were employed: Hydraulic Piston Corer or Extended Core Barrel, respectively. For processing raw sediment samples, a sterile scoopula was used to transfer up to 20 cm^3 of raw sediment into a 50 mL falcon tube. An amount of DNA lysis buffer (20 mM Tris-HCl, 2 mM EDTA, 1.2% Triton X-100, and 2% lysozyme (w/v); pH 8) was added to just cover all of the sample material (10–20 mL) and subsequently stored at –80°C until further processing. In order to sample sediment pore water (interstitial water), sediment cores were pressed with 11 µm pore-size Grade Number 1 Whatman filter paper (Whatman, Maidstone, United Kingdom) to release ~10 mL of fluid that was subsequently filtered through a 0.1 µm pore-size Supor membrane filter (Pall Corporation, Port Washington, NY, USA) via a sterile 20 mL syringe. Sterile forceps were used to transfer the membrane into a microcentrifuge tube, DNA lysis buffer was added enough to cover the membrane (~2 mL), and the sample was stored at –80°C until further processing. The pressed sediment cores, referred hereafter as squeeze cake samples, were preserved without addition of any buffers or reagents, wrapped in foil, and stored at –80°C until further processing.

DNA extraction

All samples were thawed to room temperature and environmental DNA extracted using the PowerMax Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) following the manufacturer's protocol, with the following modifications (Mason *et al.*, 2010): (i) after the addition of solution C1, two 5 min incubations at 65°C were performed with intermittent vortexing; (ii) bead beating was performed in 50 mL Falcon tubes using a Vortex Genie 2 benchtop vortexer (Scientific Industries, Bohemia, NY, USA) and a custom-made adapter for 1 h at maximum speed; and (iii) nucleic acids were eluted using ultrapure water. Bead beating for a lesser time was not attempted and therefore some DNA shearing may have occurred. Up to 10 g of sediment and squeeze cake sample were used for nucleic acid extraction. For the pore water sample, the entire filter and associated lysis buffer were added to the bead beating tube and processed identically to other samples. Negative DNA extractions consisting of only kit reagents were processed in parallel to sediment and interstitial pore water sample extractions.

Nucleic acids were concentrated as outlined in the PowerMax Soil DNA Isolation Kit manual. Sodium chloride was added to the eluted nucleic acid extracts to a final concentration

of 0.2 M and mixed, followed by the addition of 100% ethanol at a ratio of 2:1 and further mixing. The mixture was centrifuged at 2500 x g for 30 min at room temperature. The supernatant was decanted, and the resulting pellet was washed with 70% cold ethanol before evaporation overnight in a sterile laminar flow hood. Following evaporation, nucleic acid pellets were dissolved in ultrapure water and quantified via Picogreen fluorometric assay (Quant-iT dsDNA kit, Invitrogen, Carlsbad, CA, USA).

SSU rRNA gene cloning and sequencing

Small subunit ribosomal RNA gene fragments were amplified via polymerase chain reaction (PCR) using universal oligonucleotide primers 519F (5'-CAGCMGCCGCGGTAATWC-3') and 1406R (5'-ACGGGCGGTGTGTRC-3') (Lane *et al.*, 1985). Each 20 µL PCR reaction contained 0.5 U of SpeedSTAR HS DNA polymerase (Takara Bio Inc, Otsu, Shiga, Japan), 1× Fast Buffer I, 200 µM of each of the four deoxynucleoside triphosphate (dNTPs), 200 nM of both forward and reverse primer, and ~0.5–4 ng of environmental DNA template. PCR cycling conditions consisted of an initial denaturation step at 95°C for 4 min followed by 25 cycles (Samples 1363B8X1_SC, 1363G1H2_SC, 1363G3H1_SC) or 34 cycles (Samples 1363B1H1_SC, 1363B1H1_RS, 1363B7X2_IW, extraction control) of 95°C denaturation for 30 s, 55°C annealing for 1 min, 72°C extension for 2 min, and a final extension step at 72°C for 20 min. Amplification products of the anticipated length were excised from an agarose gel and subsequently purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA, USA). Products were cloned using the TOPO TA Cloning kit (Invitrogen) following the manufacturer's instructions. Clones were sequenced unidirectionally with primer T7 on an ABI 3730XL DNA Analyzer (Applied Biosystems, Carlsbad, CA, USA).

Phylogenetic analysis

DNA sequences were trimmed of vector sequence and manually curated using Sequencher version 5.1 (GeneCodes, Ann Arbor, MI, USA). Curated clone sequences were first aligned using the online SINA tool version 1.2.11 (Pruesse *et al.*, 2012) before importing into the ARB software package (Ludwig *et al.*, 2004; Westram *et al.*, 2011) for manual curation of the multiple species alignment and taxonomic classification using version SSURef_111 of the SILVA ARB database (Pruesse *et al.*, 2007, 2011). Clone sequences released to GenBank after the release of ARB database SSURef_111 that were highly related to clone sequences obtained in this study were downloaded and included in the relevant phylogenetic analyses. Maximum likelihood phylogenetic analyses were performed via RAxML using the GTR model of nucleotide substitution under the gamma model of rate heterogeneity (Stamatakis, 2006). Bootstrap

analyses for phylogenetic trees containing large (>100 sequences; *Chloroflexi* and MCG) were determined by RAxML using 1000 bootstrap iterations via the CIPRES Science Gateway version 3.3 (Miller *et al.*, 2010). All other bootstrap analyses were determined by RAxML using the rapid bootstrap analysis algorithm (1000 bootstraps) implemented within ARB (Stamatakis *et al.*, 2008). All sequences generated in this study have been deposited in GenBank under accession numbers KC990940 – KC991019.

Microbial community analysis

Microbial community analyses were performed using sequences containing >700 unambiguously aligned nucleotides (132 of 140 sequences). The UniFrac significance test (Lozupone and Knight, 2005) was used to evaluate whether the microbial community structure was different between sets of samples. A maximum likelihood-based phylogenetic tree containing all environmental sequences derived in this study was evaluated using an online implementation of the hypothesis testing approach (Lozupone *et al.*, 2006). Significant differences in community structure within pairs of samples were evaluated using 100 permutations of both the weighted and unweighted implementations of the UniFrac algorithm. Bonferroni-corrected p-values are reported. Microbial community α-diversity estimators, rarefaction curves, and community relatedness were generated or assessed using lane-masked clone sequences grouped into operational taxonomic units (OTUs) defined at 99% and 97% SSU rRNA gene sequence similarity cut-off values using the average neighbor clustering method as implemented by the mothur software package (Schloss *et al.*, 2009). Microbial richness, evenness, and diversity were assessed by the Chao1 richness estimator (S_{chao1}) (Chao, 1984), Simpson evenness index ($E_{simpson}$) (Simpson, 1949), and the non-parametric Shannon diversity index ($\hat{H}_{shannon}$) (Shannon, 1948), respectively, as implemented in mothur (Schloss *et al.*, 2009).

4.4 Results and Discussion

Universal oligonucleotide primers were used to PCR amplify, clone, and sequence SSU rRNA genes from Grizzly Bare sediment samples collected from Holes U1363B and U1363G in August to September of 2010. A total of 118 environmental gene clones were sequenced: 22 from Sample 327-U1363B-1H-1, 0–10 cm, squeeze cake (1363B1H1_SC), 19 from Sample 327-U1363B-1H-1, 130–140 cm, raw sediment (1363B1H1_RS), 23 from Sample 327-U1363B-7X-2, 125–145 cm, interstitial water (1363B7X2_IW), 19 from Sample 327-U1363B-8X-1, 130–150 cm, squeeze cake (1363B8X1_SC), 17 from Sample 327-U1363G-1H-2, 126–136 cm, squeeze cake (1363G1H2_SC), and 18 from Sample 327-U1363G-3H-1, 140–150 cm, squeeze cake (1363G3H1_SC) (Table 4.2). In addition, 22 clones were sequenced from the extraction control. The resulting microbial communities were analyzed using a variety of α-diversity calculators and

operational taxonomic units defined at 99% and 97% SSU rRNA gene sequence similarity (Table 4.3). The Shannon diversity index and Chao1 community diversity estimator indicated that Samples 1363B1H1_SC and 1363B8X1_SC had relatively high microbial diversity. The interstitial pore water sample had the lowest diversity found within all environmental samples, which may be expected due to the low sample size and therefore the amount of biomass likely contained within the pore water sample. Microbial diversity was highest in raw and pressed sediments, likely resulting from the larger amount of starting material used for nucleic acid extraction. The lowest diversity was found within the extraction control. The Simpson's evenness index revealed that squeeze cake samples had low community evenness. Microbial communities within the interstitial pore water sample were more even, due in part to putative microbial contamination, as explained below. Rarefaction curves generated using the same operational taxonomic unit definitions were for the most part steeply sloping, indicating that the clone libraries were undersampled (Figure 4.3).

Both weighted and unweighted implementations of the UniFrac distance metric were used to perform microbial community structure comparisons among samples grouped by borehole location (i.e., all Hole U1363B samples versus all Hole U1363G samples) and borehole depth (i.e., surface core [1H] versus deep cores [3H, 7X, and 8X]); comparisons were also performed using pairs of squeeze cake samples, a squeeze cake sample versus raw sediment sample from the same core section, and the interstitial pore water sample versus extraction control pair (Table 4.4). Significant differences in microbial community structure were observed between borehole locations when compared using the unweighted version of UniFrac (Table 4.4). At both locations, surface samples had significantly different microbial community structure than their respective deep sediment samples. In pairwise comparisons, individual squeeze cake sediment samples from both locations and at a range of depths did not significantly differ in microbial community structure by either the weighted or unweighted UniFrac measure (Table 4.4). Similar comparisons revealed no significant differences between the squeeze cake sample and the raw sediment sample collected from the same core (Table 4.4). Finally, microbial community structure in the sediment interstitial water sample was significantly different than the extraction control using the weighted version of UniFrac but not using the unweighted algorithm (Table 4.4).

The surface sediment samples analyzed from Hole U1363B were collected from the 0.00–0.10 m (Sample 1363B1H1_SC) and 1.30–1.40 m (Sample 1363B1H1_RS) sections. Despite the small clone library size, five different phylogenetic groups were shared between the two samples (Table 4.2). Candidate bacterial phylum BHI80-139 was the most abundant group detected in the Sample 1363B1H1_SC squeeze cake (5 of 22 clones), where it was most closely related to gene clones recovered from other samples described here and in marine sediment worldwide (e.g., Reed *et al.*, 2002; Inagaki *et al.*, 2006; Hoshino *et al.*, 2011) (Figure 4.4a).

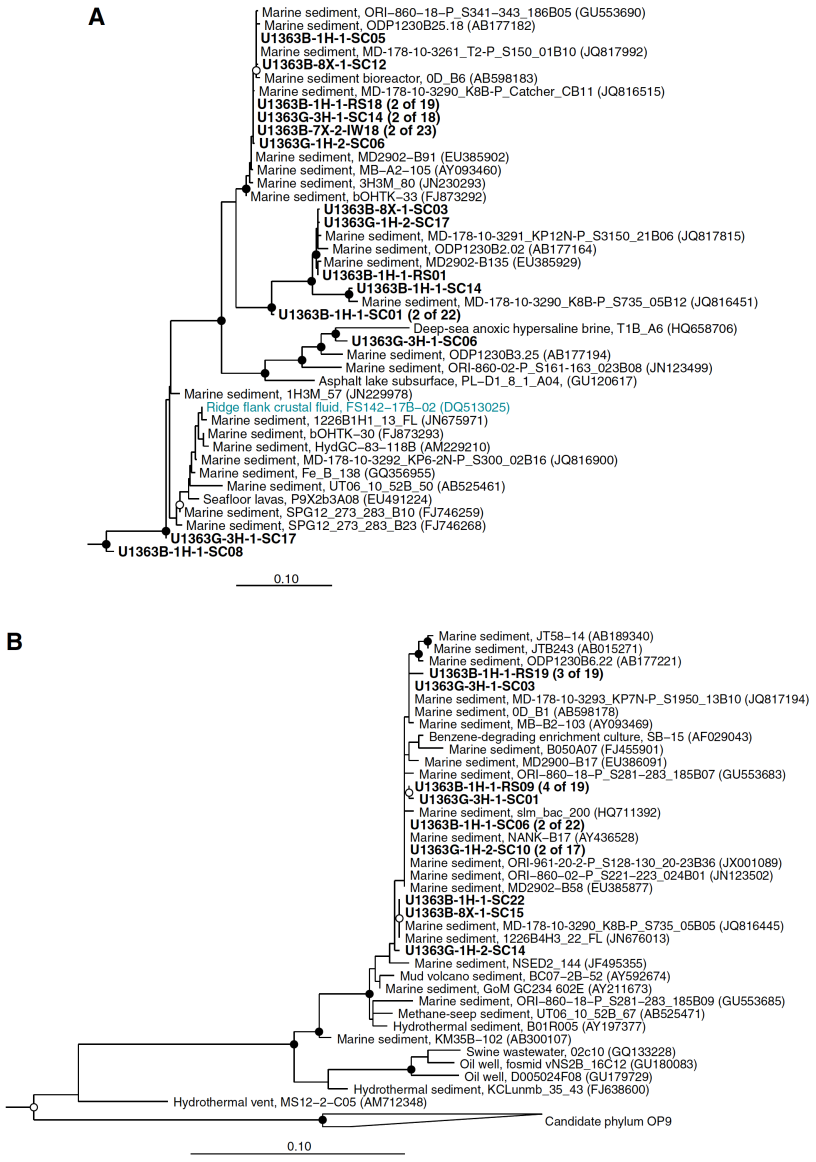

Figure 4.4 Phylogenetic relationships of Grizzly Bare sediment gene clones related to (A) candidate bacterial phylum BHI80-139 and (B) candidate bacterial phylum JS1. Lineage in blue text was previously recovered from Juan de Fuca Ridge flank Baby Bare fluids (Huber et al., 2006b). Cultivated *Dictyoglomaceae* and *Thermoanaerobacteraceae* in A and cultivated *Halanaerobiaceae* in B were used as outgroups (not shown). Open circles indicate nodes with bootstrap support between 50% and 80%, whereas solid circles indicate bootstrap support >80%, from 1000 replicates. Gene clones recovered in this study are highlighted in bold; the fractional abundance of identical clones recovered from the sample is listed in parentheses. Scale bars correspond to 0.1 substitutions per nucleotide position.

Candidate bacterial phylum JS1 formed the most abundant group detected in the surface raw sediment Sample 1363B1H1_RS (7 of 19 clones), where it was also most closely related to environmental gene clones recovered from other samples described here and in marine sediment worldwide (e.g., Li *et al.*, 1999; Newberry *et al.*, 2004; Briggs *et al.*, 2011) (Figure

4.4b). Uncultivated lineages within the bacterial phylum *Chloroflexi* were detected in surface sediment samples in relatively high abundance (Table 4.2); specifically, the vadinBA26 lineage was the most common group detected. Similar to the other groups mentioned above, highly related gene clones were detected in other samples described herein, as well as in marine sediments worldwide (e.g., Takeuchi *et al.*, 2009; Durbin and Teske, 2011) (Figure 4.5). Other less abundant clone groups recovered from Hole U1363B surface sediments include members of both the archaeal Miscellaneous Crenarchaeotal Group (MCG) and bacterial phyla *Planctomycetes* and *Proteobacteria* (Table 4.2; Figures 4.5 and 4.6).

Sediment samples analyzed from Hole U1363B originated from deeper (and older) horizons that were 8–12 m above the sediment/basement interface and consisted of interstitial pore water collected from the 45.25–45.45 m section (Sample 1363B7X2_IW) and a sediment squeeze cake collected from the 48.80–49.00 m section (Sample 1363B8X1_SC). Candidate bacterial phylum BHI80-139 was the only group shared between the two samples (two clones from each library), where it formed two distinct lineages (Figure 4.4a). The most abundant gene clone group identified from the interstitial pore water sample (11 of 23 clones) was highly related to the overwhelmingly dominant *Firmicutes* gene clone detected within the extraction control (21 of 22 clones), indicating that this group of sequences likely results from contamination; this is probably a reflection of the low amount of biomass obtained from this interstitial pore water sample. Another abundant clone group from interstitial pore water fluids (4 of 23 clones) was phylogenetically affiliated with the *Salinimicrobium* lineage of the phylum *Bacteroidetes* (Figure 4.7a), and closely related to cultivated groups recovered from marine sediment (e.g., Ying *et al.*, 2007) and terrestrial saline soil (e.g., Lim *et al.*, 2008). These isolates are both aerobic and anaerobic heterotrophs. Another abundant group in interstitial pore water (4 of 23 clones) was phylogenetically related to the *Trichocomaceae* lineage of eukaryotic *Fungi* (Figure 4.7b) and closely related to a fungus isolated from a saltern in China (GenBank acc. no. AY173080). The most abundant gene clone group identified from the deep squeeze cake sample from Hole U1363B (4 of 19 clones) was phylogenetically affiliated with archaeal lineage MCG-2, which is an uncultivated group of *Archaea* closely related to gene clones recovered from marine sediments (e.g., Colwell *et al.*, 2005) (Figure 4.6). Another abundant gene clone group recovered from the squeeze cake sample was related to the vadin26 lineage of *Chloroflexi* described above (Figure 4.5). Other less abundant clone groups recovered from Hole U1363B deep sediments include members of the bacterial phyla *Chloroflexi, Planctomycetes*, and candidate phyla BRC1, JS1, and OPB41 (Table 4.2; Figures 4.5 and 4.8).

Squeeze cake sediment samples analyzed from Hole U1363G were collected from the 2.76–2.87 m section near the surface (Sample 1363G1H2_SC) and from a deeper horizon around 10 m above the sediment/basement interface within the 17.40–17.50 m section (Sample 1363G3H1_SC). Similar to the samples described above, candidate bacterial phyla BHI80-139, JS1, and lineages within the phylum *Chloroflexi* (i.e., Napoli-4B-65, Sh765B-AG-111,

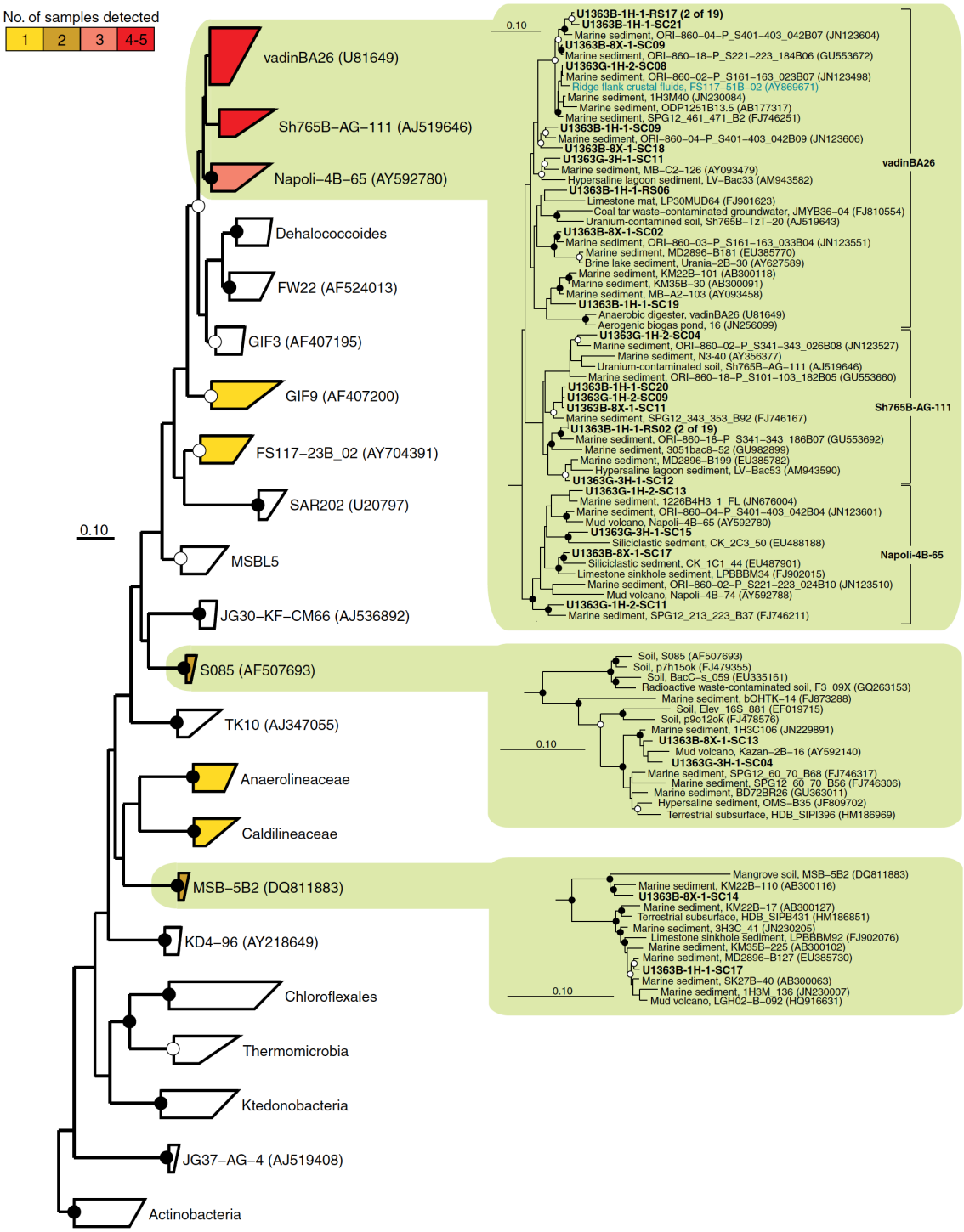

Figure 4.5 Phylogenetic relationships of Grizzly Bare sediment small subunit ribosomal RNA gene clones related to the bacterial phylum *Chloroflexi*. Lineage in blue text was previously recovered from Juan de Fuca Ridge flank Baby Bare fluids (Huber et al., 2006b). Cultivated *Actinobacteria* were used as an outgroup. See Figure 4.4 for symbol definitions and other information.

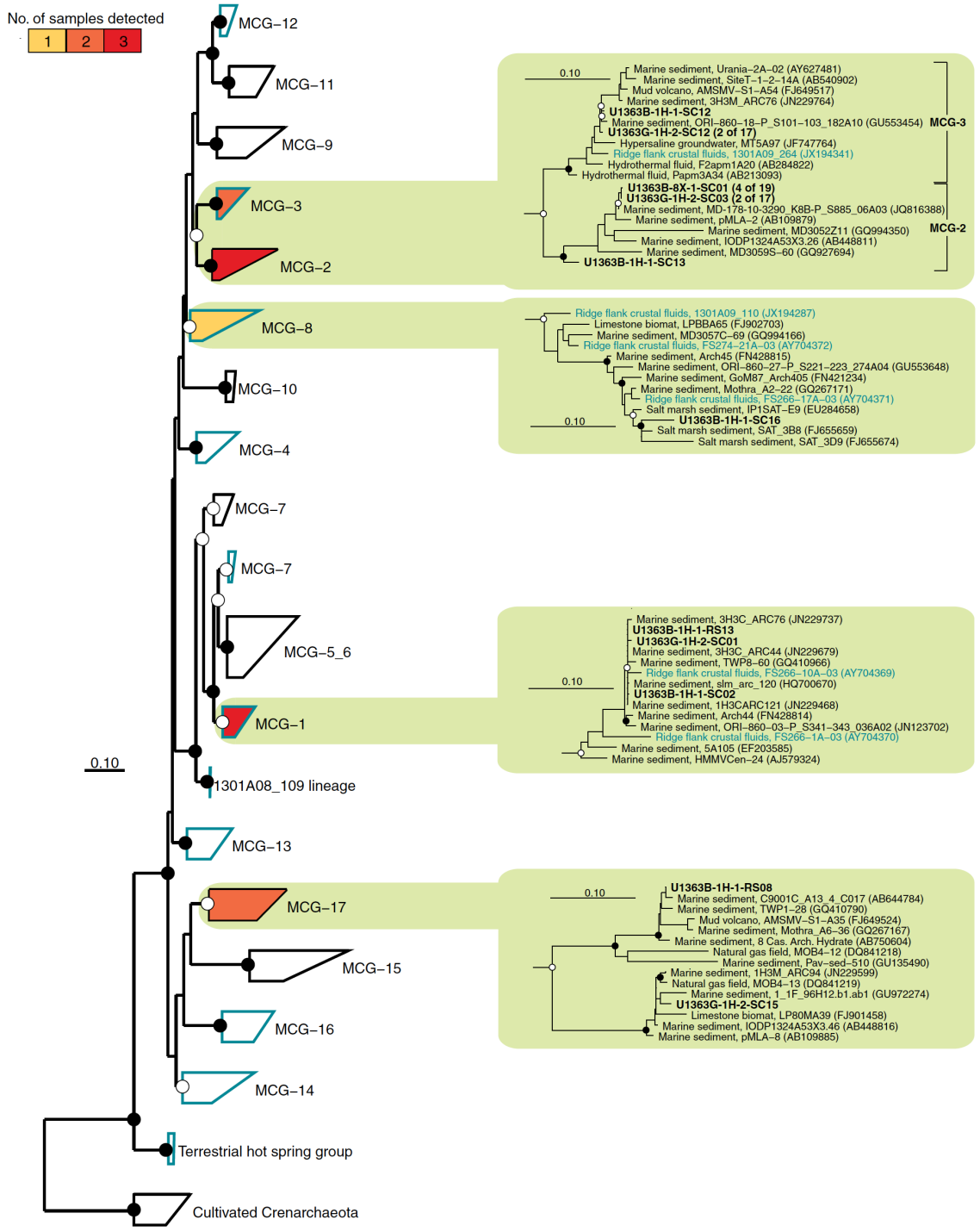

Figure 4.6 Phylogenetic relationships of Grizzly Bare sediment small subunit ribosomal RNA gene clones related to the Miscellaneous Crenarchaeotal Group. Group names are consistent with the previous naming scheme outline (Kubo et al., 2012). Blue outlines and text indicate lineages previously recovered from the Juan de Fuca Ridge flank basement environment (Cowen et al., 2003; Huber et al., 2006b, Nakagawa et al., 2006; Orcutt et al., 2011b; Jungbluth et al., 2013). Cultivated *Crenarchaeota* were used as an outgroup. See Figure 4.4 for symbol definitions and other information.

and vadinBA26) were shared between these samples, and all groups were phylogenetically related to gene clones that have previously been identified from marine sediments (e.g., Li *et al.*, 1999; Reed *et al.*, 2002; Newberry *et al.*, 2004; Inagaki *et al.*, 2006; Takeuchi *et al.*, 2009; Briggs *et al.*, 2011; Durbin and Teske, 2011; Hoshino *et al.*, 2011). Candidate bacterial phylum JS1 formed the most abundant group detected (3 of 17 clones) in the near-surface squeeze cake sample from Hole U1363G (Figure 4.4b), while the most abundant groups detected in the deeper squeeze cake sample were candidate bacterial phyla BHI80-139 and OPB41 (4 of 18 clones) (Table 4.2). Other less abundant clone groups recovered from Hole U1363G near-surface and deep sediments include members of both the archaeal group MCG and bacterial phyla *Chloroflexi*, *Planctomycetes*, *Proteobacteria*, and candidate phylum TA06 (Table 4.2; Figures 4.5, 4.6, and 4.8).

OTUs generated using similarity cut-off values of 97% and 99% were used to generate gene clone clusters, which were used to also compare overlap between microbial communities (Figures 4.9, 4.10, 4.11). Squeeze cakes and raw sediment shared an OTU cluster within the candidate phylum JS1 and multiple clusters within the candidate phylum BHI80-139 in most comparisons performed (Figures 4.9, 4.10, 4.11a, 4.11b), indicating the potential widespread distribution of these groups within Grizzly Bare sediments. The single interstitial pore water sample from Hole U1363B shared one OTU cluster in common with the extraction control, which is related to the *Tumebacillus* lineage of *Firmicutes*. However, a majority of OTUs observed in the interstitial pore water sample were not found in the extraction control (Figures 4.11c and 4.11d). In most comparisons, a majority of observed OTUs were unique to individual samples (Figures 4.9, 4.10, and 4.11).

A small subset of the gene clones identified in the samples characterized here have also been recovered from ridge flank fluids (Cowen *et al.*, 2003; Huber *et al.*, 2006b; Jungbluth *et al.*, 2013). Environmental gene clones phylogenetically related to the vadinBA26 lineage of *Chloroflexi* and candidate bacterial phyla BHI80-139 and TA06 have previously been detected from Baby Bare fluid samples (Huber *et al.*, 2006b) (Figures 4.4a, 4.5, 4.8c). In addition, lineage MCG-1 contained gene clones phylogenetically related to clones detected at Baby Bare Seamount, whereas lineages MCG-3 and MCG-8 contained clones phylogenetically related to others recovered from Hole U1301A ridge flank fluids (Jungbluth *et al.*, 2013) and Baby Bare Seamount fluids (MCG-8; Huber *et al.*, 2006b) (Figure 4.6). Seven MCG lineages recovered from CORK-derived crustal fluids previously (Cowen *et al.*, 2003; Huber *et al.*, 2006b; Nakagawa *et al.*, 2006; Orcutt *et al.*, 2011b; Jungbluth *et al.*, 2013) were not recovered from Grizzly Bare sediments in this study. Notably absent was the abundant MCG-12 (aka, lineage 1026B52) group detected in ODP Hole 1026B (Cowen *et al.*, 2003) and IODP Hole U1301A fluids (Jungbluth *et al.*, 2013) and rock chips (Orcutt *et al.*, 2011b) (Figure 4.6).

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4.6 Appendix

Table 4.1 Grizzly Bare sediment samples

IODP sample name[a]	*IODP sample identifier*	*Absolute depth (mbsf)*	*Distance from basement*[b] *(m)*	*Sample type*	*Total DNA recovered*[c] *(ng)*	*Clone prefix*
327-U1363B-1H-1, 0-10 cm	CAKE2410281	0.00-0.10	17.00-16.90	squeeze cake	153.3	1363B1H1_SC
327-U1363B-1H-1, 130-140 cm	OTHR2409681	1.30-1.40	15.70-15.60	sediment	359.8	1363B1H1_RS
327-U1363B-7X-2, 125-145 cm	LIQ2412791	45.25-45.45	11.75-11.55	interstitial pore water	8.9	1363B7X2_IW
327-U1363B-8X-1, 130-150 cm	CAKE2414001	48.80-49.00	8.20-8.00	squeeze cake	128.3	1363B8X1_SC
327-U1363G-1H-2, 126-136 cm	CAKE2425971	2.76-2.86	14.24-14.14	squeeze cake	46.6	1363G1H2_SC
327-U1363G-3H-1, 140-150 cm	CAKE2428901	17.40-17.50	<1[d]	squeeze cake	295.6	1363G3H1_SC

[a]*Naming convention: Expedition-Site/Hole-Core/Type-Section Interval.*

[b]*Basement depths of 57.0 mbsf (1363B) and 17.0 mbsf (1363G) were used (Expedition 327 Scientists, 2011b).*

[c]*Total DNA recovered from control was 5.2 ng.*

[d]*Most of Core 327-U1363G-3H is flow-in (Expedition 327 Scientists, 2011b).*

Table 4.2 Abundance and identity of SSU rRNA gene clones recovered from Grizzly Bare sediment samples

Phylogenetic affiliation[a]	*1363B1H1_SC (n=22)*	*1363B1H1_RS (n=19)*	*1363B7X2_IW (n=23)*	*1363B8X1_SC (n=19)*	*1363G1H2_SC (n=17)*	*1363G3H1_SC (n=18)*	*Extraction control (n=22)*	*Representative clones*
Archaea								
Crenarchaeota								
Misc. Crenarchaeotic Group								
MCG-1	1	1	0	0	1	0	0	1363B1H1_SC02, 1363B1H1_RS13, 1363G1H2_SC01
MCG-2	1	0	0	4	2	0	0	1363B1H1_SC13, 1363B8X1_SC01, 1363G1H2_SC03
MCG-3	1	0	0	0	2	0	0	1363B1H1_SC12, 1363G1H2_SC12
MCG-8	1	0	0	0	0	0	0	1363B1H1_SC16
MCG-17	0	1	0	0	1	0	0	1363B1H1_RS08, 1363G1H2_SC15
Bacteria								
Actinobacteria								
Propionibacterium	0	0	2	0	0	0	0	1363B7X2_IW20
Bacteroidetes								

Salinimicrobium	0	0	4	0	0	0	0	1363B7X2_IW13
Cand. phylum BHI80-139	5	3	2	2	2	4	0	1363B1H1_SC01, 1363B1H1_SC05, 1363B1H1_SC08, 1363B1H1_SC14, 1363B1H1_RS01, 1363B1H1_RS18, 1363B7X2_IW18, 1363B8X1_SC03, 1363B8X1_SC12, 1363G1H2_SC06, 1363G1H2_SC17, 1363G3H1_SC06, 1363G3H1_SC14, 1363G3H1_SC17
Cand. phylum BRC1	0	0	0	1	0	0	0	1363B8X1_SC06
Chloroflexi								
Anaerolineaceae	0	0	0	0	1	0	0	1363G1H2_SC02
Caldilineaceae	1	0	0	0	0	0	0	1363B1H1_SC15
FS117-23B-02	1	0	0	0	0	0	0	1363B1H1_SC10
GIF9	0	1	0	0	0	0	0	1363B1H1_RS12
MSB-5B2	1	0	0	1	0	0	0	1363B1H1_SC17, 1363B8X1_SC14
Napoli-4B-65	0	0	0	1	2	1	0	1363B8X1_SC17, 1363G1H2_SC11, 1363G1H2_SC13,

								1363G3H1_SC15
S085	0	0	0	1	0	1	0	1363B8X1_SC13, 1363G3H1_SC04
Sh765B-AG-111	1	2	0	1	2	1	0	1363B1H1_SC20, 1363B1H1_RS02, 1363B8X1_SC11, 1363G1H2_SC04, 1363G1H2_SC09, 1363G3H1_SC12
vadinBA26	3	3	0	3	1	1	0	1363B1H1_SC09, 1363B1H1_SC19, 1363B1H1_SC21, 1363B1H1_RS06, 1363B1H1_RS17, 1363B8X1_SC02, 1363B8X1_SC09, 1363B8X1_SC18, 1363G1H2_SC08, 1363G3H1_SC11
Firmicutes								
Tumebacillus	0	0	11	0	0	0	21	1363B7X2_IW07, ControlExtract09-17
Cand. phylum JS1	3	7	0	1	3	2	0	1363B1H1_SC06, 1363B1H1_SC22, 1363B1H1_RS09, 1363B1H1_RS19,

								1363B8X1_SC15, 1363G1H2_SC10, 1363G1H2_SC14, 1363G3H1_SC01, 1363G3H1_SC03
Cand. phylum OPB41	0	0	0	2	0	4	0	1363B8X1_SC05, 1363G3H1_SC09
Planctomycetes								
Brocadiaceae	1	0	0	0	0	0	0	1363B1H1_SC03
CCM11a	0	0	0	0	0	1	0	1363G3H1_SC13
MSBL9	0	0	0	1	0	0	0	1363B8X1_SC04
Pla3 lineage	1	0	0	0	0	0	0	1363B1H1_SC07
Proteobacteria								
Alphaproteobacteria								
Rhodobium	0	1	0	0	0	0	0	1363B1H1_RS07
Deltaproteobacteria								
Desulfarculaceae	1	0	0	0	0	0	0	1363B1H1_SC04
Nitrospinaceae	0	0	0	0	0	1	0	1363G3H1_SC08
SEEP-SRB1	0	0	0	0	0	1	0	1363G3H1_SC05
Cand. phylum TA06	0	0	0	0	0	1	0	1363G3H1_SC18
Eukaryotes								
Fungi								
Dothideomycetidae	0	0	0	0	0	0	1	ControlExtract09-13

Trichocomaceae	0	0	4	0	0	0	0	1363B7X2_IW05

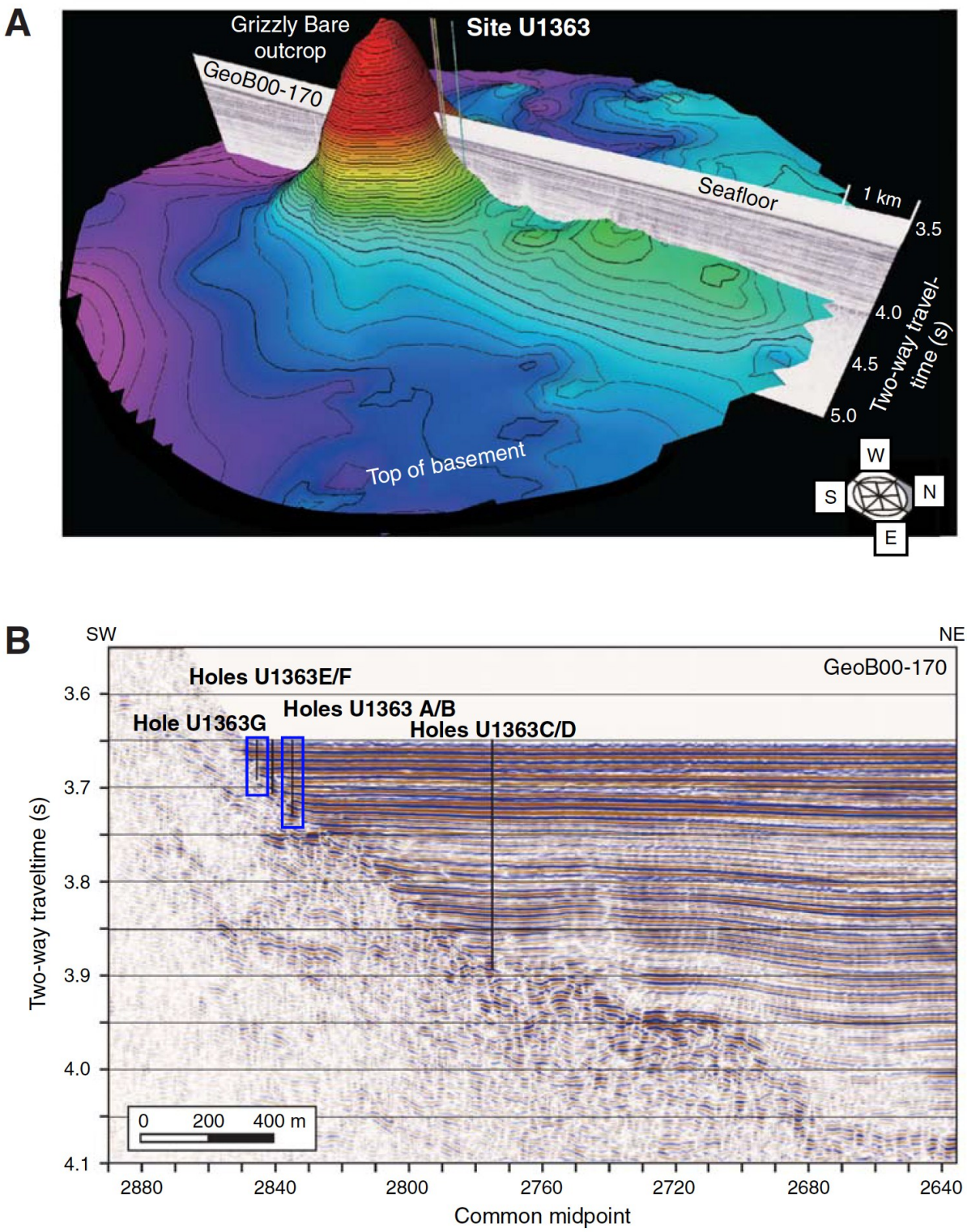
[a]Phylogenetic affiliations were determined using SILVA SSU database release 111. However, in some cases manual phylogenetic assignment was required because the SILVA taxonomy was inconsistent. In these instances, lineages were named after the first gene clone derived from the group.

Table 4.3 Microbial richness and evenness in Grizzly Bare sediment samples

	Similarity cutoff	*1363B1H1_SC*	*1363B1H1_RS*	*1363B7X2_IW*	*1363B8X1_SC*	*1363G1H2_SC*	*1363G3H1_SC*	*Extraction control*
No. of sequenced		21	19	23	17	14	17	21
Observed OTUs	99%	18	10	5	13	11	12	2
	97%	15	9	5	13	11	10	2
Estimated OTUs (Chao1)	99%	78	14	5	41	18	24	2
	97%	33	12	5	41	18	15	2
Simpson's evenness index	99%	0.02	0.14	0.27	0.05	0.03	0.06	0.90
	97%	0.04	0.15	0.27	0.05	0.03	0.08	0.90
Shannon diversity index	99%	4.26	2.53	1.45	3.47	3.29	3.15	0.31
	97%	3.39	2.33	1.45	3.47	3.29	2.66	0.31

Table 4.4 Summary of microbial community structure comparisons

Comparison	*Significance of UniFrac metric (corrected p-value)*	
	Weighted	*Unweighted*
1363B x 1363G	0.14	≤ 0.01
Surface x Deep	≤ 0.01	≤ 0.01
1363B1H1_SC x 1363B8X1_SC	1.00	1.00
1363B1H1_SC x 1363G1H2_SC	1.00	1.00
1363B1H1_SC x 1363G3H1_SC	0.96	0.06
1363B8X1_SC x 1363G1H2_SC	1.00	1.00
1363B8X1_SC x 1363G3H1_SC	0.66	0.60
1363G1H2_SC x 1363G3H1_SC	0.06	0.06
1363B1H1_SC x 1363B1H1_RS	0.87	0.80
1363B7X2_IW x Extraction Control	0.02	<0.01


A
Grizzly Bare outcrop
Site U1363
GeoB00-170
Seafloor
1 km
3.5
4.0
4.5
5.0
Two-way travel-time (s)
Top of basement
W
S
N
E
B
SW
NE
GeoB00-170
Holes U1363E/F
Hole U1363G
Holes U1363 A/B
Holes U1363C/D
3.6
3.7
3.8
3.9
4.0
4.1
Two-way traveltime (s)
0 200 400 m
2880
2840
2800
2760
2720
2680
2640
Common midpoint

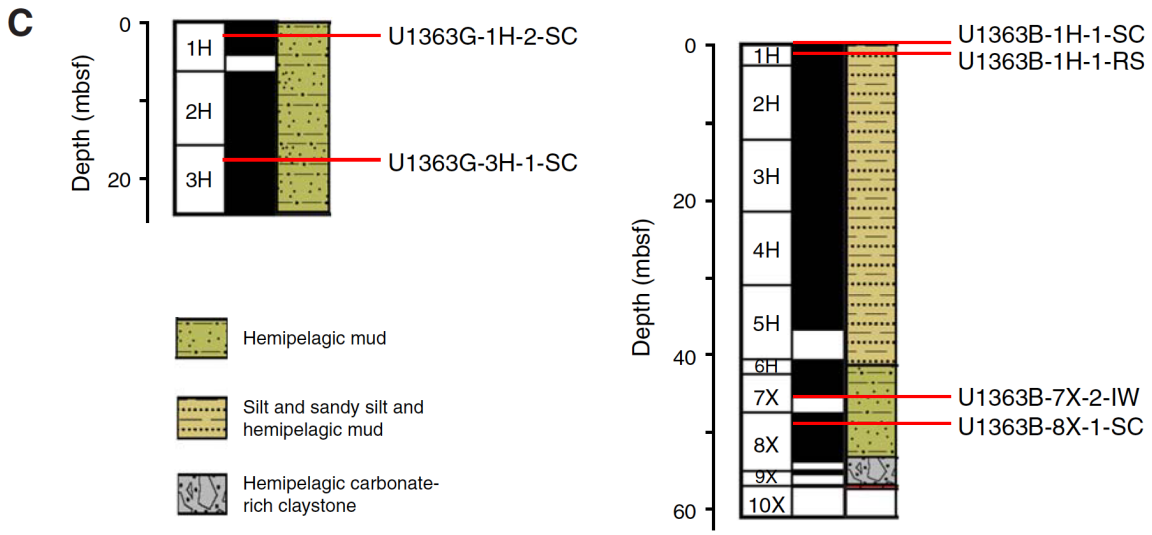


Figure 4.2 A. Three-dimensional perspective views of basement relief around Site U1363 adjacent to Grizzly Bare outcrop. Bathymetry based on digitization of seismic lines across work areas (data from Rosenberger et al., 2000; Zühlsdorff et al., 2005; Hutnak et al., 2006). Relief is shown as two-way traveltime, with no conversion to sediment thickness or depth. Part of Line GeoB00-170 (as shown in Figure F1C) is overlaid here to approximate sediment thickness (modified from Expedition 327 Scientists, 2011a). B. Seismic Line GeoB00-170 across Holes U1363A–U1363G. The sediment/basalt interface is clearly visible, but internal structure of the Grizzly Bare edifice is less resolved. Blue boxes indicate the locations of Holes U1363G and U1363B, which are shown in C (modified from Expedition 327 Scientists, 2011c).

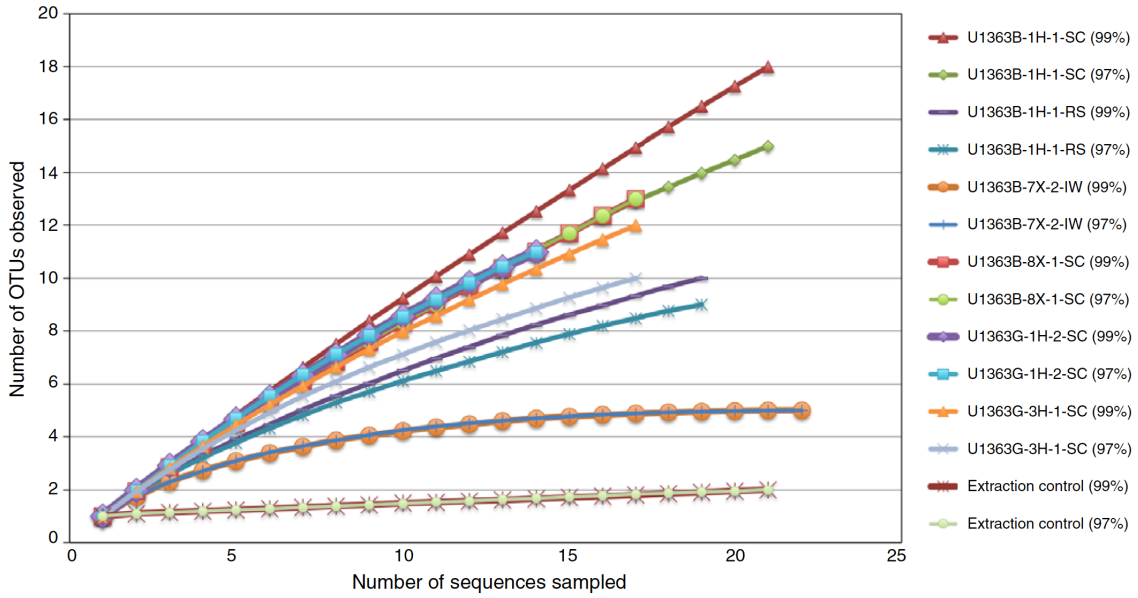


Figure 4.3 Rarefaction curves displaying the number of operational taxonomic units (OTUs) observed given the sequencing effort for Grizzly Bare sediment samples, Holes U1363B and U1363G. Data clustered at either 99% or 97% similarity cut-off values.

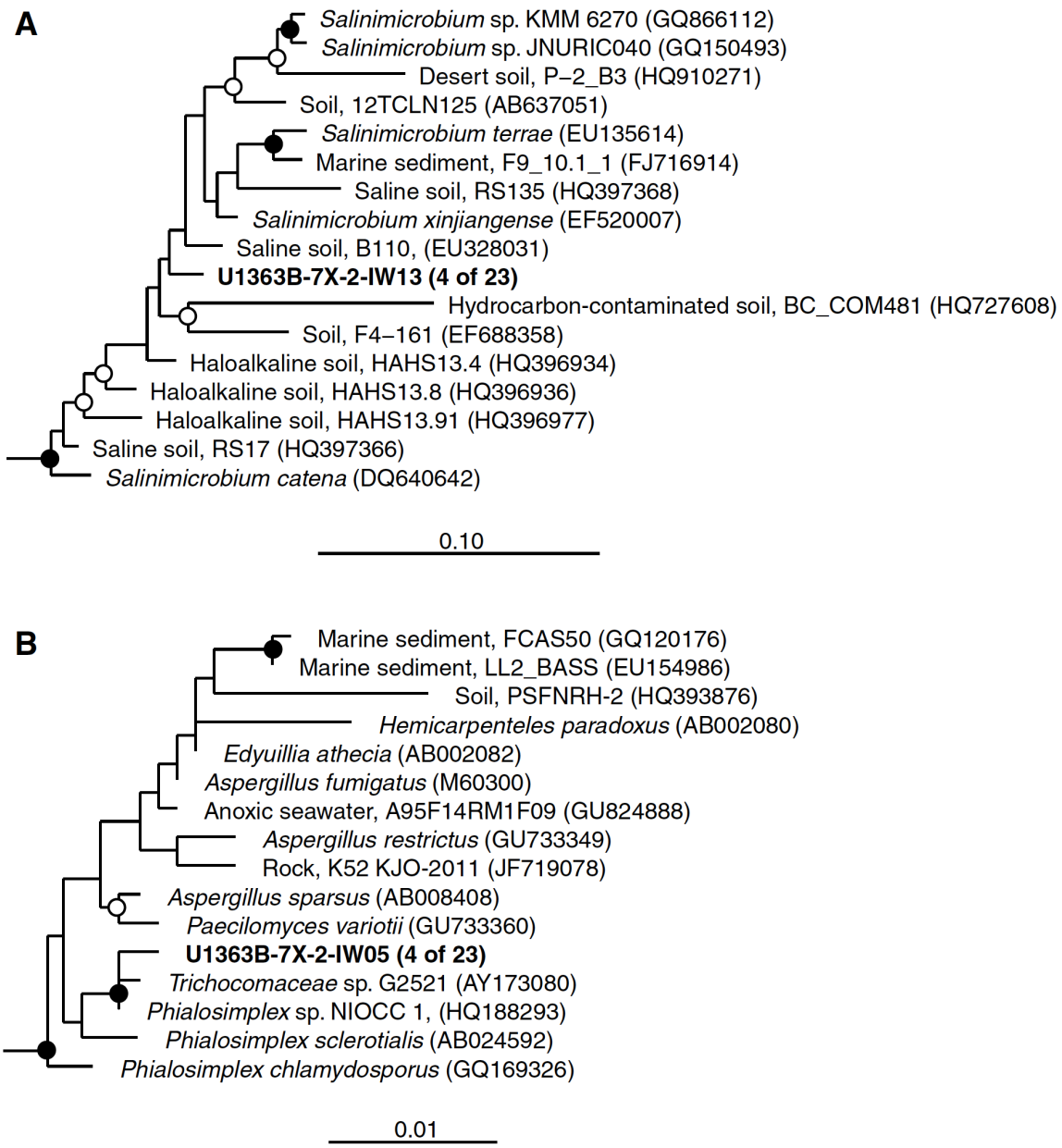
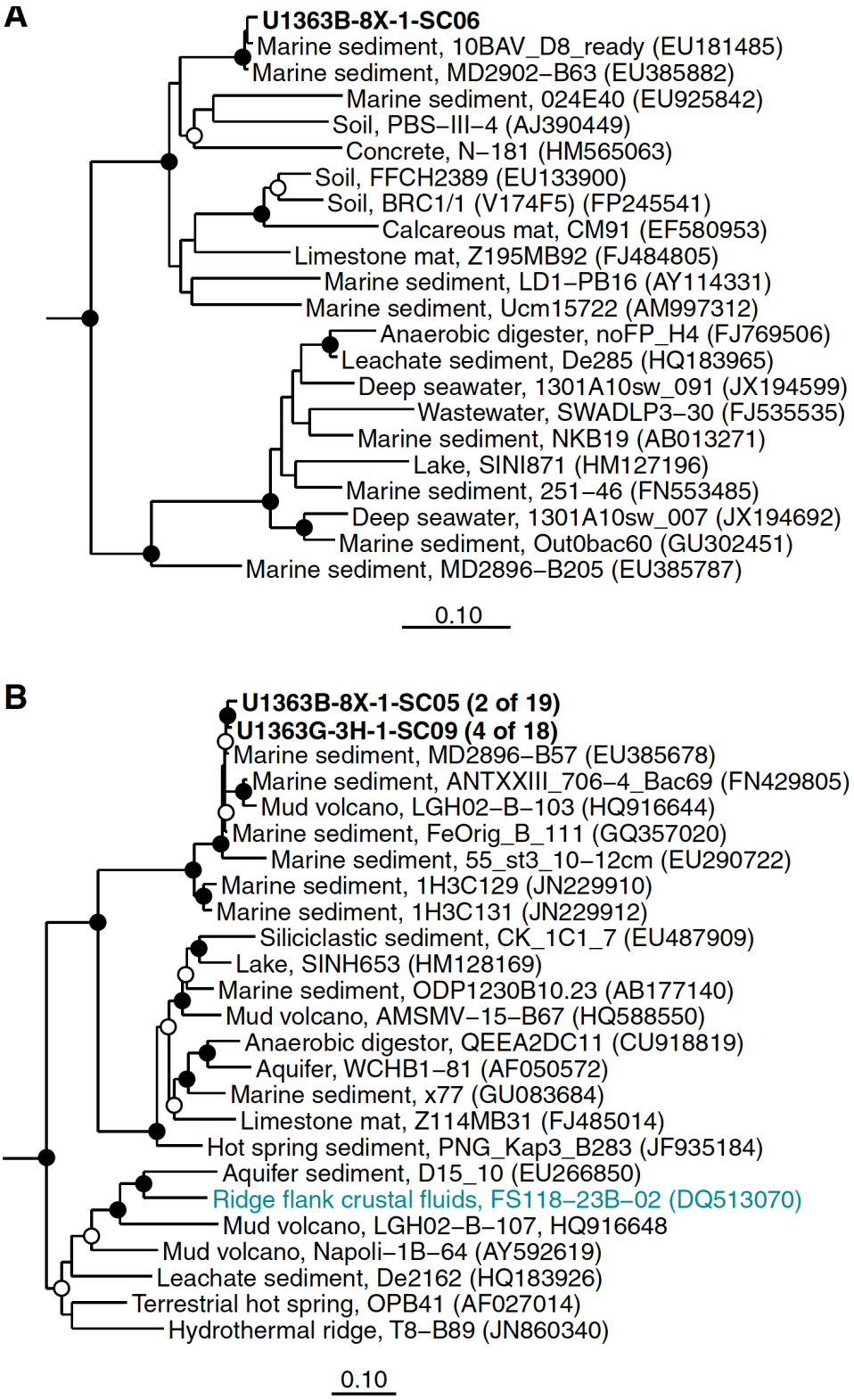


Figure 4.7 Phylogenetic relationships of Grizzly Bare sediment small subunit ribosomal RNA gene clones related to (A) the *Salinimicrobium* lineage within the bacterial phylum *Bacteroidetes* and (B) the *Trichocomaceae* lineage of eukaryotic *Fungi*. Cultivated members of the *Bacteroidetes* in A and *Fungi* in B were used as outgoups (data not shown). Scale bars correspond to (A) 0.1 and (B) 0.01 substitutions per nucleotide position. See Figure 4.4 for symbol definitions and other information.


A
U1363B-8X-1-SC06
Marine sediment, 10BAV_D8_ready (EU181485)
Marine sediment, MD2902–B63 (EU385882)
Marine sediment, 024E40 (EU925842)
Soil, PBS–III–4 (AJ390449)
Concrete, N–181 (HM565063)
Soil, FFCH2389 (EU133900)
Soil, BRC1/1 (V174F5) (FP245541)
Calcareous mat, CM91 (EF580953)
Limestone mat, Z195MB92 (FJ484805)
Marine sediment, LD1–PB16 (AY114331)
Marine sediment, Ucm15722 (AM997312)
Anaerobic digester, noFP_H4 (FJ769506)
Leachate sediment, De285 (HQ183965)
Deep seawater, 1301A10sw_091 (JX194599)
Wastewater, SWADLP3–30 (FJ535535)
Marine sediment, NKB19 (AB013271)
Lake, SINI871 (HM127196)
Marine sediment, 251–46 (FN553485)
Deep seawater, 1301A10sw_007 (JX194692)
Marine sediment, Out0bac60 (GU302451)
Marine sediment, MD2896–B205 (EU385787)
0.10
B
U1363B-8X-1-SC05 (2 of 19)
U1363G-3H-1-SC09 (4 of 18)
Marine sediment, MD2896–B57 (EU385678)
Marine sediment, ANTXXIII_706–4_Bac69 (FN429805)
Mud volcano, LGH02–B–103 (HQ916644)
Marine sediment, FeOrig_B_111 (GQ357020)
Marine sediment, 55_st3_10–12cm (EU290722)
Marine sediment, 1H3C129 (JN229910)
Marine sediment, 1H3C131 (JN229912)
Siliciclastic sediment, CK_1C1_7 (EU487909)
Lake, SINH653 (HM128169)
Marine sediment, ODP1230B10.23 (AB177140)
Mud volcano, AMSMV–15–B67 (HQ588550)
Anaerobic digestor, QEEA2DC11 (CU918819)
Aquifer, WCHB1–81 (AF050572)
Marine sediment, x77 (GU083684)
Limestone mat, Z114MB31 (FJ485014)
Hot spring sediment, PNG_Kap3_B283 (JF935184)
Aquifer sediment, D15_10 (EU266850)
Ridge flank crustal fluids, FS118–23B–02 (DQ513070)
Mud volcano, LGH02–B–107, HQ916648
Mud volcano, Napoli–1B–64 (AY592619)
Leachate sediment, De2162 (HQ183926)
Terrestrial hot spring, OPB41 (AF027014)
Hydrothermal ridge, T8–B89 (JN860340)
0.10

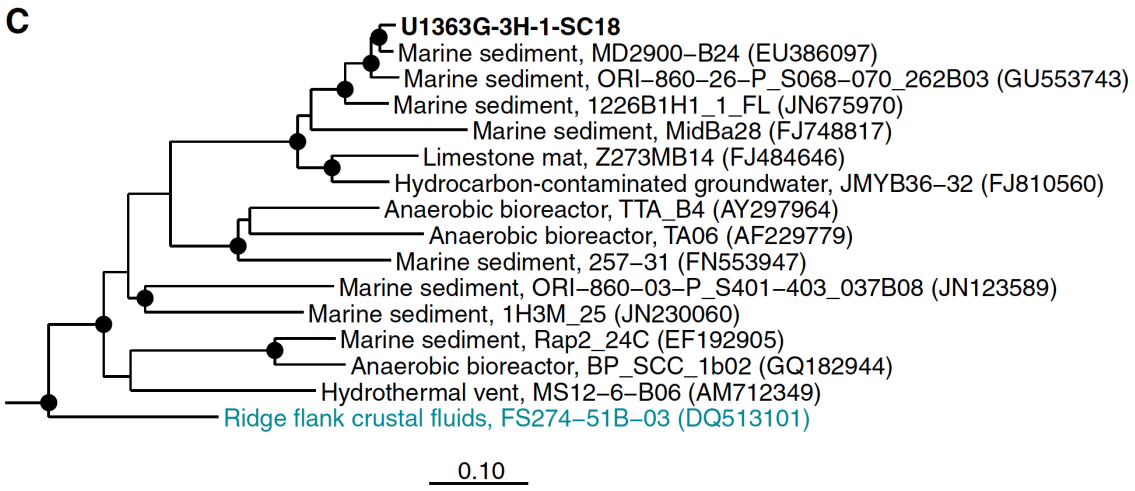


Figure 4.8 Phylogenetic relationships of Grizzly Bare sediment small subunit ribosomal RNA gene clones related to the candidate bacterial phyla (A) BRC1, (B) OPB41, and (C) TA06. Lineages in blue text are related to candidate bacterial phyla OPB41 and TA06 and were previously recovered from Juan de Fuca Ridge flank Baby Bare fluids (Huber et al., 2006b). Cultivated *Thermus* and *Deinococcus* in A, a variety of cultivated *Bacteria* in B, and cultivated *Caldithrix* in C were used as outgoups (data not shown). See Figure 4.4 for symbol definitions and other information.

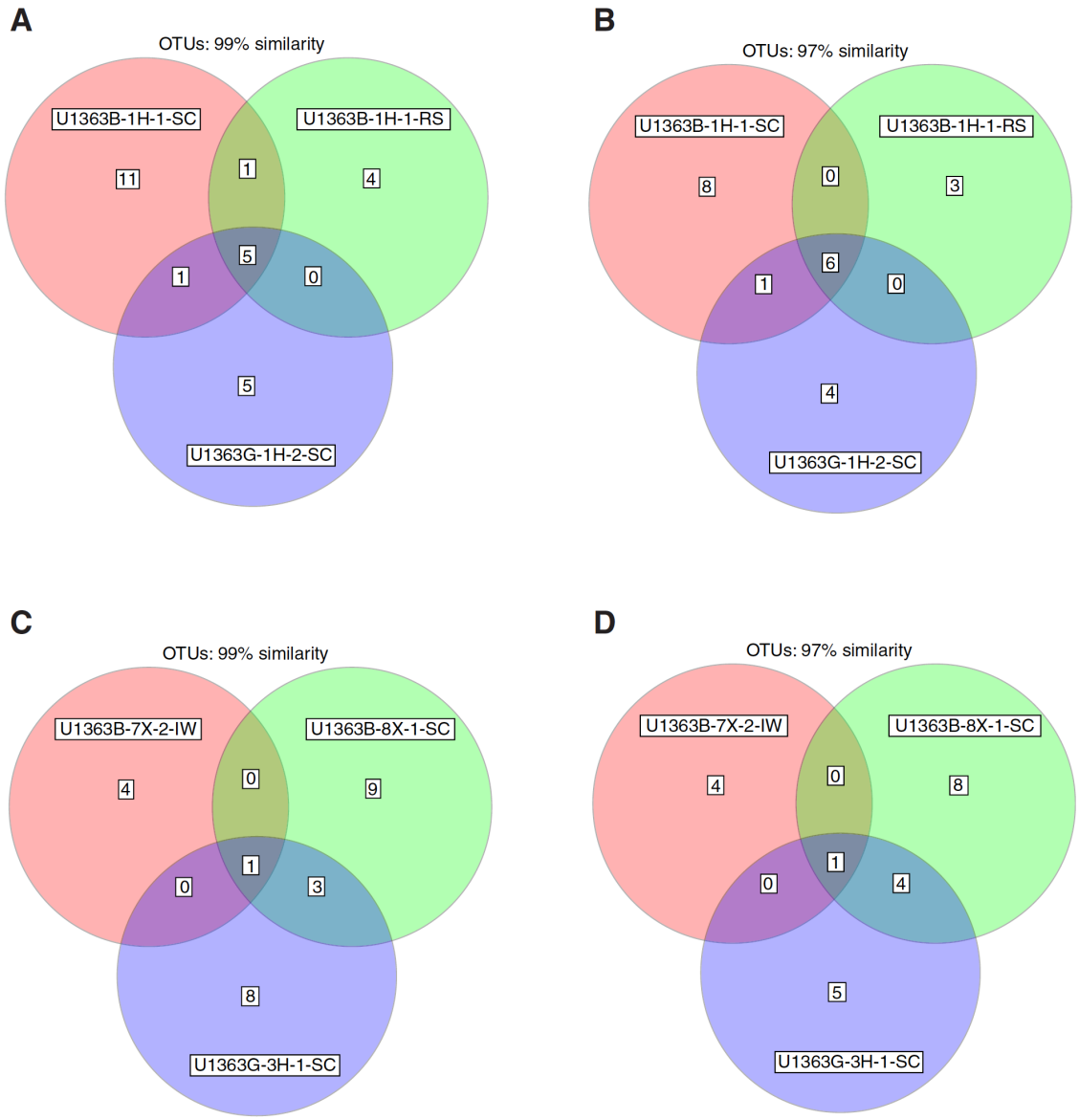


Figure 4.9 Microbial community relatedness between Hole U1363B sediment or fluid-derived small subunit ribosomal RNA (SSU rRNA) gene clones from each individual sample collected clustered in to operational taxonomic units (OTUs) at (A) 99% or (B) 97% similarity cut-off values. Hole U1363G sediment-derived SSU rRNA gene clones from both samples clustered in to OTUs at (C) 99% or (D) 97%.

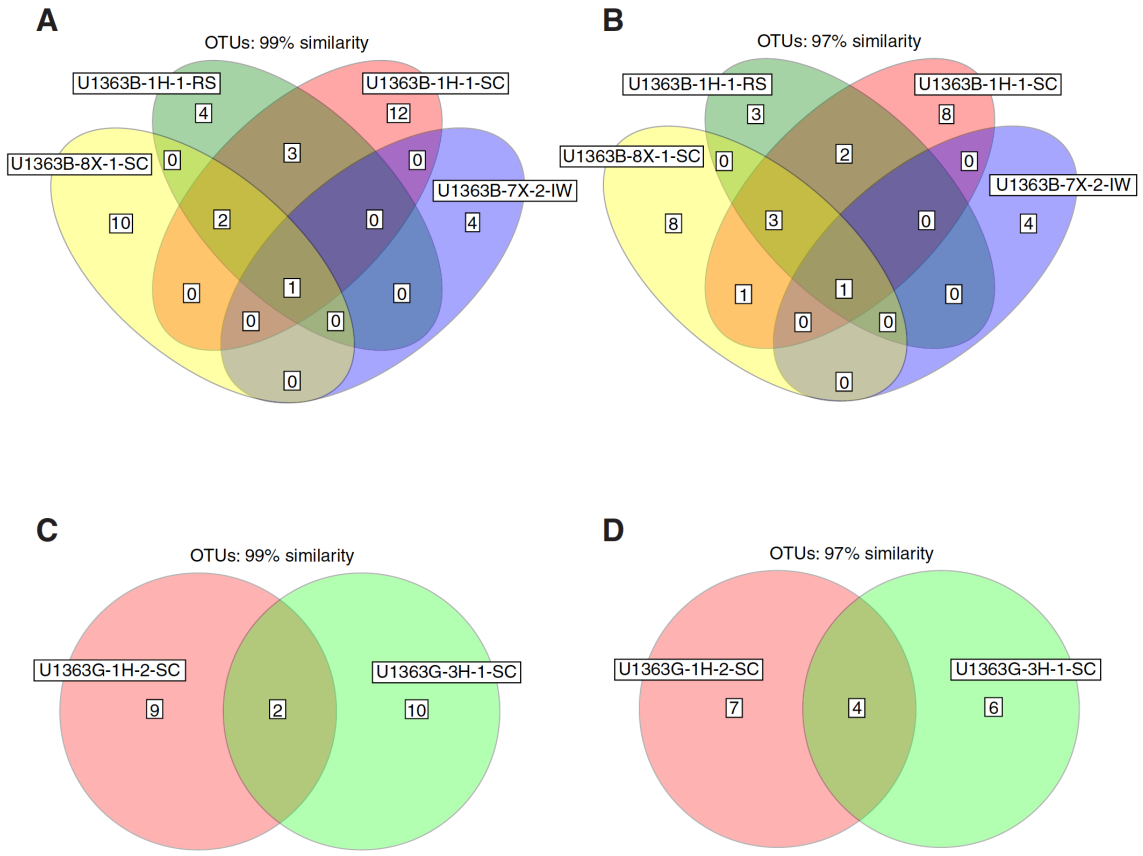


Figure 4.10 Microbial community relatedness between Hole U1363B and U1363G shallow sediment–derived small subunit ribosomal RNA (SSU rRNA) gene clones from each individual sample collected clustered into operational taxonomic units (OTUs) at (A) 99% or (B) 97% similarity cut-off values. Hole U1363B and U1363G deep sediment–derived SSU rRNA gene clones from each individual sample clustered in to OTUs at (C) 99% or (D) 97% similarity cut-off values.

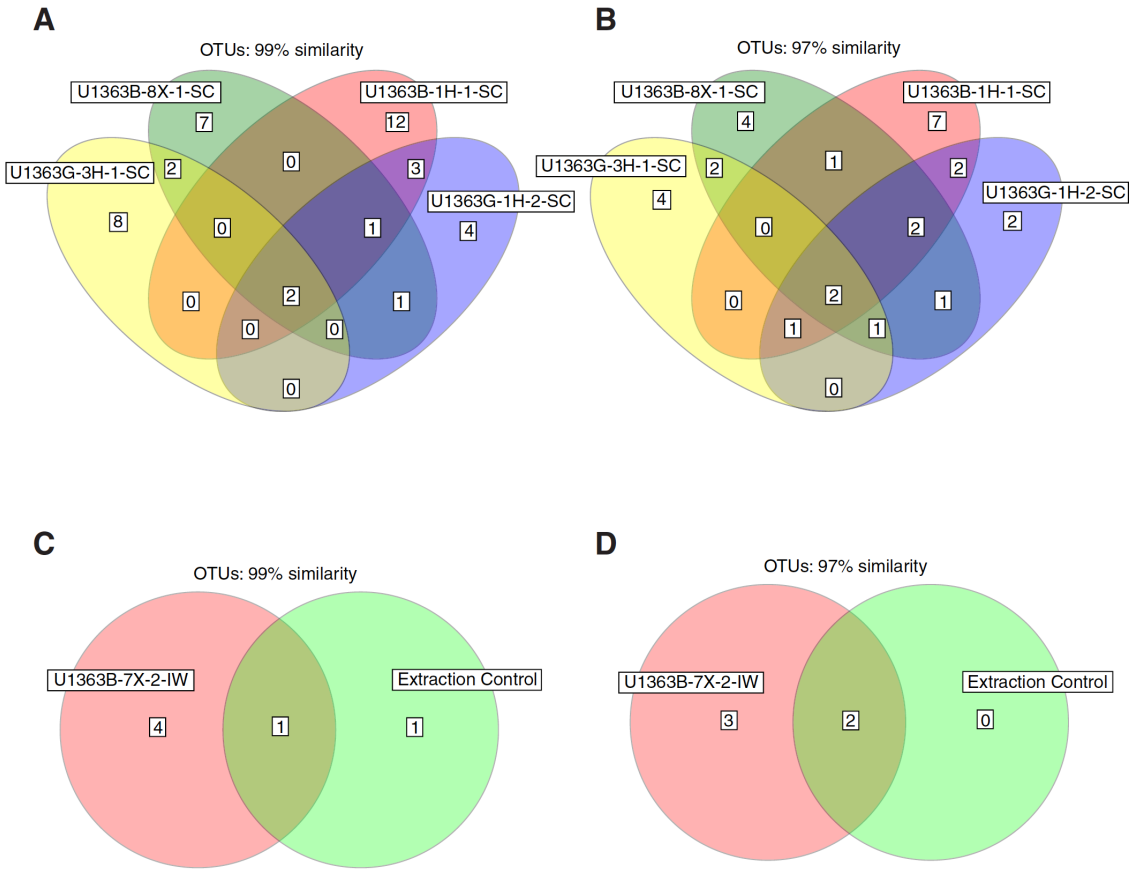


Figure 4.11 Microbial community relatedness between Hole U1363B and U1363G squeeze cake–derived small subunit ribosomal RNA (SSU rRNA) gene clones from each individual sample clustered in to operational taxonomic units (OTUs) at (A) 99% or (B) 97% similarity cut-off values. Hole U1363B interstitial water and extraction control–derived SSU rRNA gene clones from both samples clustered in to OTUs at (C) 99% or (D) 97% similarity cut-off values.

CHAPTER 5

Aging mid-ocean ridge flanks support a distinct microbial community

By Sean Jungbluth

Bob Bowers, Huei-Ting Lin, James P Cowen, and Michael S Rappé (anticipated co-authors)

5.1 Abstract

By analyzing ~1.7 million reads of the small subunit ribosomal RNA (SSU rRNA) gene by next-generation Illumina sequencing from marine sediment, deep seawater, and 24 crustal fluid samples spanning multiple years and boreholes (1025C, U1301A, U1362A, and U1362A/B), a distinct subseafloor biosphere dominated by the domain *Bacteria* was uncovered. Fluids from borehole U1301A sampled annually over the course of three years each had distinct microbial community structure, but the amplicons from each sample were dominated by members of the phylum *Proteobacteria*. In contrast, fluids from boreholes U1362A and U1362B, located ~500 meters to the northeast, contained lineages phylogenetically related to the phylum *Nitrospirae* in highest abundance. The domain *Archaea* was dominated by either the phylum *Crenarchaeota* (borehole U1301A) or the *Euryarchaeota* (boreholes 1025C, U1362A and U1362B). SSU rRNA genes phylogenetically affiliated with known lineages of methane-producing *Euryarchaeota* (e.g. *Methanobacteria*) were the most abundant archaeal group detected in fluids from Holes U1362A and U1362B, predominantly within samples originating from the deepest subsurface sampling horizon (~200 meters sub-basement) found at borehole U1362A. This window of access to the marine basement biosphere reveals a microbial landscape more spatially and temporally heterogeneous than previously recognized.

5.2 Introduction

The volume of oceanic lithosphere habitable by microbial life is a substantial portion of the Earth's crust - one that potentially extends thousands of meters below the seafloor (Biddle *et al.*, 2014). While the actual depth limit for life is likely quite deep, extrusive basalts within aging igneous oceanic crust are thought to be the most habitable subsurface microbial environments because of their relatively high porosity and extensive hydrothermal circulation (Baross *et al.*, 2004). Despite a handful of studies investigating life in the subsurface crustal environment (e.g. Cowen *et al.*, 2003; Huber *et al.*, 2006; Lever *et al.*, 2013, Jungbluth *et al.*, 2013a), most estimates and reviews of microbial biomass and diversity in the subseafloor focus on sediment samples and do not include discussions of microbial life in the basaltic crust, perhaps because there are few high-quality samples from this environment.

The uppermost (40-500 m) subseafloor basement aquifer is estimated to contain ~2% of the current world ocean volume that circulates a volume equivalent to the entire ocean every 10^5 - 10^6 years (Johnson and Prius, 2003; Wheat *et al.*, 2003). Mid-ocean ridge environments are the locus of extensive hydrothermal circulation deep into the ocean crust, and have been shown to support novel subseafloor microbial habitats (Summit and Baross, 2001). However, mid-ocean ridges make up only a small portion of the seafloor. Fluid transport through the ocean crust can occur laterally over long distances (>50 km; Fisher *et al.*, 2003) and varying

timescales (1-10^5 years), and is generally guided by thermal equilibration of exposed rocky outcrops protruding through sedimented regions. In contrast to subseafloor fluid circulation at mid-ocean ridges, the hydrothermal circulation through the sediment-covered deep ocean crust at ridge flanks and basins occurs through a more vast interconnected network of fluid flow paths that are highly convoluted (Becker *et al.*, 2013). This flow can be isolated from deep seawater for a very long time (>10^5 years) by thick and impermeable layers of sediment. As a consequence, microbes in these waters are expected to display a wide range of unique adaptive strategies (e.g. aerobic, anaerobic, mesophilic, thermophilic, etc.).

Characterization of life inhabiting the deep subseafloor biosphere has traditionally been a challenge, but fluid sampling from recently developed borehole observatories provides one of the best windows into the subseafloor biosphere. Several generations of Circulation Obviation Retrofit Kit (CORK) borehole observatories placed on the Juan de Fuca Ridge (JdFR) eastern flank (Figures 5.1a and 5.1b) were iteratively engineered to provide progressively cleaner fluid samples over time (Wheat *et al.*, 2011) (Figure 5.2). The latest generation of borehole observatories to be installed features stainless steel and Teflon-lined fluid delivery lines wider in diameter than any that have been sampled previously. These advancements, together with improvements in the sampling equipment that interfaces with the observatory, means that large volumes of high quality fluids can be acquired repetitively on predetermined time scales (Cowen *et al.*, 2012; Lin *et al.*, 2012; Jungbluth *et al.*, 2013a).

JdFR borehole observatories permit access to crustal fluids at different alteration states, ranging from aerobic to anaerobic, within the basaltic crust and represent windows into the wider spatial diversity of crustal fluids worldwide. Here, we sampled borehole fluids from locations in 1.2-3.5 million-years old ocean crust annually over the summer from 2008-2011 (Table 5.1) and use small subunit ribosomal RNA (SSU rRNA) Illumina tag sequencing and epifluorescence microscopy to provide insight into the phylogenetic diversity in the deep subseafloor biosphere. In total, 68 samples of crustal fluids, seawater, sediments, and appropriate controls were analyzed in this study, representing the most comprehensive sample suite from the disparate deep subseafloor environments (i.e. basement vs. sediment vs. deep seawater). High-quality crustal fluid samples collected in 2011 from three locations, U1301A, U1362A, and U1362B, all located in close proximity, provide the first assessment of local scale (~1 km) connectedness of subsurface fluids. Furthermore, samples collected from the newest borehole observatories installed in 2010 (U1362A/U1362B) represent the first time that a borehole has successfully provided samples so close to the time of drilling and installation of the CORK (<1 year), finally revealing, perhaps, the true makeup of the deep basaltic fluid microbial biosphere.

5.3 Materials and Methods

Sample collection and preparation

During R/V Atlantis cruises ATL15_35 (28 July 2008 – 13 August 2008), ATL15_51 (20 August 2009 – 6 September 2009), ATL15_66 (13 June 2010 – 1 July 2010) and ATL18_07 (28 June 2011 – 14 July 2011), samples of basement crustal fluids were collected from CORK observatories located east of the Juan de Fuca spreading center (Figure 5.1a). One borehole observatory (1025C) is located in cooler (~39°C) 1.2 million years old ocean crust while all other locations visited (1026B, U1301A, 1301B, U1362A, U1362B) are located within 1-2 km of each other in warmer (~65°C) 3.5 million years old ocean crust (Figure 5.1b). The borehole observatories penetrate more than 100-260 meters of sediments and another 46-280 meters of basement rock (Figure 5.2). Hole U1301A (47°45.209'N, 127°45.833'W) was the primary target for the duration of this investigation; fluids were sampled over four consecutive years 2008-2011 from the dedicated microbiological sampling line featured on a CORK-II borehole observatory (Fisher *et al.*, 2005; Wheat *et al.*, 2011). Locations 1025C (47°53.247'N, 128°38.919'W), 1026B (47°45.7571'N, 127°45.5482'W), and 1301B (47°45.2286'N, 127°45.8262'W) were sampled in years 2009-2010 and provide points of comparison despite the contamination noted from old CORK boreholes observatories (i.e. 1025C, 1026B), early sampling techniques, or compromised fluid delivery lines (i.e. 1301B). During the course of this study, two new borehole locations, U1362A (47°45.6628'N, 127°45.6720'W) and U1362B (47°45.4997'N, 127°45.7312'W), were drilled and fitted with lateral CORKs (L-CORKs) equipped with both stainless steel and Teflon-lined microbiological sampling ports (Expedition 327 Scientists, 2011a). Following one year of fluid equilibration within the new boreholes, these locations were sampled in the summer of 2011. Fluids were sampled using a deep sea pumping system or the GeoMICROBE fluid sampling systems, which are described elsewhere (Cowen *et al.*, 2012; Lin *et al.*, 2012; Jungbluth *et al.*, 2013a). Borehole fluids were typically allowed to flush for an amount of time require for expulsion of at least 3x the fluid delivery line volume. First generation CORK observatory at hole 1025C required overnight flushing to clear the larger void with the casing and is described in elsewhere (Jungbluth *et al.*, 2014). Following flushing, fluids were obtained in custom acid-washed 15 l (Medium Volume Bag Samples, MVBS) Tedlar and foil bags or 60 l (Large Volume Water Samples, LVWS) Tedlar bags protected by rigid boxes (Table 5.1). *In situ* filtrations were performed to maximize biomass collection using the GeoMICROBE sled or using the on-basket fluid sampling system (Table 5.1).

Seawater samples were collected in the vicinity of the borehole locations sampled in order to provide background controls for the borehole fluid samples (Table 5.1). Seawater was collected mostly using a Niskin rosette from within the nepheloid layer (5-10 meters above seafloor) and just above the nepheloid layer (~100 meters above seafloor). In 2010 and 2011, a

5 l Niskin bottle fitted to the ROV *Jason II* was used to collect seawater from a depth of approximately 2650 m in the vicinity of CORK U1301A. Seawater in the vicinity of borehole 1025C was sampled 35 meters above the seafloor using the LVWS pump and bag/box combination. Sediment samples were collected from site 1363 located nearby Grizzly Bare seamount during IODP expedition 327 (Expedition 327 Scientists, 2011a) and are described in detail elsewhere (Expedition 327 Scientists, 2011b; Jungbluth *et al.*, 2013b; Figure 5.3).

In 2008, retrieved fluids and GeoMICROBE-collected samples were filtered onto 25 mm-diameter 0.1 µm-pore sized and 47 mm-diameter 0.2 µm-pore sized polyethersulfone membrane filters (Pall Corporation, Port Washington, NY) and stored in 0.5 ml and 3 ml of DNA lysis buffer [20 mM Tris-HCl, 2 mM EDTA, 1.2% Triton X-100, 2% lysozyme (w/v), pH 8]. Fluid samples retrieved in years 2009-2011 were filtered through 0.22 µm-pore sized Sterivex-GP filter cartridges (Millipore Corporation, Billerica, MA) and stored in 2 ml of DNA lysis buffer. Steripak cartridge filters (Millipore Corporation) were employed in year 2011 to collected fluids *in situ* at the final portion of submersible dives and were stored in 25 ml of DNA lysis buffer, which was required to completely cover the filter membranes. Volumes of fluid samples ranged from <1 liter to ~30 liters while *in situ* filtration, specifically employed in year 2011, permitted 70 liter and 124 liter samples to be collected from the newest boreholes (U1362A/U1362B) (Table S1). A single 1.87 l sample of *Atlantis* shipboard distilled water (C1) was collected for control purposes and filtered onto a Sterivex membrane and preserved in an identical fashion to the other samples. Cells for single cell sorting were preserved in a final solution of 10% glycerol (w/v) and stored at -80°C until further processing

Analytical methods for geochemistry

Analytical methods are partially described elsewhere (Lin *et al.*, 2012; Jungbluth *et al.*, 2014). High-integrity samples were distinguished from low-quality subsurface samples *in situ* by temperature, flowrate, oxygen, and electrochemical profiles and also shipboard based on indicative chemical parameters such as magnesium, calcium, sulfate, etc. Basement fluid and seawater geochemistry parameters are listed on Table 5.2.

DNA extraction

All sediment samples were thawed to room temperature and environmental DNA was extracted and concentrated using the PowerMax Soil DNA isolation kit (MO BIO Laboratories, Carlsbad, CA, USA) following the manufacturers protocol with slight modifications that are described in detail elsewhere (Jungbluth *et al.*, 2013b). A negative DNA extraction consisting of only kit reagents (C2) was processed in parallel to sediment sample extractions. Membrane filter samples of subsurface fluids and seawater were thawed to room temperature prior to nucleic acid extraction. Environmental DNA from filter membranes was extracted using the

PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA) following the manufacturer's protocol. Samples SSF11, SSF12, SSF21/22, SSF23/24 were extracted using a modified lysis and purification method similar to (Frias-Lopez *et al.*, 2008) in order to retain high-integrity genomic DNA for future analyses. A 40 µl solution of 50 mg ml^{-1} lysozyme (Sigma-Aldrich, St. Louis, MO, USA) in DNA lysis buffer was added to the Sterivex filter and incubated at 37°C rotating for 45 minutes. Proteinase K (Qiagen, Valencia, CA, USA) was added to a final concentration of >0.55 µAU and 20% SDS (Fisher Scientific, Waltham, MA, USA) was added to a final concentration of 1% and incubated at 55°C rotating for 2 hours. Lysate was transferred to a 30 ml Oak Ridge tube using a sterile syringe. An additional 1 ml of lysis buffer was added to the filter for washing at 55°C for 15 minutes and pooled with above lysate. Phenol:chloroform:isoamyl alcohol (25:24:1; pH 8.0) totaling 3 ml was added and the mixture vortexed for 30 seconds and spun for 5 minutes at 2500 x g. The aqueous phase was transferred to a new Oak Ridge tube and 3 ml of chloroform:isoamyl alcohol (24:1) was added and the mixture vortexed for 30 seconds and spun for 5 minutes at 2500 x g. The aqueous phase was concentrated by spin dialysis using an Amicon Ultracel-30K filter (Millipore) and spinning at 1000 x g for 20 minutes. Flow-through was decanted and the Amicon filter was spun at 1000 x g for 20 minutes. A 1 ml volume of TE buffer [10mM Tris-HCl (pH 8.0), 1mM EDTA (pH 8.0)] was added over the Amicon filter membrane and spun at 1000 x g for 10 minutes; ~700 µl remained on the column and was transferred to a new tube. The filter column was washed twice with 700 µl of TE buffer and pooled with above DNA concentrate. Nucleic acids were concentrated using a vacuum centrifuge, resuspended in 50 µl of PCR-grade water and pooled together. Quantification of genomic DNA was performed using a Quant-iT™ dsDNA Assay High Sensitivity Kit (Q33120, Life Technologies, Carlsbad, CA, USA).

SSU rRNA gene PCR amplification and Illumina sequencing

An Illumina sequencing approach that has been previously described (Caporaso *et al.* 2011; Caporaso *et al.*, 2012) was used to characterize samples of boreholes fluids, sediment, and seawater. Briefly, this technique involves amplification of the V4 region of the small subunit ribosomal RNA gene using primers 515F and 806R specific to *Bacteria* and *Archaea* and which were modified to include the Illumina flowcell adapter sequences. Reverse primer 806R contained an additional 12-bp barcode, which was used to assign individual sequences to samples. Taxonomic coverage of primer pair 515/806 was assessed using PrimerProspector (Walters *et al.* 2011) and found to be nearly universal, as described previously (e.g. Bates *et al.* 2011; Walters *et al.* 2011) (Figures 5.4 and 5.5). Triplicate PCR reactions were pooled at equimolar concentrations and PCR cleanup was performed on the final pooled product using the UltraClean PCR clean up kit (MoBio Laboratories, Carlsbad, CA). Sequencing was performed on an Illumina MiSeq sequencer at the University of Colorado BioFrontiers Institute. Reads

were thrown out if sequence length was shorter than 75 bases and/or if the barcode did not match the expected barcode.

Sequence read-processing and read-pairing

An overview of the bioinformatic workflow used to process SSU rRNA Illumina sequencing reads is shown in Figure 5.6. Demultiplexing of sequence data was performed using the QIIME pipeline (Caporaso *et al.* 2010) split_libraries_fastq.py script (version 1.7) with a maximum of 1.5 errors in the barcode, and the following quality-filtering parameters adapted from Bokulich *et al.* (2013): r=3, p=0.75, q=3, and n=0. Forward reads (515F) were in higher abundance than reverse reads (806R) (Table 5.3) and were of generally higher quality (Figure 5.7). Sample and sequence hash combinations corresponding to the 1,734,012 sequences in the forward read library using the aforementioned quality-filtering parameters were drawn from the original fastq reads one and two files for the purpose of read-pairing. Sequences files and metadata for the samples used in this study will be deposited into MG-RAST.

Read pairing was performed using a variety of tools specialized to handle non-gapped sequencing errors typical of the Illumina sequencing platform and parameter values selected to control for differences in the pairing methods (i.e. base quality within the overlapping region) (Table 5.3). Where possible, parameter values for the length of overlapping regions remained low to increase the chances of successful pairing following truncation of low quality bases, which were relatively more abundant in the overlapping portion of the reads. All methods utilized default parameters, unless otherwise noted. Pairing using USEARCH (version 7.0.959; Edgar, 2010) fastq_mergepairs script used parameters: fastq_truncqual=3, fastq_minovlen=5 and fastq_filter script with fastq_maxee parameters=0.05, 1.0, and 5.0. Pairing using FLASH (version 1.2.7; Magoč and Salzberg, 2011) used parameters: m=5 and x=0.25. Pairing using PANDAseq (version 2.5; Masella *et al.* 2012) used parameters: o=5 and t=0.25. Pairing using merge_illumina_pairs script (Eren *et al.* 2013) used parameters: ignore-Ns, o=5 or 48, min-qual-score=3, and m/o=0.25. Multiple overlap sizes and stringency requirements were used in association with the merge_illumina_pairs script due to high sensitivity of the pairing to the specific length of the overlapping region and the number of mismatches in the overlap. In contrast to the other methods used, the merge_illumina_pairs script performed superiorly when the parameter for length of the overlapping region was near the full overlap length expected (o=48). A final quality check was implemented using the enforce-Q30-check (Minoche *et al.* 2011) in association with the merge_illumina_pairs script due to the quality scoring stripping that occurs following the scripts conclusion, which prohibits the use of further quality control.

Paired reads were demultiplexed using the QIIME pipeline (Caporaso *et al.* 2010) split_libraries_fastq.py script (version 1.7) with a maximum of 1.5 errors in the barcode, and the

following quality-filtering parameters: r=10, p=0.75, q=3, and n=0. A range of values for parameter r - maximum number of low bases before read truncation - were tested due to the expected increased sensitivity for this parameter to the longer read lengths, which is due to the relatively low phred scores in the overlapping portion of the reads. Parameter value r=10 was ultimately selected because it represents a balance between complete sequence retention (all pairs obtained) and no sequences obtained. Reads generated using the merge_illumina_pairs script (Eren *et al.*, 2013) were not processed this way due to the lack of quality scores; instead the reads were extra scrutinized, as described above, and directly passed to the next phase of analysis.

Sequences longer than 254 bases constituted a minor portion of the paired-reads using all methods except for the merge_illumina_pairs script with length of the overlapping region equal to 5. Queries against the NCBI non-redundant nucleotide database using BLAST (Altschul *et al.* 1990) revealed amplicons larger than 254 base pairs to have relatively few full-length sequence matches. This corresponds to an analysis of expected fragment length from Silva SEED databases of *Archaea* and *Bacteria* (Schloss, 2009), and a manually curated database of filtered sequences (>100 bp) obtained from Juan de Fuca Ridge basement basalt (Cowen *et al.* 2003; Huber *et al.* 2006; Jungbluth *et al.* 2013a; Jungbluth *et al.* 2014; this study), sediment (Jungbluth *et al.* 2013b), and bottom seawater (Jungbluth *et al.* 2013a) gene clones (Figure 5.8). Due to the relatively few numbers of reads expected and the incorrect read pairing observed, paired sequences longer than 254 bases were excluded from further analysis.

Chimeric sequences were first detected with USEARCH using the UCHIME (Edgar *et al.*, 2011) de novo chimera function and excluded from further analysis. This method was followed by UCHIME (Edgar *et al.*, 2011) chimera checking against the Silva SEED reference database with Juan de Fuca gene clones, as described above.

OTU clustering

Clustering was performed using several methods for comparative purposes (Table 5.4) and included the use of unpaired and paired reads due to the noted effects on clustering accuracy (Werner *et al.*, 2012). Custom scripts were used as necessary to reformat files and facilitate the usage of different software. The QIIME open reference picking script (pick_open_reference_otus.py) was used to perform uclustref (Edgar, 2010) and denovo clustering using the Greengenes v13.5 and Silva v111 databases clustered at 97% or 99% OTU similarity. Clustering performed using the USEARCH package involved: sequence dereplication, abundance sorting without removal of singletons, and clustering using UPARSE (Edgar, 2013). UPARSE clustering with reverse reads uniquely required 64-bit USEARCH (version 7.0.1090). Re-screening for chimeric OTUs was disabled using the command –parse_break -999 due to the prior chimeric screening performed. Following clustering, reads were mapped onto OTU

clusters using the –usearch_global script and a 0.97 sequence identity threshold. Clustering using mothur (Schloss *et al.*, 2009) was used and has been described previously (Kozich *et al.*, 2013). Briefly, reads were aligned against the Silva SEED and Juan de Fuca clone database described above, given temporary (i.e. for clustering only) taxonomic assignment using the Silva SEED *Bacteria* and *Archaea* databases combined, and finally clustered using the cluster.split command with taxonomic splitting at the order-level using a hard 0.1 genetic distance cutoff and the average-neighbor clustering algorithm. Distribution-based clustering (Preheim *et al.*, 2013) was performed using scripts associated with the software and in similar fashion to the protocol listed in the associated manual (https://github.com/spacocha/Distribution-based-clustering). USEARCH progressive clustering beginning at 98% sequence similarity level and iterating each single percentage down to 90% was used to generate a full fasta and sequence by library matrix to be used for downstream analysis. Sequences were aligned with mothur (Schloss *et al.*, 2009) using the Silva SEED reference database (Schloss, 2009) with Juan de Fuca gene clones, as described above, and distance matrices were generated using FastTree (Price *et al.*, 2010) with default options. Clustering was performed in parallel using the distribution_clustering.pl script with parameters: a=0, p=0.0005, and a range of distance parameters (d=0.1, 0.05, 0.03, 0.01) to assess differential OTU clustering that will result. Evaluation of the clustering results using the evaluate_parallel_results.pl script was unattempted due to the unique nature of required files as input, and so results were evaluated using custom bash scripts to check for the criteria described in evaluate_parallel_results.pl. Final files were produced using the scripts included within clean_up_parallel_ultra.csh, which included custom perl and bash scripts and additional sequence alignment using mothur (Schloss *et al.*, 2009) and the Silva SEED reference database with Juan de Fuca gene clones described above. Finally, sequences were analyzed in the absence of clustering (Tikhonov *et al.*, 2014) using a fasta file and community by sample matrix of unique sequences generated from forward and reverse reads and each of the read pairing methods mentioned above. Unique sequence community matrices and fasta files were generated using perl scripts fasta2unique_table4.pl and OTU2lib_count_trans1.pl associated with the distribution-based clustering pipeline (Preheim *et al.*, 2013).

Taxonomic assignment

Unique (i.e. non-clustered) paired reads from the merge-illumina-pairs script (Eren *et al.*, 2013) were selected for classification and further analysis due to the longer sequence lengths and the relatively high numbers of reads retained post processing. Sequences were aligned and taxonomy was assigned via the SINA aligner (v1.2.11; Pruesse *et al.*, 2012) using the non-redundant SSURef_115 database pre-clustered with UCLUST at a 99% sequence similarity level available online. Parameters used with the SINA tool included –lca-fields tax_slv to assign

Silva taxonomy and –search-min-sim 0.80 to expand searches for difficult-to-classify (i.e. divergent) sequences. Group names *Marinimicrobia* (SAR406), *Aminicenantes* (OP8), and *Aerophobetes* (BHI80-139) were adapted from Rinke *et al.* (2013) and *Bathyarchaeota* (MCG) is from Meng *et al.* (2014).

Silva-identified *Bathyarchaeota* (Miscellaneous Crenarcheotal Group; MCG) sequences were further classified using the Phyloassigner tool (Vergin *et al.* 2013) and a manually-curated *Bathyarchaeota* (MCG) database that follows naming schemes described in Kubo *et al.* (2012). The MCG database was generated by removing sequences using arb from the Silva SSU Ref 99 version 115 base tree. The final tree was composed of 3160 sequences and included: (1) archaeal sequences that were randomly selected to encompass all of the major archaeal lineages, (2) all non-chimeric unclassified archaeal sequences, (3) all sequences from groups MCG, C3, THSC, and (4) bacterial lineages *Chloroflexus aggregans* (CP001337), *Vibrio vulnificus* (X76333), and *Thermotoga maritime* (M21774) as outgroups. MCG groups were classified according to Kubo *et al.* (2012) whenever monophyletic lineages were consistent between the slightly varying topologies. Sequences identified as being within a monophyletic group by Kubo *et al.* (2012) but not having consistent placement in the Silva base tree were reclassified only when up to two sequences required renaming for inclusiveness in a group or when up to three sequences required renaming to be included in a group that was overwhelmingly (>99%) monophyletic in nature. Unique lineages (i.e. JX194242; JX194261) detected in Juan de Fuca ridge flank fluids (Jungbluth *et al.*, 2013a) after the classification by Kubo *et al.* (2012) were filtered and added via the parsimony insertion tools in arb. MCG-14 sequences were highly disruptive to MCG 8 monophyly and were removed and added again to the tree using the "ssuref_v115:archaea" filter. In some instances the polyphyletic nature of groups was unavoidable and so unique groups names were used to indicate mixing of groups (e.g. MCG-11 and MCG-12; MCG-15 and Group C3). Results were plotted using ggplot2 (Wickham, 2009). The *Bathyarchaeota* (MCG) database used here is available on GitHub (https://github.com/seano30)

Community analyses

Because of the low expected sequencing error rates characteristic of Illumina platforms, a relatively modest number of total sequence reads permitting easy processing, and interest in phylotype-level (i.e. single base-pair) differences among sequences, ultimately the unique read analysis method was selected for processing enroute to all further analyses. Community analyses were performed using unpaired forward reads or paired-end reads generated using the merge-illumina-pairs script (Eren *et al.*, 2013). Alpha diversity analyses (Chao1, observed species, Goods coverage, Shannon, Simpson evenness) were done in QIIME using 100 randomly-generated sets of rarefied unpaired forward reads sampled to an even depth and for

using the Bray-Curtis and abundance weighted Jaccard distance indices but only results of the Bray-Curtis index are shown. Procrustes shape analysis was performed using the Bray-Curtis dissimilarity index (Figure 5.10) and the abundance weighted Jaccard distance (data not shown) to assess differences in read-pairing and clustering techniques; no major observable differences in the clustering patterns were detected when comparing forward and reverse reads, forward reads versus paired reads, or forward reads clustered using the different techniques (Figure 5.10 and data not shown). Principle coordinates analyses (PCoA), and Procrustes analyses were performed in QIIME following screening and removal of singleton OTUs (OTUs with one member) and rarefying samples to an even sequencing depth. PCoA and Procrustes plots were visualized using EMPeror (Vázquez-Baeza *et al.*, 2013). Jackknifed beta diversity analysis was performed in QIIME using unique forward read clusters and 100 sequences randomly selected from each sample. Unweighted paired group mean average (UPGMA) was generated in QIIME using unique forward read clusters rarefied to an even sampling depth across all samples. Venn diagrams were generated in mothur (Schloss *et al.*, 2009) using unique unpaired forward reads and plotted using venneuler (Wilkinson, 2011). Paired-end unique read clusters taxonomically assigned as described above were imported into R for generation of OTU heatmaps and Dufrene-Legendre indicator species analysis. Heat maps and indicator taxa plots were generated using R packages Phyloseq (McMurdie and Holmes, 2013), vegan (Oksanen *et al.*, 2013), and labdsv (Roberts, 2013). Following identification of reads to be included heat map and indicator taxa plots, the taxonomic identity of those reads was honed by adding filtered sequences into the Silva SSU Ref 99 version 115 using the parsimony insertion tools within arb (Ludwig *et al.*, 2004).

Statistical analysis of sample groupings

Statistical analyses were performed using unpaired forward reads rarefied to an even sampling depth across all samples. Dissimilarity among community matrices and associated chemical metadata were explored using Mantel tests with 1000 replications performed in QIIME (version 1.8) using scripts distance_matrix_from_mapping.py and compare_distance_matrices.py; chemical data reported as less than detection limits were assumed to be zero for this analysis. Comparisons were also performed using two sets of categories: sample type (seawater, sediment, subsurface fluids; mixed and controls removed), and location: (seawater, sediment, U1301A, U1362A, U1362B; mixed, controls, 1025C, removed) using the compare_categories.py script in QIIME. Statistical tests performed include ANOSIM implemented through QIIME and adonis, MRPP, PERMDISP, PERMANOVA, and db-RDA implemented through QIIME using the R package vegan (Okasanen *et al.*, 2013); all based on 1000 permutations.

SSU rRNA gene cloning and sequencing and single cell flow sorting analysis

Borehole U1362A sample SSF19 was sent for single cell isolation and processing at the Bigelow Laboratory Single Cell Genomics Center (www.bigelow.org/scgc) using Fluorescent Activated Cell Sorting in a 384-well plate. Briefly, cells were lysed and the DNA was amplified using multiple displacement amplification as described previously (Swan *et al.*, 2011). DNA from sorted single cells was screened with bacterial (27F-M13: 5'-AGRGTTYGATYMTGGCTCAG-3'/907R_degen-M13: 5'-CCGTCAATTCMTTTRAGTTT-3') and archaeal (Arc_344F-M13:5'-ACGGGGYGCAGCAGGCGCGA-3'/Arch_915R-M13R:5'-GTGCTCCCCCGCCAATTCCT-3') small-subunit rRNA (SSU rRNA) primers (Lane, 1991). Additionally, SSU rRNA genes were amplified from nucleic acids extracted from selected environmental samples (SSF11, SSF12, SSF21/22, SSF23/24) using the primer pair 519F (5'-CAGCMGCCGCGGTAATWC-3') and 1406R (5'-ACGGGCGGTGTGTRC-3') (Lane *et al.* 1985). PCR, cloning reactions, and DNA sequencing were as performed by Jungbluth *et al.* (2013a).

Phylogenetic analysis of gene clones

DNA sequences were trimmed of vector sequence and manually curated using Sequencher version 5.1 software (GeneCodes, Ann Arbor, MI). Paired-end sequences resulting from the single cell genomic analysis were assembled using Sequencer default parameters. Curated clone sequences were first aligned using the online SINA tool version 1.2.11 (Pruesse *et al.*, 2012) before importing into the ARB software package (Ludwig *et al.*, 2004) for manual curation of the multiple species alignment and taxonomic classification using version SSURef_115 of the SILVA ARB database clustered to a 99% level of similarity (Pruesse *et al.*, 2007). Additional sequences that were most similar to clone sequences obtained in this study, as revealed by BLAST search against the non-redundant nucleotide database (Altschul *et al.*, 1990), were aligned as described above and included in relevant phylogenetic analyses. A statistical selection of best-fit models of nucleotide substitution was performed on the near-full length sequences (>1200 nucleotides) within the multiple species alignment using jModelTest (Price *et al.*, 2010; Darriba *et al.*, 2012) version 2.1.1. Phylogenetic analyses were performed using near-full length sequences (>1200 nucleotides) with the RAxML maximum likelihood method using the GTR model of nucleotide substitution under the gamma- and invariable-models of rate heterogeneity (Stamatakis *et al.*, 2006). Trees with the highest log likelihood score were selected by performing 100 iterations of the RAxML method. Sequences of short length were added to the maximum likelihood-derived phylogenies using the parsimony insertion tool in ARB; using appropriately sized alignment filters, gene clones and sequences derived from GenBank were added first, following was the addition of Illumina tag sequences.

All non-redundant sequences generated in this study will be submitted to GenBank following peer-reviewed publications of data.

Sample preparation for microscopy and fluorescence microscopy

Samples collected in 2009-2011 were prepared in similar fashion to the protocol outlined by Jungbluth *et al.* (2013a). Briefly, 40 ml or 120 ml sub-samples were fixed with a final concentration of 3% formaldehyde (0.2 µm-filtered) for 2 to 4 hours at 4°C, and subsequently filtered through 0.2 µm pore-sized polycarbonate membranes (Whatman, Maidstone, United Kingdom). After air-drying, membranes were stored desiccated at -80°C until microscopic analysis. In 2009 only, 40 ml seawater and borehole fluid sub-samples were fixed as above, but subsequently stored at -80°C until thawed and filtered on 0.2 µm pore-sized polycarbonate membranes (Whatman).

Fluorescence microscopy was performed as described in Jungbluth *et al.* 2013a with different equipment. Citifluor/VectaShield/PBS/DAPI stained filter sections were inspected with a Nikon Eclipse 90i (Tokyo, Japan) equipped with a 100x objective, filter sets appropriate for DAPI fluorescence, a camera Nikon DS-fi1 and Nikon NIS Element software version 3.22.11.

5.4 Results and Discussion

Borehole fluid sampling and geochemistry

Subseafloor crustal fluids were sampled from five different boreholes along the JdFR (Figures 5.2 and 5.3) during the summers of 2008 to 2011 using pumping equipment to both collect whole water and filtered water *in situ* (Table 5.1). The boreholes penetrate ~100 to 260 m of sediments and another ~48 to 300 meters of basement; new boreholes 1326A and U1362B penetrate ~240 meters of sediment and another 120-300 meters of basement (Figures 5.2 and 5.3). The majority of borehole fluid samples, particularly those collected in years 2010-2011, had chemical concentrations characteristic of high-integrity fluids (near-neutral pH, depleted in dissolved organic carbon, total organic carbon, carbon dioxide, potassium, nitrate, sulfate and phosphate) and microbial cell abundances (i.e. <~10^4 cells ml^{-1}) indicative of basement fluids (Mottl and Wheat, 1994; Jungbluth *et al.*, 2013a) (Figure 5.11). A subset of borehole fluid samples were found to have high (>90%) contamination with bottom seawater – these samples are designated as 'mixed' or low-quality subsurface fluids (LQSSF) (Tables 5.1 and 5.2). Crustal fluids collected from borehole 1025C located in the hydrothermal transition zone are of intermediate chemical composition between the borehole fluids sampled from 3.5 Ma ocean crust and bottom seawater (Figure 5.11a). Within the high-integrity crustal fluid samples from 3.5 Ma ocean crust, relatively slight variations in chemical characteristics (e.g. Mg, DOC, TOC,

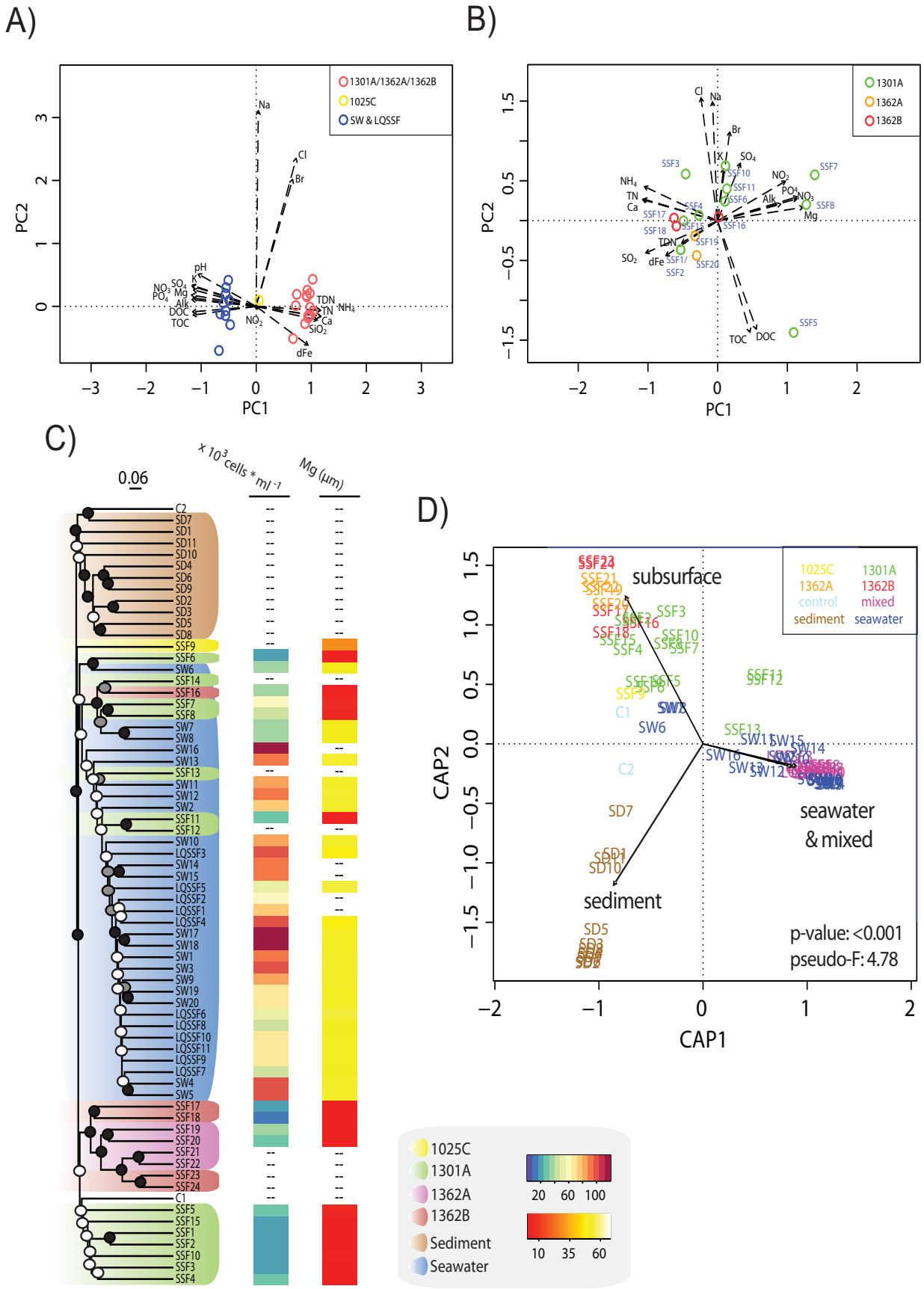

A)
1301A/1362A/1362B
1025C
SW & LQSSF
PC1
PC2
B)
1301A
1362A
1362B
PC1
PC2
C)
0.06
x 10³ cells * ml⁻¹
Mg (µm)
1025C
1301A
1362A
1362B
Sediment
Seawater
20 60 100
10 35 60
D)
subsurface
seawater
& mixed
sediment
1025C
1301A
1362A
1362B
control
mixed
sediment
seawater
p-value: <0.001
pseudo-F: 4.78
CAP1
CAP2

Figure 5.11 (A) Principle components analysis biplot of (A) all samples and (B) borehole U1301A, U1362A, and U1362B subsurface fluid samples with corresponding chemical data. Dashed arrows indicate the direction of increase for chemical parameters measured and arrow length indicates the degree of correlation with the ordination axis. Samples are scaled by eigenvalues derived from the chemical parameters. Alk, alkalinity; DOC, dissolved organic carbon; TOC, total organic carbon; TDN, total dissolved nitrogen; TN, total nitrogen; dFe, dissolved iron. (C) Unweighted pair group method with arithmetic mean (UPGMA) analysis of Bray-Curtis dissimilarity indices. Consensus phylogeny is shown and support for nodes, based on 100 replicates, is indicated using circles: black (100%), gray (>80%), and white (>50%). The scale bar corresponds to a Bray-Curtis dissimilarity value of 0.06. Each sample is colored according to location in sediment, seawater, and borehole fluid observatory sampled (1025C, U1301A, U1362A, U1362B). Cellular abundance of microbial groups inhabiting borehole fluids and background seawater and corresponding magnesium concentrations are indicated using a heat map. Gradient scales are indicated and unperformed analyses are indicated using dash lines. (D) Distance-based redundancy analysis (db-rda) showing grouping of all samples based on categories: subsurface, sediment, seawater, and mixed. Sample names correspond to those described in detail on Table 5.1 and are colored according to origin.

nitrite, nitrate, phosphate, carbon dioxide, sulfate) and total microbial cell abundances were revealed and likely indicate varying degrees of seawater intrusion into otherwise high-integrity crustal fluid samples (Figure 5.11b).

Microbial community structure

A total of ~1.7 million SSU rRNA amplicons were sequenced in the forward and reverse directions resulting in 150-bp-long reads with an expected overlap of ~24 bases. The average number of reads per sample was 25,029 and 13,488, respectively, in the unpaired and paired sequences used for further analysis. Subsurface fluids have a distinct microbial community composition different from sediments, seawater, and seawater-contaminated samples. Microbial community differences were explored in higher-dimensional space with PCoA analysis of all microbial sequence tags together (data not shown), and separating sequences into domains *Archaea* and *Bacteria* (Figure 5.12a); results were highly consistent with average linkage analysis and support the distinction of at least three separate microbial community end members (sediments, seawater, subsurface fluids). Stability of clusters generated from analysis of all sequence tags was assessed using jackknifed UPGMA clustering and supports a stable result (Figure 5.12b). PERMDISP was used to assess multivariate homogeneity of group dispersions and reveals nearly significant differences between sample types (Table 5.5); notably this analysis included samples that were of questionable microbiological quality (i.e. SS7, SSF8, SSF11, SSF16) because the fluid sample geochemistry did not indicate overwhelming seawater contamination. A small proportion of sequences were found in common among the samples of seawater, sediment, and subsurface fluids and provides further evidence for a distinct microbial community in aging basement fluids (Figure 5.12c).

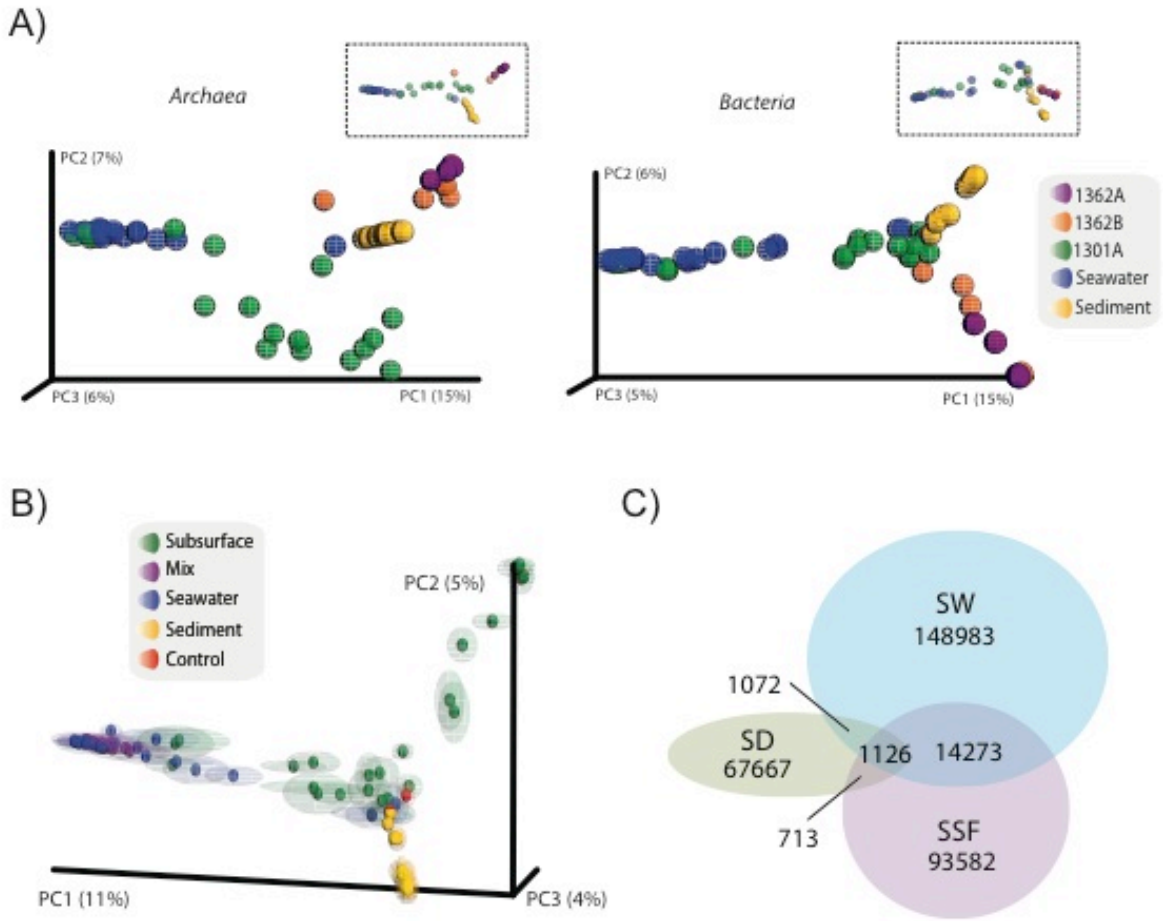


Figure 5.12 (A) Principle coordinates analysis (PCoA) of Bray-Curtis dissimiliarity indices using rarefied unique paired-reads representing the domains *Archaea* and *Bacteria*. Rarefaction of archaeal sequences was performed to the lowest number of sequences (190) detected in the subsurface fluid samples and removing samples C1, C2, SW6, and SW8. Rarefaction of bacterial sequences was performed to the lowest number of sequences (2803) represented in a single sample. Hole 1025C and all low-quality subsurface samples are not shown. Samples are colored according to location in sediment, seawater, and borehole fluid observatory sampled (1301A, U1362A, U1362B). Plot axes are scaled according to the percentage variation explained via the top three principal components. Rotated plots are shown inset to reveal additional features occluded in depiction of the maximum variation within PC1 and PC2. (B) Jackknifed beta diversity analysis of Bray-Curtis dissimiliarity indices using unique unpaired forward reads and rarefying to an even sequence depth across samples. Jackknifed subsets consisted of 100 sequences per sample. Cluster stability is indicated using semi-transparent ovals encompassing opaque-colored sample mid-points. Samples are colored according to type (subsurface, mix, seawater, sediment, control) and as described in Table 5.1. (C) Venn diagram of unique unpaired forward reads showing the overlap in microbial communities between seawater, sediment, and subsurface fluids.

Breaks from the major environmental grounding trend in samples are predictable using information about microbial cell abundance and chemical composition (Figures 5.11c, 5.11d and Tables 5.1 and 5.5). Average linkage analysis reveals differences in the microbial communities that track observed differences in the sample chemical characteristics, specifically, all low-quality subsurface fluids identified as chemically indistinguishable from bottom seawater (Figure 5.11a) cluster with other bottom seawater samples (Figure 5.11c). A subset of higher-quality fluid samples cluster with bottom seawater samples; these samples exhibit chemical characteristics (i.e. elevated magnesium and phosphate) and cell concentrations (i.e. $>2 \times 10^4$ cells ml^{-1}) indicative of bottom seawater (Figures 5.11b and 5.11c); Crustal fluids from U1301A were distinct from borehole U1362A and U1362B fluids (Figure 5.11c). Chemical parameters that distinguish high-quality crustal fluids were identified using Mantel correlation analysis and include: pH, calcium, magnesium, potassium, silicate, ammonium, phosphate, nitrate, sulfate, dissolved iron, DOC, TDN, and alkalinity (Table 5.5). Mantel analysis was repeated on subsurface fluid samples only and revealed calcium, magnesium, ammonium, nitrate, dissolved, iron, and to a lesser degree, potassium to be correlated with differences in the observed microbial communities (Table 5.5). Grouping samples by type (subsurface, sediment, seawater) is statistically significant using the rank-based ANOSIM test (p-value=0.001; alpha=0.05); an intermediate-high value for the R statistic (R=0.6795) indicates dissimilarity between the categorical groupings. Sample grouping again by type (control, subsurface, sediment, seawater, mixed) is statistically significant using the db-RDA method (p-value=<0.001; alpha=0.05; pseudo-F=4.78) (Figure 5.11d) and results are highly consistent with UPGMA (Figure 5.11c). db-RDA analysis revealed sediments from deeper (25—225 meters below seafloor) horizons (i.e. SD1, SD7, SD10, SD11) to be most similar to subsurface fluid samples (Figure 5.11d). Other tests (adonis, MRPP, PERMANOVA) performed using similar categorical groups were also significant using alpha=0.05 (data not shown).

Microbial communities in subsurface fluid are taxonomically distinct from overlying sediments and bottom seawater and contain novel lineages of *Archaea* and *Bacteria* that have been linked to metabolic functions characteristic of chemolithoautotrophs and chemoheterotrophs. Groups identified in fluids collected from the newest borehole observatories (U1362A and U1362B) contain unique archaeal lineages of Marine Benthic Group E (MBG-E) and *Archaeaglobus* and bacterial lineages of *Nitrospirae*, *Calescamantes*(EM19/KB1), *Aminicenantes* (OP8), and EM3 (Figures 5.13, 5.14a, 5.14b, and 5.15). Notably, samples SSF21/SSF22 and SSF23/SSF24 are the largest biomass samples collected and analyzed to date from this environment, ~124 liters and ~70 liters, respectively; archaeal groups *Archaeglobus*, MBG-E, and bacterial group *Nitrospirae* were in high abundance. Other notable groups in U1362A and U1362B fluids include *Methanococci*, *Deltaproteobacteria – Desulfovibrio*, Deep Hydrothermal Vent Euryarchaeota Group 2 (DHVEG-2), and *Thermococci – Palaeococcus*. Microbial communities recovered in borehole U1301A fluids, including from samples that have

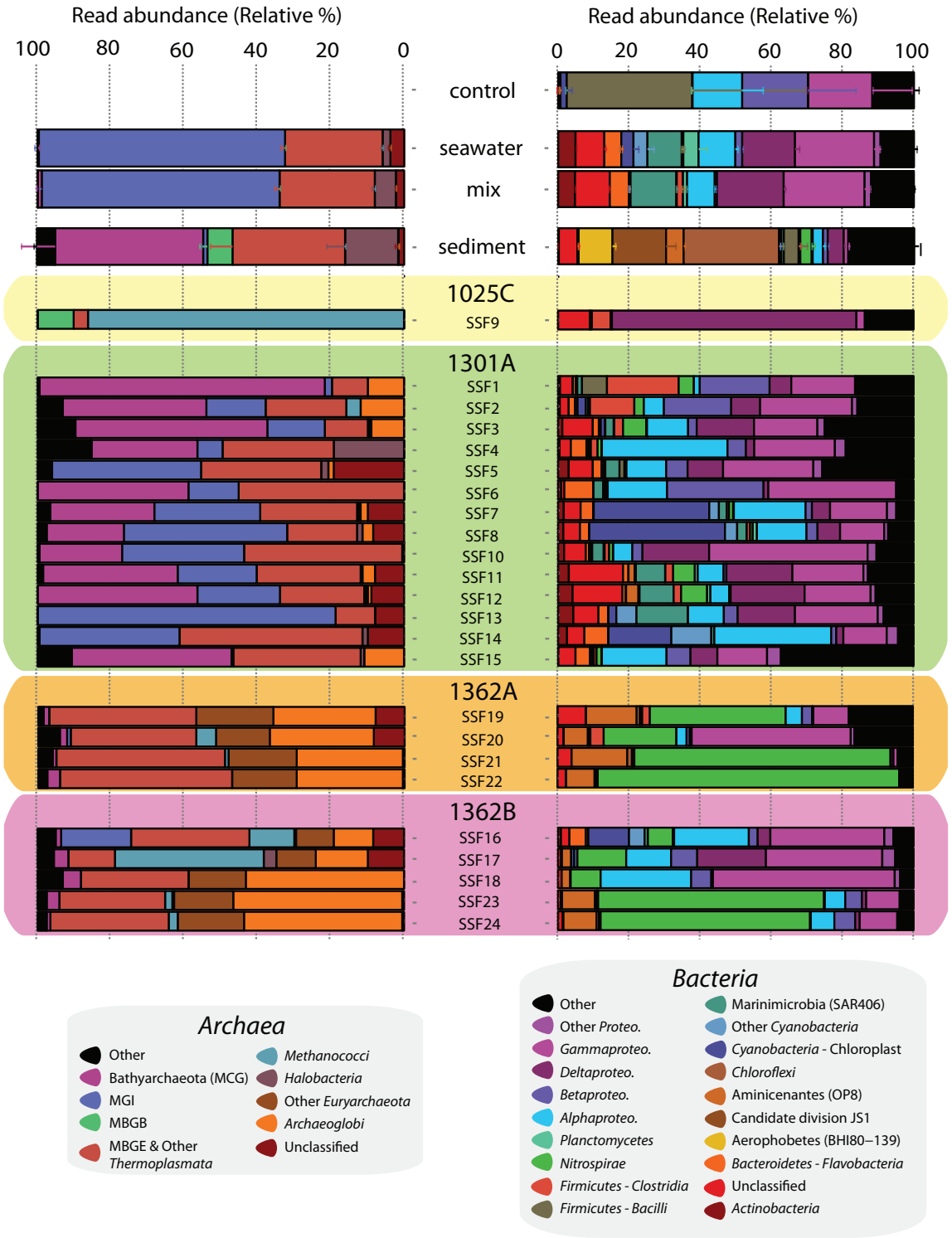


Figure 5.13 Phylum- and class-level taxonomic distributions of *Archaea* and *Bacteria* within control, seawater, seawater/subsurface mixed, sediment, and subsurface fluid samples. Subsurface fluid samples are plotted individually; the taxonomic distribution of other samples is averaged and standard deviation of the mean is indicated. Archaeal sequences in controls were either absent (sample C2) or present in very low (<0.01%) abundance (sample C1), so are not included.

been sequenced previously (i.e. SSF1/SSF2, SSF4) (Jungbluth *et al.* 2013a) were populated by archaeal groups *Bathyarchaeaota* (MCG), Marine Group I (MG-I), and other *Thermoplasmata* [e.g. South Africa Goldmine Euryarchaeota Group (SAGMEG)] and bacterial groups *Gammaproteobacteria* (*Thiotrichaceae*, *Sulfurovum*, *Thiomicrospira*), *Nitrospirae*, Aminicenantes (OP8), and other numerous other lineages (e.g. *Firmicutes – Desulforudis, Lachnospiracea*, , *Alkaliphilus*). Overall, lineages recovered in borehole U1301A fluids were not as distinct as seen in U1362A/U1362B fluids. Groups recovered in borehole 1025C were highly similar to the groups that have been recovered previously with gene cloning and sequencing (Jungbluth *et al.*, 2014), although new groups have been uncovered with the deeper sequencing depth. MG-I was the most abundant archaeal groups recovered followed by Marine Benthic Group B (MBG-B) and bacterial groups from the *Deltaproteobacteria* phylum (e.g. *Desulfocapsa*, *Desulfobulbus*, *Desulfovibrio*), *Firmicutes – Desulforudis* lineage, and *Bacteroidetes* group SB-1 (Figures 5.13, 5.14a, 5.15). Indicator taxa analysis of subsurface samples revealed archaeal groups Terrestrial Hydrothermal Spring Crenarchaeal Group (THSCG), MBGE, DHVEG-2, *Methanothermococcus*, *Archaeaoglobus*, distinct *Bathyarchaeota* (MCG) lineages and bacterial lineages of *Gammaproteobacteria* (*Pseudomonas*), *Nitrospirae*, *Aminicenantes* (OP8), *Calescamantes* (EM19/KB1), and *Chloroflexi – Anaerolineaceae* to be characteristic of these sample types (Figure 5.14b).

Microbial communities observed in designated control samples types (controls, seawater, mixed, sediment) are taxonomically distinct from subsurface fluids. The most abundant groups within seawater and mixed samples were affiliated with the archaeal MG-I and bacterial class *Gammaproteobacteria* (Figure 5.13). Other abundant groups in seawater and mixed samples include other archaeal *Thermoplasmata* [e.g. Marine Group II (MG-II)], and bacterial groups *Deltaproteobacteria*, *Deferribacteres* (SAR406)*, Alphaproteobacteria* and several other groups (Figure 5.13). Groups recovered are highly similar to groups recovered previously in seawater samples (i.e. SW1, SW3, SW9, SW15) using gene cloning and sequencing (Jungbluth *et al.*, 2013a). Archaeal groups *Bathyarchaeota* (MCG), and *Thermoplasmata* (e.g. SAGMEG) and bacterial lineages JS1 and *Chloroflexi* were in high abundance with sediment samples and, in general, groups recovered were highly similar to groups recovered previously (i.e. SD2, SD4, SD5, SD6, SD9) using gene cloning and sequencing (Jungbluth *et al.*, 2013b). Other lineages in sediment samples include archaeal groups MBG-B and *Halobacteria* and bacterial groups *Aerophobetes* (BHI80-139), *Aminicenantes* (OP8), *Firmicutes – Clostridia*, and *Proteobacteria*. Archaeal sequences were detected in very low (i.e. single sequence) abundance with both control samples (i.e. C1 and C2). Bacterial groups identified in control samples include *Firmicutes – Bacilli*, *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria* (*Enterobacteriaceaea, Moraxellaceae*, *Stenotrophomonas*, *Pseudomonas*), and several other groups (e.g. *Acidobacteria*, *Bacteroidetes, Alphaproteobacteria*). Of particular note was the presence of *Betaproteobacteria* lineage

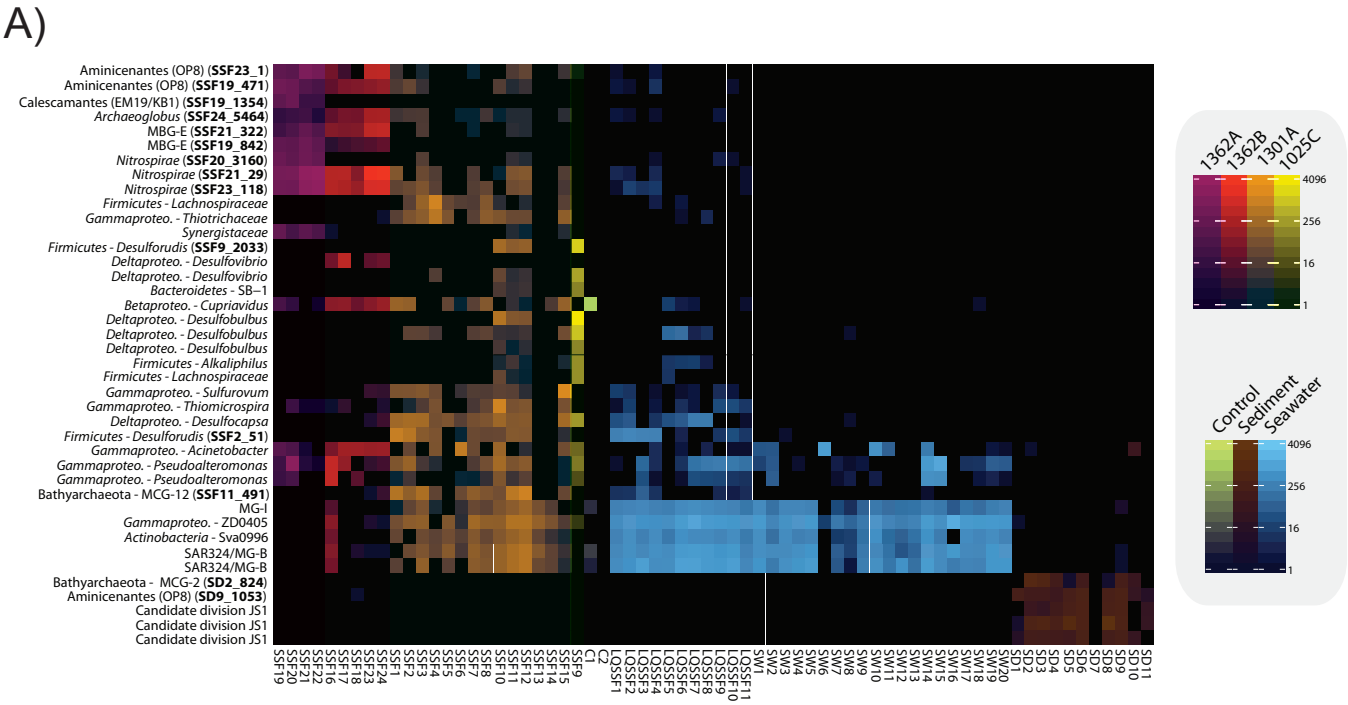


Figure 5.14 Heat map of unique sequence reads representing the top five lineages detected in control, sediment, and seawater samples and the top 10 lineages detected in boreholes 1025C, U1301A, U1362A, and U1362B. The total number of reads detected is indicated in the scale bar along with color of sample set. (B) Dufrêne-Legendre indicator species analysis of *Archaea* and *Bacteria* based on unique reads rarefied as described in Figure 1 and using 1000 randomized iterations to calculate probabilities. Abundance of unique reads representing taxonomic lineages is indicated.

Cupriavidus, and MG-I and MG-II, which were found in the control sample of shipboard water (Figure 5.14a). Indicator taxa analysis reveals archaeal groups MG-I, MG-II, MG-III, MBGB, distinct *Bathyarchaeota* (MCG), SAGMEG and bacterial lineages of *Gammaproteobacteria* (JL-ETNP-Y6, E01-9C-26, *Pseudomonas*, *Marinobacter*, *Pseudospirillum*), *Alphaproteobacteria* (i.e. *Pelagibacter* - SAR11), *Flavobacteriaceae*, SAR324/Marine Group B, and *Cyanobacteria* to be characteristic of seawater and sediment communities (Figure 5.14b).

Variation in microbial lineages characterized in borehole fluids previously and described here show patterns that are describable in part through variation in sample location and integrity. Novel lineages were detected mostly from borehole U1362A and U1362B fluids in archaeal groups THSCG, *Archaeoglobus*, MBG-E and bacterial groups *Nitrospirae*, and *Aminicenanates* (OP8) using Illumina tag sequencing and either gene cloning and sequencing or single cells isolation and SSU rRNA gene sequencing (Figures 5.14a and 5.15). Single cell isolation provided a separate method for assessing major community members and provides supporting evidence for the relatively high abundance of *Archaeoglobus*, MBG-E, *Nitrospirae*, *Calescamantes* (EM19/KB1), *Aminicenantes* (OP8), and novel support for the lineage *Aquifex* (Figure 5.15). Near full length gene clones and isolated single cells from U1362A/U1362B fluids in groups Aminicenantes (OP8), and *Nitrospirae* lineages are distinct, having closest relatives in GenBank that are relatively distant: 91%, 88%, 90% similar identity (Figure 5.15; data not shown). Single cell isolates SCGC_AC-708_J03 and SCGC_AC-708_O07 from the archaeal group *Archaeoglobus* were relatively dissimilar from other characterized sequences in GenBank, 95% and 96%, respectively, given the relatively well-classified nature of this group (Figure 5.15; data not shown). Group EM3 was detected from borehole U1362A and U1362B fluids only using gene cloning and sequencing. There is relatively little overlap in the major microbial lineages detected using Illumina sequencing in boreholes U1362A/U1362B and U1301A; specifically, borehole U1301A fluids contain many groups of *Proteobacteria* and a more constant background of seawater samples is evident (e.g. *Acinetobacter*, *Pseudoalteromonas*) (Figure 5.14a). *Candidatus* Desulforudis gene clones related to the lineage previously detected in borehole 1025C were detected for the first time here in U1301A borehole fluids; this is supported by Illumina tag sequencing (Figure 5.15). Lineages from borehole 1025C are similar to previous characterizations (Jungbluth *et al.* 2014). Interestingly, a handful of apparently real subsurface fluid microbial groups [e.g. *Aminicenantes* (OP8), *Archaeglobus*, *Nitrospirae*, *Deltaprotebacteria – Desulfbulbus*, and *Firmicutes – Alkaliphilus*] were detected using the Illumina sequencing depth afforded in trace amounts within the long tail of the abundance distributions, which expected given that low-quality samples were at least attempts to connect to and sample fluids from the CORK observatories (Figure 5.14a).

Illumina sequence tags recovered from groups *Aminicenantes* (OP8) and *Bathyarchaeota* (MCG) reveal sub-lineages that appear to prefer either sediment or subsurface fluids (Figures 5.15 and 5.16). Phylogenetic analysis of *Aminicenantes* (OP8) reveals Illumina

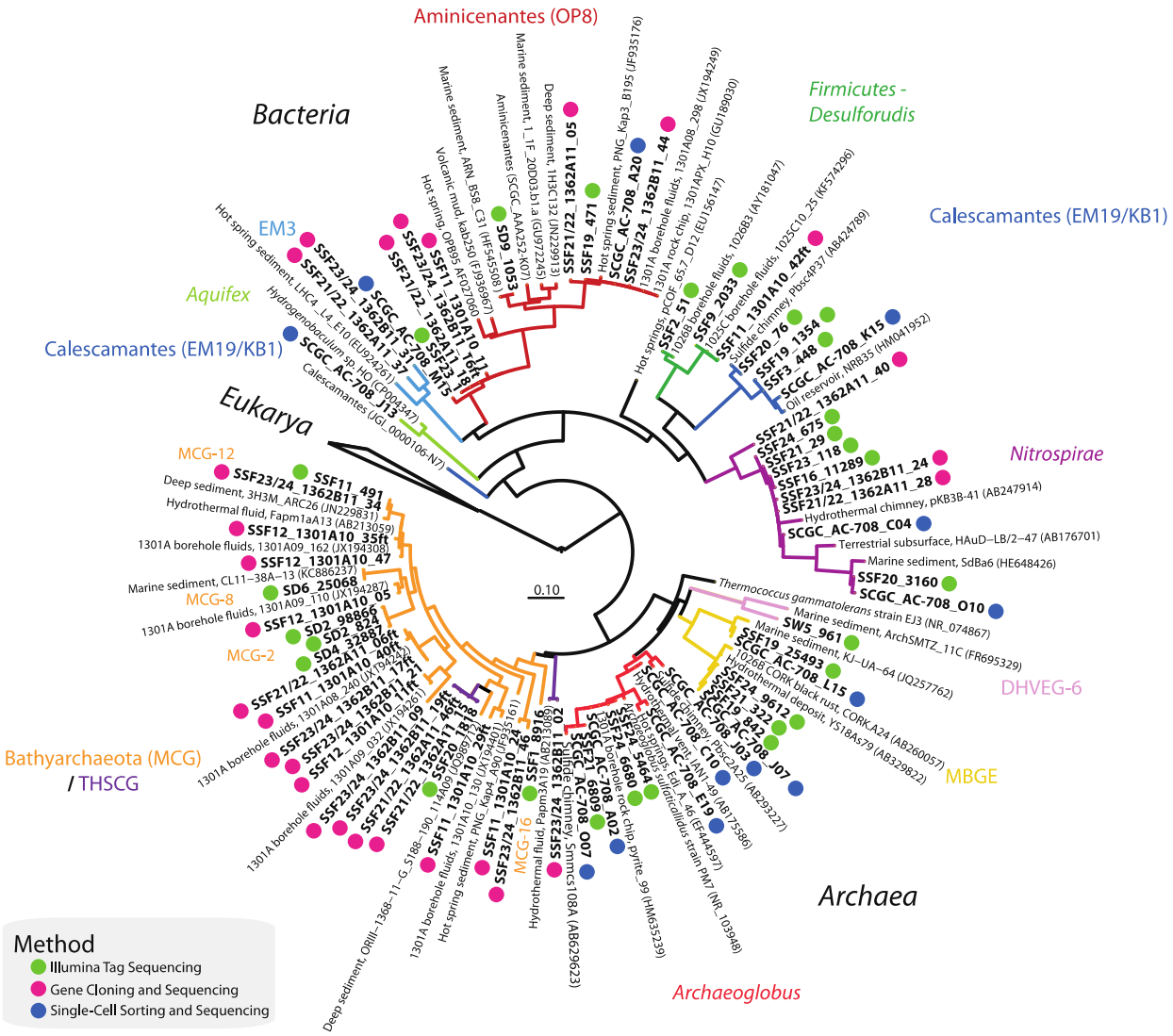


Figure 5.15 Phylogenetic relationships of subsurface fluid gene clones, single cell SSU rRNA gene clones, or Illumina sequence tags related to groups: *Calescamantes* (EM19/KB1), *Aquifex*, EM3, *Aminicenantes* (OP8), *Firmicutes* lineage *Desulforudis*, *Nitrospirae*, DHVEG-6, MBGE, *Archaeglobus*, *Bathyarchaeota* (MCG), and THSCG. SSU rRNA sequences recovered in this study are in bold-font and colored according to source: Illumina tag sequencing (green), gene cloning and sequencing (red), single-cell sorting and sequencing (blue). Cultivated *Eukarya* sequences were used as outgroups. The scale bar corresponds to 0.1 substitutions per nucleotide position. Tree was visualized using iTOL (Letunic and Bork, 2007; Letunic and Bork, 2011).

tags from sediments to show high relation to groups detected in sediments and tags from subsurface fluids to be similar to 1) lineages described previously (1301A08_298; Jungbluth *et al.*, 2013a) and 2) novel microbial sequences detected in U1362A/U1362B here through gene might be cloning and single cell isolation (Figure 5.15). Diverse members of archaeal group *Bathyarchaeaota* (MCG) were detected in tag sequencing and subjected to fine-scale phylogenetic classification of groups using Phyloassigner and a custom database consistent with

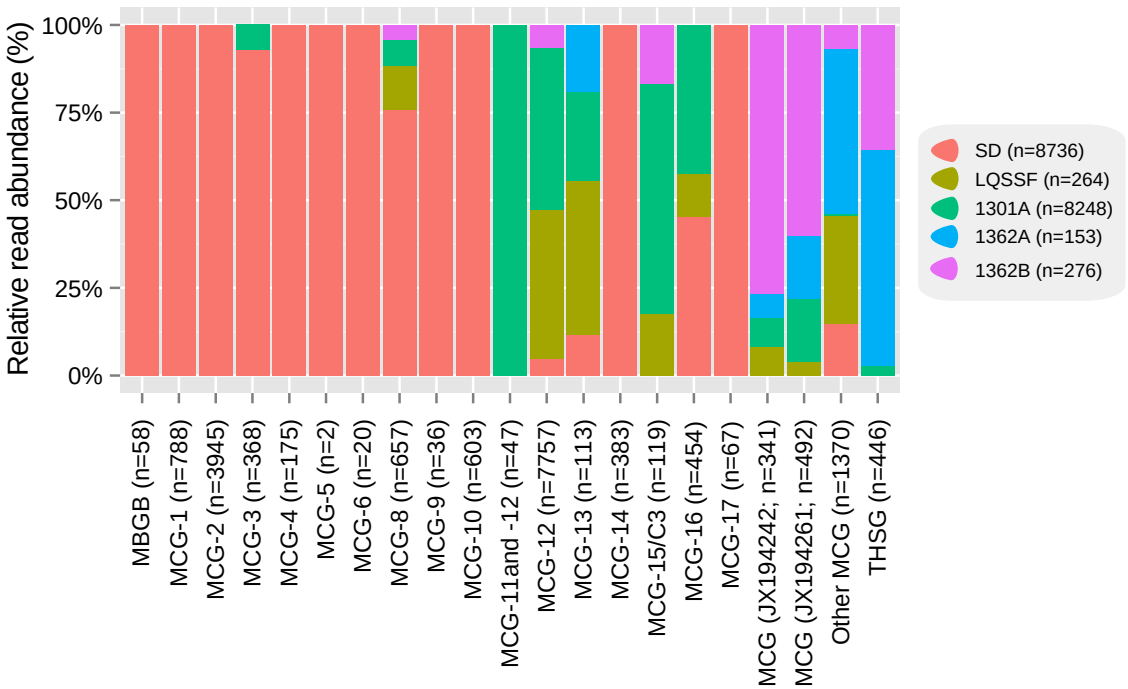


Figure 5.16 High-resolution Phyloassigner-based taxonomic analysis of unique reads classified as *Bathyarchaeaota* - Miscellaneous Crenarchaeota Group (MCG). Naming schemes are adopted from Kubo et al. 2012, but modified to include two divergent lineages (JX194242 and JX194261) detected previously in subsurface crustal fluids (Jungbluth et al. 2013a). The number of reads in each environmental group were normalized for comparative purposes and the number of total reads detected in each environmental group and "MCG" lineage is indicated.

MCG naming schemes described by (Kubo *et al.*, 2012) and supplemented with novel groups described previously (Jungbluth *et al.* 2013a). *Bathyarchaeota* (MCG) are a highly diverse group present in both sediment and subsurface fluid sample types; clear lineages indicative of either sediments, borehole U1301A, or borehole U1362A/U1362B fluids (Figure 5.16). Lineages MCG-1 – MCG-10, MCG-14, and MCG-17 are sediment-associated groups (Figure 5.16). MCG detected in borehole U1301A fluids in multiple groups, but mostly restricted to MCG-11 – MCG-13, MCG-15/C3, and MCG-16 (Figure 5.16). MCG were not particularly abundant in borehole U1362A and U1362B fluids, but were commonly in novel lineages (JX194242, JX194261) detected by Jungbluth *et al.* (2013a) and other novel, unnamed MCG groups. Fine-scale phylogenetic analysis using a hand-curated database of MCG sequences (Kubo *et al.*, 2012) revealed sequences classified previously by the SINA tools to actually be in groups MBGB and THSCG; THSCG sequences may be abundant in boreholes U1362A and U1362B samples as evident by detection via both Illumina tag sequencing and gene cloning and sequencing (Figures 5.15 and 5.16). Notably, gene cloning and sequencing was used to recover groups of *Bathyarchaeaota* (MCG) in subsurface fluids that were not recovered using Illumina sequencing (Figure 5.15).

Discussion

This study is the first to employ the latest generation of CORK observatories equipped with Teflon-lined sampling ports and fluid delivery lines (i.e. U1362A/U1362B) to sample fluids from a depth >290 meters sub-basement, representing the deepest depth horizon from which fluids have been retrieved from the subseafloor. Borehole fluids collected from older CORK observatories (i.e. 1025C, 1026B, U1301A, 1301B) were also analyzed here, and together comprise an expansive catalogue of deep crustal fluid samples. Differences between high- and low-integrity borehole fluid samples were clearly discernible using chemical signatures (e.g. elevated magnesium) and microbial cell abundances (i.e. elevated; typically > 2 x 10^4 cells ml^{-1}) indicative of bottom seawater contamination. Chemical characteristics of high-integrity borehole U1362A and U1362B fluid samples are largely consistent with previous results from basement fluids collected from boreholes or exiting seamounts (e.g. Mottl *et al.*, 1998; Wheat *et al.*, 2000; Lin *et al.*, 2012). Higher average concentrations of total nitrogen, total dissolved nitrogen, ammonium, calcium, silicate, and dissolved iron reveal the potential for fluid samples from borehole U1362A and U1362B to be relatively more altered than crustal fluids described previously. Microbial cell abundances in selected high-integrity samples from U1301A (SSF15) and U1362B (SSF17) were 5.6-5.8 x 10^3 cells ml^{-1}, which is ~62-64% the previously observed lowest number of cells observed in borehole U1301A fluids collected in 2009 (Jungbluth *et al.*, 2013a). Sample SSF18 contained cells in lower abundance (2.64 x 10^3 cells ml^{-1}) and has a high chemical integrity; however, the microbial community in this sample was uniquely dominated by *Proteobacteria* and contaminated by lineages (e.g. *Xanthomonas*) previously identified in pre-drilling mud fluids (Masui *et al.*, 2008), making any inference from this sample suspect. The low microbial cell abundances obtained here represent the best low-end estimates for microbial biomass in this environment; however, a range of microbial cell abundances were observed in "high" integrity samples and extend up to 26.1 x 10^3 cells ml^{-1}. Notably, the highest cell counts were observed in a sample (SSF19) with relatively high concentrations of aqueous iron. In spite of seawater or other unknown sources of contamination trickling into a subset of samples, the relatively large number of high-integrity fluid samples collected here help further constrain the magnitude of biomass in deep ocean crustal hydrothermal fluids than has previously been estimated (Jungbluth *et al.*, 2013a) and reveal a range of microbial cell concentrations in basement fluids that are, perhaps, dictated by relatively minute changes in basement fluid chemistry.

Microbial communities in deep crustal fluids from an anoxic basement aquifer are demonstrably different from bottom seawater, sediments. Greater than 12 high-integrity Juan de Fuca Ridge flank crustal fluid samples have been collected from three boreholes and provide the statistical power needed to assert the distinctness of a basement fluid microbial community. Differences among microbial communities from the different environments were

more apparent in “cleaner” samples collected from boreholes U1362A/U1362B; although a subset of crustal fluid samples from U1301A do also appear quite distinct. Of the total number of reads identified in subsurface fluids, greater than 85% (93582/109694) were unique to that environment. A non-negligible number of reads overlapped with seawater but are explained by simple end member mixing assuming very low amounts of seawater contamination during sampling. Low sample numbers from borehole U1362A and U1362B likely contributed to there being no significant difference from seawater. Some sediment samples collected from intermediate depths or nearby the sediment basement interface (i.e. SD1, SD7, SD10, SD11) were comparatively more similar to basement fluid microbial communities, although sediments and subsurface fluids are overall highly distinct and very few reads were found to overlap. Notably, sediments were collected ~50 km away from the location of borehole fluid sampling; sediments from locations U1301A, U1362A, and U1362B would serve as a beneficial addition to this study. Notwithstanding, the distinctness between the basement fluid and sediment environment would likely be supported if deep sediments from the exact fluid sampling area followed trends described here and for other deep sediments found worldwide, specifically the presence of bacteria groups *Gammaproteobacteria*, *Epsilonproteobacteria*, *Betaproteobacteria*, *Chloroflexi*, and OP9/JS1 and archaeal groups MCG, DSAG/MBGB (e.g. Inagaki *et al.*, 2003; Kormas *et al.*, 2003; Webster *et al.* 2006; Webster *et al.*, 2009). Subsurface fluid groups detected here, including Nitrospirae and MBGE are rare in sediment studies and are most often detected in studies of hydrothermally influenced sediments or deposits (e.g. Takai and Horikoshi, 1999; Suzuki *et al.*, 2004; Nercessian *et al.* 2005). In particular, *Archaeoglobus* is not common in sedimented environments and is largely affiliated with higher temperature hydrothermal systems; temperatures typical for growth of *Archaeoglobus* in the U1301A/U1362A/U1362B location are encountered only in the basement environment. Though not detected using Illumina tag sequencing, groups EM3 (*Thermotoga*) and *Aquifex* were observed in crustal fluids and indicate that fluid samples are originating from deep with the crust. *Aquifex* has never previously been detected outside of the ridge crest hydrothermally associated environment, so its detection here reveals samples truly distinct from the sediments. Microbial groups associated with drilling and coring on the D/V *Joides Resolution* – the vessel used to drill all JdFR holes - have never been analyzed; however, microbiological contamination assessments carried out on board the D/V *Chikyu* indicate contaminating groups include *Gammaproteobacteria* (*Pseudomonas*, *Vibrio*, *Marinobacter*), *Bacteroidetes*, *Alphaproteobacteria*, *Firmicutes* (*Acholeplasma*), and *Verrucomicrobia* (Masui *et al.*, 2008; Yanagawa *et al.*, 2013). Samples collected from locations U1362A, U1362B are relatively free of contaminating microbial groups induced or implanted during drilling; however all U1301A/U1362A/U1362B samples do have some background of *Gammaproteobacteria* lineages *Pseudomonas* and *Xanthomonas* (Masui *et al.*, 2008). Overall, no microbial communities previously described have the combination of novel microbial diversity and

unique community structure that has been detected here in basement crustal fluids, which indicates the potential for a novel biosphere in the sealed anoxic basaltic aquifer.

There appear to be distinct and reproducible borehole fluid microbial communities in crustal fluids from Holes U1362A and U1362B collected over the span of multiple days and from Hole U1301A over the span of days and years. Borehole U1362A, which penetrates the deepest depth extent into the basement rock, is the most distinct of the subsurface fluid samples and was the only crustal fluid sample significantly different from sediments. Microbial communities in Hole U1301A are relatively similar to microbes from Holes U1362A/B, which is consistent with connectedness in the subsurface and has been described previously for this location (e.g. Jungbluth *et al.*, 2013a, Jungbluth *et al.*, 2014) and for deep subsurface oil reservoirs (Lewin *et al.*, 2014); however, notably there are differences in the microbial communities found between the locations. Significant differences were found between U1301A and U1362A microbial communities, though it is likely that microorganisms from U1362B would have also been found to be significantly different if low-quality sample SSF16 was removed from analysis. Major members in borehole U1362A and U1362B microbial communities [e.g. OP8, *Archaeoglobus*, *Nitrospirae*, *Calescamantes* (EM19/KB1)] are found in Hole U1301A here or previously (Jungbluth *et al.*, 2013a), though in relatively low abundance. Hole U1301A fluids had a significantly larger proportion of archaeal groups MCG and bacteria groups *Proteobacteria* and *Firmicutes*, which is consistent with previous studies with from this location (Orcutt *et al.*, 2011; Smith *et al.*, 2011; Jungbluth *et al.*, 2013a). *Deltaproteobacteria* and *Firmicutes* lineage *Desulforudis* were again detected in U1301A and 1025C fluids but these groups were notably in very low abundance or absent in U1362A and U1362B. The very low abundance of microbial groups indicative of seawater contamination [e.g. Marine groups I, II, and SAR324 (Marine Group B)] in locations U1362A/B is in contrast to all borehole U1301A samples. Differences between subsurface sites could be real or could be associated with differences of the CORK materials used, but perhaps most notably is the relative quickness with which boreholes U1362A and U1362B were positively pressured and producing fluids following drilling. Due to the enhanced fluid flow expected in these borehole locations from drilling-induced disturbances or difficult-to-seal borehole observatories, it may very well be that samples collected closer to the time of drilling will provide the best snapshots into the *in situ* deep subseafloor community, specifically before secondary colonization and biofilms occur in association with the observatories.

Unique read analysis and high-resolution phylogenetic classification using sequencing of Illumina tags, gene clones, and flow sorted single cells - three methods invoking different primer sets – reveal a comprehensive view of microbial groups in subsurface fluids (Figure 5.5). Specifically, the use flow sorted single cells provides independent confirmation for the relatively high abundance of microbial groups *Archaeaoglobus*, MBGE, *Nitrospirae*, *Calescamantes*, and *Aminicenantes*, which were also detected using Illumina tag sequencing. Lineages of

Archaeoglobus detected here are highly related to cultivated groups, likely linking them to a sulfate-reduction metabolic lifestyle. Deep branching lineages of *Nitrospirae* detected here were major indicator taxa for subsurface samples and are highly divergent from the next uncultivated gene clones and even further from cultivated representatives. Speculation into the metabolic lifestyle of the *Nitrospirae* is difficult, though notably sulfate-reduction is a theme within this group (e.g. Sekiguchi *et al.*, 2008). Other highly abundant groups are uncultivated and information about metabolic lifestyle has to be inferred from metagenomic and single cell genomic analyses. *Bathyarchaeaota* (MCG) classified according to the subgroups described in Kubo *et al.* (2012) revealed distinct sediment and subsurface fluid clades, some of which were originally detected in Hole U1301A fluids (Figure 5.16; Jungbluth *et al.*, 2013a). Recent evidence suggests this group is involved in catabolism of aromatic compounds (Meng *et al.*, 2014) and peptide degredation (Lloyd *et al.*, 2013), though the diversity of this group suggests a more complex role in carbon remineralization processes. Subgroup MCG-12 was found in relatively high abundance in U1301A fluids, but MCG were not particularly abundant from U1362A/B; even though total organic carbon is roughly similar across these sites, this is suggestive that there may be differences in the lability of organic matter between borehole locations. Single cell genomic analyses of *Calescamantes* and *Aminicenantes* have recently been performed, providing insight into these groups (Rinke *et al.*, 2013). A relatively high amount of inter-group divergence in SSU rRNA genes for these groups was observed; however, *Aminicenantes* has been implicated in amino acid degradation. Despite genomic sequencing efforts, metabolic association of *Calescamantes* is still unknown; but these groups will likely prove to be important in the subsurface ocean fluids due to their wide distribution across thermophilc locations in the terrestrial and marine subsurface. There is currently no genetic information known about Marine Benthic Group E so its metabolic features are unknown; however, at least two lineages were characterized here, and one lineage is highly similar to a gene clone identified in the black rust sample from CORK 1026B (Nakagawa *et al.* 2006). Overall, this study provides evidence for the importance of some microbial groups that have only previously been detected in the subsurface crustal fluids but not in high abundance (e.g. MBGE and *Calescamantes*; Nakagawa *et al.*, 2006; Jungbluth *et al.*, 2013a), groups that have been found previously within some samples in higher abundance (e.g. MCG, OP8, *Archaeoglobus*; Orcutt *et al.*, 2011; Jungbluth *et al.*, 2013a), and some novel lineages that are described here for the first time (e.g. *Nitrospirae*).

The subseafloor ocean crust is largely ignored during discussions of microbial habitats in the deep subseafloor, which may be attributable to a lack of evidence demonstrating the extent and existence of a distinct deep subseafloor biosphere. Sampling opportunities from the sediment-buried ocean crust, especially those with any significant temporal or spatial scope, are rare due to the expensive operations costs and the inaccessible nature of the environment. The low biomass in the deep subsurface environment makes it difficult to acquire adequate sample volume for molecular analysis to overcome contamination problems associated with

drilling and resolve partitioning of the surface-attached and planktonic deep subsurface microbial communities (Jungbluth *et al.*, 2013). Analyses constrained to the use of rocks are highly biased because of the low samples amounts that can be retrieved and problems with the recovery of highly porous sections. This invariably means that studies of the subsurface biosphere rocky environment (e.g. Lever *et al.*, 2013) will also need to engage in the analysis of fluids (e.g. Jungbluth *et al.*, 2013a) to fully characterize the resident microbial community.

Borehole observatories built for the purpose of microbiological sampling have been iteratively improved during several decades of Ocean Drilling Program operations, and data shown here indicate that this high-risk scientific agenda has paid off in the form of characterizing novel microbial communities in aging mid-ocean ridge flanks. Nevertheless, many possible variables which might cause changeability in community composition and function about life in the ocean crust remain; to name a few: connectedness between sites, short-term variation at single sites, impact of distance from seawater injection points, thickness of the sediment cap, partitioning between surface-attached and planktonic microbial communities that can be expected (e.g. Flynn *et al.*, 2012), and rates of metabolic processes. Fluid circulation through the sediment-covered basement is likely to be a common feature of the seafloor (Fisher *et al.*, 2003) and discovery of additional hydrothermally active seafloor locations fueling chemosynthetic microbial ecosystems will surely continue. Additional locations will need to be drilled and explored and more samples will need to be collected from extant borehole observatories to understand temporal changes in this environment.

5.5 References

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5.6 Appendix

Table 5.1 Summary of samples used for analysis

Sample ID	*Sample type*	*Location (Depth in meters)*	*Sample year*	*Sample date*	*Collection method (Bag type)*	*Biological replicates*	*Technical replicates*	*Volume filtered (Liters)*	*Sequenced before?*	*Cell concentration (DAPI) (x 10^3 cells ml^{-1})*
SSF1	subsurface	U1301A	2009	30-Aug-09	LVWS (Tedlar)	biorep_3	techrep_1	2.61	yes[a]	8.98[a]
SSF2	subsurface	U1301A	2009	30-Aug-09	LVWS (Tedlar)	biorep_3	techrep_1	2.54	no	8.98[a]
SSF3	subsurface	U1301A	2010	19-Jun-10	LVWS (Tedlar)	biorep_8	n.a.	13.2	low	7.39
SSF4	subsurface	U1301A	2010	19-Jun-10	LVWS (Tedlar)	biorep_8	n.a.	11.2	yes[a]	15.3[a]
SSF5	subsurface	U1301A	2010	20-Jun-10	LVWS (Tedlar)	biorep_9	n.a.	2.41	no	16.1
SSF6	subsurface	U1301A	2010	20-Jun-10	LVWS (Tedlar)	biorep_9	n.a.	5.30	no	3.99
SSF7	subsurface	U1301A	2010	23-Jun-10	LVWS (Tedlar)	biorep_10	n.a.	14.9	no	50.7
SSF8	subsurface	U1301A	2010	23-Jun-10	LVWS (Tedlar)	biorep_10	n.a.	3.15	no	30.5
SSF9	subsurface	1025C	2010	27-Jun-10	LVWS (Tedlar)	n.a.	n.a.	1.95	yes[c]	n.a.
SSF10	subsurface	U1301A	2010	30-Jun-10	LVWS (Tedlar)	biorep_14	n.a.	21.1	no	11.2
SSF11	subsurface	U1301A	2010	30-Jun-10	LVWS (Tedlar)	biorep_14	n.a.	29.2	low	15.4
SSF12	subsurface	U1301A	2010	30-Jun-10	Insitu (n.a.)	biorep_14	n.a.	~120	low	n.a.
SSF13	subsurface	U1301A	2011	04-Jul-11	LVWS (Tedlar)	biorep_15	n.a.	0.90	no	n.a.
SSF14	subsurface	U1301A	2011	04-Jul-11	LVWS (Tedlar)	biorep_15	n.a.	1.25	no	n.a.
SSF15	subsurface	U1301A	2011	04-Jul-11	MVBS (Tedlar)	biorep_15	n.a.	14.1	no	5.82
SSF16	subsurface	U1362B	2011	08-Jul-11	LVWS (Tedlar)	biorep_18	n.a.	9.42	no	21.8
SSF17	subsurface	U1362B	2011	08-Jul-11	LVWS (Tedlar)	biorep_18	n.a.	24.7	no	5.57
SSF18	subsurface	U1362B	2011	10-Jul-11	MVBS (foil)	biorep_19	n.a.	14.4	no	2.64
SSF19	subsurface	U1362A	2011	12-Jul-11	MVBS (foil)	biorep_20	n.a.	13.8	no	26.1
SSF20	subsurface	U1362A	2011	12-Jul-11	MVBS (foil)	biorep_20	n.a.	15.7	no	15.6
SSF21	subsurface	U1362A	2011	12-Jul-11	Insitu (n.a.)	biorep_20	techrep_3	~124	low	n.a.
SSF22	subsurface	U1362A	2011	12-Jul-11	Insitu (n.a.)	biorep_20	techrep_3	~124	low	n.a.
SSF23	subsurface	U1362B	2011	10-Jul-11	Insitu (n.a.)	biorep_19	techrep_2	~70	low	n.a.
SSF24	subsurface	U1362B	2011	10-Jul-11	Insitu (n.a.)	biorep_19	techrep_2	~70	low	n.a.
LQSSF1	mix	U1301A	2008	07-Aug-08	GeoMicrobe (n.a.)	n.a.	n.a.	10.0	no	67.6
LQSSF2	mix	U1301A	2008	07-Aug-08	GeoMicrobe (n.a.)	n.a.	n.a.	10.0	no	54.3
LQSSF3	mix	U1301A	2008	07-Aug-08	LVWS (Tedlar)	biorep_2	n.a.	2.95	no	99.6
LQSSF4	mix	U1301A	2008	07-Aug-08	LVWS (Tedlar)	biorep_2	n.a.	2.82	no	99.6

LQSSF5	mix	1026B	2009	31-Aug-09	LVWS (Tedlar)	biorep_4	n.a.	4.92	no	41.2
LQSSF6	mix	1026B	2009	31-Aug-09	LVWS (Tedlar)	biorep_4	n.a.	4.76	no	41.2
LQSSF7	mix	1301B	2009	03-Sep-09	LVWS (Tedlar)	biorep_5	n.a.	4.98	no	36.8
LQSSF8	mix	1301B	2009	03-Sep-09	LVWS (Tedlar)	biorep_5	n.a.	5.01	no	36.8
LQSSF9	mix	U1301A	2009	04-Sep-09	LVWS (Tedlar)	biorep_6	n.a.	4.18	no	63.8
LQSSF10	mix	U1301A	2009	04-Sep-09	LVWS (Tedlar)	biorep_6	n.a.	3.98	no	63.8
LQSSF11	mix	U1301A	2009	04-Sep-09	LVWS (Tedlar)	biorep_6	n.a.	4.30	no	63.8
SW1	seawater	near U1301A (2644)	2008	08-Aug-08	CTDNiskin (n.a.)	n.a.	n.a.	2.63	yes[a]	87.8[a]
SW2	seawater	near U1301A (2515)	2008	08-Aug-08	CTDNiskin (n.a.)	n.a.	n.a.	3.91	no	72.2
SW3	seawater	near U1301A (~2650)	2009	04-Sep-09	CTDNiskin (n.a.)	biorep_7	n.a.	3.76	yes[a]	95.0[a]
SW4	seawater	near U1301A (~2650)	2009	04-Sep-09	CTDNiskin (n.a.)	biorep_7	n.a.	3.59	no	95.0[a]
SW5	seawater	near U1301A (~2650)	2009	04-Sep-09	CTDNiskin (n.a.)	biorep_7	n.a.	3.69	no	95.0[a]
SW6	seawater	near 1025C (2571)	2010	24-Jun-10	LVWS (Tedlar)	biorep_11	n.a.	4.01	low	22.0
SW7	seawater	near 1025C (2571)	2010	24-Jun-10	LVWS (Tedlar)	biorep_11	n.a.	4.25	no	22.0
SW8	seawater	near 1025C (2571)	2010	24-Jun-10	LVWS (Tedlar)	biorep_11	n.a.	9.00	no	22.0
SW9	seawater	near U1301A (2648)	2010	28-Jun-10	CTDNiskin (n.a.)	biorep_12	n.a.	5.00	yes[a]	76.0[a]
SW10	seawater	near U1301A (2648)	2010	28-Jun-10	CTDNiskin (n.a.)	biorep_12	n.a.	5.00	no	76.0[a]
SW11	seawater	near U1301A (2648)	2010	28-Jun-10	CTDNiskin (n.a.)	biorep_12	n.a.	5.00	no	76.0[a]
SW12	seawater	near U1301A (2575)	2010	28-Jun-10	CTDNiskin (n.a.)	biorep_13	n.a.	5.00	no	84.2
SW13	seawater	near U1301A (2575)	2010	28-Jun-10	CTDNiskin (n.a.)	biorep_13	n.a.	5.00	no	84.2
SW14	seawater	near U1301A (~2655)	2010	29-Jun-10	JasonNiskin (n.a.)	n.a.	n.a.	4.91	yes[a]	88.6[a]
SW15	seawater	near U1301A (~2655)	2010	30-Jun-10	JasonNiskin (n.a.)	n.a.	n.a.	4.90	no	88.6[a]
SW16	seawater	near U1301A (2661)	2011	04-Jul-11	JasonNiskin (n.a.)	n.a.	n.a.	5.22	low	120
SW17	seawater	near U1301A (2661)	2011	07-Jul-11	CTDNiskin (n.a.)	biorep_16	n.a.	3.90	no	120
SW18	seawater	near U1301A (2661)	2011	07-Jul-11	CTDNiskin (n.a.)	biorep_16	n.a.	6.05	no	120
SW19	seawater	near U1301A (2500)	2011	07-Jul-11	CTDNiskin (n.a.)	biorep_17	n.a.	4.45	no	61.8
SW20	seawater	near U1301A (2500)	2011	07-Jul-11	CTDNiskin (n.a.)	biorep_17	n.a.	5.3	no	61.8
SD1	sediment	1363F (~32)	2010	03-Sep-10	HPC (n.a.)	n.a.	n.a.	n.a.	no	n.a.
SD2	sediment	1363B (~38)	2010	31-Aug-10	XCB (n.a.)	n.a.	n.a.	n.a.	low[b]	n.a.
SD3	sediment	1363F (~10)	2010	03-Sep-10	HPC (n.a.)	n.a.	n.a.	n.a.	no	n.a.
SD4	sediment	1363G (~2)	2010	04-Sep-10	HPC (n.a.)	n.a.	n.a.	n.a.	low[b]	n.a.
SD5	sediment	1363G (~18)	2010	04-Sep-10	HPC (n.a.)	n.a.	n.a.	n.a.	low[b]	n.a.
SD6	sediment	1363B (~2)	2010	31-Aug-10	HPC (n.a.)	n.a.	n.a.	n.a.	low[b]	n.a.
SD7	sediment	1363D (~225)	2010	02-Sep-10	XCB (n.a.)	n.a.	n.a.	n.a.	no	n.a.
SD8	sediment	1363B (~45)	2010	31-Aug-10	XCB (n.a.)	n.a.	n.a.	n.a.	no	n.a.
SD9	sediment	1363B (~1)	2010	31-Aug-10	HPC (n.a.)	n.a.	n.a.	n.a.	low[b]	n.a.
SD10	sediment	1363F (~25)	2010	03-Sep-10	HPC (n.a.)	n.a.	n.a.	n.a.	no	n.a.

SD11	sediment	1363D (~205)	2010	02-Sep-10	XCB (n.a.)	n.a.	n.a.	n.a.	no	n.a.
C1	control	Shipboard distilled water	2009	25-Aug-09	n.a.	n.a.	n.a.	1.87	no	n.a.
C2	control	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	low[b]	n.a.

[a]Jungbluth *et al.*, 2013a

[b]Jungbluth *et al.*, 2013b

[c]Jungbluth *et al.*, 2014

Table 5.2 Summary of basement and seawater fluid biogeochemistry

Sequence Sample IDs	*Location*	*pH*	*Ca^{2+} (mM)*	*Mg^{2+} (mM)*	*K^{+} (mM)*	*Na^{+} (mM)*	*Cl^{-} (mM)*	*Br^{-} (mM)*	*Si (μM)*	*NH_4^{+} (μM)*	*PO_4^{2-} (μM)*	*NO_2^{-} (μM)*	*NO_3^{-} (μM)*	*SO_4^{2-} (mM)*	*Fe_{aq} (μM)*	*Mn^{2+} (μM)*	*Dissolved H_2S*	*DOC (μM)*	*TDN (μM)*	*Alkalin (meq/*
SSF1, SSF2	U1301A	7.4	54.1	2.0	6.0	462.0	547	0.83	1150	102	0.10	0	0.61	17.2	1.1	4.1	0.17	13	102	0.42
SSF3	U1301A	7.6	54.3	2.7	7.0	470.8	558	0.88	1154	102	0.10	0	0.90	18.2	0.7	<1	<0.1	9	104	0.48
SSF4	U1301A	7.4	54.0	2.7	6.4	469.6	552	0.88	1153	102	0.10	0	0.90	18.0	0.5	<1	<0.1	15	102	0.48
SSF5	U1301A	7.5	49.6	5.6	6.2	450.6	530	0.84	1051	91	0.22	0	2.69	17.9	0.5	<1	<0.1	26	102	0.57
SSF6	U1301A	7.4	53.2	3.7	6.4	468.8	553	0.89	1126	99	0.20	0	1.76	18.3	0.5	1.5	<0.1	14	100	0.53
SSF7	U1301A	7.5	51.9	6.9	6.6	468.1	554	0.88	259	92	0.43	0.06	3.18	19.0	0.2	4.1	<0.1	12	103	0.65
SSF8	U1301A	7.5	51.9	5.7	6.5	463.6	546	0.88	358	95	0.48	0.06	2.91	18.5	0.3	3.6	<0.1	16	100	0.62
SSF9	1025C	7.9	30.4	28.9	9.4	468.7	539	0.85	590	43	0.05	0	6.4	26.2	0.1	<1	<0.1	22	49	0.88
SSF10	U1301A	7.3	51.9	4.0	6.3	474.8	566	0.91	1124	100	0.15	0	2.1	18.8	1.0	1.9	<0.1	12	99	0.55
SSF11	U1301A	7.3	51.9	3.9	6.0	469.1	557	0.89	1128	99	0.16	0	2.0	18.4	0.5	1.4	<0.1	10	101	0.56
SSF15	U1301A	7.4	55.3	1.9	6.4	459.0	551	0.91	1149	98	0.09	0	0.08	18.1	0.8	N.D.	N.D.	13	104	0.43
SSF16	U1362B	7.3	53.3	4.0	6.6	462.4	548	0.84	1093	95	0.06	0	2.0	19.1	1.4	N.D.	N.D.	11	100	0.60
SSF17	U1362B	7.3	55.4	2.2	6.4	462.6	549	0.88	1144	100	0.06	0	0.03	18.8	1.7	N.D.	N.D.	12	105	0.51
SSF18	U1362B	7.3	55.4	2.5	6.4	462.4	547	0.85	1144	100	0.06	0	0.03	18.6	1.3	N.D.	N.D.	12	104	0.48
SSF19	U1362A	7.5	53.7	2.2	6.5	460.4	548	0.87	1071	98	0.12	0	0.03	18.8	2.4	N.D.	N.D.	15	102	0.59
SSF20	U1362A	7.5	54.3	2.8	6.5	461.4	544	0.81	1067	98	0.11	0	0.03	18.7	1.9	N.D.	N.D.	16	103	0.60
LQSSF3, LQSSF4	U1301A	7.6	15.4	47.4	9.8	455.6	537	0.78	284	15.7	2.53	<0.05	35.4	26.9	<0.1	N.D.	N.D.	N.D.	45	2.27
LQSSF5, LQSSF6	1026B	7.7	10.2	54.6	9.98	461.7	538	0.78	177	0.10	2.74	<0.05	41.4	27.8	0.3	N.D.	N.D.	40	43	2.39
LQSSF7, LQSSF8	1301B	7.6	10.5	52.4	10.1	463.8	539	0.79	181	0.10	2.85	<0.05	41.4	27.8	<0.1	N.D.	N.D.	39	42	2.38
LQSSF9, LQSSF10, LQSSF11	U1301A	7.8	13.0	49.9	9.9	466.2	542	0.83	235	6.2	2.69	<0.05	39.0	27.3	<0.1	N.D.	N.D.	37	45	2.30
SW1, SW2	above U1301A seawater	7.7	10.4	53.4	10.0	445.3	517	0.76	162	<0.05	2.93	<0.05	40.7	27.5	<0.1	N.D.	N.D.	N.D.	40	2.56
SW3, SW4, SW5	above U1301A seawater	7.8	10.4	53.6	9.9	468.2	543	0.78	183	<0.05	2.95	<0.05	41.3	28.0	<0.1	N.D.	N.D.	40	43	2.42
SW6, SW7, SW8	above 1025C seawater	7.9	10.2	51.1	10.2	459.9	531	0.84	104	<0.05	2.14	<0.05	27.3	27.5	N.D.	0.7	N.D.	48	32	2.39
SW9, SW10, SW11	above U1301A seawater	7.8	10.5	53.3	10.5	473.6	545	0.84	174	0	2.80	<0.05	40.3	28.2	<0.1	0	0	40	44	2.46

SW12, SW13	above U1301A seawater	7.8	10.6	53.9	10.4	471.6	550	0.89	166	<0.05	2.80	<0.05	41.0	28.6	<0.1	N.D.	N.D.	40	44	2.47
SW19, SW20	above U1301A seawater	N.D.	10.6	52.8	N.D.	N.D.	541	0.86	177	<0.05	N.A.	<0.05	42.8	28.1	<0.1	N.D.	N.D.	42	44	N.A.
SW17, SW18	above U1301A seawater	N.D.	10.4	53.0	N.D.	N.D.	541	0.86	188	<0.05	N.A.	<0.05	42.4	28.1	<0.1	N.D.	N.D.	52	45	N.A.

Table 5.3 Illumina read statistics

SampleID	*Non-Paired Quality Filtering*		*Read Pairing and Quality Filtering*[a]						
	Qiime default: forward (515F) read	*Qiime default: reverse (806R) read*	*USEARCH*[c]			*FLASH*	*Pandaseq*	*Eren merge-illumina-pairs*	
			e=5.0	*e=1.0*	*e=0.05*			*m/o=0.25, o=48*	*m/o=0.25, o=5*
Total Reads (all read lengths)	1734012 (100%)	1513711 (87.3%)	912101 (52.6%)	664404 (38.3%)	35152 (2.0%)	767046 (44.2%)	194913 (11.2%)	965443 (55.7%)	56545 (3.2%)
Mode Read Length (bp)	~150	~150	~250	~250	~250	~250	~250	~250	~290
Total Paired-reads ≤254bp	NA	NA	849375 (49.0%)	609512 (35.2%)	35132 (2.0%)	758504 (43.7%)	193399 (11.2%)	965443 (55.7%)	17405 (1.0%)
UCHIME_denovo non-chimeras[i]	1723431 (99.4%)	1511248 (87.2%)	837419 (48.3%)	600094 (34.6%)	34270 (2.0%)	748440 (43.2%)	189437 (10.9%)	952375 (54.9%)	NP
UCHIME_ref non-chimeras (total reads)	1701960 (98.2%)	1490603 (86.0%)	804762 (46.4%)	577257 (33.3%)	33189 (1.9%)	720495 (41.6%)	181747 (10.5%)	917211 (52.9%)	NP
Average Reads per Sample	25029	21921	11835	8489	488	10596	2673	13488	NP
Min Reads per Sample	6108	5317	2832	1950	107	2584	711	3250	NP
Max Reads per Sample	43629	38261	19158	14083	864	17195	4575	23098	NP
Standard Deviation per Sample	9169	8080	4292	3165	196	3841	1020	4895	NP

[a]1,734,012 overlapping read pairs were used for pairing

Table 5.4 Illumina sequence clustering statistics

SampleID		*Non-Paired Quality Filtering*		*Read Pairing and Quality Filtering*[a]					
		Qiime default: forward (515F) read	*Qiime default: reverse (806R) read*	*USEARCH*[c] *e=5.0*	*USEARCH*[c] *e=1.0*	*USEARCH*[c] *e=0.05*	*FLASH*	*Pandaseq*	*Eren merge-illumina-pairs*
Total Reads (no. of unique sequences; percentage)		1701960 (335762; 19.7%)	1490603 (1394851; 93.6%)	804762 (283058; 35.2%)	577257 (180619; 31.3%)	33189 (13045; 39.3%)	720495 (280670; 39.0%)	181747 (74206; 40.8%)	917211 (303095; 33.0%)
Unique[a]		77055	21956	44893	33226	2336	40753	11721	51239
Uclustref_greengenes_v13.5 + de novo	97	15806	148819	9529	7389	1595	9528	4243	11693
Uclustref_greengenes_v13.5 + de novo	99	16390	149505	10451	8300	1798	10284	4790	10552
Uclustref_Silva_v111 + de novo	97	15871	150403	9727	7716	1709	9664	4468	11411
Uclustref_Silva_v111 + de novo	99	16869	150735	10723	8527	1826	10523	4977	10320
UPARSE[b]		21145	N.P.	11277	7336	1429	11571	4283	12463
mothur	97	23155	N.P.	10737	8043	1481	14610	5103	10923
mothur	99	50897	N.P.	23025	15963	1809	22344	6720	23001
Distribution-based clustering	0.01	37589	N.P.	N.P.	N.P.	1647	N.P.	N.P.	20879
Distribution-based clustering	0.03	18910	N.P.	N.P.	N.P.	1348	N.P.	N.P.	10539
Distribution-based clustering	0.05	15381	N.P.	N.P.	N.P.	1132	N.P.	N.P.	8167
Distribution-based clustering	0.1	12477	N.P.	N.P.	N.P.	877	N.P.	N.P.	5918

Table 5.5 Microbial community test statistics

Mantel correlation

	All samples		*Subsurface fluids*	
Variable	*r statistic*	*p-value*	*r statistic*	*p-value*
pH	0.412	0.001**	0.184	0.155
Calcium	0.548	0.001**	0.338	0.002**
Magnesium	0.543	0.001**	0.318	0.002**
Potassium	0.470	0.001**	0.246	0.063*
Sodium	-0.067	0.473	0.135	0.257
Chloride	0.154	0.129	0.073	0.540
Bromide	0.033	0.669	0.024	0.809
Silicate	0.559	0.001**	0.140	0.270
Ammonium	0.535	0.001**	0.314	0.004**
Phosphate	0.513	0.001**	0.118	0.372
Nitrite	0.275	0.008*	0.027	0.872
Nitrate	0.583	0.001**	0.436	0.001**
Sulfate	0.531	0.001**	0.311	0.006*
Dissolved Iron	0.552	0.001**	0.467	0.001**
DOC	0.577	0.001**	0.179	0.152
TDN	0.558	0.001**	0.290	0.024
Alkalinity	0.488	0.001**	0.335	0.003**

PERMDISP

	Sample type		
Sample type	*seawater*	*sediment*	*subsurface*
seawater	--	0.084*	0.001**
sediment	0.090*	--	0.064*
subsurface	<0.001***	0.071*	--

	Location				
Location	*U1301A*	*U1362A*	*U1362B*	*seawater*	*sediment*
U1301A	--	<0.001***	0.053*	0.038**	0.823
U1362A	<0.001***	--	0.321	0.164	0.004**
U1362B	0.061*	0.329	--	0.930	0.140
seawater	0.035**	0.160	0.932	--	0.074*
sediment	0.824	0.005**	0.152	0.090*	--

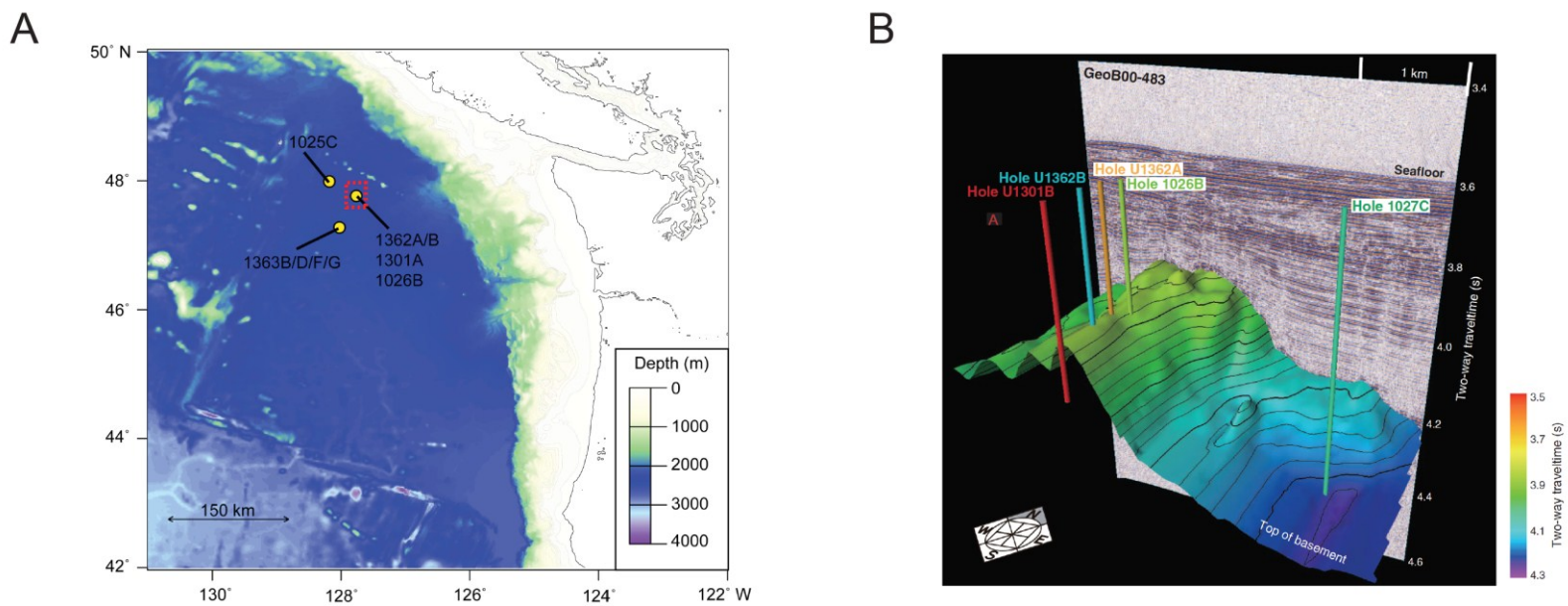

Figure 5.1 (A) Map of CORK observatory sampling sites on the Juan de Fuca Ridge flank, Pacific Ocean generated using marmap (Pante and Simon-Bouhet, 2013). Bathymetric details of boreholes located in 3.5 million-year crust are shown in higher detail within panel B. (B) Three-dimensional view of basement relief at CORK sites 1026B, U1301A, U1362A, and U1362B as shown in two-way travel time.

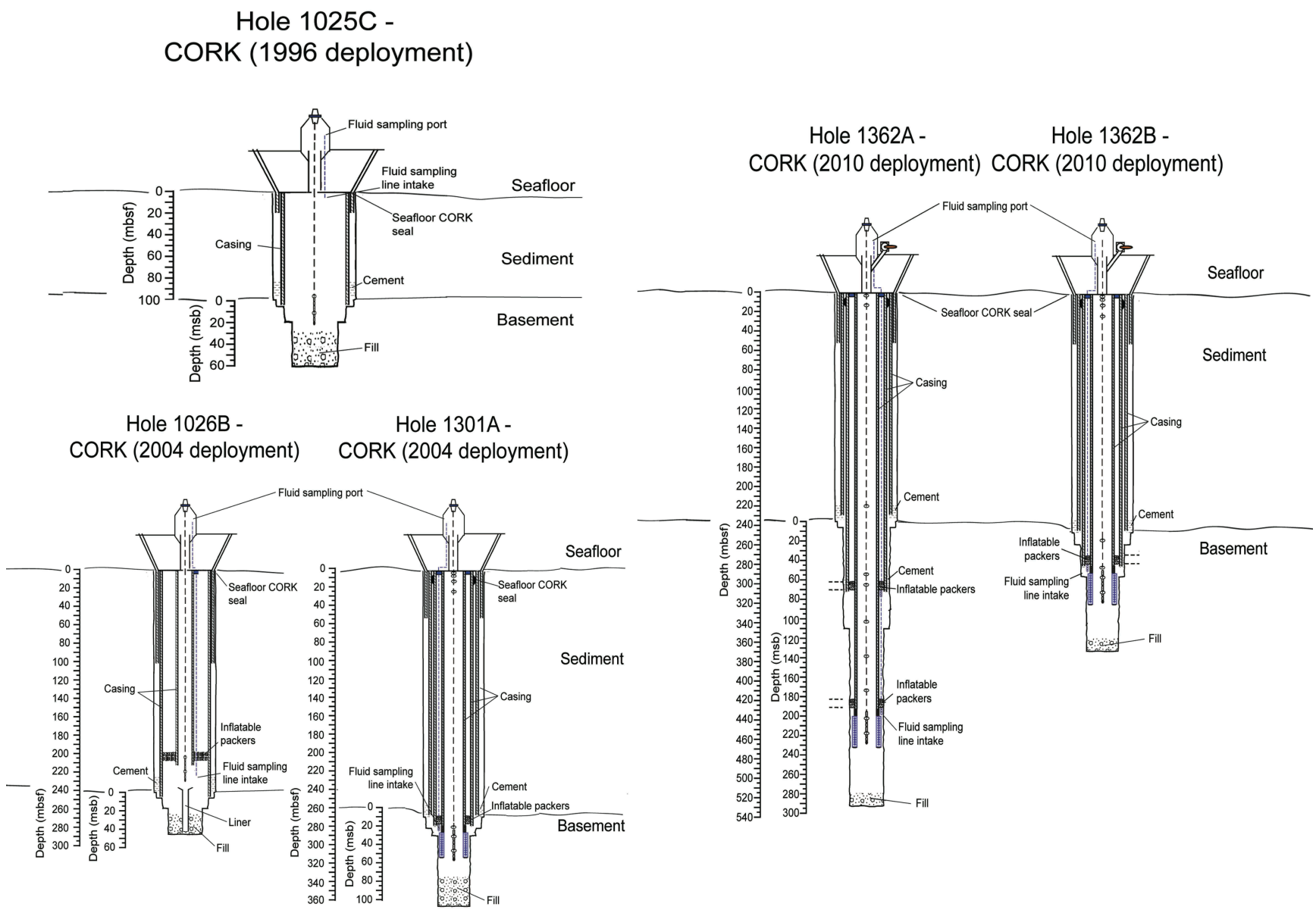

Figure 5.2 Multiple generations of CORK installations located within boreholes on the Juan de Fuca Ridge flank. Borehole 1025C contains a first generation CORK, boreholes 1026B and U1301A contain second-generation CORKs, and boreholes U1362A and U1362B contain third-generation CORKs. The fluid sampling line associated with each CORK is indicated with a purple color dashed lines. (Adapted and modified from Expedition 327 Scientists, 2011a).

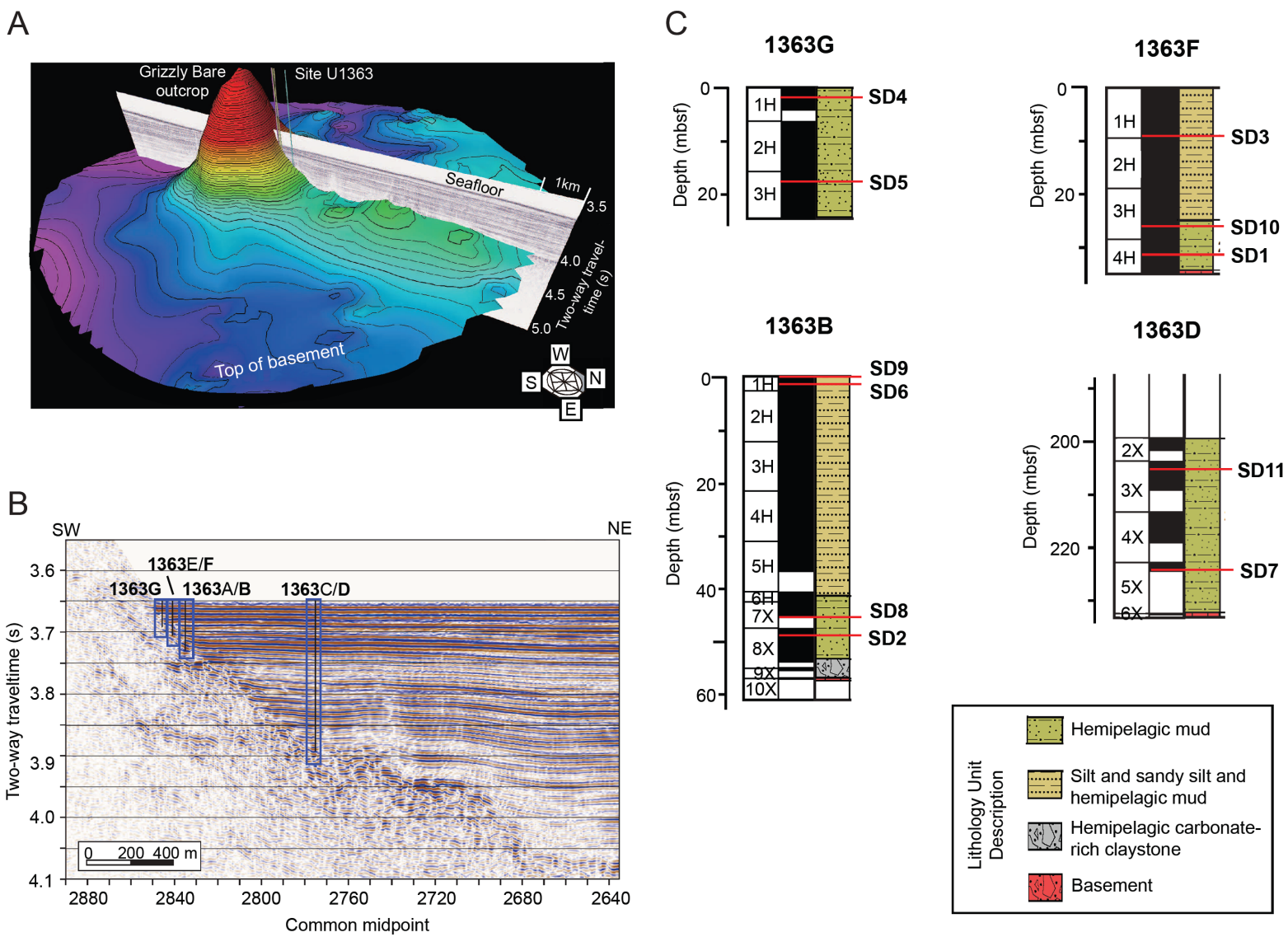

Figure 5.3 (A) Three-dimensional map of Grizzly Bare outcrop. (B) Cross-section of Grizzly Bare drilling locations. (C) Lithostratigraphy of Hole U1363B/D/F/G. Core recovery: black = recovered, white=not recovered. Red lines indicate locations of samples within the sediment column (modified from Expedition 327 Scientists, 2011b)

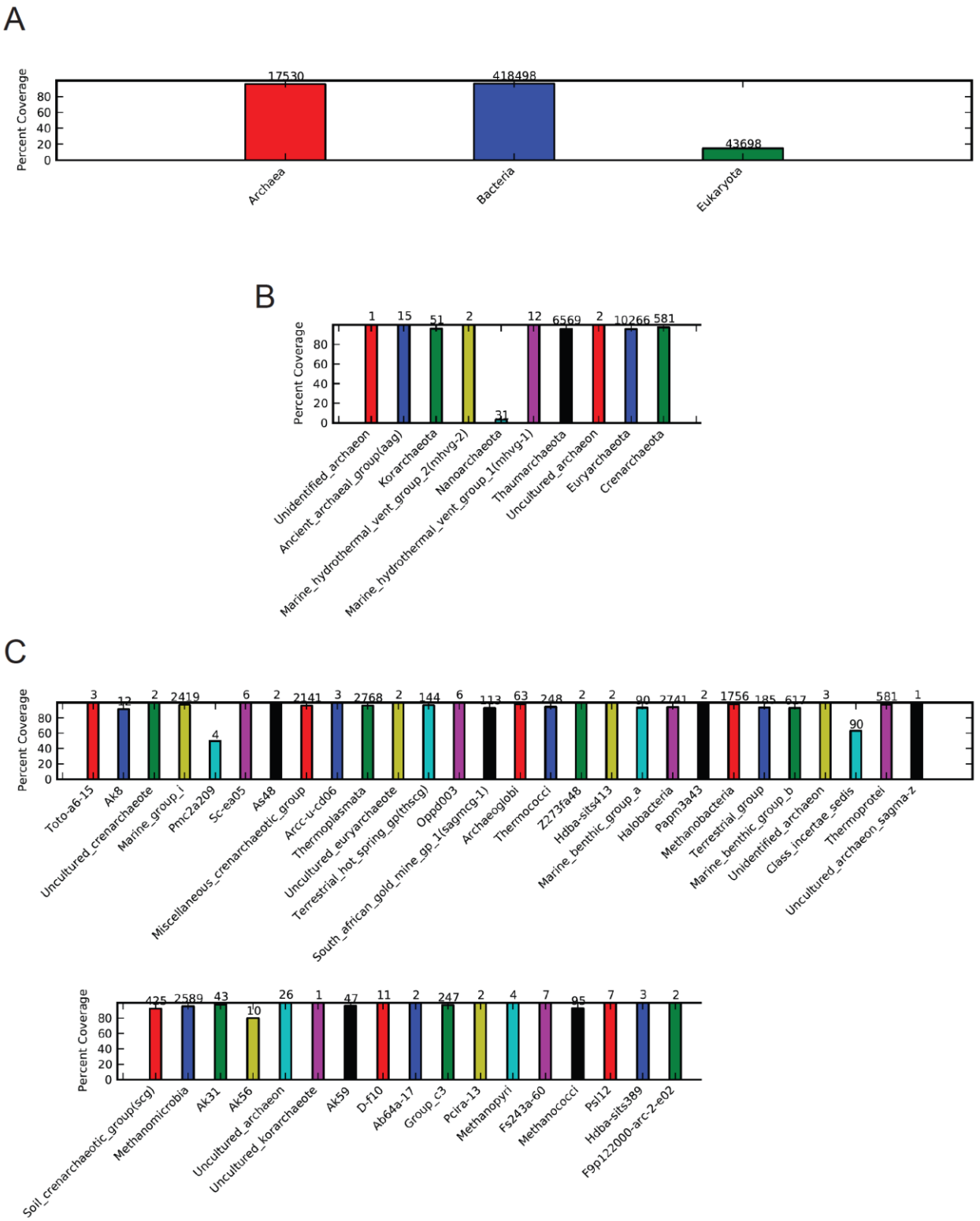

Figure 5.4 Assessment of primer 515F/806R coverage for (A) domain level, (B) phylum level of the *Archaea* domain, and (C) class level of the *Archaea* domain.

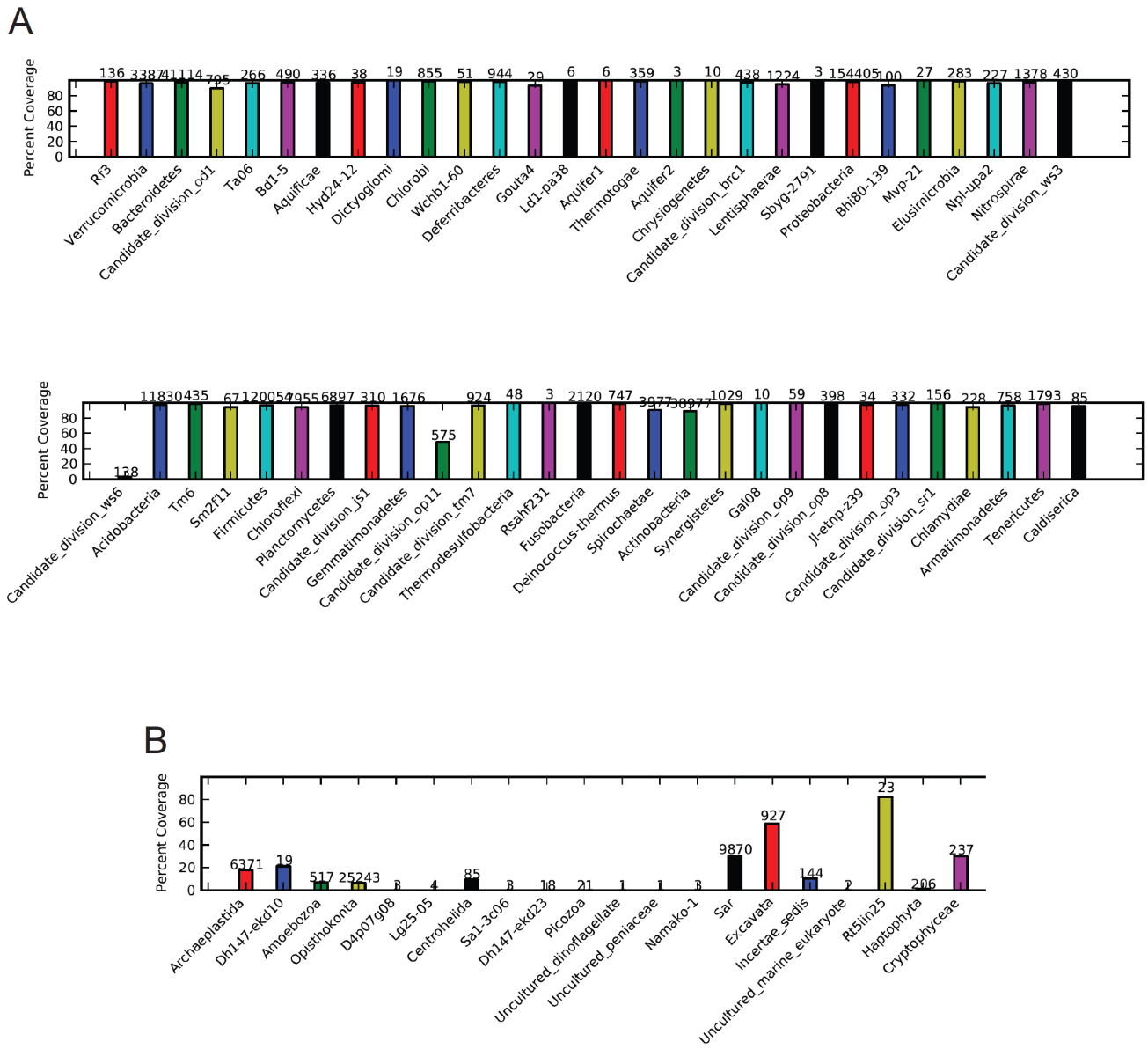

Figure 5.5 Assessment of primer 515F/806R coverage for (A) phylum level of the *Bacteria* domain, and (B) phylum level of the *Eukarya* domain.

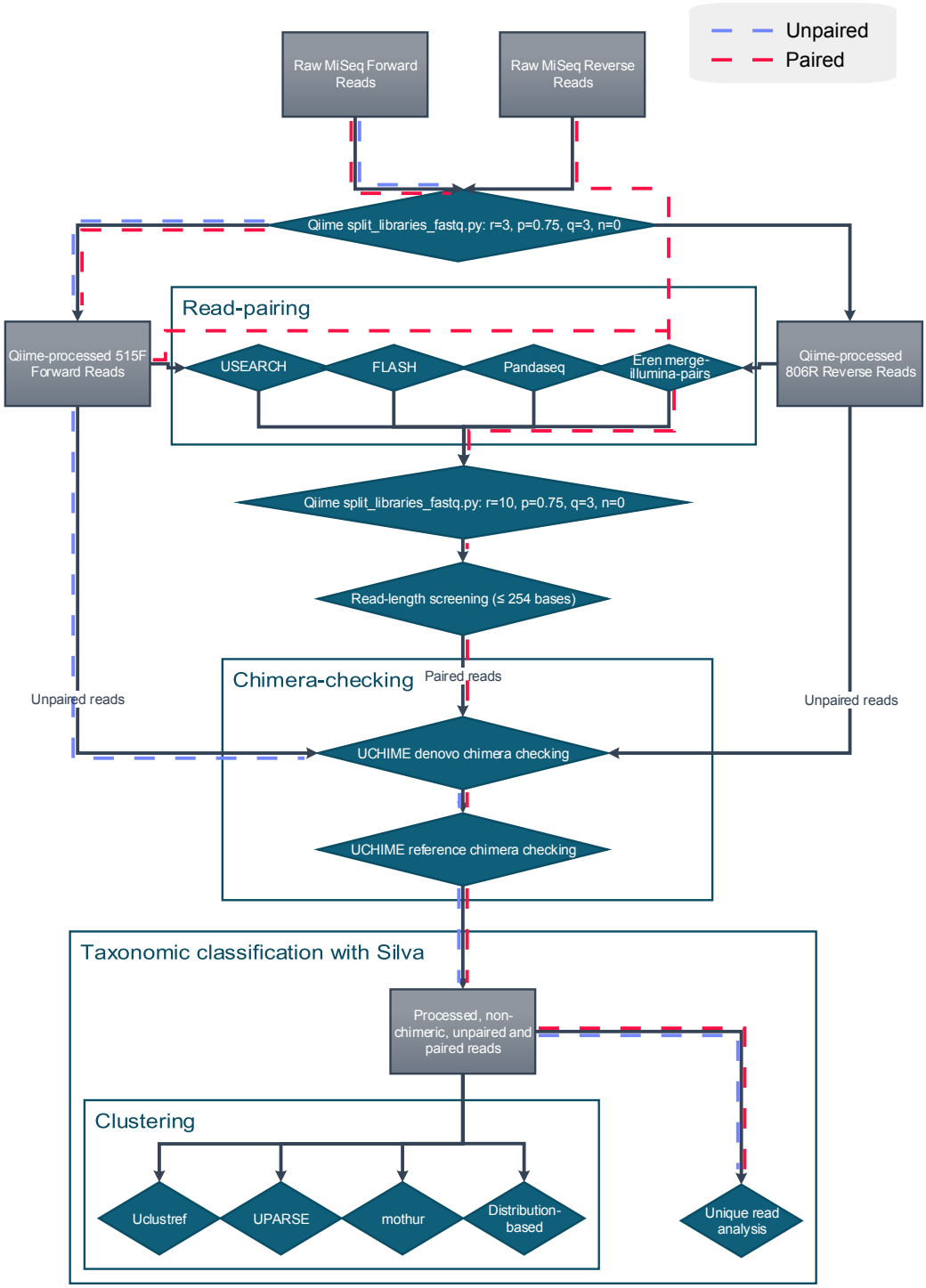

Figure 5.6 Bioinformatic workflow used to process SSU rRNA Illumina sequencing reads. Dashed lines highlight the schemes used to process unpaired and paired reads that were used for primary (non-experimental) alpha- and beta-diversity analyses.

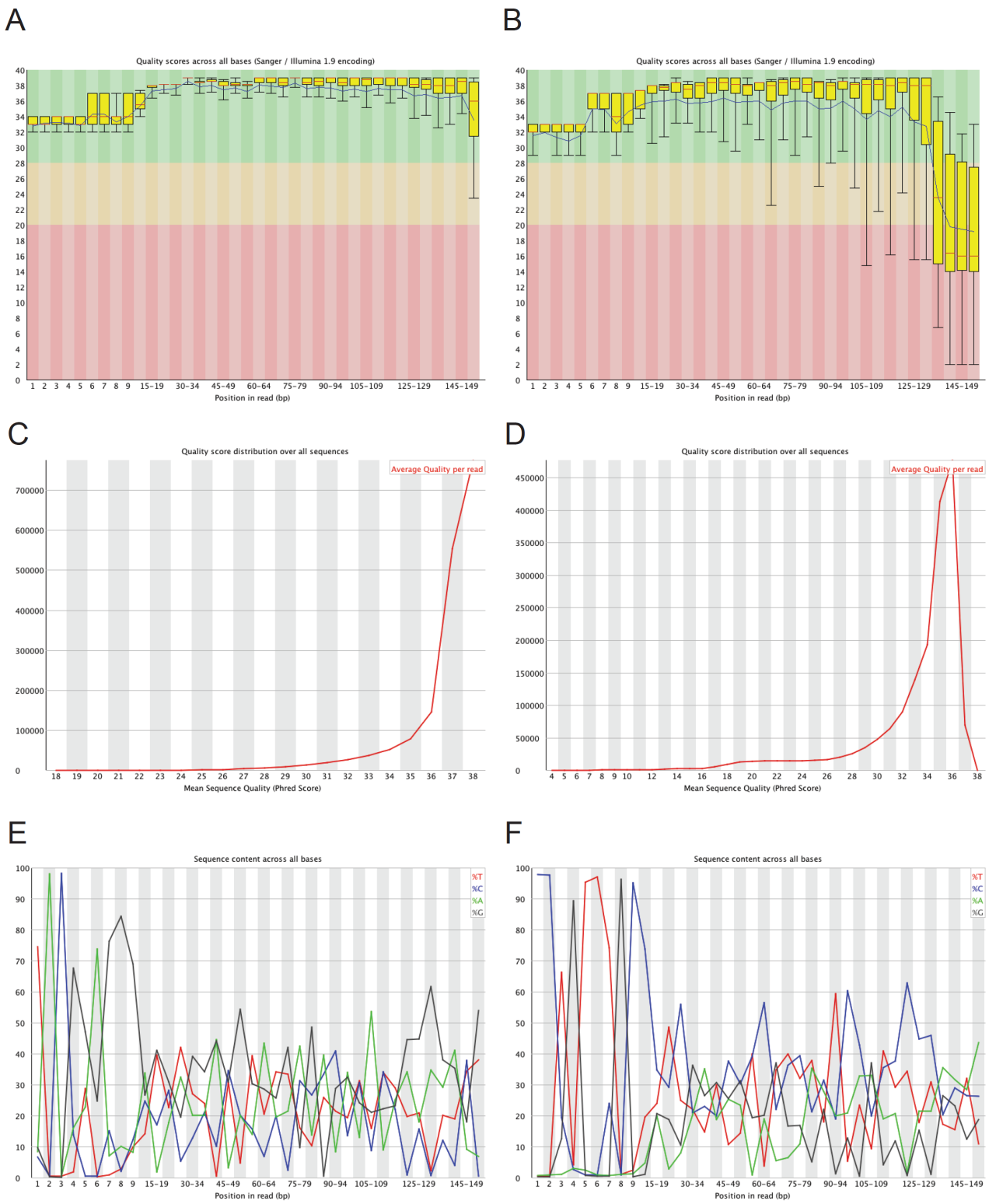

Figure 5.7 (A) Quality scores of Illumina reads across all bases for (A) forward reads and (B) reverse reads. Quality distribution over all samples for (C) forward reads and (D) reverse reads. Base pair distribution across all bases for (E) forward reads and (F) reverse reads

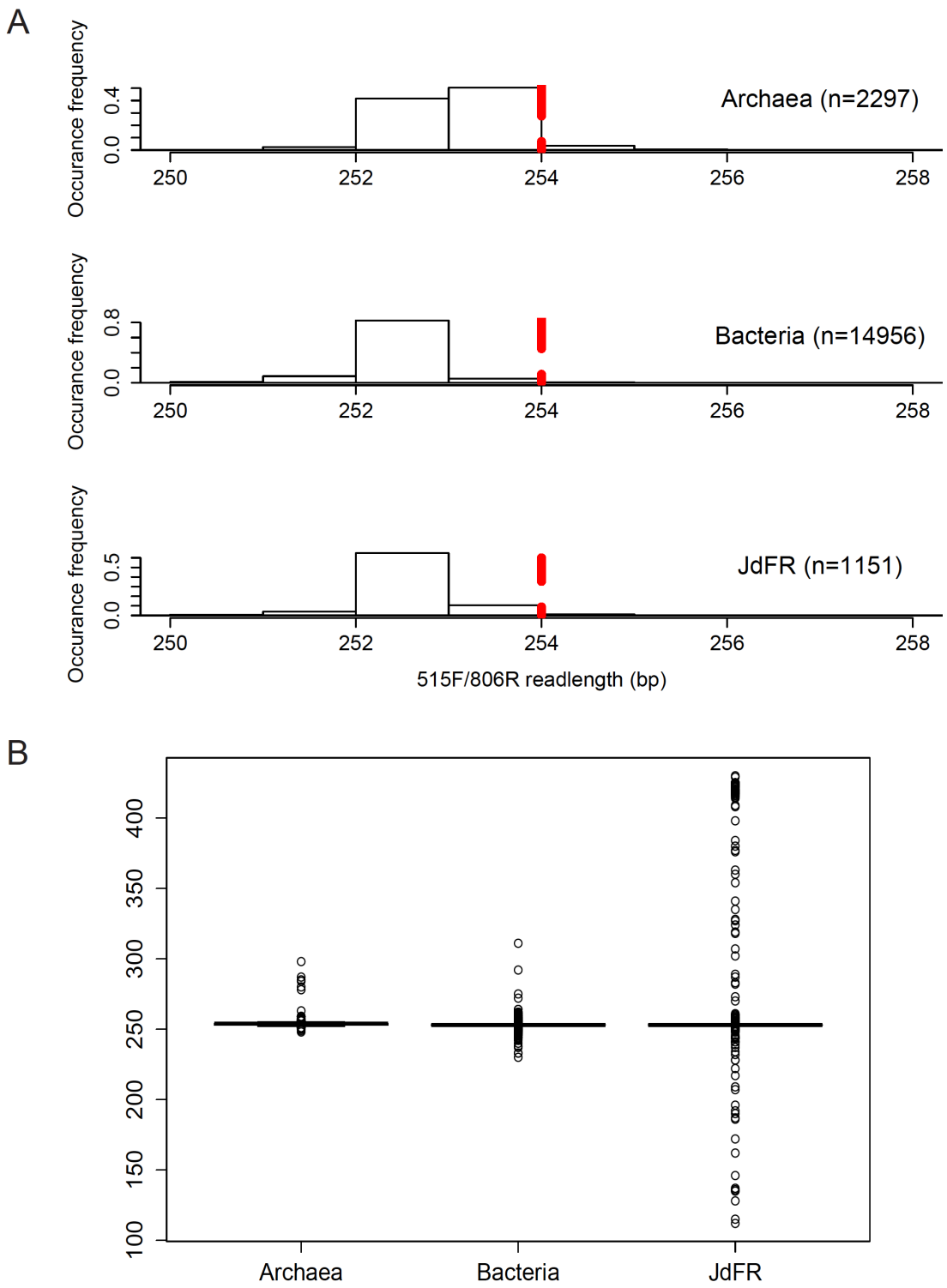

Figure 5.8 (A) Histogram and (B) boxplot diagram of read-length distribution for sequences used in the database during chimera checking. Length cutoff defined for this database was 254 base pairs and is indicated with the red dashed line in panel (A)

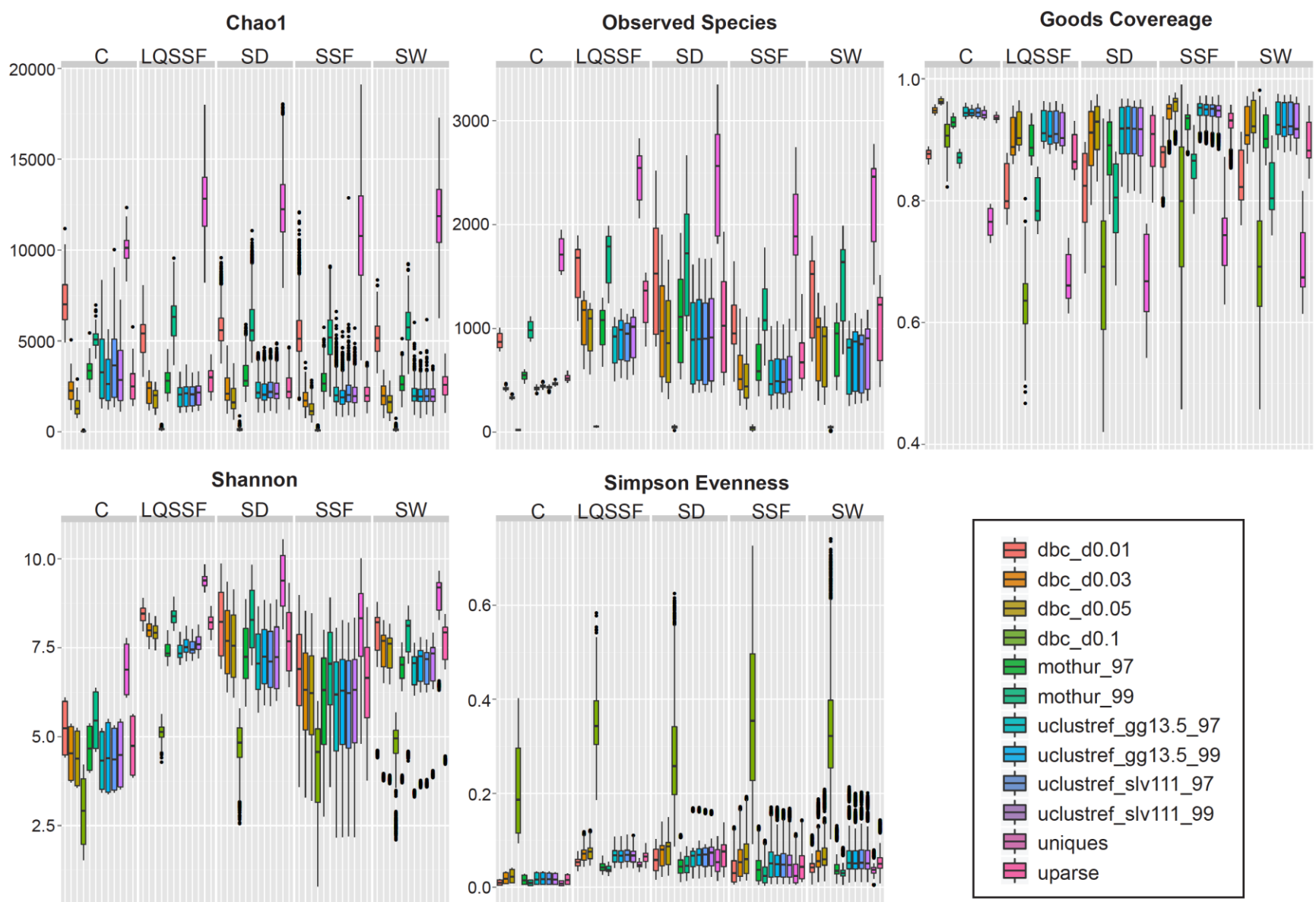

Figure 5.9 Box-plot diagram representing 1000 replications of Alpha-diversity metrics: Chao1, Observed Speices, Good Coverage, Shannon diversity, and Simpson Evenness. Colors of the bars correspond to the different clustering methods or unique read analysis performed and percentages listed in the legend refer to the OTU similarity cutoff used. DBC, distribution-based clustering; gg, greengenes; slv, silva; C, controls; LQSSF, low-quality subsurface fluids; SD, sediment; SSF, subsurface fluids; SW, seawater.

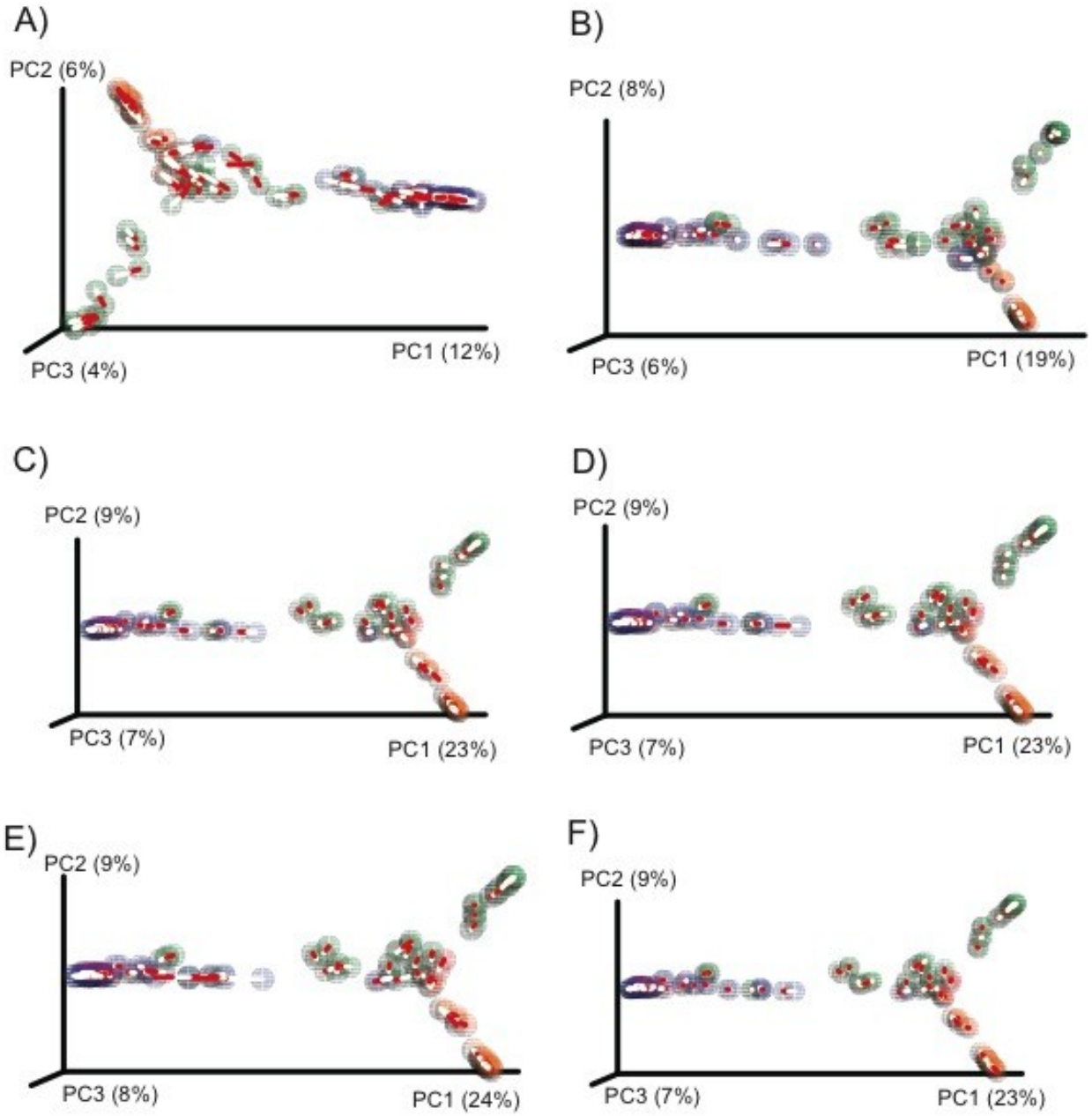

Figure 5.10 For brevity, "uniques" corresponds to reads resulting from Unique read analysis. Procrustes PCoA biplot of forward uniques and (A) reverse uniques, (B) paired uniques [merge-paired-reads; Eren et al. 2013], (C) forward read clusters [UCLUSTref with SILVA 99% OTU database], (D) forward read clusters [UPARSE], (E) forward read clusters [mothur; 97% OTUs], (F) forward read clusters [distribution-based clustering; d=0.1]. Green, subsurface fluids; yellow/brown, sediments; purple/blue, seawater and low-quality subsurface fluids.

CHAPTER 6

High-resolution temporal analysis of deep subseafloor microbial communities from the Juan de Fuca Ridge flank

By Sean Jungbluth

Huei-Ting Lin, Chih-Chiang Hsieh, and Michael S Rappé (anticipated co-authors)

6.1 Abstract

Illumina sequencing technology was used to generate ~1.6 million suitable small subunit ribosomal RNA (SSU rRNA) reads from sediment, seawater, and subseafloor fluids collected from the Juan de Fuca eastern flank in 2013. Borehole U1362A and U1362B observatories, featuring fluid delivery lines composed of stainless steel or PTFE, were sampled here and reveal the potential for differences in the observed microbial communities based on the material types used. Sampling from Hole U1362A was performed at depth horizons extending to 30, 70, and ~200 meters sub-basement and reveal differences in the observed microbial communities, which indicates depth-specific zonation of microorganisms in the basaltic basement fluids. Archaeal groups (e.g. THSCG, MCG, SAGMEG, MBGE, *Methanococcaceae*, *Archaeoglobus*) and bacterial groups (e.g. *Nitrospiraceae*, OP8, KB1, *Desulfobulbus*, and *Desulfohalobiaceae*) detected in previous years of basement fluid sampling on the Juan de Fuca Ridge flank were found here. Three short-term time-series sample collection events from the biolines associated with borehole U1362A and U1362B reveal the potential for hourly changes in borehole fluid microbial communities. Direct cell enumeration of samples collected from U1362A and U1362B revealed an elevated biomass compared to samples at these locations from previous years and indicate a range of microbial cell abundances up to ~45,000 cell x ml^{-1} can be expected in high-quality borehole fluid samples.

6.2 Introduction

Adequate characterization of the subseafloor biosphere inhabiting aging crustal fluids has remained a challenge to the Ocean Drilling Program and Integrated Ocean Drilling Program for over thirty years because of environmental heterogeneity and the difficulty of retrieving pristine samples uncontaminated with seawater or sediments (Biddle *et al.*, 2014). The reliable access to high-integrity crustal fluids is only very recently (year 2011) established (Expedition 327 Scientists, 2011a) and is enabled using custom-built subseafloor sampling systems and instrumented borehole observatories featuring biofouling-resistant materials (Wheat *et al.*, 2011). Improvements in the quality of samples harvested for microbiological purposes have helped to constrain estimates of microbial cell abundances in the basement environment (Cowen *et al.*, 2012), and allowed descriptions of novel microbial community diversity and structure (Jungbluth *et al.*, 2013a).

Juan de Fuca Ridge borehole observatories permit access to crustal fluids at different alteration states, ranging from aerobic to anaerobic, within the basaltic crust and represent windows into the wider spatial diversity of crustal fluids worldwide (Jungbluth *et al.*, 2014). Reliable access to high-intergrity fluid samples, at this moment, is however restricted to the two newest locations to be drilled in 3.5 mya ocean crust – borehole observatories U1362A and

U1362B. Borehole observatories U1362A and U1362B are the deepest penetrating of all the Juan de Fuca locations fitted with CORK observatories and feature a combination of stainless steel and polytetrafluoroethylene fluid delivery lines (Expedition 327 Scientists, 2011b). These latest borehole observatory features enable a much-needed comparison of materials used in fluid delivery line construction and provide the first ever assessments of the microbiology within fluids collected from multiple isolated depth horizons at a single location.

Previous studies relying on the vehicle-assisted collection of whole water samples have revealed temporal changes in microbial communities collected annually from a single borehole (Jungbluth *et al.*, 2013a) and prelimary – statistically weak - evidence for changes occurring during single, hour-long, sampling events. Autonomous fluid sampling instruments, called GeoMICROBE sleds (Cowen *et al.*, 2012), provide a more versatile option that vastly expands sampling capabilities and allows for targeted, time-resolved collection of fluids for timescales close to that of the expected tidal cycle (Figure 6.1). In combination with the newest boreholes providing demonstrably reliable access to pristine samples of basement fluids, the autonomous fluid sampling packages permit investigations into the fine-scale (i.e hourly) temporal changes within basement fluid microorganisms. *In situ* filtrations targeting the microbial-sized fraction within basement fluids performed in serial with episodic collection of small volumes of fluids helps to ensure high chemical integrity and enables the retrieval of relatively large amounts of biomass at multiple time points. Taken together, this sampling method is a promising route that can be used to investigate the temporal variation of the deep biosphere on an sub-hourly time scale.

In this study, samples were collected in July 2013 from Juan de Fuca borehole observatories U1362A, U1362B, and U1301A, all located in 3.5 mya ocean crust. Small subunit RNA (SSU rRNA) Illumina tag sequencing and epifluorescence microscopy were then used to provide insight into the phylogenetic diversity and biomass variation in the deep subseafloor biosphere. Three sampling time series lasting ~8 hours were conducted at holes U1362A and U1362B with samples being collected from multiple fluid delivery line types (i.e. stainless steel or PTFE) and depth horizons. In total, 138 samples of crustal fluids, seawater, and sediments were analyzed in this study, representing the most comprehensive sample suite collected in a single year from disparate deep subseafloor environments (i.e. basement vs. sediment vs. deep seawater). In contrast to sediments collected in previous years, which were located ~50 km away from the borehole observatories, sediment cores were collected here at various distances from a borehole observatory and provide, perhaps, a more accurate view of the sediment microbial diversity proximal to the location of crustal fluid sampling. This dataset can be compared to previous SSU rRNA Illumina tag sequencing efforts to provide a comprehensive characterization of the microorganisms inhabiting ridge flank crustal fluids.

6.3 Materials and Methods

Sample collection and preparation

During R/V Atlantis cruises ATL26_03 (13 July 2013 – 26 July 2013), samples of basement crustal fluids were collected from CORK observatories located east of the Juan de Fuca spreading center (Figure 5.1). Borehole observatories visited (U1301A, U1362A, U1362B) are located within 1-2 km of each other in warmer (~65°C) 3.5 million years old ocean crust (Figure 5.1). The borehole observatories penetrate more than 230 meters of sediment and another 100-280 meters of basement rock (Figure 5.2). Locations U1362A (47°45.6628'N, 127°45.6720'W) and U1362B (47°45.4997'N, 127°45.7312'W) were the primary targets for this investigation and are fitted with lateral CORKs (L-CORKs) equipped with both stainless steel and Teflon-lined microbiological sampling ports (Expedition 327 Scientists, 2011b). Whole water fluid samples were collected using previously described sampling equipment (Lin *et al.*, 2012; Jungbluth *et al.*, 2013a) from all combinations of fluid delivery line types and depth horizons that are available for sampling: (1) U1362A deep horizon stainless steel line, (2) U1362A deep horizon bioline, (3) U1362A shallow horizon stainless steel line, (4) U1362B stainless steel line, and (5) U1362B bioline. Fluids were also sampled from the biolines attached to both U1362A and U1362B using the GeoMICROBE fluid sampling systems, which is described elsewhere (Cowen *et al.*, 2012). In addition, a large-volume fluid sample was collected from a CORK-II borehole observatory located at Hole U1301A (47°45.209'N, 127°45.833'W) using previously described sampling equipment (Lin *et al.*, 2012; Jungbluth *et al.*, 2013a). Borehole fluids were typically allowed to flush for an amount of time require for expulsion of at least 3x the fluid delivery line volume. Following flushing, fluids were obtained in acid-washed, custom-fabricated 15 l Tedlar and foil bags (Medium Volume Bag Samples, MVBS) or 60 l Tedlar bags (Large Volume Water Samples, LVWS) (Table 6.1). The bags were housed in rigid boxes during deployment and recovery for protection. *In situ* filtrations were also performed to maximize biomass collection using the GeoMICROBE sled (Cowen *et al.*, 2012).

Seawater samples were collected in the vicinity of the borehole locations in order to provide background controls for the borehole fluid samples (Table 6.1). Seawater was collected mostly using a Niskin rosette from within the nepheloid layer (5-10 meters above seafloor), above the nepheloid layer (~140 meters above seafloor), and at a intermediate depth (~60 meters above seafloor). In addition, a 5 l Niskin bottle fitted to the ROV *Jason II* was used to collect seawater from a depth of approximately 2650 m in the vicinity of CORK U1362B. Sediment samples were collected using push cores from nearby Hole 1301B from a distances of ~5, ~15, and ~55 meters away from the center of the CORK observatory and span the full visible range of drilling influence.

GeoMICROBE-collected samples were filtered in parallel through four 0.22 µm-pore sized Sterivex-GP filter cartridges (Millipore Corporation, Billerica, MA) and stored in 2 ml of DNA lysis buffer [20 mM Tris-HCl, 2 mM EDTA, 1.2% Triton X-100, 2% lysozyme (w/v), pH 8]. Fluid samples retrieved were filtered through 0.22 µm-pore sized Sterivex-GP filter cartridges (Millipore Corporation, Billerica, MA) and stored in DNA lysis buffer. Sediment core samples were decanted of overlying seawater, sectioned (top/middle/bottom) according to visibly distinct horizons over the ~6-8 inch core length, and stored without the addition of DNA lysis buffer at -80°C in sterile Whirlpak bags (Nasco, Fort Atkinson, Wisconsin, USA). Some cores were fully fluidized and sectioning could not be performed. Fluid samples for microscopy were collected and preserved as described previously (Jungbluth *et al.* 2013a). Briefly, 40-80 ml sub-samples were fixed with a final concentration of 3% formaldehyde (0.2 µm-filtered) for 2 to 4 hours at 4°C, and subsequently filtered through 0.2 µm pore-sized polycarbonate membranes (Whatman, Maidstone, United Kingdom). After air-drying, membranes were stored desiccated at -80°C onboard the ship and at -20°C in the laboratory for microscopic analysis. Sediment microscopy samples were not collected.

Flourescence microscopy

Fluorescence microscopy was performed as described in Jungbluth *et al.* 2013a with different equipment. Citifluor/VectaShield/PBS/DAPI stained filter sections were inspected with a Nikon Eclipse 90i (Tokyo, Japan) equipped with a 100x objective, filter sets appropriate for DAPI fluorescence, a camera Nikon DS-fi1 and Nikon NIS Element software version 3.22.11.

DNA extraction

All sediment samples were thawed to room temperature and environmental DNA was extracted from each sediment core or core subsample using the PowerSoil DNA isolation kit (MO BIO Laboratories, Carlsbad, CA, USA) following manufacturers protocols. Environmental DNA was also extracted from selected whole cores using equal portions of core subsections or full cores totaling 10 g and using the PowerMax Soil DNA isolation kit (MO BIO Laboratories) following the manufacturers protocols with slight modifications that are described in detail elsewhere (Jungbluth *et al.*, 2013b). Environmental DNA from filter membranes was extracted using the PowerSoil DNA isolation kit following the manufacturer's protocol.

SSU rRNA gene PCR amplification and Illumina sequencing

An Illumina sequencing approach that has been previously described (Caporaso *et al.* 2011; Caporaso *et al.*, 2012) was used to characterize samples of boreholes fluids, sediment,

and seawater. Briefly, this technique involves amplification of the V4 region of the small subunit ribosomal RNA gene using primers 515F and 806R specific to *Bacteria* and *Archaea* and which were modified to include the Illumina flowcell adapter sequences. Reverse primer 806R contained an additional 12-bp barcode, which was used to assign individual sequences to samples. Taxonomic coverage of primer pair 515/806 was assessed using PrimerProspector (Walters *et al.* 2011) and found to be nearly universal, as described previously (e.g. Bates *et al.* 2011; Walters *et al.* 2011) (Figures 5.4 and 5.5). Triplicate PCR reactions were pooled at equimolar concentrations and PCR cleanup was performed on the final pooled product using the UltraClean PCR clean up kit (MoBio Laboratories, Carlsbad, CA). Sequencing was performed on an Illumina MiSeq sequencer at the Hawaii Institute for Marine Biology.

Sequence read-processing and read-pairing

Read quality for forward and reverse reads was assessed using fastQC. Demultiplexing of sequence data was performed using the QIIME pipeline (Caporaso *et al.* 2010) split_libraries_fastq.py script (version 1.8) with a maximum of 1.5 errors in the barcode, and the following quality-filtering parameters adapted from Bokulich *et al.* (2013): r=3, p=0.75, q=3, and n=0. Forward reads (515F) were in higher abundance than reverse reads (806R) (Table 6.2) and generally higher quality (Figure 6.2). Read pairing was performed in QIIME (version 1.8) using join_paired_ends.py and SeqPrep with default parameters. Sequences were aligned and taxonomy was assigned via the SINA aligner (v1.2.11; Pruesse *et al.*, 2012) using the non-redundant SSURef_115 database pre-clustered with UCLUST at a 99% sequence similarity level available online. Parameters used with the SINA tool included –lca-fields tax_slv to assign Silva taxonomy and –search-min-sim 0.80 to expand searches for difficult-to-classify (i.e. divergent) sequences. Paired reads from negative PCR control reactions were dereplicated using USEARCH (Edgar, 2010; version 7.0.1090) and all sequences that represented greater than 250 identical reads were excluded from environmental sample libraries using QIIME script exclude_seqs_by_blast.py with parameters: –e 1e-10, -p 0.95, -m 3, –w 28. A relatively small number of groups identified in negative PCR controls were manually screened and discluded from the removal processes (e.g. groups OP8, Nitrospirae) due to an expected background of barcode sequencing errors. The full list of taxa identified in negative controls and either kept or removed from further analysis is listed in Table 6.3. A total of 1,596,710 reads were retained following filtering of 364,777 contaminating sequence reads. Clustering and removal of chimeric OTUs (Edgar *et al.*, 2011) was performed using UPARSE (Edgar, 2013) in the USEARCH software package with default parameters. Reads from all samples were mapped to OTUs USEARCH –usearch_global with parameters: -stand plus, and -id 0.97.

Community and Phylogenetic Analysis

Singleton OTUs were removed using QIIME script filter_otus_from_otu_table.py and samples with less than 2000 sequence reads were screened and removed using QIIME script filter_samples_from_otu_table.py. Sequences were aligned using pynast with the QIIME script align_seqs with default parameters and a phylogenetic tree was generated using fastree using QIIME script make_phylogeny.py with default parameters. Beta diversity analyses were performed after removing singleton OTUs and using the Bray-Curtis, abundance weighted Jaccard, and the weighted and unweighted unifrac distance indices, but patterns were highly similar across methods and so only results of the Bray-Curtis index are shown, unless otherwise stated. PCoA plots were visualized using EMPeror (Vázquez-Baeza *et al.*, 2013). Unweighted paired group mean average (UPGMA) alaysis was performed using QIIME script upgma_cluster.py using the unweighted unifrac distance index. Heat maps were generated using R package Phyloseq (McMurdie and Holmes, 2013).

6.4 Results and Discussion

Direct cell counts

After staining with the DNA-specific dye DAPI (4', 6-diamidino-2-phenylindole), microscopic examination of borehole U1362A, U1362B, and U1301A fluid samples revealed average microbial cell abundances that ranged from $5.38 \times 10^3 – 4.50 \times 10^4$ cells x ml^{-1} (Table 6.4). Cell concentrations detected in Hole U1362A ranged from $1.49 – 3.88 \times 10^4$ cells x ml^{-1} and no clear differences were observed between the different depth horizons or the type of fluid delivery line used during sample collection. Cell counts in the first U1362A fluid samples collected on 7/16 and 7/17 were elevated compared to the following three samples retrieved. Microbial cell abundances in U1362B revealed a different pattern; average cell abundances in samples collected early in the week were 4.18×10^4 cells x ml^{-1} and one week following dropped to 6.97×10^3 cells x ml^{-1} (Table 6.4). Cell abundances in the first U1362B fluid sample collected on 7/21 were 174% larger than the average of the other samples collected during the same sampling event. A single sample collected from borehole U1301A was characterized by a microbial cell concentration of 2.22×10^4 cells x ml^{-1}. Microbial cell concentrations in all fluid samples were less than those detected in bottom seawater and ranged from 4.5% to 38% of the average cellular concentration in seawater samples.

Table 6.4 Microbial cell abundances – year 2013

Date	*Location*	*Depth/Line*	*Sample*	*Cell Concentration (cells x ml^{-1})*	*Standard Deviation*
7/14/13	U1362B	BioLine	JdFR #03	45024	7132
7/14/13	U1362B	BioLine	JdFR #04	41597	4371
7/14/13	U1362B	BioLine	JdFR #05	44151	2236
7/14/13	U1362B	BioLine	JdFR #06	36423	2877
7/21/13	U1362B	BioLine	JdFR #27	10243	1762
7/21/13	U1362B	BioLine	JdFR #28	5376	1431
7/21/13	U1362B	BioLine	JdFR #29	6636	1946
7/21/13	U1362B	BioLine	JdFR #30	5600	1597
7/16/13	U1362A	Deep; BioLine	JdFR #09	38842	2609
7/16/13	U1362A	Deep; BioLine	JdFR #10	20440	2443
7/16/13	U1362A	Deep; BioLine	JdFR #11	17304	1929
7/16/13	U1362A	Deep; BioLine	JdFR #12	14924	1898
7/17/13	U1362A	Deep; Stainless	JdFR #15	34340	3359
7/17/13	U1362A	Deep; Stainless	JdFR #16	21924	2595
7/17/13	U1362A	Deep; Stainless	JdFR #17	22445	3046
7/17/13	U1362A	Deep; Stainless	JdFR #18	18215	2649
7/19/13	U1362A	Shallow; Stainless	JdFR #21	22638	3149
7/19/13	U1362A	Shallow; Stainless	JdFR #22	26467	4732
7/19/13	U1362A	Shallow; Stainless	JdFR #23	29960	6182
7/19/13	U1362A	Shallow; Stainless	JdFR #24	18155	3216
7/22/13	U1301A		JdFR #31	22159	3603
7/14/13	Nearby U1362A/B	~2648 m	seawater	139105	9586
7/19/13	Nearby U1362A/B	~2648 m	seawater	101594	7669
7/21/13	Nearby U1362A/B	~2648 m	seawater	172585	18148
7/23/13	Nearby U1362A/B	~2647 m	seawater	107659	6324
7/23/13	Nearby U1362A/B	~2589 m	seawater	107924	5131
7/23/13	Nearby U1362A/B	~2515 m	seawater	77953	6196
7/16/13	Nearby U1362A/B	~2648 m	seawater	131982	5261

Microbial community structure

Oligonucleotide PCR primers were used to amplify archaeal and bacterial SSU rRNA genes from all subsurface fluid samples, samples of nearby background seawater, and marine sediments collected from nearby borehole observatory U1301B. A total of ~7.4M SSU rRNA amplicons were sequenced in the forward and reverse directions resulting in 150-bp-long reads with an expected overlap of ~24 bases. Read-pairing was performed using SeqPrep and resulted in ~6.1M successfully paired reads. Contaminating paired reads detected in negative PCR

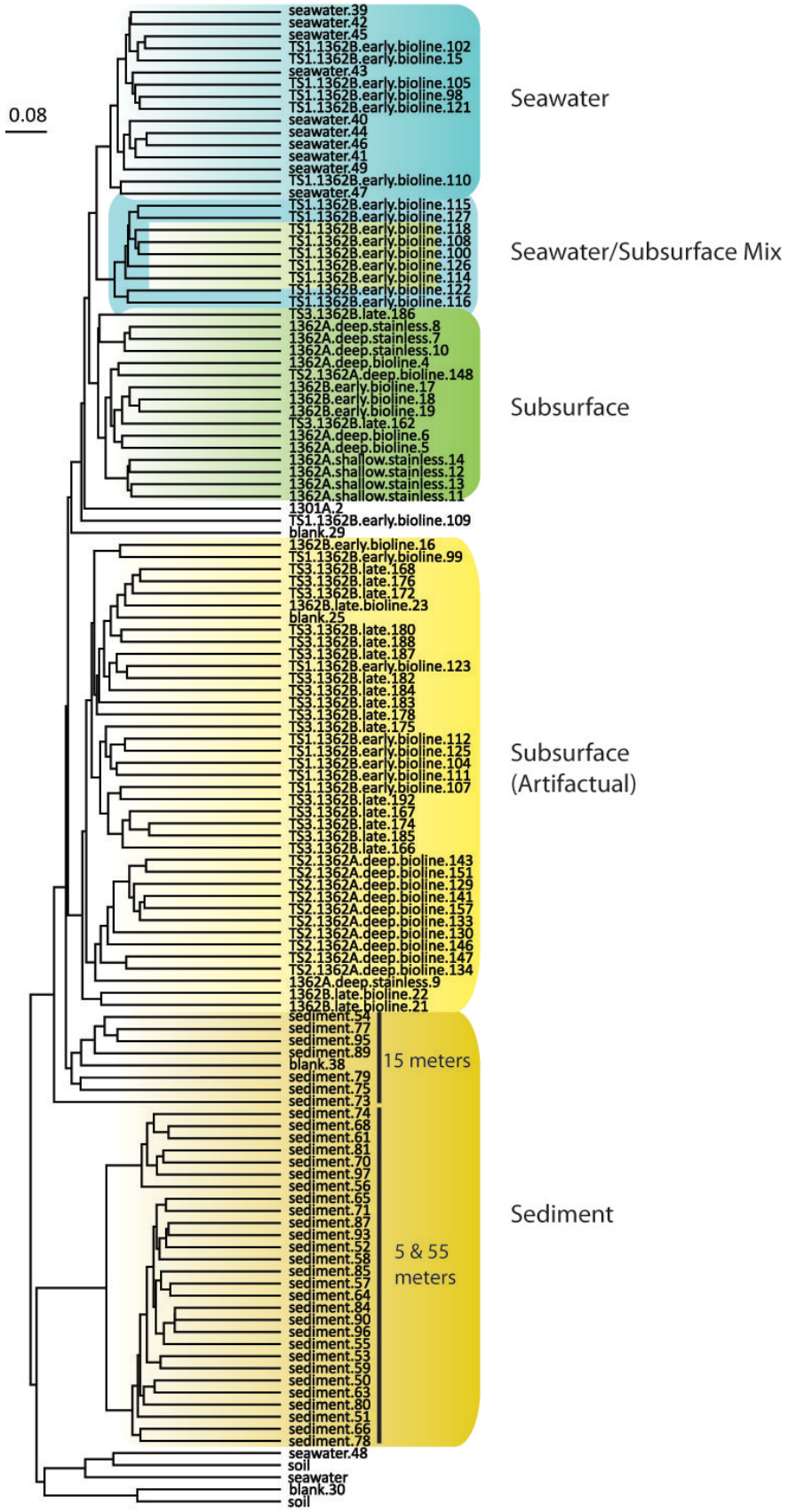

Figure 6.3 Unweighted pair group method with arithmetic mean (UPGMA) analysis of unweighted UniFrac dissimilarity indices. The scale bar corresponds to a Bray-Curtis dissimilarity value of 0.06.

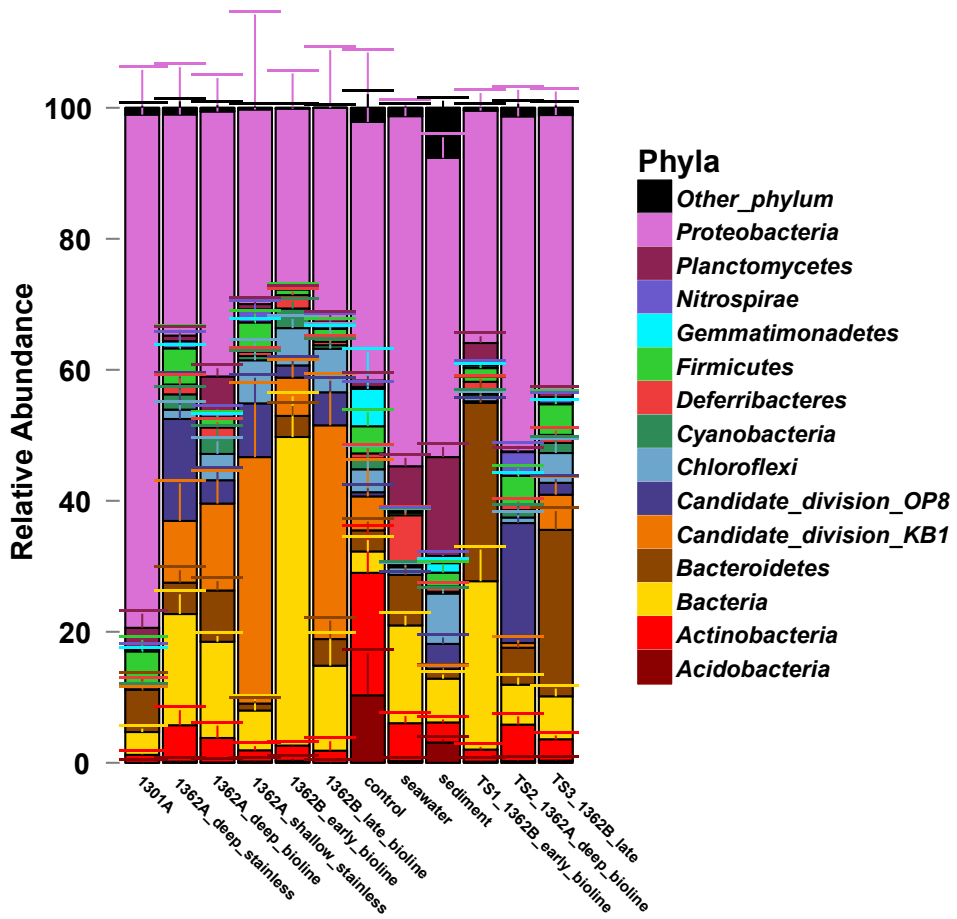

Figure 6.5 Phylum-level taxonomic distribution of *Bacteria* within 2013 samples of basement fluids, seawater, sediment, and negative PCR controls. Samples are binned according to location/depth and/or time-series deployment. Error bars represent standard deviation of taxa across samples of the sample type. TS1 = time series 1, TS2 = time series 2, TS3 = time series 3.

control samples were screened and removed from all environmental samples using a BLAST similarity search and manual curation, which yielded in 3.6M uncontaminated reads, or roughly 48% of the total unpaired reads sequenced. Large variation in the average number of reads per sample was observed and sediment samples were overrepresented as evidenced by representation in the form of 20 out of the top 21 sample library ranks. Read cluster analysis and chimera checking were performed using UPARSE and identified 51,314 OTUs.

Subsurface fluids have a distinct microbial community composition different from sediments and seawater, with limited exceptions (Figures 6.3 and 6.4). Unweighted paired group arithmetic mean average using the unweighted UniFrac analysis revealed a distinctness between the microbial communities sampled from the different environments and the different boreholes (Figure 6.3). Basement fluid microbial communities largely exhibit a clustering pattern consistent with the depth horizon and fluid delivery line type used for sampling (Figure 6.3). Time-series samples collected using the GeoMICROBE sled deployments were

overwhelmingly distinct from whole water bag samples, although some overlap is noted. The first three sets of samples collected during time-series #1 conducted at U1362B were overwhelmingly contaminated with background seawater, which is consistent with the dive events recorded for this equipment deployment; this included an inability to connect to the CORK observatory prior to start of fluid sampling (Figure 6.3). Following collection of the first three samples, the microbial community changed to being more closely related to borehole fluid communities, which is again consistent with the events recorded (i.e. no connection) during early sampling. Within the second-half of time-series #1 and the other two time-series deployments, no clear observable differences or patterns emerge with respect to the temporal ordering of microbial communities. Technical replicates of samples collected during all time-series did not always cluster together. Sediment samples collected from 15 meters away from wellhead U1301B were distinct from all other sediment samples (Figure 6.3).

Taxonomic identification of microbial groups in borehole fluid samples reveals distinct composition differences compared to sediment and bottom seawater (Figures 6.5, 6.6, 6.7, 6.8, and 6.9). Borehole fluids from U1362A and U1362B are enriched in bacterial groups such as OP8, KB1, and *Nitrospirae* compared to seawater and sediments, although sample-to-sample differences are noted (Figures 6.5, 6.7, and 6.9). *Proteobacteria*, *Planctomycetes*, *Actinobacteria*, and additional unclassified *Bacteria* were generally common to seawater, sediment, and subsurface fluids (Figure 6.7). Archaeal groups found in U1362A and U1362B subsurface fluids include *Archaeoglobaceae*, SAGMEG, MCG, MBGE, and THSCG (Figures 6.6 and 6.8). Although overlap is noted, seawater and sediments generally contained archaeal groups *Candidatus* Parvarchaeum, MBGB, Marine Group I, Marine Group II, and Marine Group III (Figure 6.6). Borehole U1301A fluids were highly enriched in *Proteobacteria* (Figure 6.9). Archaeal groups were in very low abundance in negative control PCRs; however, bacterial groups *Acidobacteria*, *Actinobacteria*, *Proteobacteria*, and other phyla were detected.

Microbial groups retrieved from borehole U1362A were different depending on the depth horizon sampled, although large sample-to-sample variations were observed (Figures 6.10, 6.11, and 6.12). Bacterial group KB1 was in notably higher abundance in the U1362A shallow horizon and archaeal group MBGE was in relatively high abundance in the deep horizon. The deep horizon fluids had relatively similar microbial communities despite the different types of fluid delivery line materials used. Microrganisms from the time series deployment at the U1362A deep bioline include bacterial phyla *Proteobacteria* and OP8 in relatively high abundance and other groups such as *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Nitrospirae*. Archaeal groups observed during the same time-series deployment include MBGE, *Archaeoglobus*, MCG, and SAGMEG. Hourly temporal changes in the microbial community during a single time-series deployment are suggested; however, notable differences are present in the samples meant to serve as the technical replicates collected for each timepoint (Figure 6.13).

Microbial phyla retrieved from the single depth horizon at borehole U1362B were different depending on the date of sampling in the cruise (Figures 6.5, 6.14, and 6.15). Fluid samples collected early in the expedition contained a higher proportion of unclassified bacterial groups and significantly fewer microorganisms from candidate division KB1 (Figures 6.5 and 6.15). Other bacterial groups identified in fluid samples from U1362B included OP8, *Bacteroidetes*, *Chloroflexi*, *Proteobacteria*, and several other phyla. Archaeal groups from Hole U1362B include MCG, *Archaeoglobaceae*, DHVEG-6, and potential seawater lineages Marine Group I, Marine Group II, and Marine Group III (Figure 6.14). Microorganisms retrieved during time-series deployments at U1362B were enriched in *Bacteroidetes* compared to groups detected in whole fluids retrieved (Figure 6.15). Similar to as observed for the PCoA analysis, the first three sets of samples collected during time-series #1 conducted at U1362B were highly respresented by groups commonly retrieved in seawater (Figure 6.16) due to the inability to connect to the CORK prior to sampling. These 'contaminating' groups were still recovered after the GeoMICROBE sled was attached to the borehole observatory around halfway through the deployment. The second time series conducted at U1362B was mostly free of lineages detected in seawater, although some overlap is noted. Hourly temporal changes in the microbial community during the second time-series deployment are suggested; however, notable differences are present in the technical replicate samples collected for each timepoint (Figure 6.16) and differences between *in situ* filters and whole water samples are noted (Figures 6.17 and 6.18).

Discussion

Access to the microbial communities within the deep subseafloor sediment-covered basement is highly restricted and enabled at just a few locations worldwide (e.g. Juan de Fuca, Costa Rica margin, Mid-Atlantic Ridge) (e.g., Nigro *et al.*, 2012; Edwards *et al.*, 2012; Jungbluth *et al.*, 2013a). The borehole observatories U1362A and U1362B sampled extensively in 2013 were constructed to provide alternate sampling features compared to older observatories (Expedition 327 Scientists, 2011a). These features include fluid delivery lines composed of different types of inert materials that extend to multiple isolated depth horizons, which enables an assessment of biofoulding effects on materials and characterization of depth specific differences that might be present in the deep subsurface microbiota. This study is the first to provide a comparison between stainless steel and Teflon fluid delivery lines used to sample fluids and the first evidence for differences in the microorganisms retrieved from different depth horizons at the same borehole. Furthermore, multiple time-series sampling deployments were conducted and provide the first sample suite of that kind for the deep subsurface basement fluids.

A relatively large number of samples for microscopy were collected here and permit investigations into the magnitude of microbial cell abundances in the basement rock. Multiple fluid delivery lines sampled from borehole U1362A reveal an amount of microbial biomass that is similar to what was previous described in 2011 from the two samples that were observed (Jungbluth *et al.*, Chapter 5). Cellular abundances appear to be mostly constant despite the depth horizon sampled (~200 and ~70 meters sub-basement) and the type of fluid delivery line used, and these numbers are consistently ~2x higher than those described previously for "high-integrity" samples collected from nearby Hole U1301A (Jungbluth *et al.*, 2013a). Borehole U1362B permits sampling at a single depth horizon (~30 meters sub-basement) and the patterns in microbial cell abundance are different. Samples collected on July 14 contained cell abundances higher on average than those found in all U1362A samples and dropped by nearly an order of magnitude one week later to numbers that are consistent with those previously observed in high-integrity samples collected from U1362B. These numbers, if reliable, provide the most robust estimates of biomass in the basement environment yet observed. Concomitant samples were collected for biogeochemical analysis, which will be used to verify sample integrity.

All molecular data generated as part of this project must be considered suspect due to the sequence read screening procedures that were needed to drop the contamination signal in the libraries (Salter *et al.*, 2014). Negative PCR controls yielded discernable bands when run on an agarose gel and were included in the sequencing run in order to provide a mechanism to remove potential contaminants; however, contamination in real samples was so widespread that any 'real' signals were too depleted for proper statistical analysis. The PCR bias produced by contaminating DNA caused *Archaea* to be undetectable in nearly half of the environmental samples collected, which is inconsistent with previous observations from similar suites of samples. Despite these setbacks, screening and removal procedures allowed for the noise induced by contaminating sequences to be decreased. Low-biomass samples collected during the time-series deployments at U1362B were disproportionately affected by contamination with exogenous DNA (e.g. *Pseudomonas* SSU rRNA amplicons) due to the relatively low levels of "real" sequence reads expected in the PCR reactions, and those libraries are considered highly suspect. Microbial diversity and community structure in all other samples should be considered suspect, but overall, beta diversity patterns are consistent with those that are described previous, which reveal basic differences in subsurface fluids, sediments, and bottom seawater. To minimize the effects of bias that accumulate in the sequencing library during PCR, the unweighted UniFrac metric was used (Lozupone and Knight, 2005), which relies only on the presence/absence data for microbial groups.

Overall microbial community beta diversity patterns were highly similar to the patterns described previously that indicate differences between the subsurface fluids, sediments, and bottom seawater (Jungbluth *et al.*, Chapter 5). Nearly all whole water samples retrieved and

filtered shipboard clustered together and with borehole fluid samples retrieved previously from boreholes U1362A and U1362B (Jungbluth *et al.*, Chapter 5). A significant proportion of subsurface samples, the majority of which were collected during the time series deployments, exhibit clustering patterns not previously seen and are believed here to be attributed to undesirable artifacts in the sequencing libraries. Sediment samples collected from nearby borehole U1301B clustered closely with Grizzly Bare sediment samples described previously (Jungbluth *et al.*, Chapter 5), with exception of sediments collected ~15 meters away for the borehole observatory, which were in a distinct cluster. Sediments from ~15 meters away from the wellhead were highly fluidized and unlike sediments collected from ~5 and ~15 meters away from the wellhead; the reason for distinct sediment alteration in this zone is unclear.

Despite the limitations imposed from screening contaminants, borehole U1362A and U1362B samples tended to cluster according to sampling location and depth. If confirmed, this further suggests horizontal spatial heterogeneity in ocean crustal microorganisms and represents the first example of vertical heterogeneity within fluids collected from different isolated intervals at a single borehole. In some locations, shallow ocean crust has been shown to be less permeable than deep ocean crust (Becker *et al.*, 2013), so this is perhaps explainable based on porosity differences in the different layers within subseafloor basalt. The taxonomic composition of major groups identified in borehole U1362A and U1362B fluids include Nitrospirae, OP8, KB1, MBGE, and *Archaeoglobus*, which is highly consistent with samples collected from these locations in 2011 (Jungbluth *et al.*, Chapter 5). Other groups detected here (e.g. *Cloacibacterium*) require further investigation to confirm their presence in the deep subsurface.

Samples collected during the time series deployment of the GeoMICROBE sled successfully retrieved groups that are indicative of deep subsurface microorganisms (e.g. *Nitrospirae*, OP8, KB1, MBGE, and *Archaeoglobus*); however, the low biomass in these samples made them disproportionately sensitive to sequencing contaminants and so any data is suspect. Samples collected from the time series deployment at U1362A were distinct from the two time series deployments conducted at U1362B. Differences were observed in the technical replicates collected during each time series, which further indicates the presence of PCR or sequencing bias. Notwithstanding, the microbial diversity and community structure observed throughout the time series deployments does not appear to change.

Juan de Fuca Ridge flank borehole fluids collected from CORK observatories in July 2013 comprise the largest sample suite collected in a single year and reveal the temporal variation in basement crust microorganisms on multiple scales (i.e. hours, days, and weeks). Fluids from multiple locations, depth horizons, and collected using different fluid delivery line types were investigated and helped to resolve the three-dimensional spatial structure of basaltic crust microorganisms and investigate borehole material fouling effects. The diversity of sampling opportunities employed here were not available prior to installation of the most recently

developed CORK observatories and the autonomous GeoMICROBE sampling sled. Sampling capabilities of borehole observatories will continue to improve, which further indicates that retrieval of borehole crustal fluids is the best window available into the deep subsurface oceanic crust.

6.5 References

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6.6 Appendix

Table 6.1 Summary of year 2013 samples used for analysis

Sample Name	Sample Type	Location (Depth in meters)	Replicate	Sample Date	Sample ID	Collection Method	Volume Filtered (Liters)
1301A.1	subsurface	U1301A		7/22/13	JdFR#31	LVBS	22.5
1301A.2	subsurface	U1301A		7/22/13	JdFR#31	LVBS	17.8
1362A.deep.bioline.3	subsurface	U1362A		7/16/13	JdFR#9	MVBS	12.8
1362A.deep.bioline.4	subsurface	U1362A		7/16/13	JdFR#10	MVBS	12.8
1362A.deep.bioline.5	subsurface	U1362A		7/16/13	JdFR#11	MVBS	15.5
1362A.deep.bioline.6	subsurface	U1362A		7/16/13	JdFR#12	MVBS	15.5
1362A.deep.stainless.10	subsurface	U1362A		7/17/13	JdFR#16	MVBS	12.8
1362A.deep.stainless.7	subsurface	U1362A		7/17/13	JdFR#17	MVBS	12.7
1362A.deep.stainless.8	subsurface	U1362A		7/17/13	JdFR#18	MVBS	12.7
1362A.deep.stainless.9	subsurface	U1362A		7/17/13	JdFR#15	MVBS	12.8
1362A.shallow.stainless.11	subsurface	U1362A		7/19/13	JdFR#21	MVBS	11.9
1362A.shallow.stainless.12	subsurface	U1362A		7/19/13	JdFR#22	MVBS	11.9
1362A.shallow.stainless.13	subsurface	U1362A		7/19/13	JdFR#23	MVBS	11.9
1362A.shallow.stainless.14	subsurface	U1362A		7/19/13	JdFR#24	MVBS	9.5
1362B.early.bioline.16	subsurface	U1362B		7/14/13	JdFR#3	MVBS	12.6
1362B.early.bioline.17	subsurface	U1362B		7/14/13	JdFR#4	MVBS	12.6
1362B.early.bioline.18	subsurface	U1362B		7/14/13	JdFR#5	MVBS	12.6
1362B.early.bioline.19	subsurface	U1362B		7/14/13	JdFR#6	MVBS	12.6
1362B.late.bioline.23	subsurface	U1362B		7/21/13	JdFR#26	LVBS	61.3
1362B.late.bioline.20	subsurface	U1362B		7/21/13	JdFR#28	MVBS	13.7
1362B.late.bioline.21	subsurface	U1362B		7/21/13	JdFR#29	MVBS	13.6
1362B.late.bioline.22	subsurface	U1362B		7/21/13	JdFR#30	MVBS	13.6
1362B.late.bioline.24	subsurface	U1362B		7/21/13	JdFR#27	MVBS	13.7
TS1.1362B.early.bioline.101	subsurface	U1362B	T1_set1	7/14-7/15/13		*in situ*	

TS1.1362B.early.bioline.105	subsurface	U1362B	T1_set1	7/14-7/15/13	*in situ*
TS1.1362B.early.bioline.109	subsurface	U1362B	T1_set1	7/14-7/15/13	*in situ*
TS1.1362B.early.bioline.15	subsurface	U1362B	T1_set1	7/14-7/15/13	*in situ*
TS1.1362B.early.bioline.113	subsurface	U1362B	T1_set2	7/14-7/15/13	*in situ*
TS1.1362B.early.bioline.117	subsurface	U1362B	T1_set2	7/14-7/15/13	*in situ*
TS1.1362B.early.bioline.121	subsurface	U1362B	T1_set2	7/14-7/15/13	*in situ*
TS1.1362B.early.bioline.125	subsurface	U1362B	T1_set2	7/14-7/15/13	*in situ*
TS1.1362B.early.bioline.102	subsurface	U1362B	T1_set3	7/14-7/15/13	*in situ*
TS1.1362B.early.bioline.106	subsurface	U1362B	T1_set3	7/14-7/15/13	*in situ*
TS1.1362B.early.bioline.110	subsurface	U1362B	T1_set3	7/14-7/15/13	*in situ*
TS1.1362B.early.bioline.98	subsurface	U1362B	T1_set3	7/14-7/15/13	*in situ*
TS1.1362B.early.bioline.114	subsurface	U1362B	T1_set4	7/14-7/15/13	*in situ*
TS1.1362B.early.bioline.118	subsurface	U1362B	T1_set4	7/14-7/15/13	*in situ*
TS1.1362B.early.bioline.122	subsurface	U1362B	T1_set4	7/14-7/15/13	*in situ*
TS1.1362B.early.bioline.126	subsurface	U1362B	T1_set4	7/14-7/15/13	*in situ*
TS1.1362B.early.bioline.103	subsurface	U1362B	T1_set5	7/14-7/15/13	*in situ*
TS1.1362B.early.bioline.107	subsurface	U1362B	T1_set5	7/14-7/15/13	*in situ*
TS1.1362B.early.bioline.111	subsurface	U1362B	T1_set5	7/14-7/15/13	*in situ*
TS1.1362B.early.bioline.99	subsurface	U1362B	T1_set5	7/14-7/15/13	*in situ*
TS1.1362B.early.bioline.115	subsurface	U1362B	T1_set6	7/14-7/15/13	*in situ*
TS1.1362B.early.bioline.119	subsurface	U1362B	T1_set6	7/14-7/15/13	*in situ*
TS1.1362B.early.bioline.123	subsurface	U1362B	T1_set6	7/14-7/15/13	*in situ*
TS1.1362B.early.bioline.127	subsurface	U1362B	T1_set6	7/14-7/15/13	*in situ*
TS1.1362B.early.bioline.100	subsurface	U1362B	T1_set7	7/14-7/15/13	*in situ*
TS1.1362B.early.bioline.104	subsurface	U1362B	T1_set7	7/14-7/15/13	*in situ*
TS1.1362B.early.bioline.108	subsurface	U1362B	T1_set7	7/14-7/15/13	*in situ*
TS1.1362B.early.bioline.112	subsurface	U1362B	T1_set7	7/14-7/15/13	*in situ*
TS1.1362B.early.bioline.116	subsurface	U1362B	T1_set8	7/14-7/15/13	*in situ*
TS1.1362B.early.bioline.120	subsurface	U1362B	T1_set8	7/14-7/15/13	*in situ*
TS1.1362B.early.bioline.124	subsurface	U1362B	T1_set8	7/14-7/15/13	*in situ*
TS1.1362B.early.bioline.128	subsurface	U1362B	T1_set8	7/14-7/15/13	*in situ*

TS2.1362A.deep.bioline.129	subsurface	U1362A	T2_set1	7/18-7/19/13	*in situ*
TS2.1362A.deep.bioline.133	subsurface	U1362A	T2_set1	7/18-7/19/13	*in situ*
TS2.1362A.deep.bioline.137	subsurface	U1362A	T2_set1	7/18-7/19/13	*in situ*
TS2.1362A.deep.bioline.141	subsurface	U1362A	T2_set1	7/18-7/19/13	*in situ*
TS2.1362A.deep.bioline.145	subsurface	U1362A	T2_set2	7/18-7/19/13	*in situ*
TS2.1362A.deep.bioline.149	subsurface	U1362A	T2_set2	7/18-7/19/13	*in situ*
TS2.1362A.deep.bioline.153	subsurface	U1362A	T2_set2	7/18-7/19/13	*in situ*
TS2.1362A.deep.bioline.157	subsurface	U1362A	T2_set2	7/18-7/19/13	*in situ*
TS2.1362A.deep.bioline.130	subsurface	U1362A	T2_set3	7/18-7/19/13	*in situ*
TS2.1362A.deep.bioline.134	subsurface	U1362A	T2_set3	7/18-7/19/13	*in situ*
TS2.1362A.deep.bioline.138	subsurface	U1362A	T2_set3	7/18-7/19/13	*in situ*
TS2.1362A.deep.bioline.142	subsurface	U1362A	T2_set3	7/18-7/19/13	*in situ*
TS2.1362A.deep.bioline.146	subsurface	U1362A	T2_set4	7/18-7/19/13	*in situ*
TS2.1362A.deep.bioline.150	subsurface	U1362A	T2_set4	7/18-7/19/13	*in situ*
TS2.1362A.deep.bioline.154	subsurface	U1362A	T2_set4	7/18-7/19/13	*in situ*
TS2.1362A.deep.bioline.158	subsurface	U1362A	T2_set4	7/18-7/19/13	*in situ*
TS2.1362A.deep.bioline.131	subsurface	U1362A	T2_set5	7/18-7/19/13	*in situ*
TS2.1362A.deep.bioline.135	subsurface	U1362A	T2_set5	7/18-7/19/13	*in situ*
TS2.1362A.deep.bioline.139	subsurface	U1362A	T2_set5	7/18-7/19/13	*in situ*
TS2.1362A.deep.bioline.143	subsurface	U1362A	T2_set5	7/18-7/19/13	*in situ*
TS2.1362A.deep.bioline.147	subsurface	U1362A	T2_set6	7/18-7/19/13	*in situ*
TS2.1362A.deep.bioline.151	subsurface	U1362A	T2_set6	7/18-7/19/13	*in situ*
TS2.1362A.deep.bioline.155	subsurface	U1362A	T2_set6	7/18-7/19/13	*in situ*
TS2.1362A.deep.bioline.159	subsurface	U1362A	T2_set6	7/18-7/19/13	*in situ*
TS2.1362A.deep.bioline.132	subsurface	U1362A	T2_set7	7/18-7/19/13	*in situ*
TS2.1362A.deep.bioline.136	subsurface	U1362A	T2_set7	7/18-7/19/13	*in situ*
TS2.1362A.deep.bioline.140	subsurface	U1362A	T2_set7	7/18-7/19/13	*in situ*
TS2.1362A.deep.bioline.144	subsurface	U1362A	T2_set7	7/18-7/19/13	*in situ*
TS2.1362A.deep.bioline.148	subsurface	U1362A	T2_set8	7/18-7/19/13	*in situ*
TS2.1362A.deep.bioline.152	subsurface	U1362A	T2_set8	7/18-7/19/13	*in situ*
TS2.1362A.deep.bioline.156	subsurface	U1362A	T2_set8	7/18-7/19/13	*in situ*

TS2.1362A.deep.bioline.160	subsurface	U1362A	T2_set8	7/18-7/19/13	*in situ*
TS3.1362B.late.161	subsurface	U1362B	T3_set1	7/20-7/21/13	*in situ*
TS3.1362B.late.165	subsurface	U1362B	T3_set1	7/20-7/21/13	*in situ*
TS3.1362B.late.169	subsurface	U1362B	T3_set1	7/20-7/21/13	*in situ*
TS3.1362B.late.173	subsurface	U1362B	T3_set1	7/20-7/21/13	*in situ*
TS3.1362B.late.177	subsurface	U1362B	T3_set2	7/20-7/21/13	*in situ*
TS3.1362B.late.180	subsurface	U1362B	T3_set2	7/20-7/21/13	*in situ*
TS3.1362B.late.183	subsurface	U1362B	T3_set2	7/20-7/21/13	*in situ*
TS3.1362B.late.186	subsurface	U1362B	T3_set2	7/20-7/21/13	*in situ*
TS3.1362B.late.162	subsurface	U1362B	T3_set3	7/20-7/21/13	*in situ*
TS3.1362B.late.166	subsurface	U1362B	T3_set3	7/20-7/21/13	*in situ*
TS3.1362B.late.170	subsurface	U1362B	T3_set3	7/20-7/21/13	*in situ*
TS3.1362B.late.174	subsurface	U1362B	T3_set3	7/20-7/21/13	*in situ*
TS3.1362B.late.178	subsurface	U1362B	T3_set4	7/20-7/21/13	*in situ*
TS3.1362B.late.181	subsurface	U1362B	T3_set4	7/20-7/21/13	*in situ*
TS3.1362B.late.184	subsurface	U1362B	T3_set4	7/20-7/21/13	*in situ*
TS3.1362B.late.187	subsurface	U1362B	T3_set4	7/20-7/21/13	*in situ*
TS3.1362B.late.163	subsurface	U1362B	T3_set5	7/20-7/21/13	*in situ*
TS3.1362B.late.167	subsurface	U1362B	T3_set5	7/20-7/21/13	*in situ*
TS3.1362B.late.171	subsurface	U1362B	T3_set5	7/20-7/21/13	*in situ*
TS3.1362B.late.175	subsurface	U1362B	T3_set5	7/20-7/21/13	*in situ*
TS3.1362B.late.179	subsurface	U1362B	T3_set6	7/20-7/21/13	*in situ*
TS3.1362B.late.182	subsurface	U1362B	T3_set6	7/20-7/21/13	*in situ*
TS3.1362B.late.185	subsurface	U1362B	T3_set6	7/20-7/21/13	*in situ*
TS3.1362B.late.188	subsurface	U1362B	T3_set6	7/20-7/21/13	*in situ*
TS3.1362B.late.164	subsurface	U1362B	T3_set7	7/20-7/21/13	*in situ*
TS3.1362B.late.168	subsurface	U1362B	T3_set7	7/20-7/21/13	*in situ*
TS3.1362B.late.172	subsurface	U1362B	T3_set7	7/20-7/21/13	*in situ*
TS3.1362B.late.176	subsurface	U1362B	T3_set7	7/20-7/21/13	*in situ*
TS3.1362B.late.189	subsurface	U1362B	T3_set8	7/20-7/21/13	*in situ*
TS3.1362B.late.190	subsurface	U1362B	T3_set8	7/20-7/21/13	*in situ*

TS3.1362B.late.191	subsurface	U1362B	T3_set8	7/20-7/21/13	*in situ*	
TS3.1362B.late.192	subsurface	U1362B	T3_set8	7/20-7/21/13	*in situ*	
seawater.43	seawater	nearby U1362A (2648)		7/19/13	Niskin on Jason	2.5
seawater.47	seawater	nearby U1362A (2648)		7/16/13	Niskin on Jason	5.0
seawater.44	seawater	nearby U1362B (2515)		7/23/13	CTD-Niskin	2.0
seawater.46	seawater	nearby U1362B (2515)		7/23/13	CTD-Niskin	5.0
seawater.48	seawater	nearby U1362B (2515)		7/23/13	CTD-Niskin	5.0
seawater.41	seawater	nearby U1362B (2589)		7/23/13	CTD-Niskin	6.5
seawater.42	seawater	nearby U1362B (2589)		7/23/13	CTD-Niskin	6.5
seawater.40	seawater	nearby U1362B (2647)		7/23/13	CTD-Niskin	3.8
seawater.49	seawater	nearby U1362B (2647)		7/23/13	CTD-Niskin	5.5
seawater.39	seawater	nearby U1362B (2648)		7/14/13	Niskin on Jason	5.5
seawater.45	seawater	nearby U1362B (2648)		7/21/13	Niskin on Jason	5.0
sediment.69	sediment	nearby 1301B (~15 m.f.w.)	core4	7/22/13	push core	
sediment.83	sediment	nearby 1301B (~15 m.f.w.)	core4_whole	7/22/13	push core	
sediment.89	sediment	nearby 1301B (~15 m.f.w.)	core4_whole	7/22/13	push core	
sediment.95	sediment	nearby 1301B (~15 m.f.w.)	core4_whole	7/22/13	push core	
sediment.75	sediment	nearby 1301B (~15 m.f.w.)	core5	7/22/13	push core	
sediment.54	sediment	nearby 1301B (~15 m.f.w.)	core5_whole	7/22/13	push core	
sediment.60	sediment	nearby 1301B	core5_whole	7/22/13	push core	

		(~15 m.f.w.)			
sediment.67	sediment	nearby 1301B (~15 m.f.w.)	core5_whole	7/22/13	push core
sediment.73	sediment	nearby 1301B (~15 m.f.w.)	core6_whole	7/22/13	push core
sediment.79	sediment	nearby 1301B (~15 m.f.w.)	core6_whole	7/22/13	push core
sediment.84	sediment	nearby 1301B (~15 m.f.w.)	core6_whole	7/22/13	push core
sediment.50	sediment	nearby 1301B (~5 m.f.w.)	core1	7/22/13	push core
sediment.70	sediment	nearby 1301B (~5 m.f.w.)	core1_bottom	7/22/13	push core
sediment.76	sediment	nearby 1301B (~5 m.f.w.)	core1_bottom	7/22/13	push core
sediment.81	sediment	nearby 1301B (~5 m.f.w.)	core1_bottom	7/22/13	push core
sediment.51	sediment	nearby 1301B (~5 m.f.w.)	core1_top	7/22/13	push core
sediment.57	sediment	nearby 1301B (~5 m.f.w.)	core1_top	7/22/13	push core
sediment.64	sediment	nearby 1301B (~5 m.f.w.)	core1_top	7/22/13	push core
sediment.58	sediment	nearby 1301B (~5 m.f.w.)	core2_bottom	7/22/13	push core
sediment.65	sediment	nearby 1301B (~5 m.f.w.)	core2_bottom	7/22/13	push core
sediment.71	sediment	nearby 1301B (~5 m.f.w.)	core2_bottom	7/22/13	push core
sediment.52	sediment	nearby 1301B (~5 m.f.w.)	core2_top	7/22/13	push core
sediment.87	sediment	nearby 1301B (~5 m.f.w.)	core2_top	7/22/13	push core
sediment.93	sediment	nearby 1301B (~5 m.f.w.)	core2_top	7/22/13	push core
sediment.63	sediment	nearby 1301B (~5 m.f.w.)	core3	7/22/13	push core

sediment.66	sediment	nearby 1301B (~5 m.f.w.)	core3_bottom	7/22/13	push core
sediment.72	sediment	nearby 1301B (~5 m.f.w.)	core3_bottom	7/22/13	push core
sediment.78	sediment	nearby 1301B (~5 m.f.w.)	core3_bottom	7/22/13	push core
sediment.53	sediment	nearby 1301B (~5 m.f.w.)	core3_middle	7/22/13	push core
sediment.59	sediment	nearby 1301B (~5 m.f.w.)	core3_middle	7/22/13	push core
sediment.94	sediment	nearby 1301B (~5 m.f.w.)	core3_middle	7/22/13	push core
sediment.77	sediment	nearby 1301B (~5 m.f.w.)	core3_top	7/22/13	push core
sediment.82	sediment	nearby 1301B (~5 m.f.w.)	core3_top	7/22/13	push core
sediment.88	sediment	nearby 1301B (~5 m.f.w.)	core3_top	7/22/13	push core
sediment.86	sediment	nearby 1301B (~55 m.f.w.)	core7	7/22/13	push core
sediment.61	sediment	nearby 1301B (~55 m.f.w.)	core7_bottom	7/22/13	push core
sediment.68	sediment	nearby 1301B (~55 m.f.w.)	core7_bottom	7/22/13	push core
sediment.74	sediment	nearby 1301B (~55 m.f.w.)	core7_bottom	7/22/13	push core
sediment.55	sediment	nearby 1301B (~55 m.f.w.)	core7_top	7/22/13	push core
sediment.90	sediment	nearby 1301B (~55 m.f.w.)	core7_top	7/22/13	push core
sediment.96	sediment	nearby 1301B (~55 m.f.w.)	core7_top	7/22/13	push core
sediment.92	sediment	nearby 1301B (~55 m.f.w.)	core8	7/22/13	push core
sediment.56	sediment	nearby 1301B (~55 m.f.w.)	core8_bottom	7/22/13	push core
sediment.62	sediment	nearby 1301B (~55 m.f.w.)	core8_bottom	7/22/13	push core

sediment.80	sediment	nearby 1301B (~55 m.f.w.)	core8_bottom	7/22/13	push core
sediment.85	sediment	nearby 1301B (~55 m.f.w.)	core8_top	7/22/13	push core
sediment.91	sediment	nearby 1301B (~55)	core8_top	7/22/13	push core
sediment.97	sediment	nearby 1301B (~55 m.f.w.)	core8_top	7/22/13	push core

m.f.w.: meters from wellhead

Table 6.2 Illumina Read and Clustering Statistics

	Qiime default forward read (515F)	*Qiime default reverse (806R) read*	*Paired-end reads (SeqPrep)*	*Filtered Paired-end reads (SeqPrep)*
Total Reads (all lengths)	7403068	7404069	6137683	3556013
Unique sequences	1560243	2118938	1961487	1596710
Mode Read Length (bp)	~150	~150	~255	~255
Average Reads per Sample	38558	N.P.	31967	18521
Median Reads per Sample	27033	N.P.	22227	3403
Standard Deviation per Sample	38297	N.P.	31379	30744
UPARSE clusters	260671	N.P.	54901	51314

N.P.: not performed

Table 6.3 Contaminating taxa screened and removed from dataset

Number of identical sequences	megablast _sim	Keep or remove	SILVA Taxonomy
59092	100%	Remove	*Bacteria*;*Proteobacteria*;*Gammaproteobacteria*;*Pseudomonadales*;*Pseudomonadaceae*;*Pseudomonas*;
8301	100%	Remove	*Bacteria*;*Proteobacteria*;*Gammaproteobacteria*;*Xanthomonadales*;*Xanthomonadaceae*;*Xanthomonas*;
4110	100%	Remove	*Bacteria*;*Proteobacteria*;*Alphaproteobacteria*;*Rickettsiales*;mitochondria;
3309	100%	Remove	*Bacteria*;*Proteobacteria*;*Betaproteobacteria*;*Burkholderiales*;*Oxalobacteraceae*;*Herbaspirillum*;
2630	100%	Remove	*Bacteria*;*Proteobacteria*;*Alphaproteobacteria*;*Sphingomonadales*;*Sphingomonadaceae*;*Sphingobium*;
2502	98%	Keep	*Bacteria*;Candidate division KB1;
2124	100%	Remove	*Bacteria*;*Proteobacteria*;*Gammaproteobacteria*;*Pseudomonadales*;*Pseudomonadaceae*;*Pseudomonas*;
1872	91%	Keep	*Bacteria*;*Chloroflexi*;*Anaerolineae*;*Anaerolineales*;*Anaerolineaceae*;uncultured;
1773	100%	Remove	*Bacteria*;*Proteobacteria*;*Betaproteobacteria*;*Burkholderiales*;*Comamonadaceae*;*Ottowia*;
1531	100%	Remove	*Bacteria*;*Firmicutes*;*Bacilli*;*Bacillales*;*Planococcaceae*;*Solibacillus*;
1378	100%	Remove	*Bacteria*;*Proteobacteria*;*Gammaproteobacteria*;*Pseudomonadales*;*Pseudomonadaceae*;*Pseudomonas*;
751	99%	Keep	*Bacteria*;*Proteobacteria*;*Deltaproteobacteria*;*Myxococcales*;*Polyangiaceae*;*Sorangium*;
726	99%	Keep	*Bacteria*;*Synergistetes*;*Synergistia*;*Synergistales*;*Synergistaceae*;uncultured;
717	100%	Remove	*Bacteria*;*Proteobacteria*;*Gammaproteobacteria*;*Pseudomonadales*;*Moraxellaceae*;*Acinetobacter*;
684	99%	Keep	*Bacteria*;Candidate division KB1;
657	100%	Remove	*Bacteria*;*Proteobacteria*;*Alphaproteobacteria*;*Rickettsiales*;mitochondria;
630	100%	Remove	*Bacteria*;*Proteobacteria*;*Gammaproteobacteria*;*Xanthomonadales*;*Xanthomonadaceae*;*Stenotrophomonas*;
589	100%	Remove	*Bacteria*;*Acidobacteria*;*Acidobacteria*;*Acidobacteriales*;*Acidobacteriaceae* (Subgroup 1);uncultured;
547	99%	Keep	*Bacteria*;*Proteobacteria*;*Deltaproteobacteria*;*Desulfovibrionales*;*Desulfovibrionaceae*;*Desulfovibrio*;
518	96%	Keep	*Bacteria*;TA06;
509	100%	Remove	*Bacteria*;*Acidobacteria*;*Acidobacteria*;*Acidobacteriales*;*Acidobacteriaceae* (Subgroup 1);uncultured;
501	99%	Keep	*Bacteria*;*Proteobacteria*;*Alphaproteobacteria*;*Rickettsiales*;mitochondria;
497	99%	Keep	*Bacteria*;*Verrucomicrobia*;*Opitutae*;*Opitutales*;*Opitutaceae*;*Opitutus*;
467	100%	Remove	*Bacteria*;*Proteobacteria*;*Betaproteobacteria*;*Burkholderiales*;*Comamonadaceae*;*Delftia*;
453	100%	Remove	*Bacteria*;*Proteobacteria*;*Alphaproteobacteria*;*Rickettsiales*;mitochondria;
450	100%	Remove	*Bacteria*;*Proteobacteria*;*Betaproteobacteria*;*Burkholderiales*;*Comamonadaceae*;*Comamonas*;

447	100%	Remove	*Bacteria*;*Verrucomicrobia*;*Opitutae*;*Opitutales*;*Opitutaceae*;*Opitutus*;
446	100%	Remove	*Bacteria*;*Actinobacteria*;*Actinobacteria*;*Micrococcales*;*Micrococcaceae*;*Micrococcus*;
443	100%	Remove	*Bacteria*;*Proteobacteria*;*Alphaproteobacteria*;*Sphingomonadales*;*Sphingomonadaceae*;*Novosphingobium*;
439	100%	Remove	*Bacteria*;*Actinobacteria*;*Thermoleophilia*;*Solirubrobacterales*;TM146;
436	100%	Remove	*Bacteria*;*Proteobacteria*;*Gammaproteobacteria*;*Enterobacteriales*;*Enterobacteriaceae*;*Erwinia*;
416	100%	Remove	*Bacteria*;*Verrucomicrobia*;*Opitutae*;*Opitutales*;*Opitutaceae*;*Opitutus*;
401	100%	Remove	*Bacteria*;*Proteobacteria*;*Gammaproteobacteria*;*Enterobacteriales*;*Enterobacteriaceae*;*Yersinia*;
367	99%	Keep	*Bacteria*;*Proteobacteria*;*Alphaproteobacteria*;*Rickettsiales*;mitochondria;
349	100%	Remove	*Bacteria*;*Proteobacteria*;*Gammaproteobacteria*;*Pseudomonadales*;*Pseudomonadaceae*;*Pseudomonas*;
347	100%	Remove	*Bacteria*;*Proteobacteria*;*Gammaproteobacteria*;*Enterobacteriales*;*Enterobacteriaceae*;*Escherichia-Shigella*;
344	100%	Remove	*Bacteria*;*Proteobacteria*;*Gammaproteobacteria*;*Pseudomonadales*;*Pseudomonadaceae*;*Pseudomonas*;
343	100%	Remove	*Bacteria*;*Acidobacteria*;*Acidobacteria*;*Acidobacteriales*;*Acidobacteriaceae* (Subgroup 1);uncultured;
318	99%	Keep	*Bacteria*;*Acidobacteria*;*Acidobacteria*;Subgroup 3;Family Incertae Sedis;*Candidatus* Solibacter;
316	100%	Remove	*Bacteria*;*Proteobacteria*;*Betaproteobacteria*;*Burkholderiales*;*Comamonadaceae*;*Mitsuaria*;
315	100%	Remove	*Bacteria*;*Bacteroidetes*;*Sphingobacteriia*;*Sphingobacteriales*;*Sphingobacteriaceae*;*Pedobacter*;
306	100%	Remove	*Bacteria*;*Proteobacteria*;*Alphaproteobacteria*;*Rickettsiales*;mitochondria;
302	92%	Keep	*Bacteria*;Candidate division OP8;
297	98%	Keep	*Bacteria*;*Synergistetes*;*Synergistia*;*Synergistales*;*Synergistaceae*;uncultured;
297	100%	Remove	*Bacteria*;*Proteobacteria*;*Alphaproteobacteria*;*Sphingomonadales*;*Sphingomonadaceae*;*Sphingomonas*;
296	100%	Remove	*Bacteria*;*Firmicutes*;*Bacilli*;*Bacillales*;*Staphylococcaceae*;*Staphylococcus*;
280	99%	Keep	*Bacteria*;*Proteobacteria*;*Gammaproteobacteria*;*Xanthomonadales*;*Xanthomonadaceae*;*Xanthomonas*;
276	99%	Keep	*Bacteria*;*Gemmatimonadetes*;*Gemmatimonadetes*;AT425-EubC11 terrestrial group;
261	100%	Remove	*Bacteria*;*Actinobacteria*;*Actinobacteria*;*Corynebacteriales*;*Corynebacteriaceae*;*Corynebacterium*;
260	100%	Remove	*Bacteria*;*Proteobacteria*;*Betaproteobacteria*;*Burkholderiales*;*Burkholderiaceae*;*Ralstonia*;
258	99%	Keep	*Bacteria*;*Acidobacteria*;*Acidobacteria*;Subgroup 2;
256	100%	Remove	*Bacteria*;*Acidobacteria*;*Acidobacteria*;*Acidobacteriales*;*Acidobacteriaceae* (Subgroup 1);uncultured;
252	92%	Keep	*Bacteria*;*Chloroflexi*;*Anaerolineae*;*Anaerolineales*;*Anaerolineaceae*;uncultured;

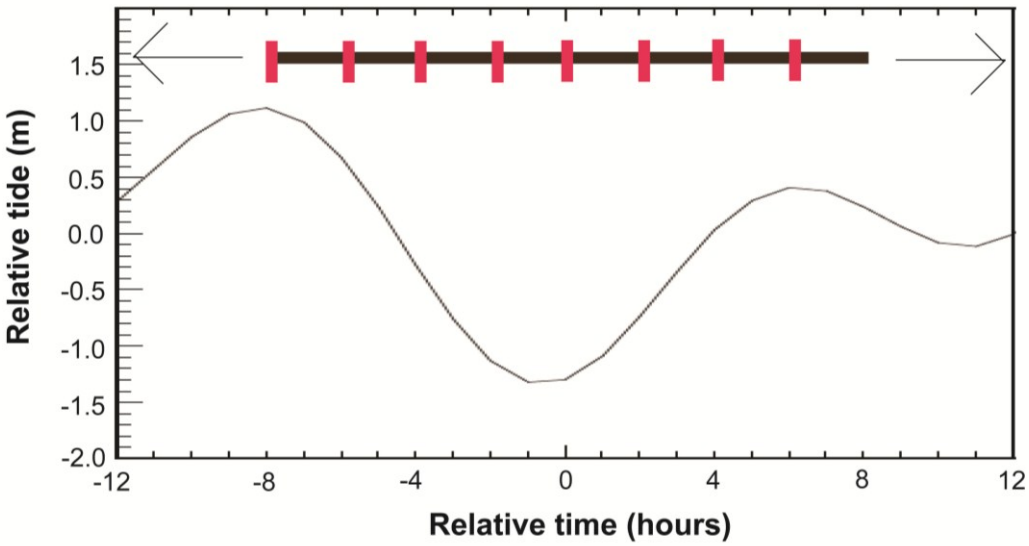

Figure 6.1 Predicted tidal cycle at boreholes U1301A, U1362A, and U1362B. Sampling timeline for *in situ* short term (~16 hr) sampling deployments is estimated with scale set above the tidal graph. Red colors represent estimated start times of sampling events over the course of entire instrument deployments (black-color line). (modified from Expedition 327 Scientists). Depending on synchronization with the tides, the sampling time series start could shift either left or right on the x-axis.

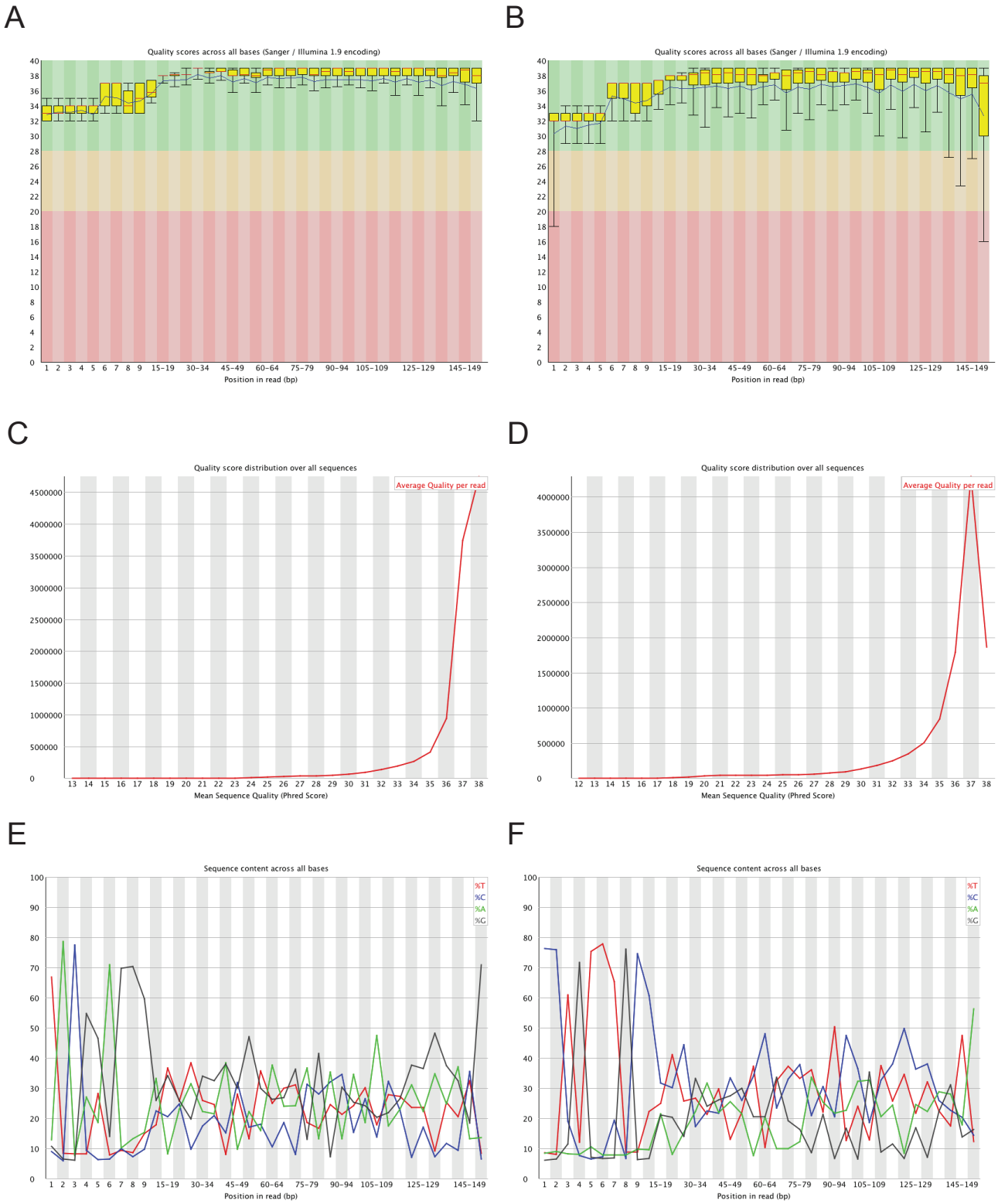

Figure 6.2 (A) Quality scores of Illumina reads across all bases for (A) forward reads and (B) reverse reads. Quality distribution over all samples for (C) forward reads and (D) reverse reads. Base pair distribution across all bases for (E) forward reads and (F) reverse reads

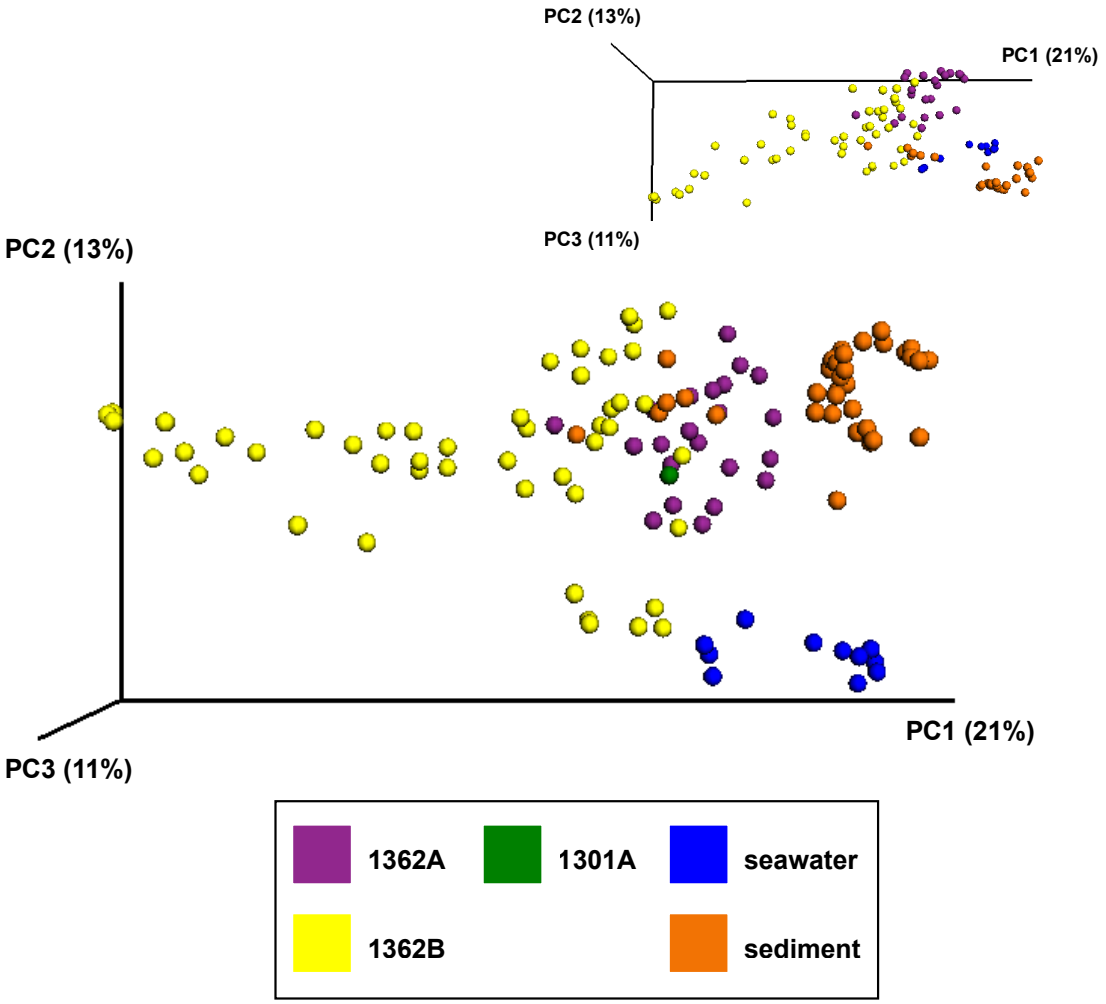

Figure 6.4 Principle coordinates analysis (PCoA) of Bray-Curtis dissimilarity indices using all UPARSE clusters. Samples are colored by location. Plots are scaled according to the percentage variation explained via the top three principal components. Rotated plots are shown above-right to reveal additional features occluded in depication of the maximum variation within PC1 and PC2.

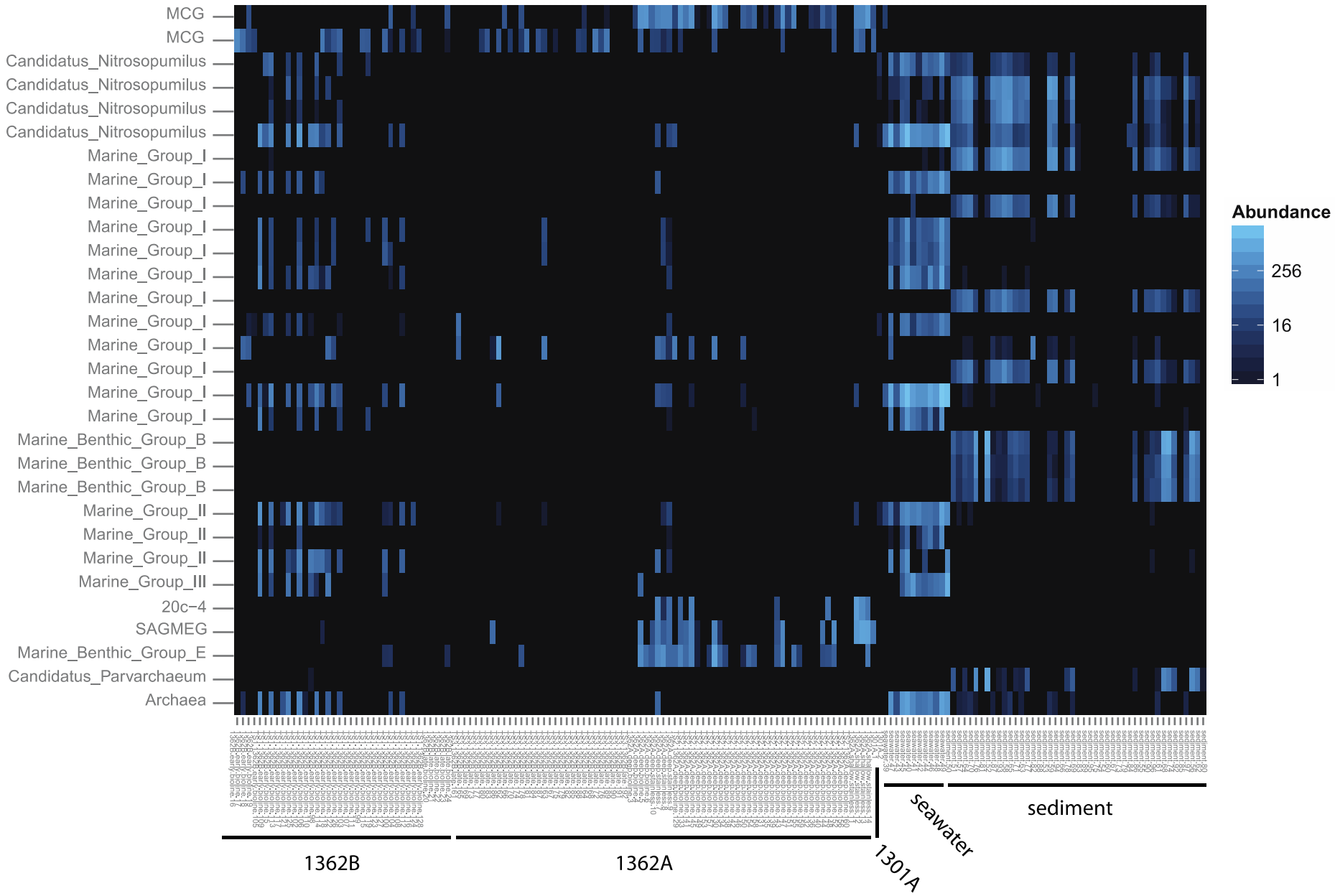

Figure 6.6 Heat map of top 30 archaeal UPARSE clusters identified in all samples. The total number of clusters detected is indicated in the scale bar.

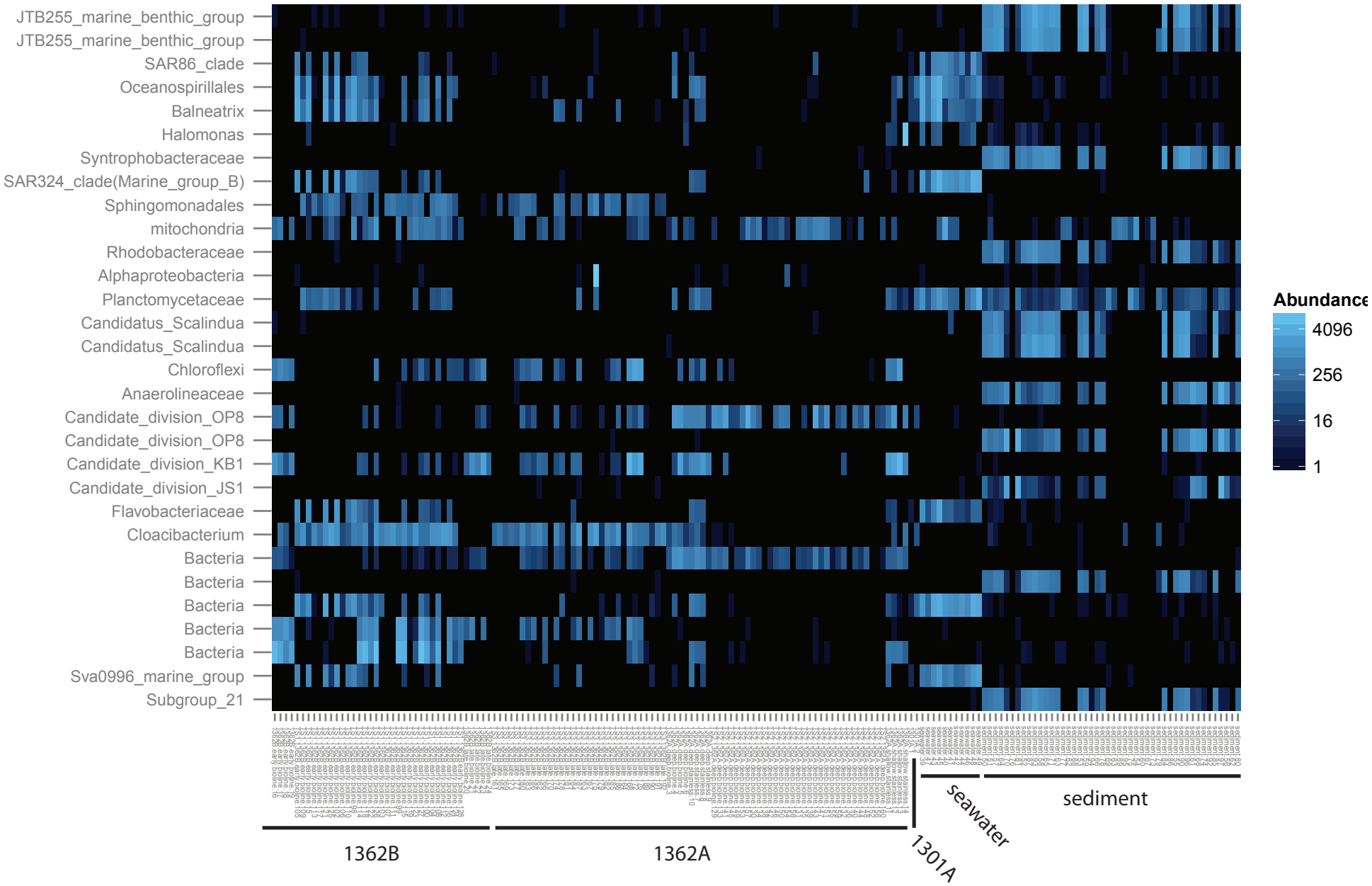

Figure 6.7 Heat map of top 30 bacterial UPARSE clusters identified in all samples. The total number of clusters detected is indicated in the scale bar.

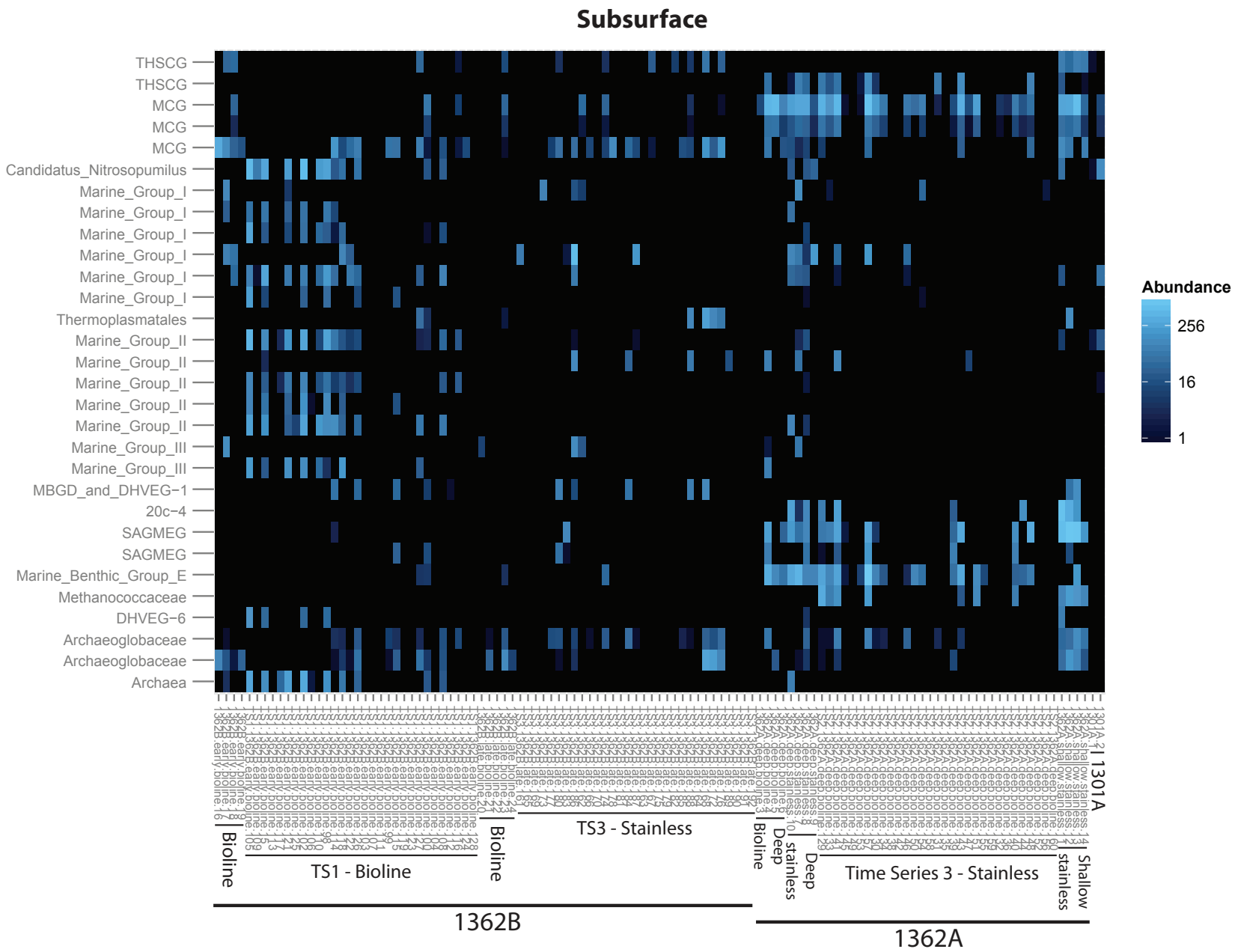

Figure 6.8 Heat map of top 30 archaeal UPARSE clusters identified in subsurface samples. The total number of clusters detected is indicated in the scale bar.

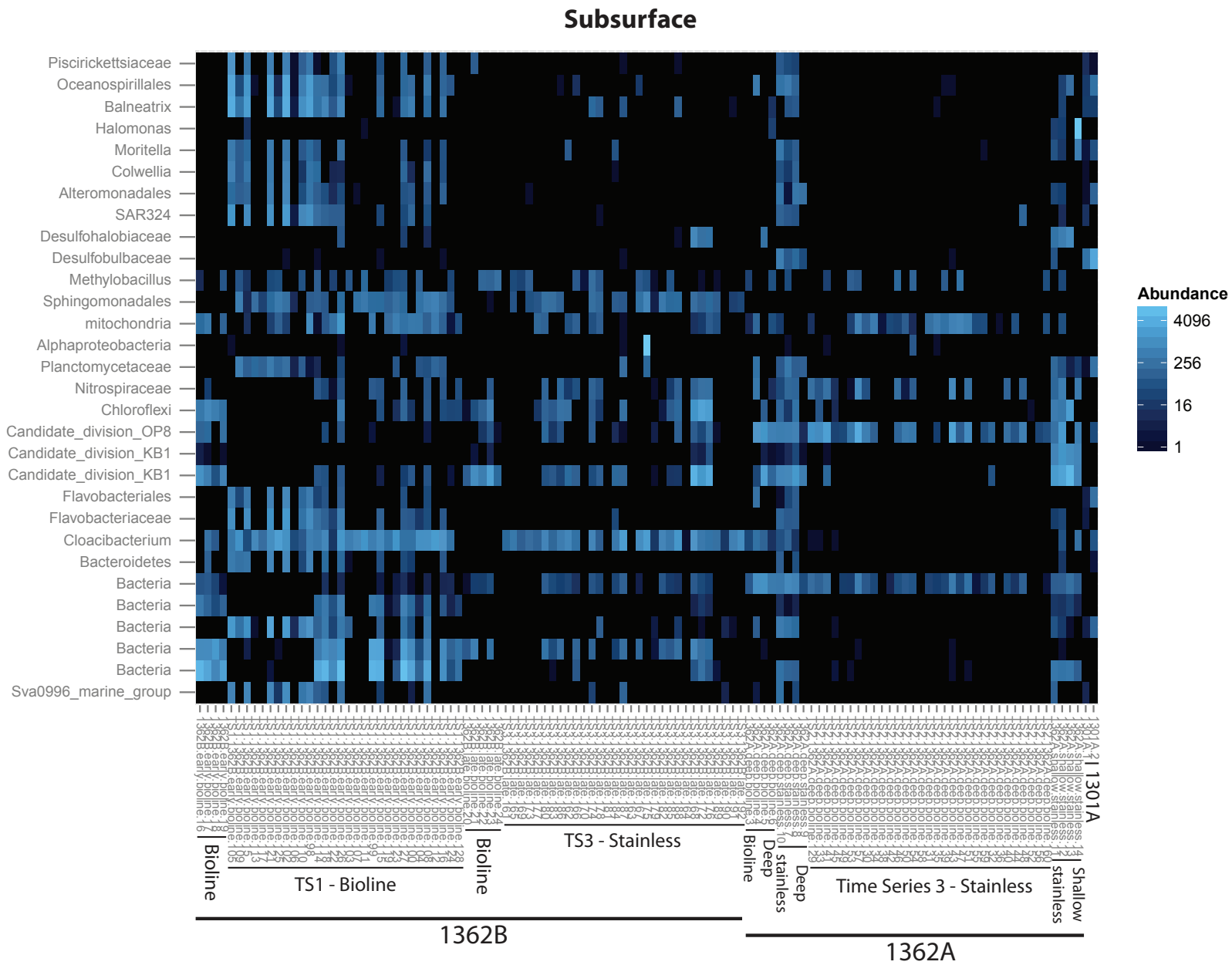

Figure 6.9 Heat map of top 30 bacterial UPARSE clusters identified in subsurface samples. The total number of clusters detected is indicated in the scale bar.

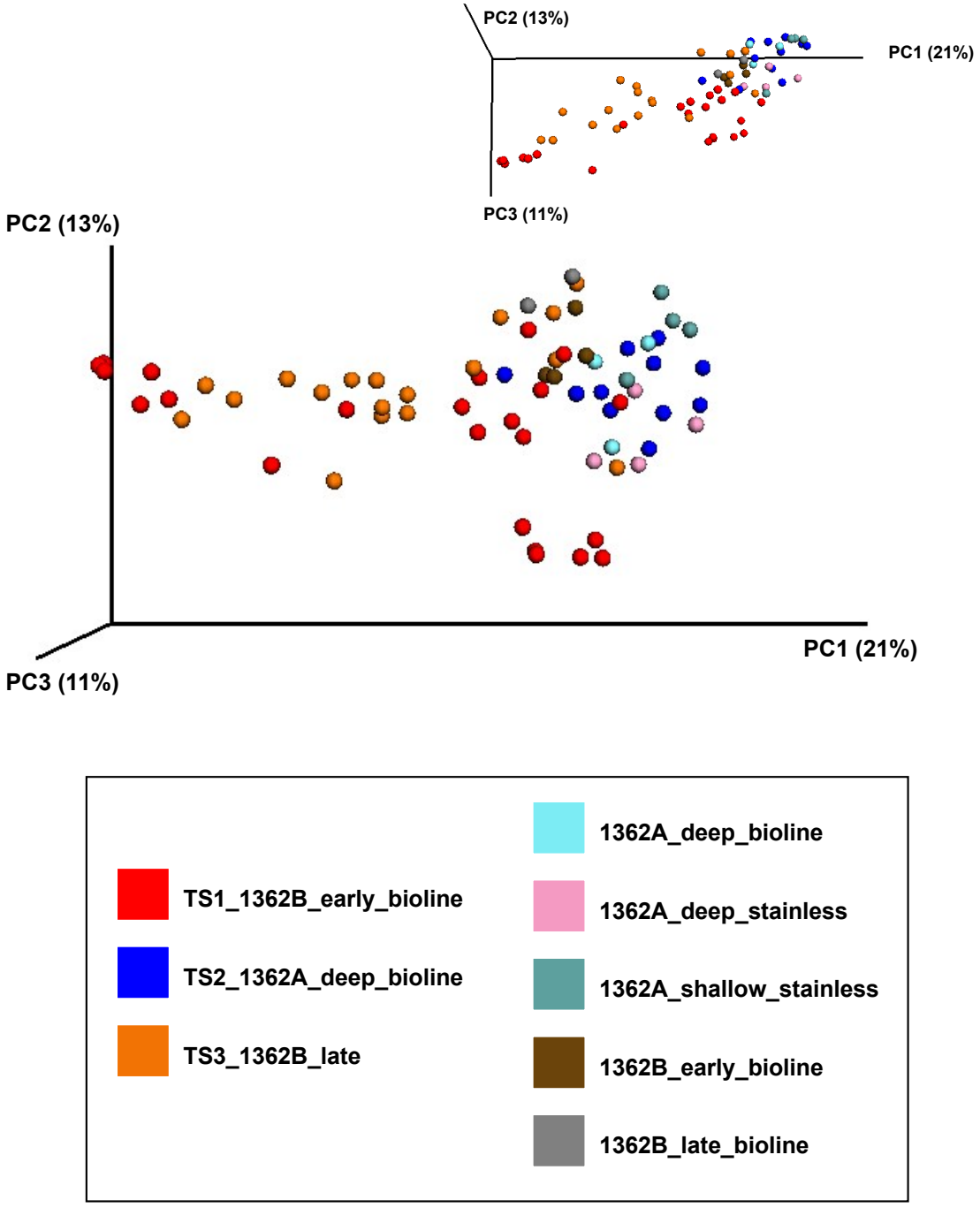

Figure 6.10 Principle coordinates analysis (PCoA) of Bray-Curtis dissimilarity indices using subsurface fluid UPARSE clusters. Samples are colored by collection event. Plots are scaled according to the percentage variation explained via the top three principal components. Rotated plots are shown above-right to reveal additional features occluded in depication of the maximum variation within PC1 and PC2. TS1 = time series 1, TS2 = time series 2, TS3 = time series 3.

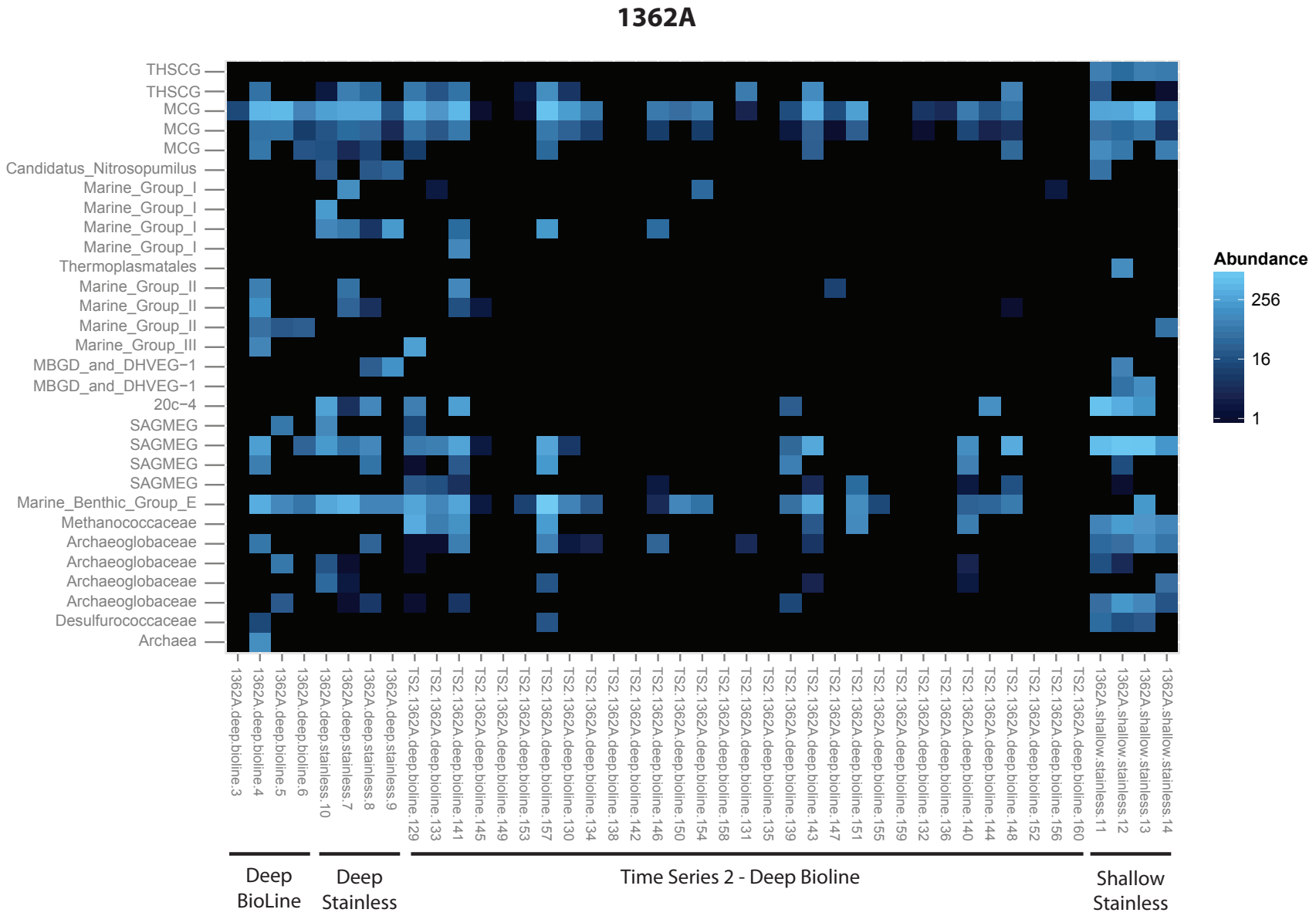

Figure 6.11 Heat map of top 30 archaeal UPARSE clusters identified in U1362A samples. The total number of clusters detected is indicated in the scale bar.

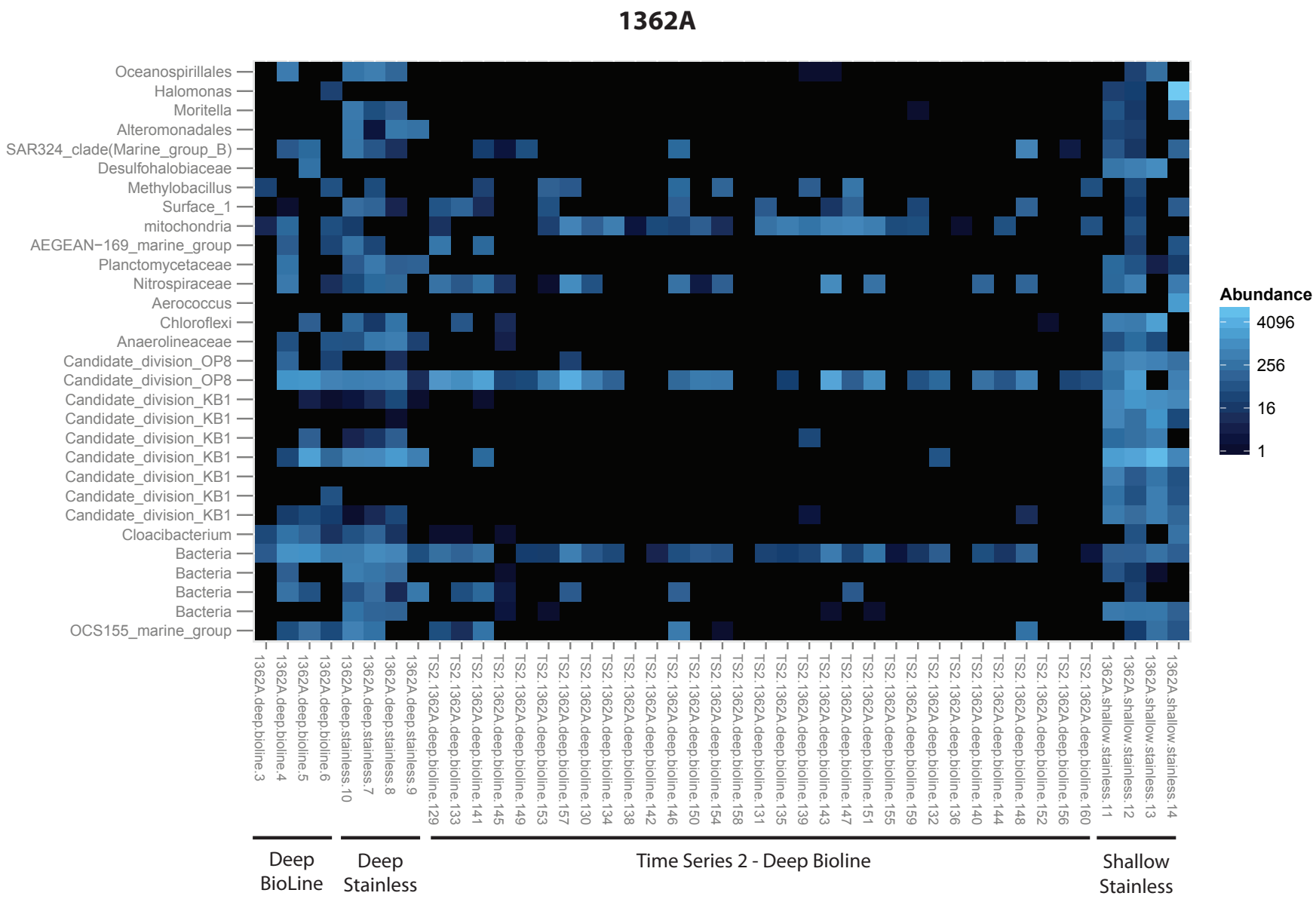

Figure 6.12 Heat map of top 30 bacterial UPARSE clusters identified in U1362A samples. The total number of clusters detected is indicated in the scale bar.

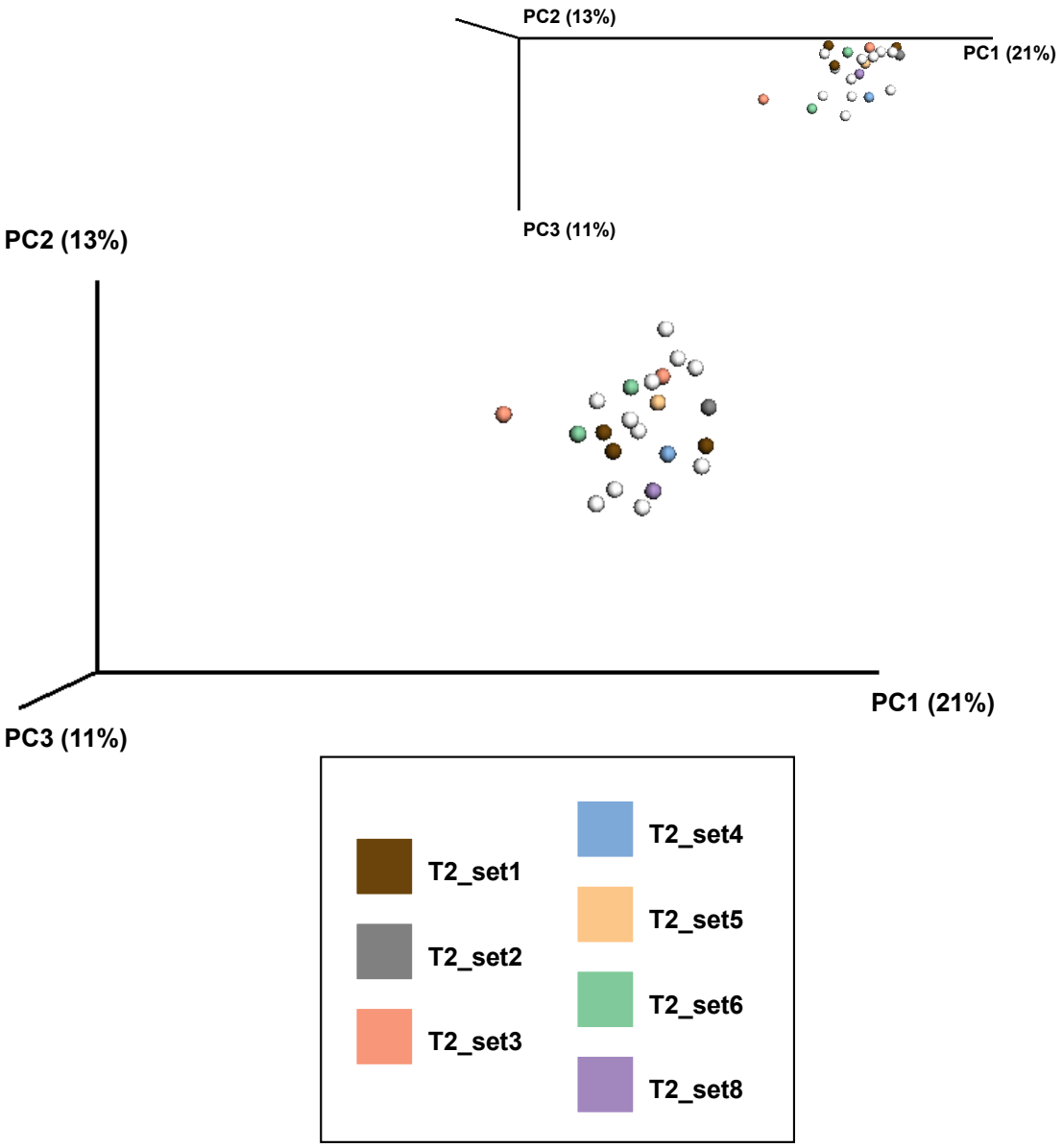

Figure 6.13 Principle coordinates analysis (PCoA) of Bray-Curtis dissimilarity indices using subsurface fluid time-series #2 UPARSE clusters. Samples are colored by time of collection – set1 is first and set8 is last. Bag samples collected from 1362A are indicated with white dots for comparison purposes. Plots are scaled according to the percentage variation explained via the top three principal components. Rotated plots are shown above-right to reveal additional features occluded in depication of the maximum variation within PC1 and PC2.

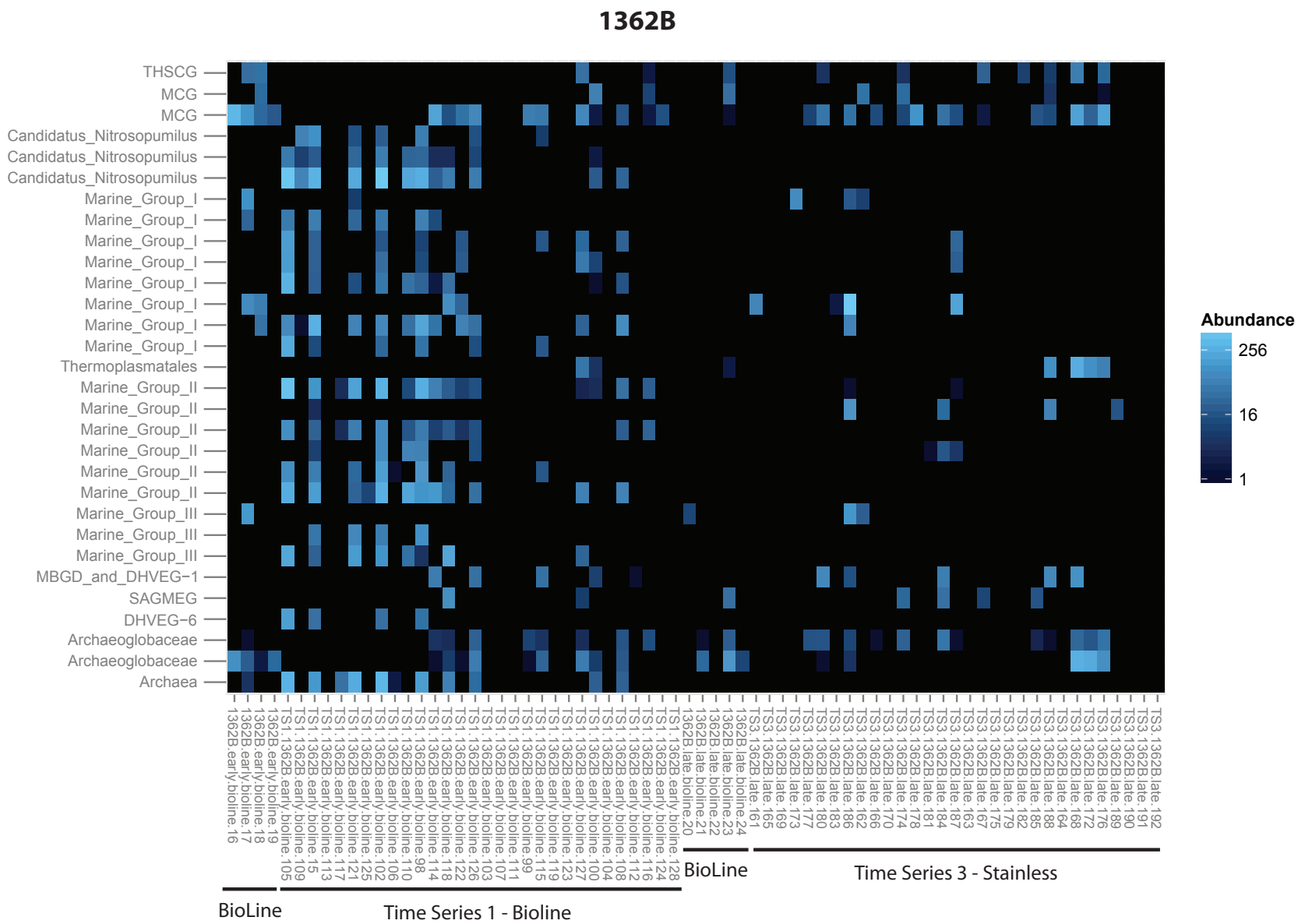

Figure 6.14 Heat map of top 30 archaeal UPARSE clusters identified in U1362B samples. The total number of clusters detected is indicated in the scale bar.

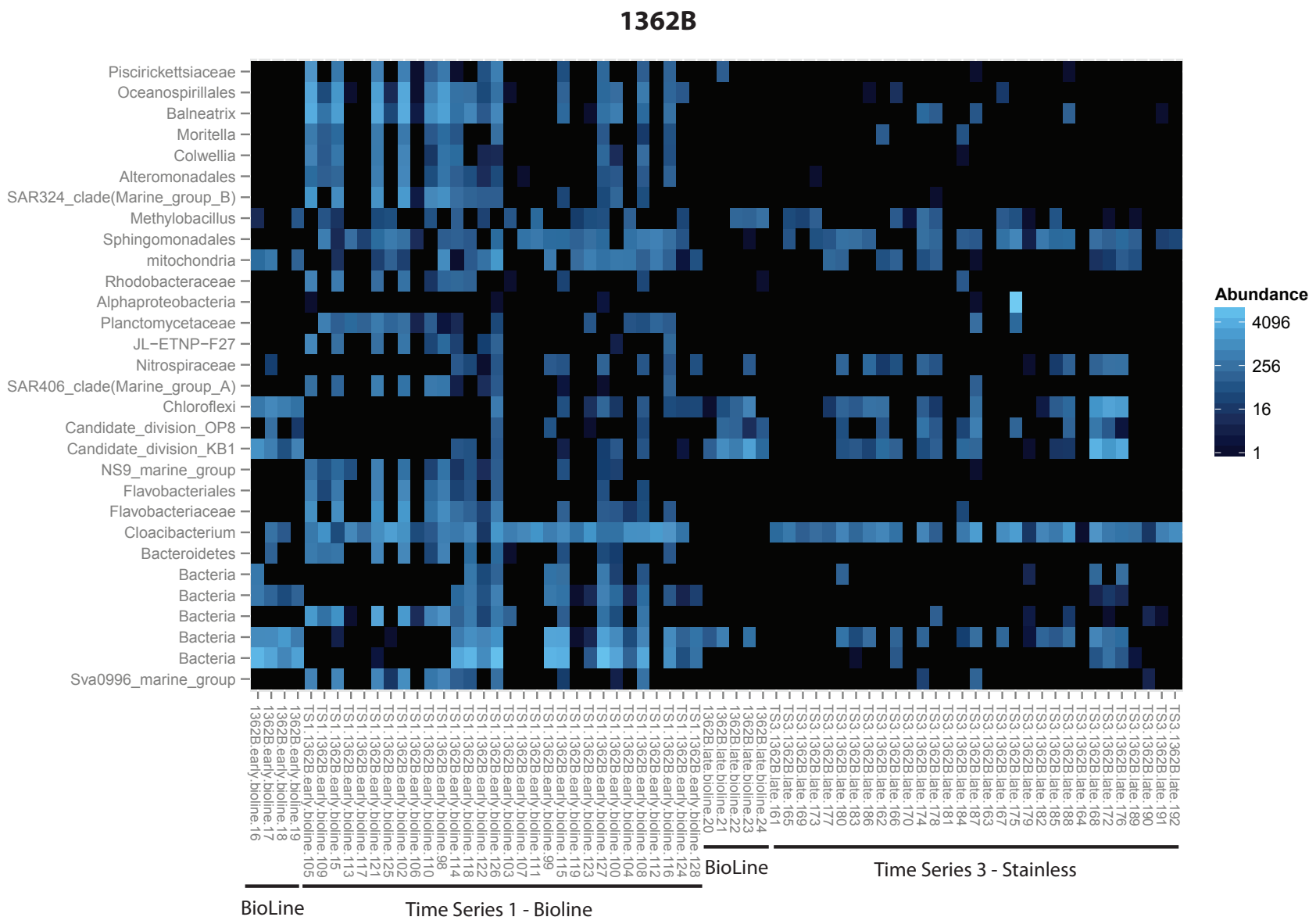

Figure 6.15 Heat map of top 30 bacterial UPARSE clusters identified in U1362B samples. The total number of clusters detected is indicated in the scale bar.

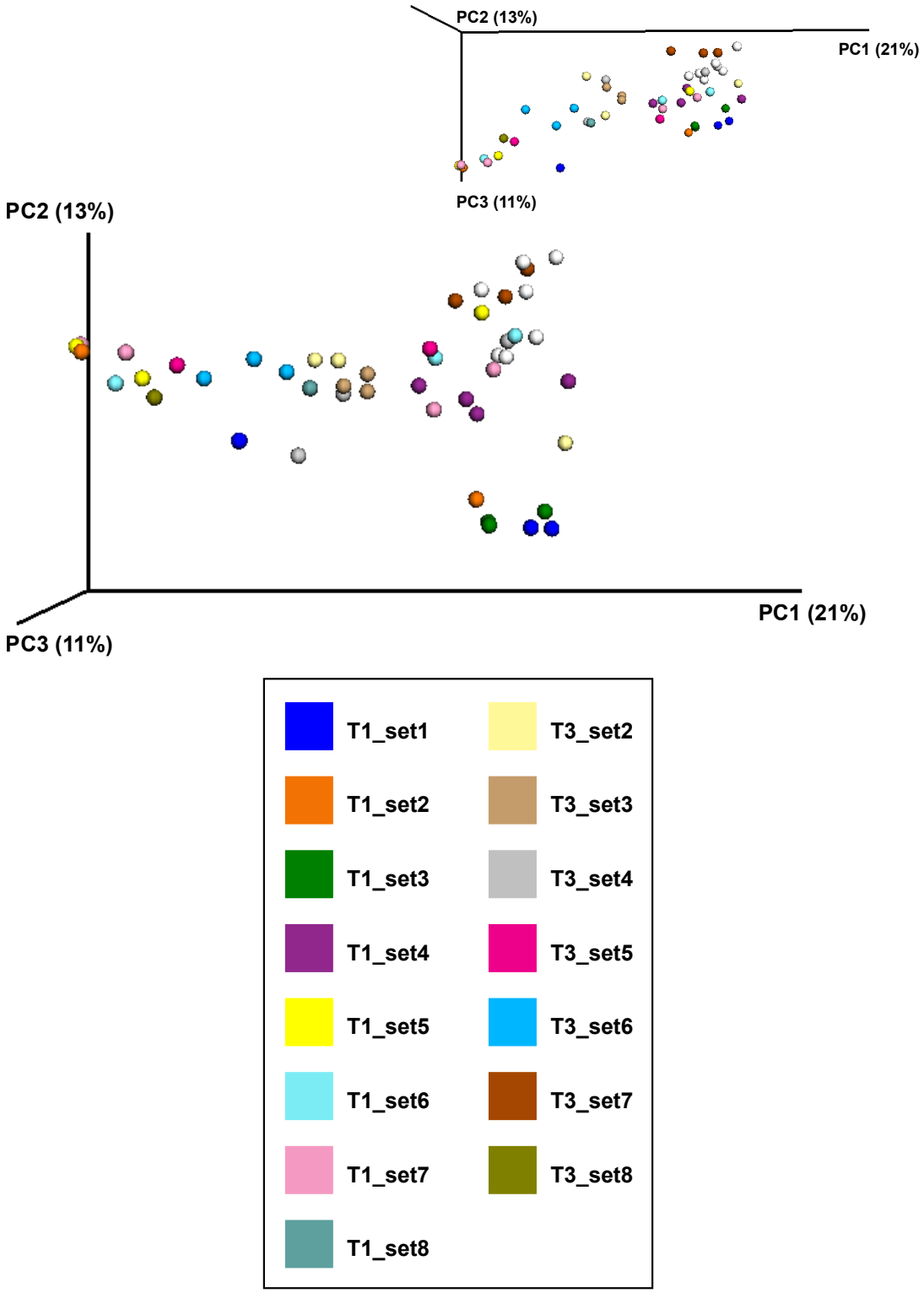

Figure 6.16 Principle coordinates analysis (PCoA) of Bray-Curtis dissimilarity indices using subsurface fluid time-series #1 and #3 UPARSE clusters. Samples are colored by time of collection – set1 is first and set8 is last. Bag samples collected from 1362B are indicated with white dots for comparison purposes. Plots are scaled according to the percentage variation explained via the top three principal components. Rotated plots are shown above-right to reveal additional features occluded in depication

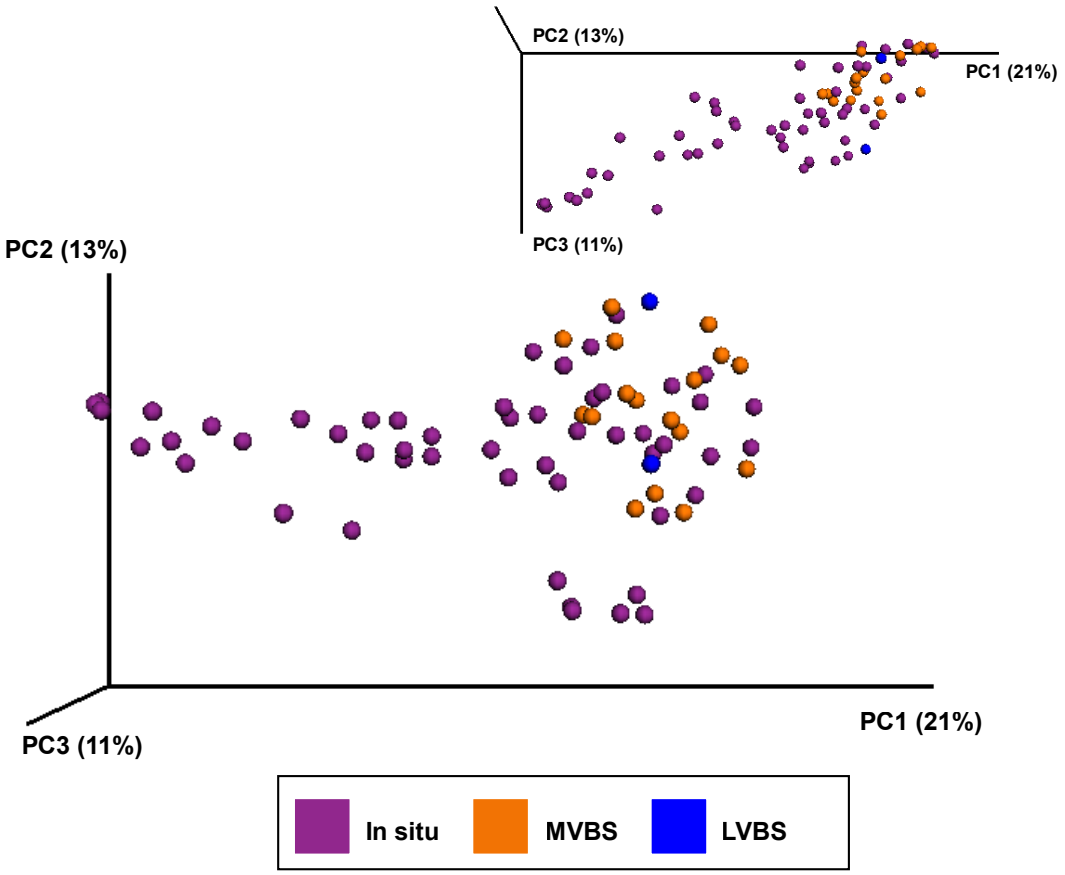

Figure 6.17 Principle coordinates analysis (PCoA) of Bray-Curtis dissimilarity indices using subsurface fluid UPARSE clusters. Samples are colored by collection method. Plots are scaled according to the percentage variation explained via the top three principal components. Rotated plots are shown above-right to reveal additional features occluded in depication of the maximum variation within PC1 and PC2.

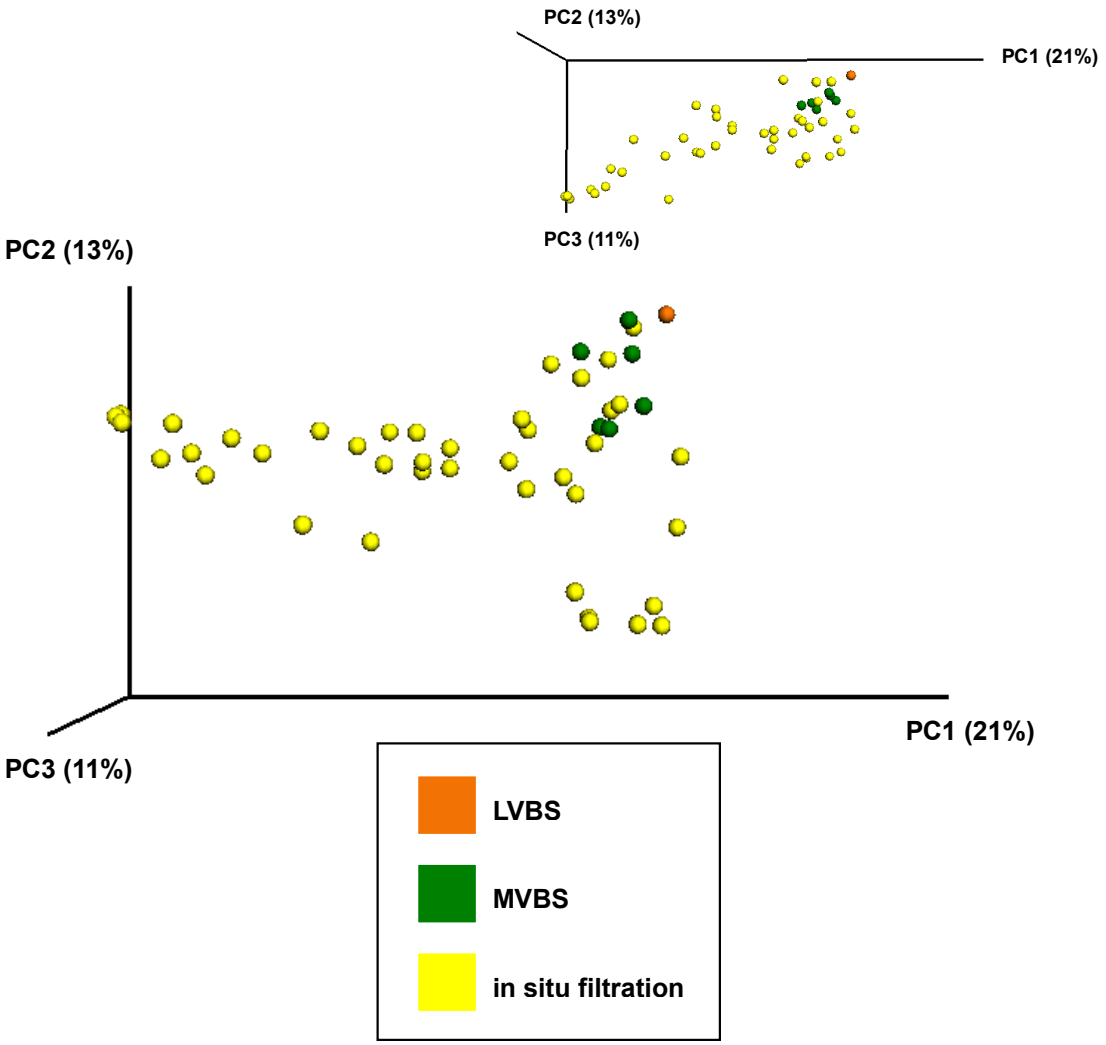

Figure 6.18 Principle coordinates analysis (PCoA) of Bray-Curtis dissimilarity indices using U1362B subsurface fluid UPARSE clusters. Samples are colored by collection method. Plots are scaled according to the percentage variation explained via the top three principal components. Rotated plots are shown above-right to reveal additional features occluded in depication of the maximum variation within PC1 and PC2.

CHAPTER 7

Conclusions and Discussion

By Sean Jungbluth

Jennifer F Biddle, Mark A Lever, and Michael S Rappé (co-authors)

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7.1 Research Synthesis

I have provided a description of the nature of the sediment-covered biosphere within the oceanic basement through the use of new borehole fluid sampling technology, which was used to conduct a multiyear (Chapters 2 and 5) and within-year time-series (Chapters 5 and 6) analysis of microbial communities inhabiting basalt-hosted ridge flank crustal fluids. By analyzing microbial communities found in fluids sampled from the latest versions of CORK observatories (Chapters 5 and 6), these sampling platforms were compared with respect to the quality of sampling for microbiology studies (Chapter 6). The significance of this work includes the first time-series analyses of ridge flank crustal fluid microbial communities from a spatial array of CORK borehole observatories penetrating young ocean basement aged ~1.2 to ~3.5 million-years (Chapters 2, 3, 5, and 6), which reveals an environment distinct from sediments (Chapters 4 and 6). This work currently represents the first application of next-generation sequencing to describe microbial communities within the deep biosphere environment (Chapters 5 and 6). Novel 16S rRNA gene diversity was described, and has expanded the catalogued diversity of microbial phylogenetic markers and indicated groups that might be ecologically-relevant in crustal fluids (Chapters 2, 3, 5, and 6). Microbial cell numbers and biomass were estimated from fluids collected from multiple CORKs, which helps to constrain the size and magnitude of the microbial life in the deep biosphere (Chapters 2, 5, and 6). Cellular morphologies included a range of shapes and sizes in subsurface fluids and seawater (Figures 7.1 – 7.46) (Chapters 2, 3, 5, and 6). In total, this work comprises the most thorough exploration to date of the microbial biosphere within sediment-covered basement fluids, because it uniquely combines advances in sampling technology with next-generation sequencing to understand microbial communities inhabiting the crustal ocean. This work serves as a basis for future studies into the biosphere within young ridge flank fluids, and should guide CORK-enabled microbiology fluid sampling efforts elsewhere.

7.2 Future Directions

So far, research on life within the oceanic crust is still in an early exploratory stage. While it is known that microbial life, consisting of *Bacteria*, *Archaea* (Table 7.1), viruses and fungi and perhaps further into the tree of life, exists deep into the crust, knowledge of these communities is still profoundly limited by access to fresh rock and fluid samples. So far, only a few sites have been sampled and analyzed, and those even have large vertical intervals missing due to poor core recovery of certain lithology types and limitations to depth of penetration. Even within rocks and rock fragments retrieved, the distribution of microbes and microbial biomass remains unclear. It is, for instance, not known what fraction of microbial biomass grows as epiliths on rock surfaces, as chasmoendoliths in rock fissures, as cryptoendoliths in

porous rock, or as euendoliths by actively penetrating into rock, let alone how the biomass of these attached-growing microbes compares to that of planktonic forms. Even less is known how and if microbial communities inhabiting different parts of rocks differ phylogenetically and in terms of their ecological niches. In the absence of analyses on the three-dimensional distribution of microbes throughout rocks and circulating fluids – both on microscopic and macroscopic scales – the global biomass of crustal microbes is a matter of speculation. It remains to be shown whether the vast ocean crust, which is distributed over 60% of the Earth's surface and ranges in thickness from 5–10 km, is a globally significant habitat in terms of its microbial biomass, or an ecological desert that is mainly of interest to microbiologists and biochemists with interest in extremophilic forms of life on Earth and beyond. The role of ocean crust-inhabiting microbes in Earth's biogeochemical cycles is also largely unknown. For instance, past studies have shown ocean crust to be a globally significant sink of carbon. Yet, the extent to which microbes play a role in this carbon sequestration remains to be shown (Orcutt *et al.*, 2011a). The same is true for seawater-derived sulfur. Sulfate from seawater is known to accumulate in parts of the ocean crust, and it is evident that microbial sulfate reduction plays a role locally (Lever *et al.*, 2013). Yet, chemical precipitation is also likely to be important (Alt and Shanks, 2011), and the relative extent to which sulfur accumulation is caused by microbes and chemical precipitation processes remains unknown. Currently, it is even uncertain what the main energy sources of life in the ocean crust are. In some locations, the main energy may come from photoautotrophically synthesized organic matter that was originally produced in illuminated surface environments, and enters crust on outcrops due to seawater circulation through crust or by mixing of bottom sediments with formation fluids. Due to the low concentrations of organic carbon in most bottom seawater and the typically highly refractory state of organic compounds at the bottom of sediment columns, one might expect microbial abundance in these places to be very small compared to other habitats in the biosphere. On the other hand, indigenous energy sources, such as electrons from microbial Fe(II) oxidation, H_2 produced by serpentinization reactions, or FTT synthesis of C1-compounds and small-chain organic molecules, might turn out to be quantitatively important (Gold, 1992; Bach and Edwards, 2003; Lever *et al.*, 2013). And based on modeled 120°C isotherms, it has been estimated that the habitable zone in crust ranges from 0.5 km in 1 million years ago (mya) old crust and increases over time to 5 km in 180 mya old crust (Heberling *et al.*, 2010). If indigenous energy sources are indeed important, and the habitable zone is as large as has been postulated, then the potential for microbial growth and proliferation in the oceanic crust is tremendous. This would mean that in addition to solar irradiance, chemical reactions within rocks are among the major energy sources of life on Earth. Ocean crust would then harbor one of the largest global pools of living biomass, with total biomass exceeding that in all aquatic and soil environments (Heberling *et al.*, 2010). The implications of a deep, rock-associated biosphere on Earth would be far-reaching with respect to the potential of crustal subsurface habitats

elsewhere in the universe to support life independent of sunlight. On a more proximal scale, while postulating questions of significance for the coming years, it is important that research remains grounded by already existing data. Based on clearly observable trends in the limited existing data sets, it is already possible to formulate hypotheses that will be testable in the near future. For instance, the discrepancy in microbes between CORK-associated fluids, downhole incubations and rocks from the same locations highlights the potentially diverse nature of life in oceanic crust, and indicates that there are many habitats and forms of crust-associated microbes. Even if these differences are due to sampling biases caused by the fact that different sampling methods pick up different habitat types, it seems likely that phylogenetic differences observed would reflect divergent ecological niches, such as fluid versus rock-associated life histories. This possibility also indicates that, currently, no one single sampling approach can satisfy all questions about crustal life. Instead, multiple techniques, including drilling, logging and borehole observatories, are needed to study endolithic, epilithic and free-living forms, respectively. Each of these tools provides complementary information that will improve our understanding of life and its role in crustal alteration and global geochemical cycles. To more exhaustively study the distribution of life in ocean crust, it will be important to further expand the use of the already existing, as well as develop innovative new research tools. With the construction of the drilling vessel *Chikyu*, which was designed to drill to 7 km below the seafloor, the obtenance of rock samples from deeper parts of oceanic crust, and even parts of the Mohorovičić discontinuity at the very boundary of crust and mantle, is within reach. Future CORK designs may enable the closing off of different borehole sections, and thereby enable microbial growth experiments throughout all major segments of the same borehole. Further developments in *in situ* fluid sampling devices, such as the Quicksilver Probe (Schlumberger Limited, USA), which can be installed within boreholes, will enable the sampling of pristine fluids from rock formations surrounding CORKs. This will eliminate the potential problem of fluid residence times within boreholes prior to sampling. Even short residence times may alter the chemical composition of fluids, making concentration measurements on molecules with high microbial turnover rates, e.g. intermediates such as H_2, VFAs and alcohols, especially problematic. Furthermore, the combination of automatic devices with sidewall coring techniques throughout the borehole, will make it possible to conduct and monitor experiments directly on rocks surrounding the boreholes, e.g. by means of isotopic tracer experiments, combined with auto-sampling devices (Jannasch *et al.*, 2004; Orcutt *et al.*, 2010) or *in situ* sample processors (Wankel *et al.*, 2010; Ussler *et al.*, 2013). Apart from questions with respect to the distribution and biomass, many unknowns concerning life in the ocean crust have yet to be addressed. These include the relationship of microbes to the age of the crust, the mechanisms by which microbes bore into crust and are involved in crustal transformations, the vertical and horizontal connectivity of ocean crust and the seeding of seafloor and subseafloor basalts (Orcutt *et al.*, 2011a). With the increasing efficiency of low biomass molecular methods

and the growing field of data on crustal life, the great challenges of these studies may be answered soon, provided that field expeditions continue to collect high-quality samples and continue to improve methods and instruments that enable the study of life in basalt using in-situ observatories.

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7.4 Appendix

Table 7.1 Phylogenetic diversity of selected small subunit ribosomal RNA gene clones recovered from multiple samples of fluids, environmental isolates, or subseafloor colonization experiments involving ocean crust boreholes

	Costa Rica Rift flank	Juan de Fuca Ridge flank				
Phylogenetic affiliation[a]	*896A*[b]	*1025C*[c]	*1026B*[d]	*U1301A*[e]	*U1362 A*	*U1362B*
Archaea						
Euryarchaeota						
Archaeoglobales	-	-	++	+	+	+
Parvarchaeum	-	-	+	+	-	-
Marine Benthic Group E (MBGE)	-	-	+	-	+	+
Methanococcales[f]	-	-	+	-	-	-
Methanomicrobia	-	-	+	+	-	-
Misc. Euryarchaeotic Group (MEG)	-	-	-	++	-	-
Thaumarchaeota						
Marine Group I			+	+	-	-
Bathyarchaeota – (Misc. Crenarchaeotic Group; MCG)	-	-	++	+++	+	+
Terrestrial Hydrothermal Spring Crenarchaeotic Group (THSCG)	-	-	-	-	+	+
Bacteria						
Actinobacteria						
Microbacteriaceae	-	-	+	+	-	-
Aminicenantes[g] **(OP8)**	-	-	-	++	+	+
Bacteroidetes						
CS_B045	-	-	-	++	-	-

Flavobacteriaceae						
Lutibacter	-	-	-	++	-	-
NB1-m	+	-	-	+	-	-
NS9	+	-	+	-	-	-
Calescamantes[g] (EM19/KB1)	-	-	-	++	+	-
Cyanobacteria						
Synechococcus	-	-	+	++	-	-
Firmicutes						
Caloranaerobacter	-	-	++	+	-	-
D8A-2	-	-	+	+	-	-
Desulforudis/Ammonifex	-	+	++	++	-	-
Desulfotomaculum	-	+	++	-	-	-
Peptococcaceae	-	-	++	-	-	-
RF3	-	-	+	++	-	+
Fusibacter	-	-	+	++	+	-
Marinimicrobia[g] (SAR406)	-	+	+	++		
Nitrospirae	-	-	-	+	+	+
Plactomycetes						
OM190	-	-	+	+	-	-
Phycisphaeraceae	+	-	-	+	-	-
Proteobacteria						
Alphaproteobacteria						
Hyphomicrobiaceae	-	-	+	+	-	-
Hyphomonadaceae	+	-	-	+	-	-
OCS116	++	-	+	-	-	-
Phyllobacteriaceae	+	-	-	+	-	-
Rhodobacteraceae						
IndB1-2	+	-	-	+	-	-
Leisingera	+	-	-	+	-	-
SAR11	-	+	+	++	-	+

SB1-18	+	-	-	+	-	-
Sphingomonadaceae	-	-	+	++	-	+
Betaproteobacteria						
Alcaligenaceae	-	-	+	+	-	-
Burkholderiaceae	-	-	++	+	-	+
Oxalobacteraceae	-	-	-	++	-	+
Deltaproteobacteria						
Desulfobacula	-	-	+	+	-	-
Desulfobulbaceae						
Desulfobulbus	-	+	+	+	-	-
Desulfocapsa	-	+	+	++	-	-
MSBL7	-	-	-	++	-	-
Desulfohalobiaceae	-	-	++	-	-	-
Desulfovibrionaceae	-	+	-	+	-	-
Desulfuromonadaceae	-	-	+	+	-	-
Nitropinaceae	-	-	+	+	-	-
SAR324	-	-	+	++	-	-
Epsilonproteobacteria						
Sulfurimonas	+	-	+	+++	-	+
Gammaproteobacteria						
9NBGBact_8	+	-	+	+	-	-
Alcanivoracaceae	-	-	+	++	-	-
Alteromonadaceae						
Marinobacter	-	-	++	++	-	-
Colwelliaceae	-	-	-	++	-	-
Ectothiorhodospiraceae	+	-	-	+	-	-
Halomonadaceae	-	-	-	++	-	-
JL-ETNP-Y6	-	-	+	++	-	-
JTB35	-	-	+	++	-	-
Methylophaga	+	-	+	+	-	-

Moraxellaceae	-	+	-	+	+	+
Oceanospirillaceae	-	-	-	++	-	-
OM182	-	-	-	++	-	-
Pseudoalteromonadaceae	-	-	++	+	-	-
Pseudomonadaceae	-	-	+	++	+	+
Xanthomonadaceae						
Thermomonas	-	-	-	++	-	-
Thiomicrospira	+	-	++	++	-	-
ZD0405	-	-	+	++	-	-
Thermotogae						
EM3	-	-	-	-	+	+
Kosmotoga	-	-	++	-	-	-
Thermosipho	-	-	++	-	-	-
Verrucomicrobia						
Arctic97B-4	-	-	++	-	-	-
Roseibacillus	+	-	-	+	-	-
Eukaryota						
Collodaria	-	-	-	++	-	-

[a]Phylogenetic affiliations were determined using SILVA SSU database release 115. However, in some cases manual phylogenetic assignment was required because the SILVA taxonomy was inconsistent. In these instances, lineages were named after the first gene clone derived from the group. Groups commonly found across all samples or that were the most abundant gene clones detected in reliable investigations of microbial community structure are indicated in boldface, with exception to plausiable contaminant groups: *Marinimicrobia*, SAR11, *Sphingomonadaceae*, *Bulkholderiaceaeare Moraxellaceae*, and *Pseudomonadaceae*.

[b](Nigro *et al.*, 2012) [c](Jungbluth *et al.*, 2014) [d](Cowen *et al.*, 2003; Huber *et al.*, 2006; Jungbluth *et al.*, 2014; Nakagawa *et al.*, 2006) [e](Orcutt *et al.*, 2011b, Jungbluth *et al.*, 2013; Smith *et al.*, 2011) [f]Included due to high abundance in black rust CORK sample (Nakagawa *et al.*, 2006)

[g]Candidate phyla names were adapted from (Rinke *et al.*, 2013; Meng *et al.*, 2014)

- Not detected; + Detected once; ++ Multiple samples taken; detected in multiple samples; +++ Multiple samples taken; detected in all samples; clones from Smith *et al.* (2011) not included in this assessment due to the paucity of SSU rRNA genes reported.

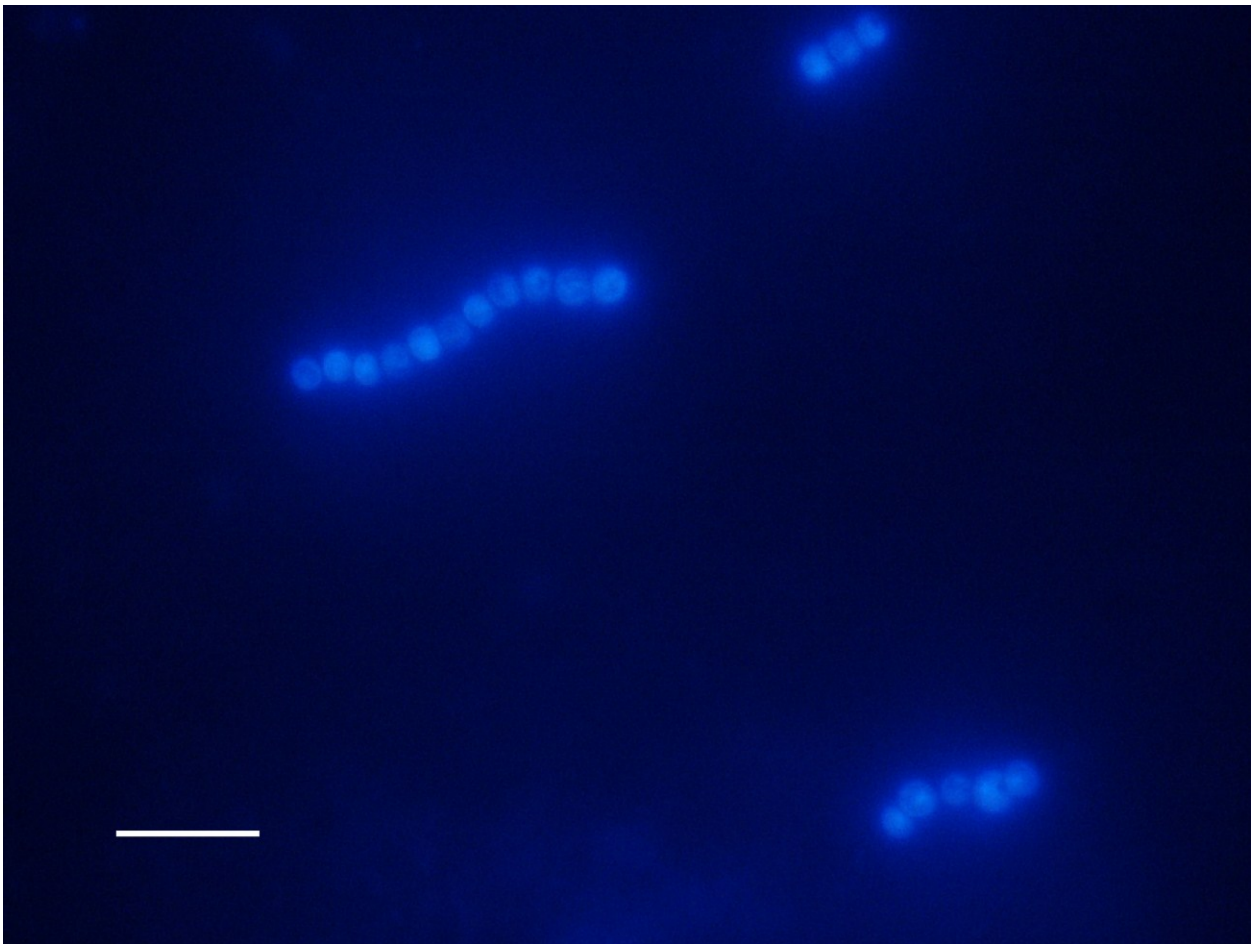

Figure 7.1 Micrograph of DAPI-stained borehole U1301A sample from June 19, 2010. Scale bar is 10 µm.

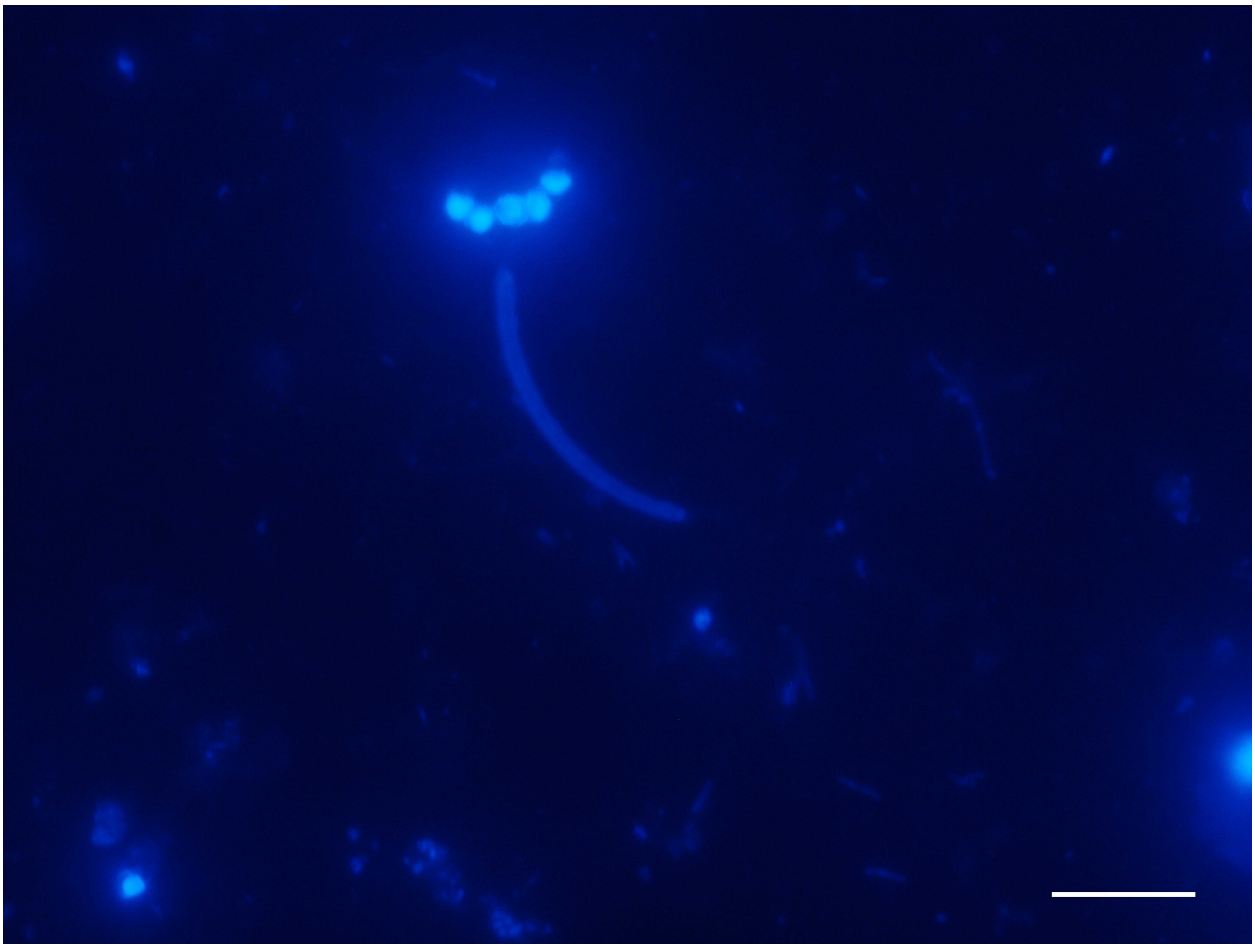

Figure 7.2 Micrograph of DAPI-stained borehole U1301A sample from June 19, 2010. Scale bar is 10 µm.

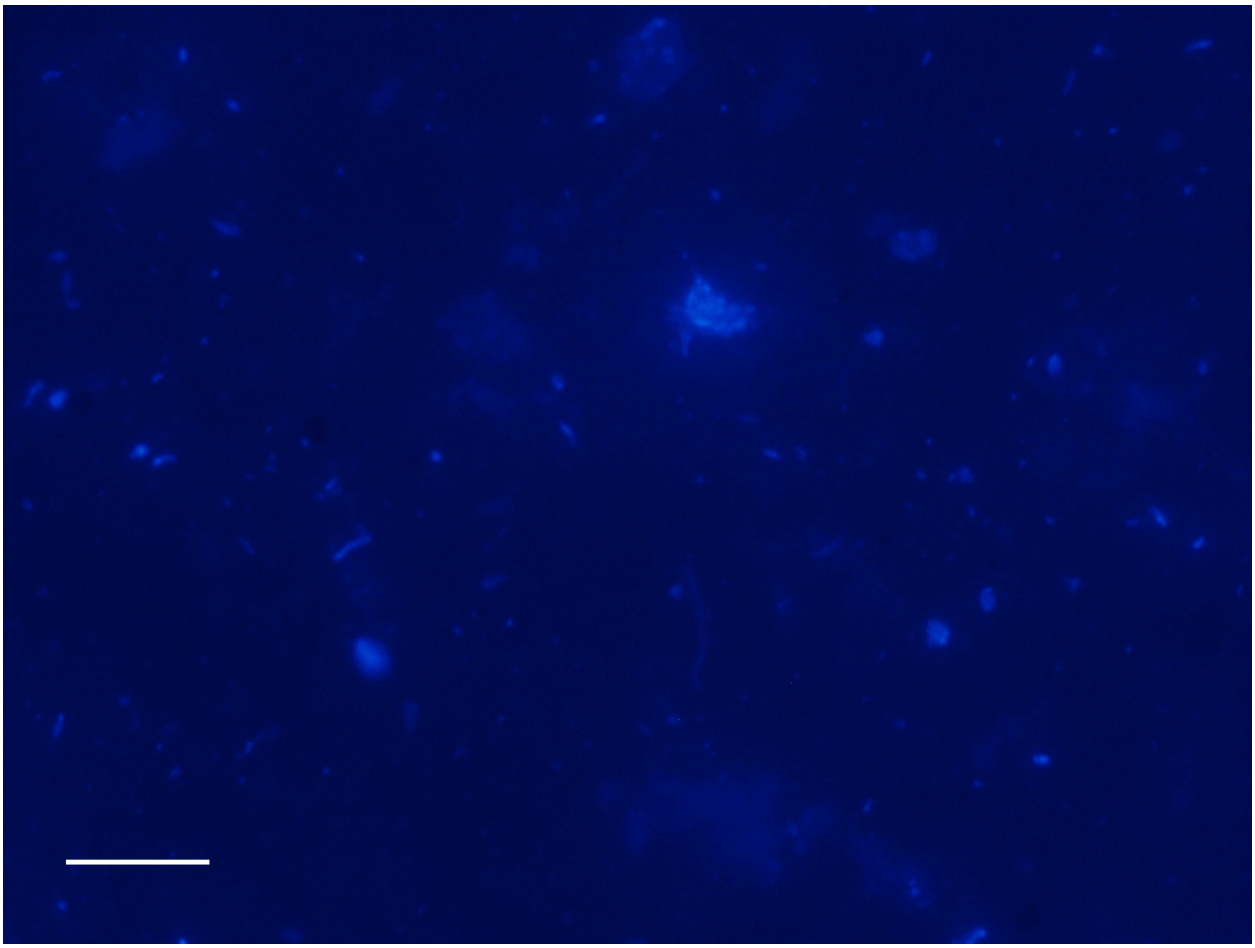

Figure 7.3 Micrograph of DAPI-stained borehole U1301A sample from June 19, 2010. Scale bar is 10 µm.

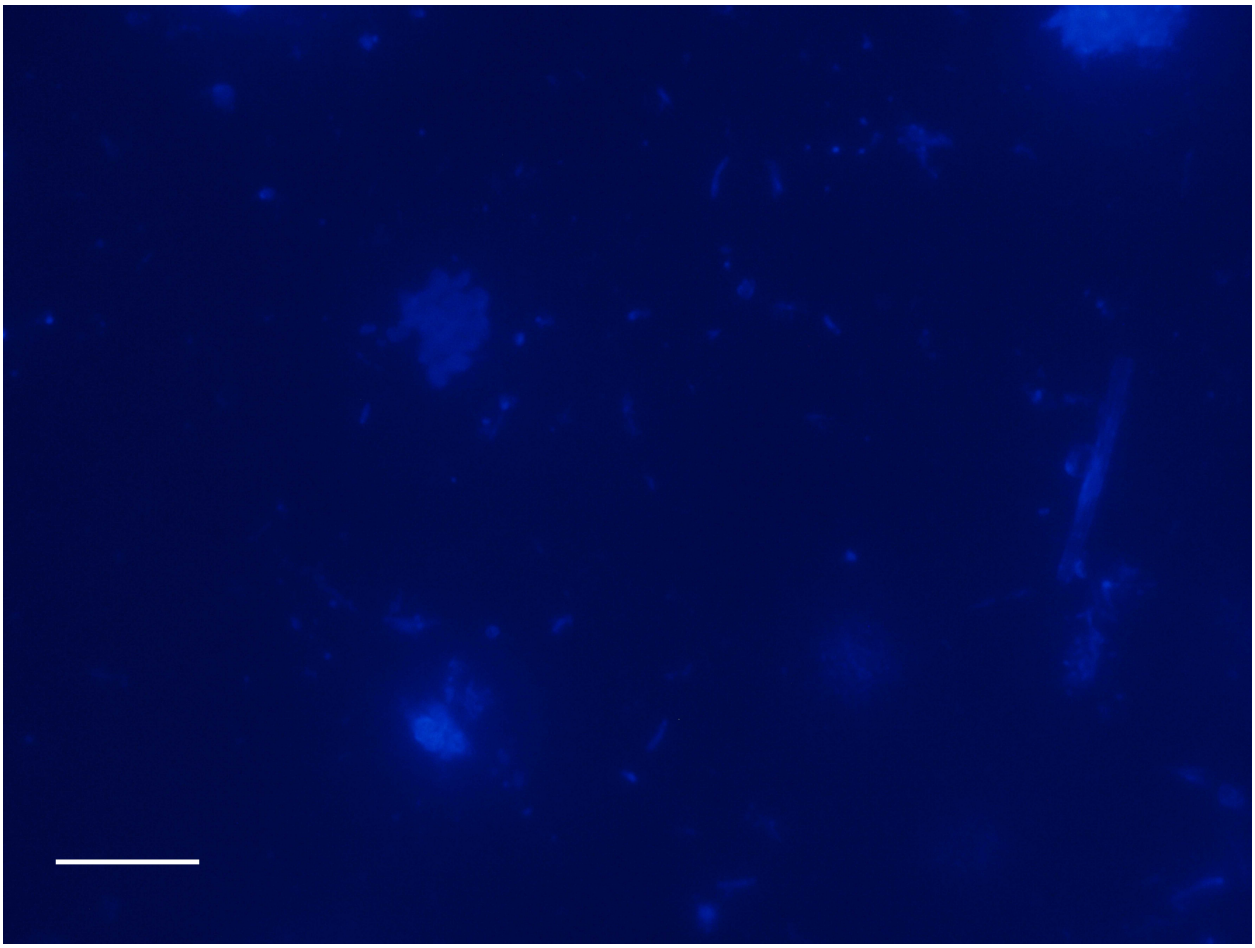

Figure 7.4 Micrograph of DAPI-stained borehole U1301A sample from June 19, 2010. Scale bar is 10 µm.

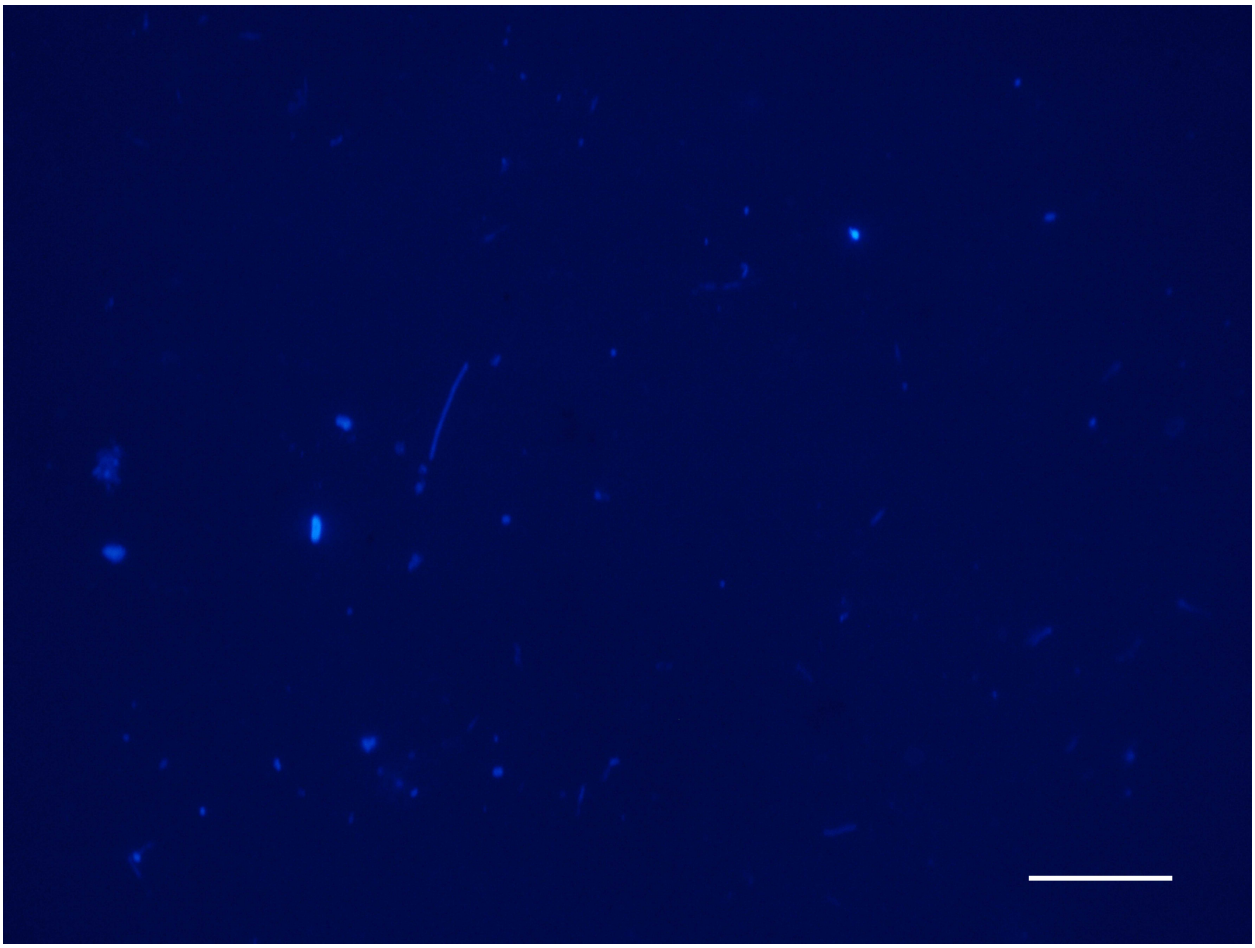

Figure 7.5 Micrograph of DAPI-stained borehole U1301A sample from June 20, 2010. Scale bar is 10 µm.

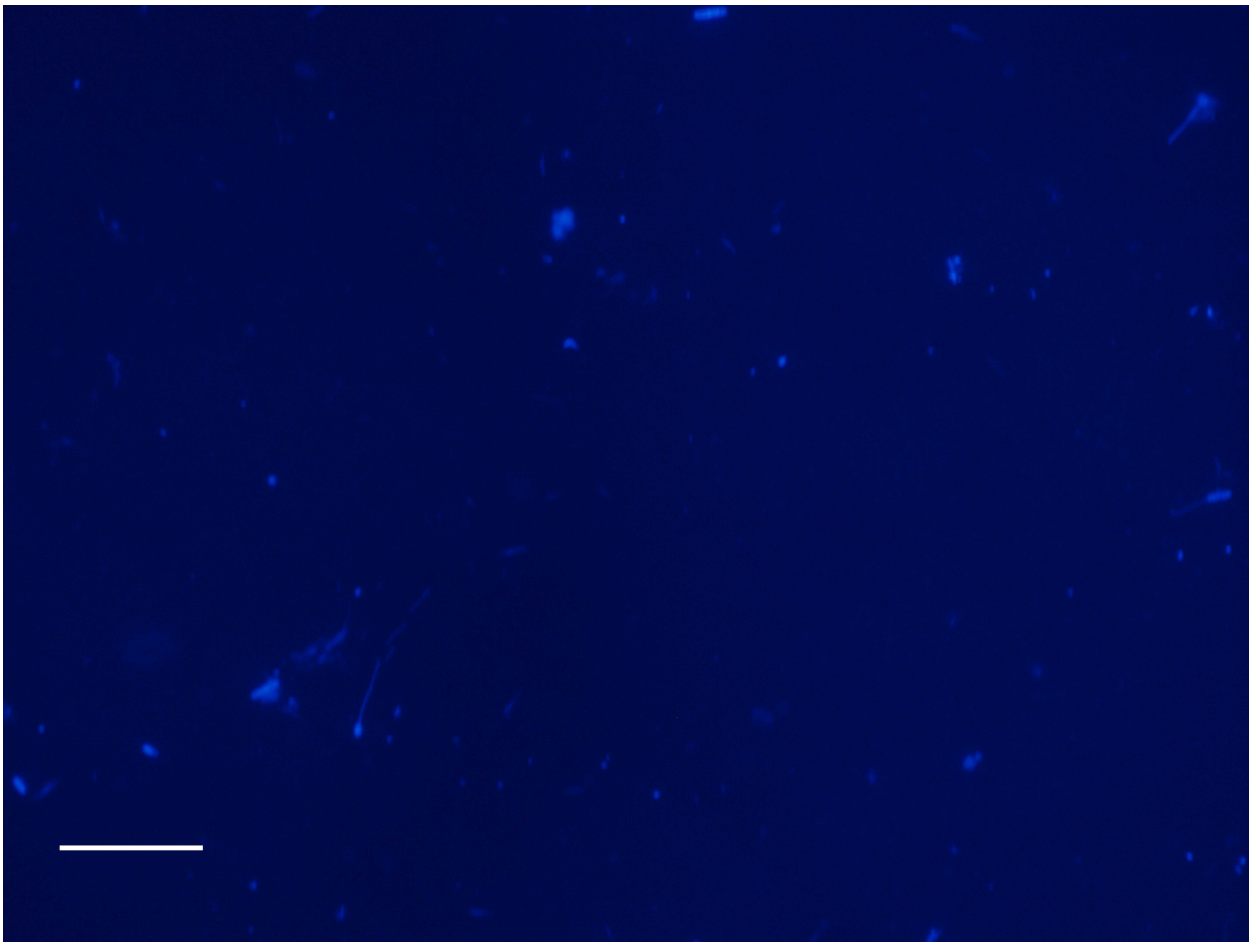

Figure 7.6 Micrograph of DAPI-stained borehole U1301A sample from June 20, 2010. Scale bar is 10 µm.

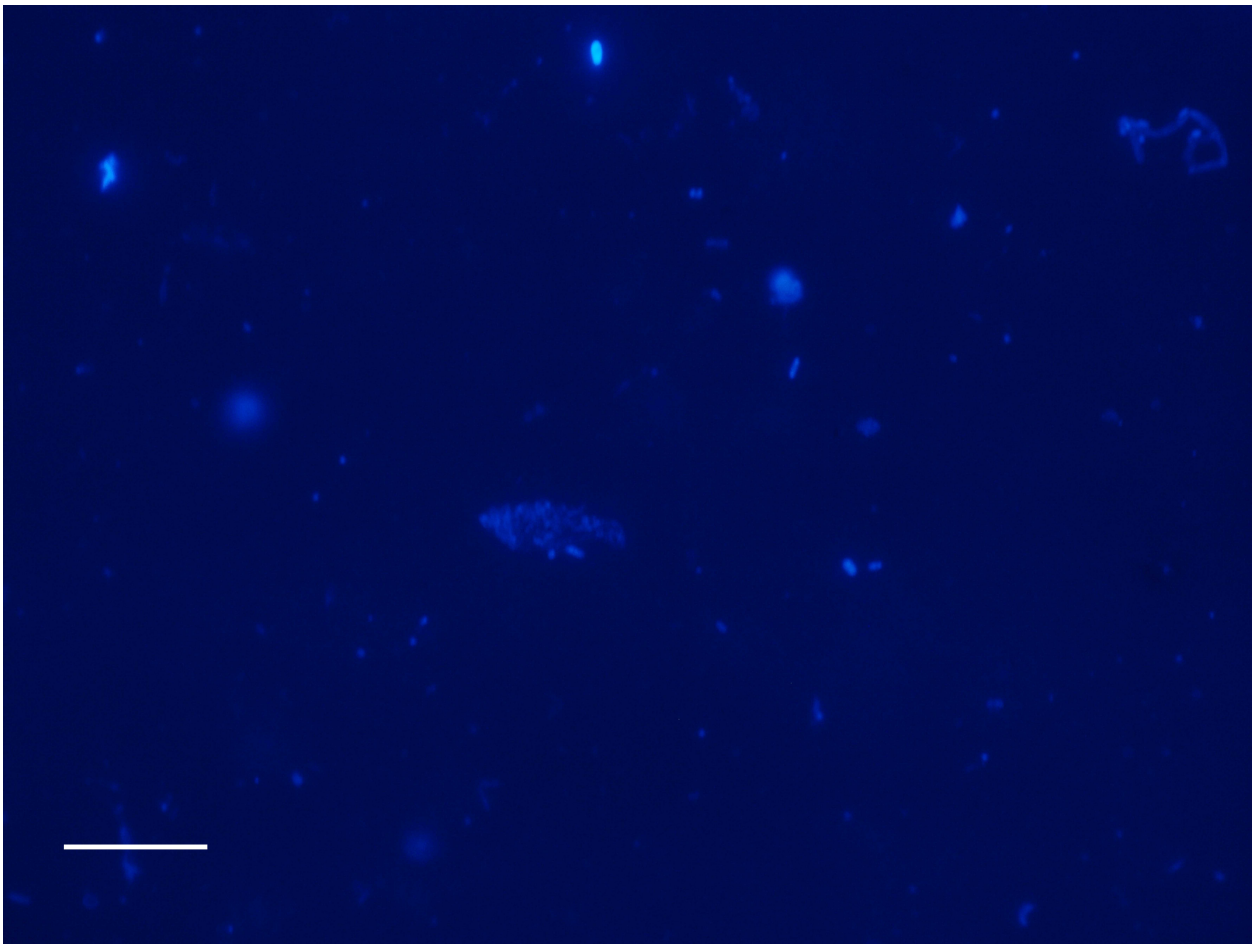

Figure 7.7 Micrograph of DAPI-stained borehole U1301A sample from June 20, 2010. Scale bar is 10 µm.

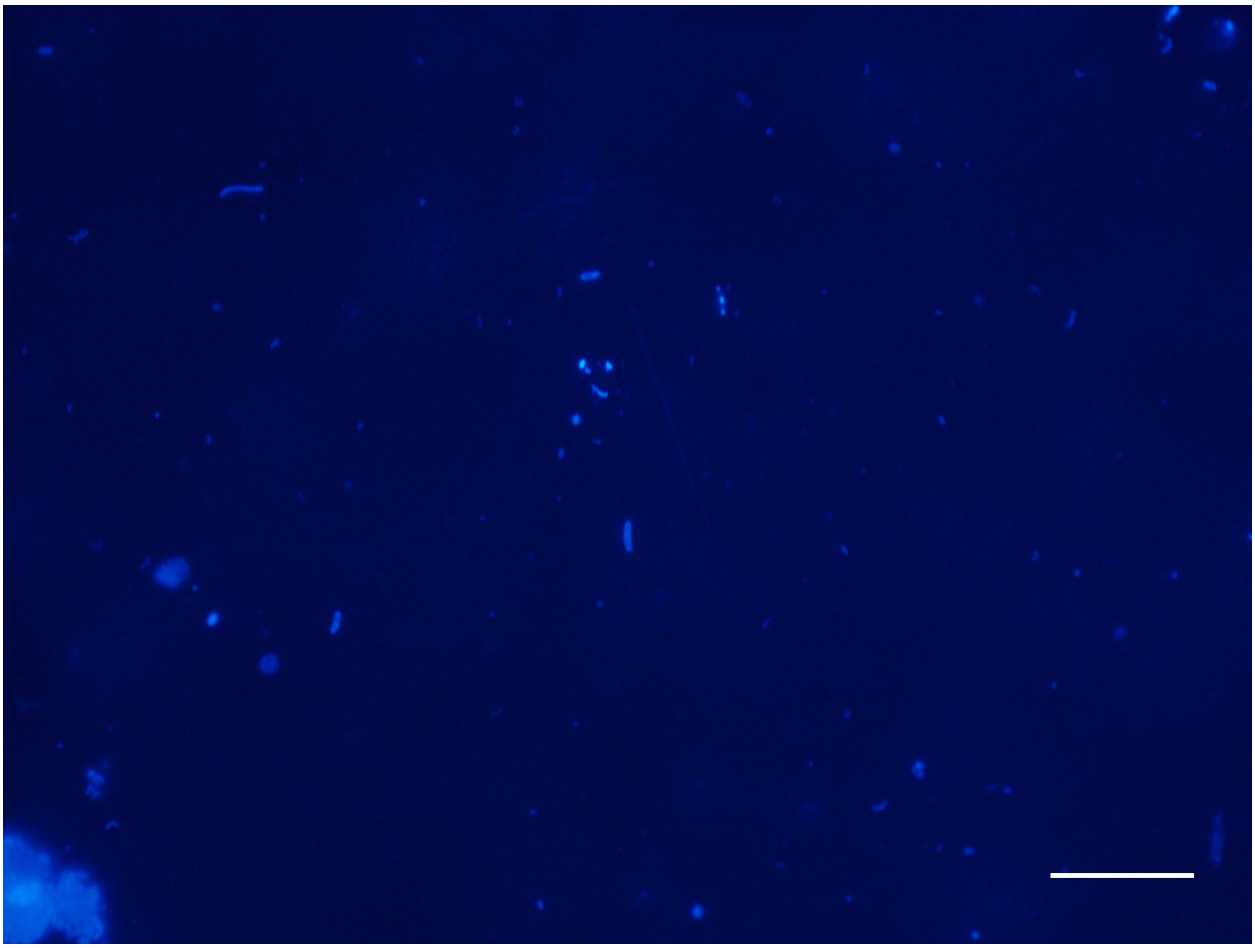

Figure 7.8 Micrograph of DAPI-stained borehole U1301A sample from June 23, 2010. Scale bar is 10 µm.

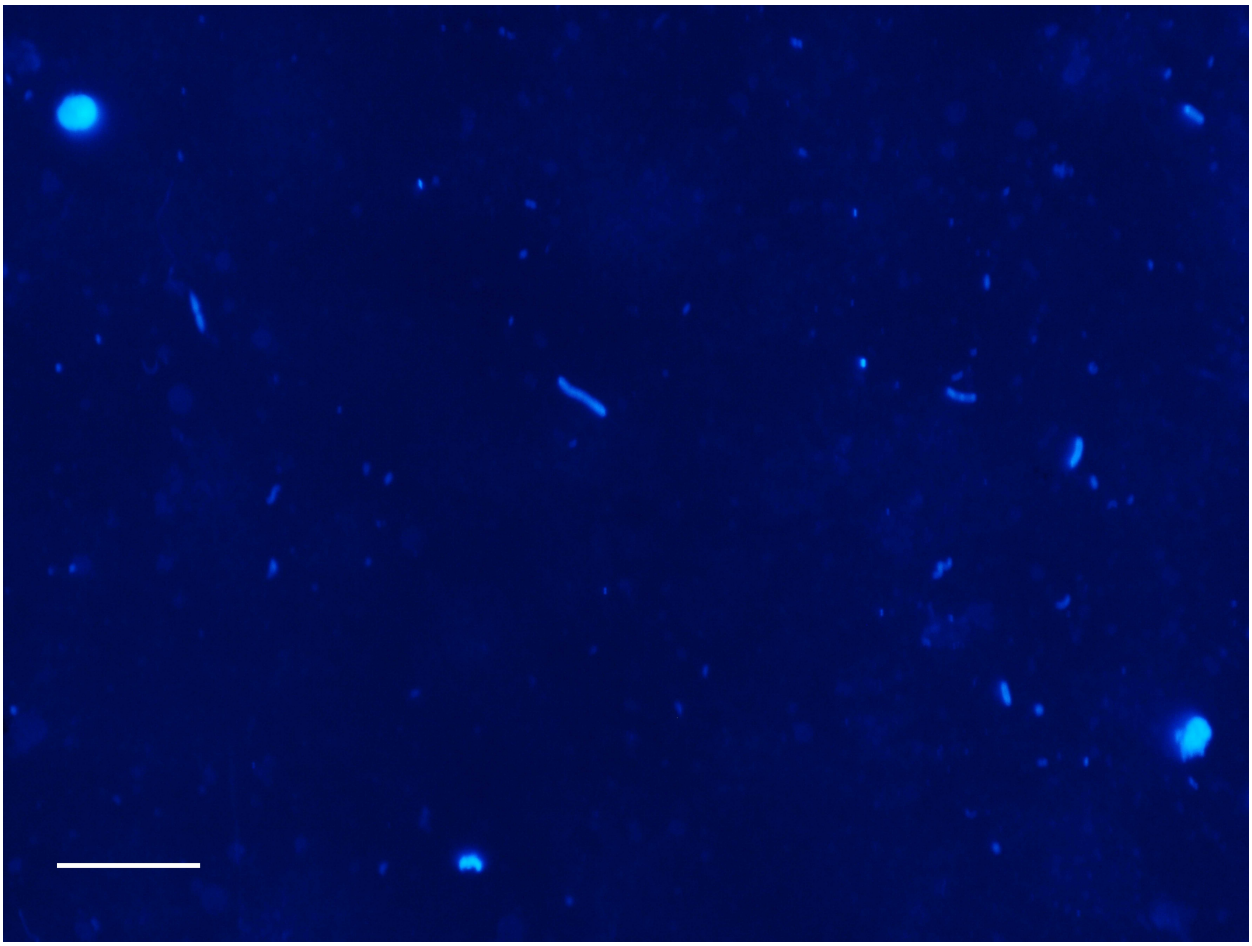

Figure 7.9 Micrograph of DAPI-stained borehole U1301A sample from June 23, 2010. Scale bar is 10 µm.

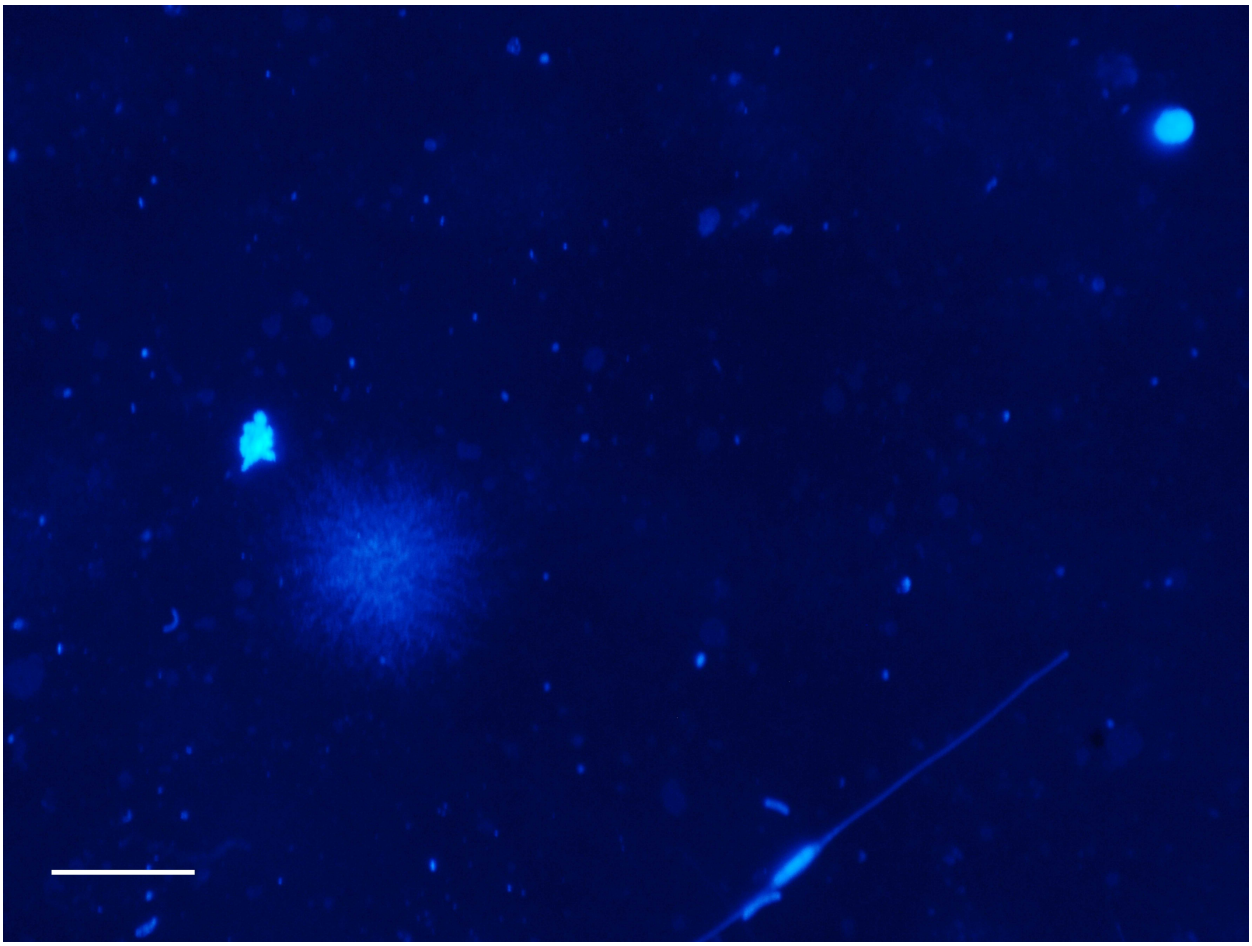

Figure 7.10 Micrograph of DAPI-stained borehole U1301A sample from June 23, 2010. Scale bar is 10 µm.

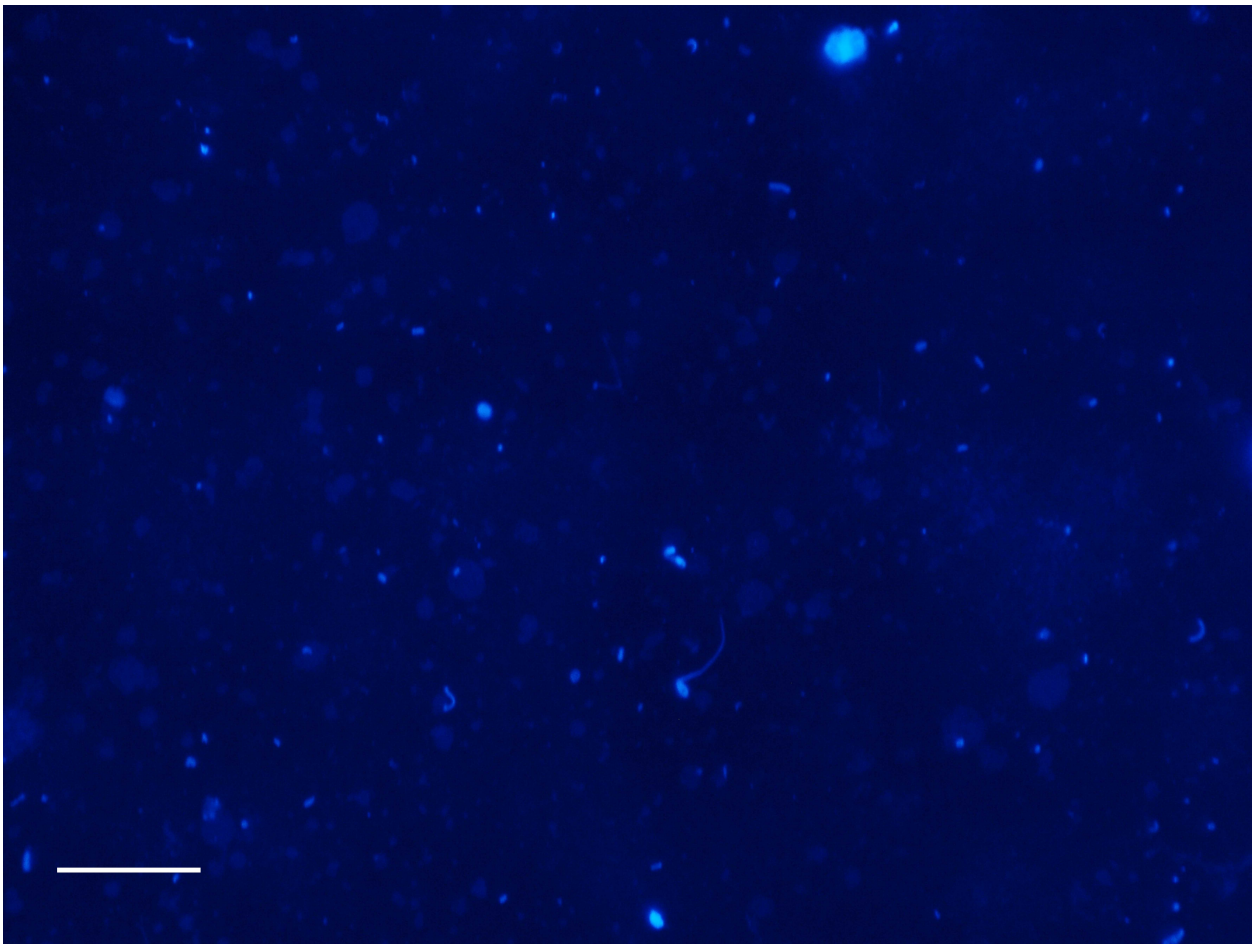

Figure 7.11 Micrograph of DAPI-stained borehole U1301A sample from June 23, 2010. Scale bar is 10 µm.

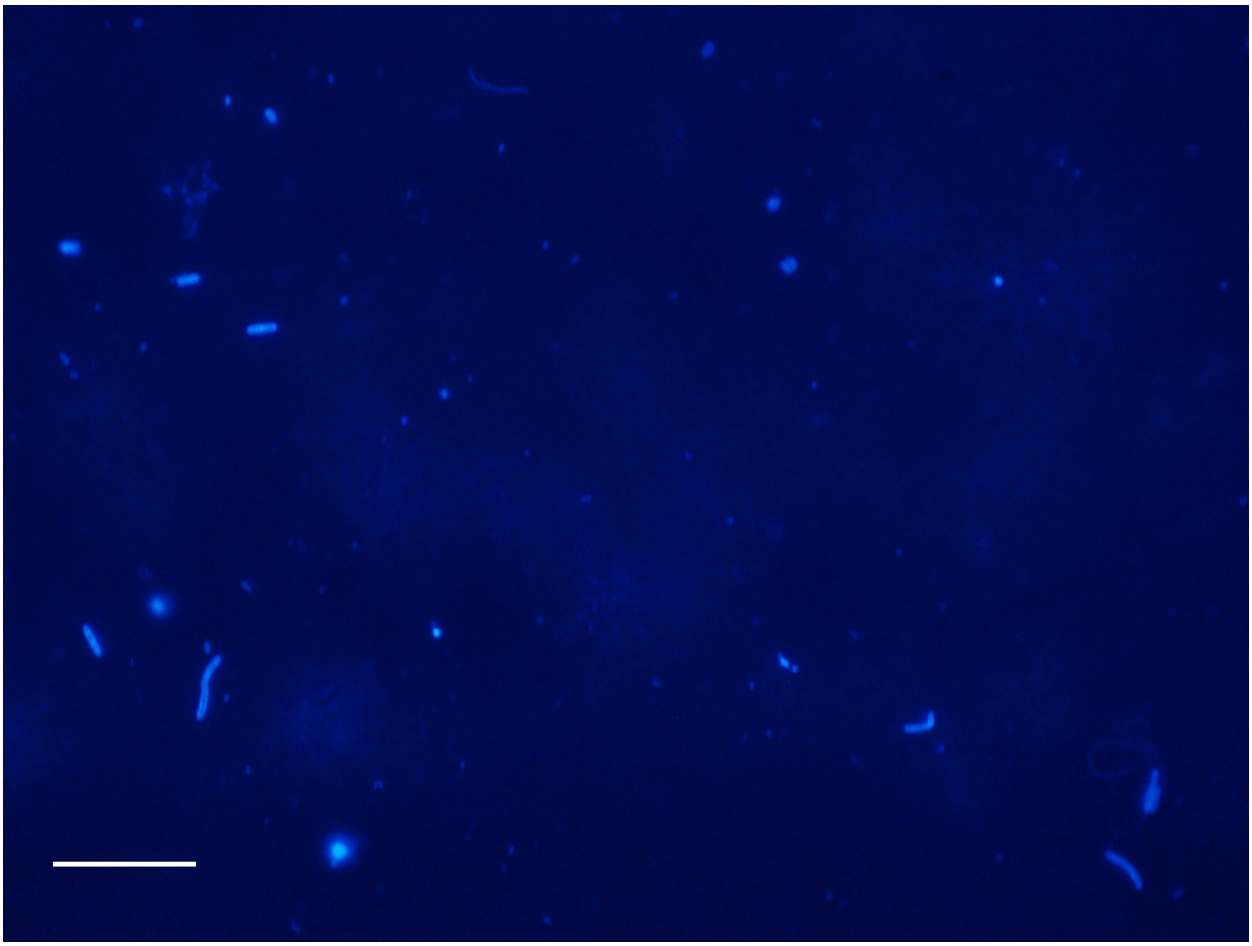

Figure 7.12 Micrograph of DAPI-stained borehole U1301A sample from June 23, 2010. Scale bar is 10 µm.

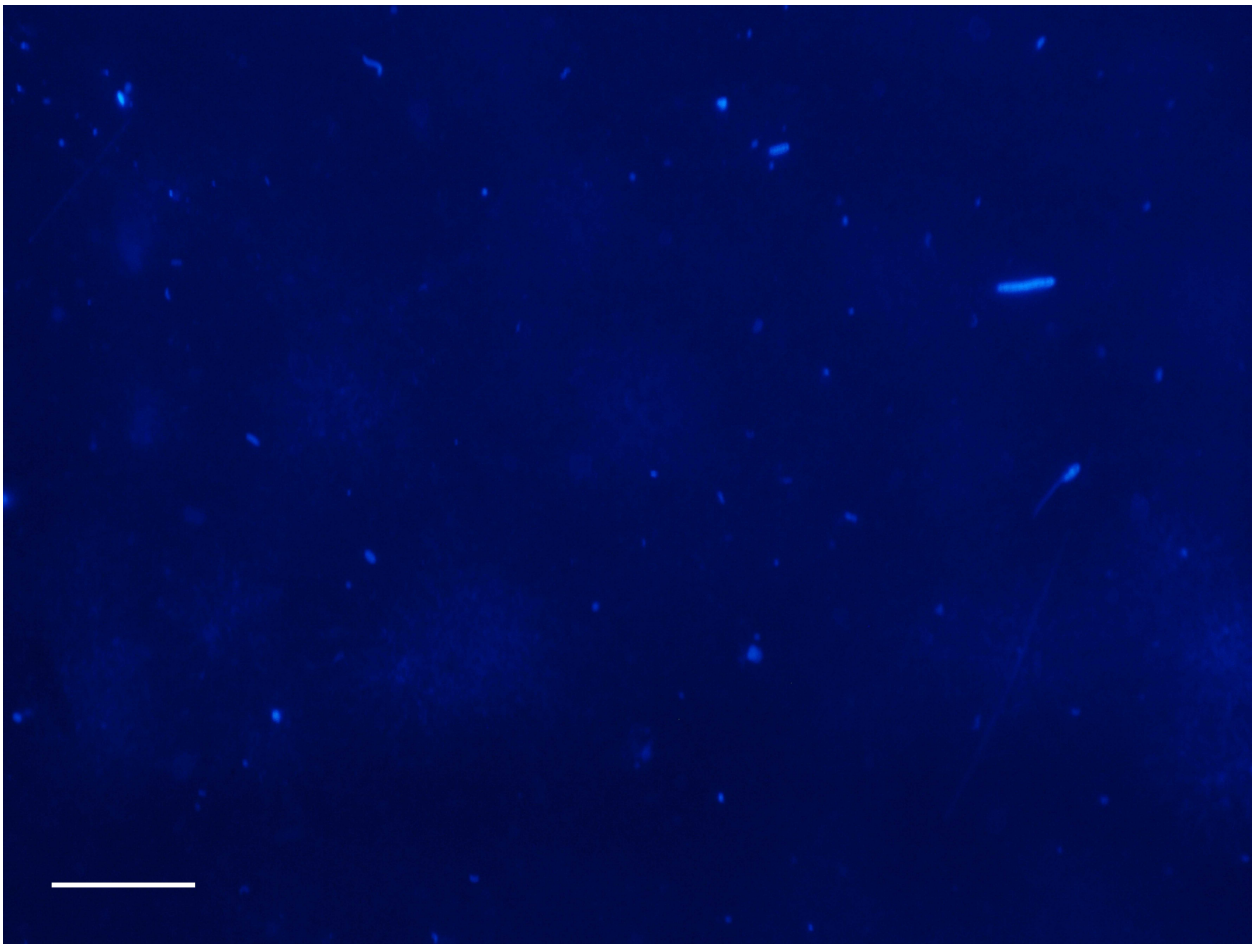

Figure 7.13 Micrograph of DAPI-stained borehole U1301A sample from June 23, 2010. Scale bar is 10 µm.

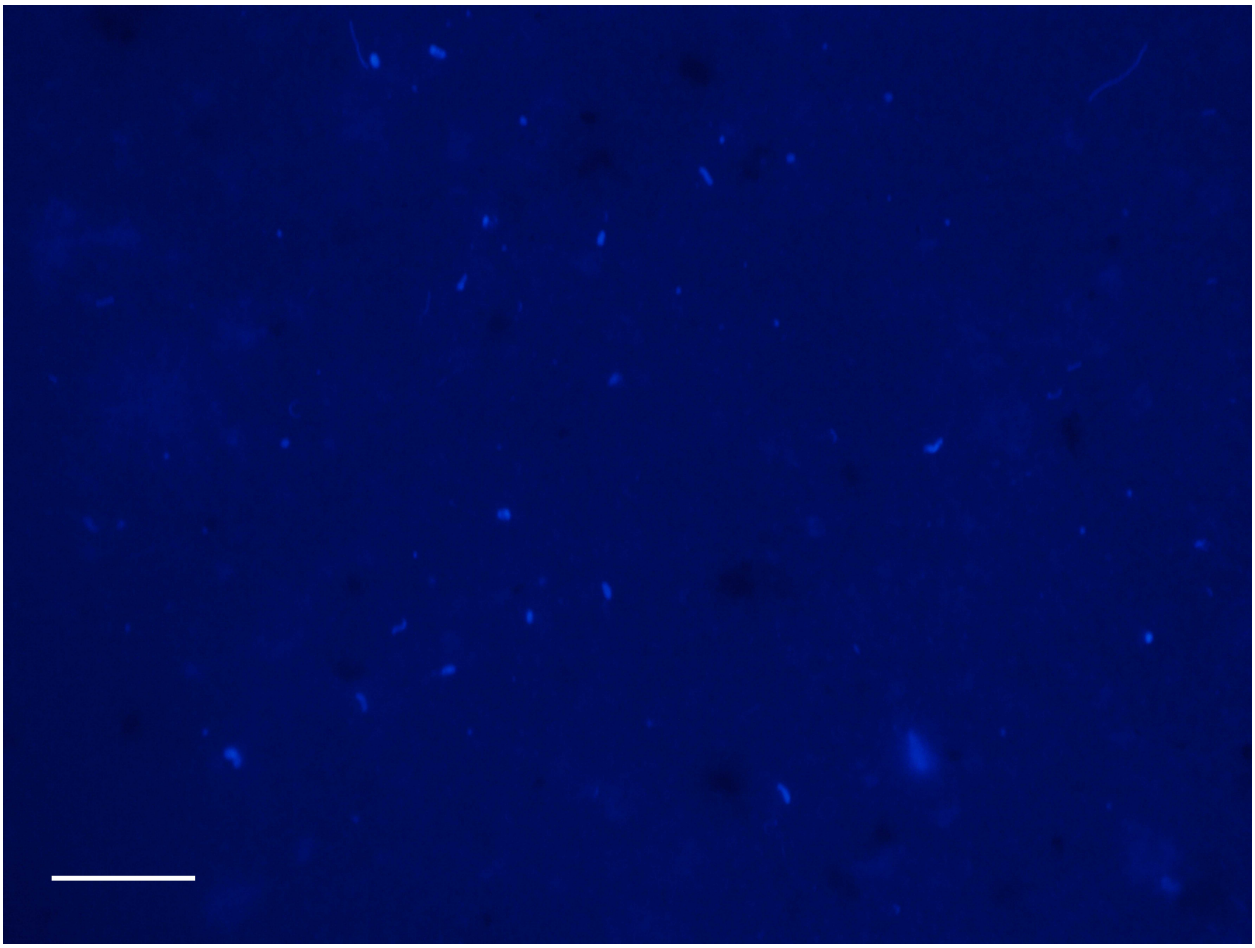

Figure 7.14 Micrograph of DAPI-stained borehole U1301A sample from June 30, 2010. Scale bar is 10 µm.

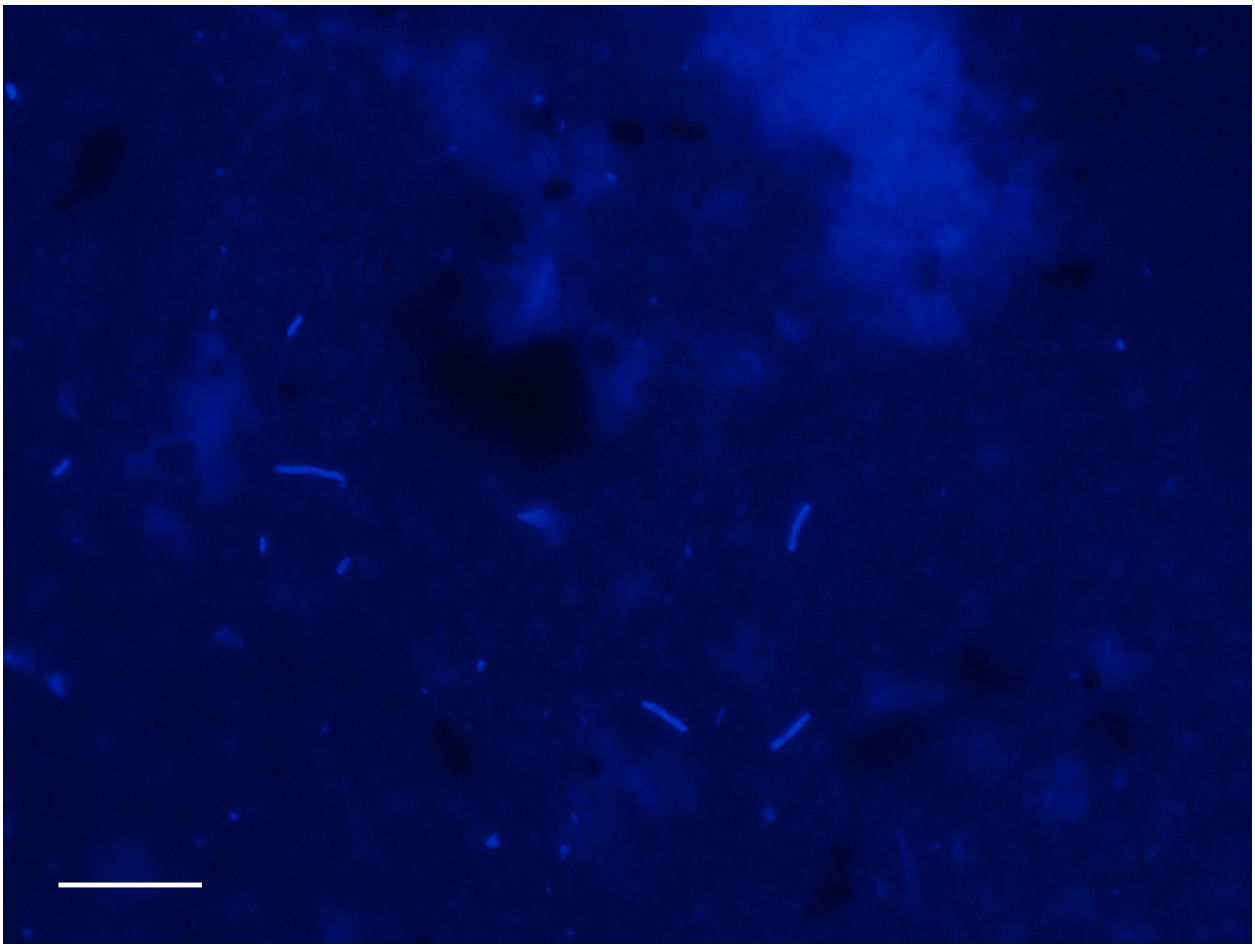

Figure 7.15 Micrograph of DAPI-stained borehole U1301A sample from June 30, 2010. Scale bar is 10 µm.

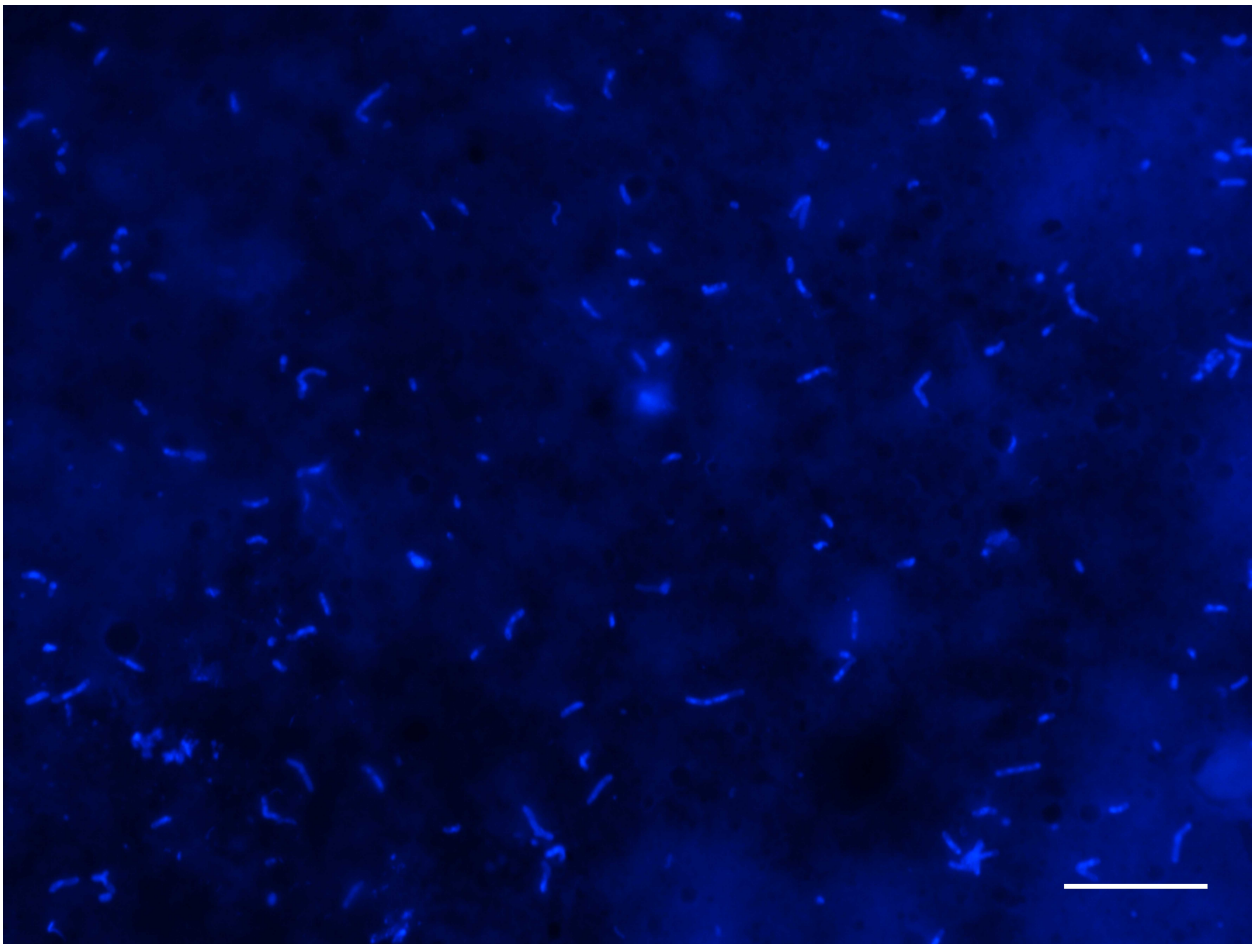

Figure 7.16 Micrograph of DAPI-stained borehole U1301A sample from June 30, 2010. Scale bar is 10 µm.

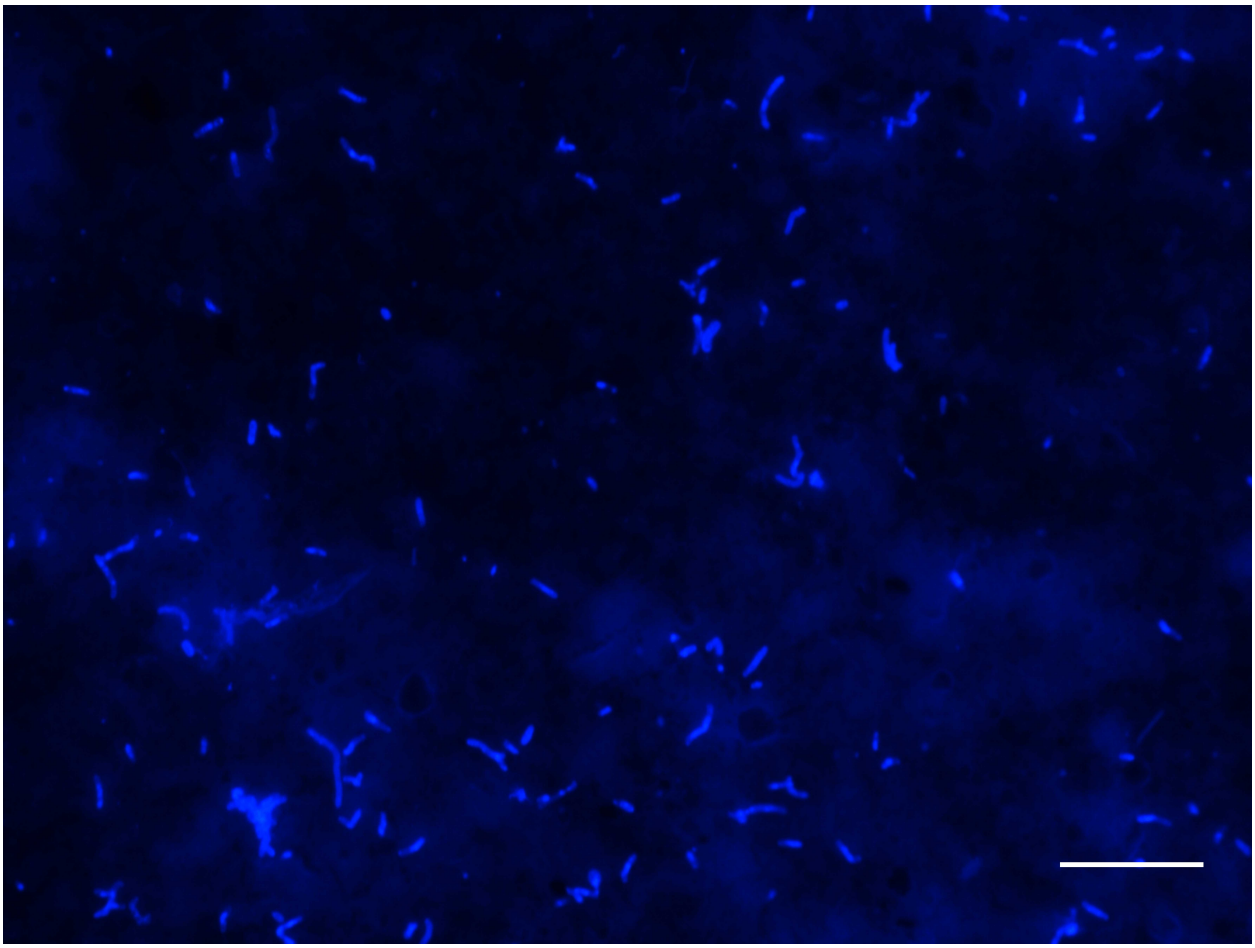

Figure 7.17 Micrograph of DAPI-stained borehole U1301A sample from June 30, 2010. Scale bar is 10 µm.

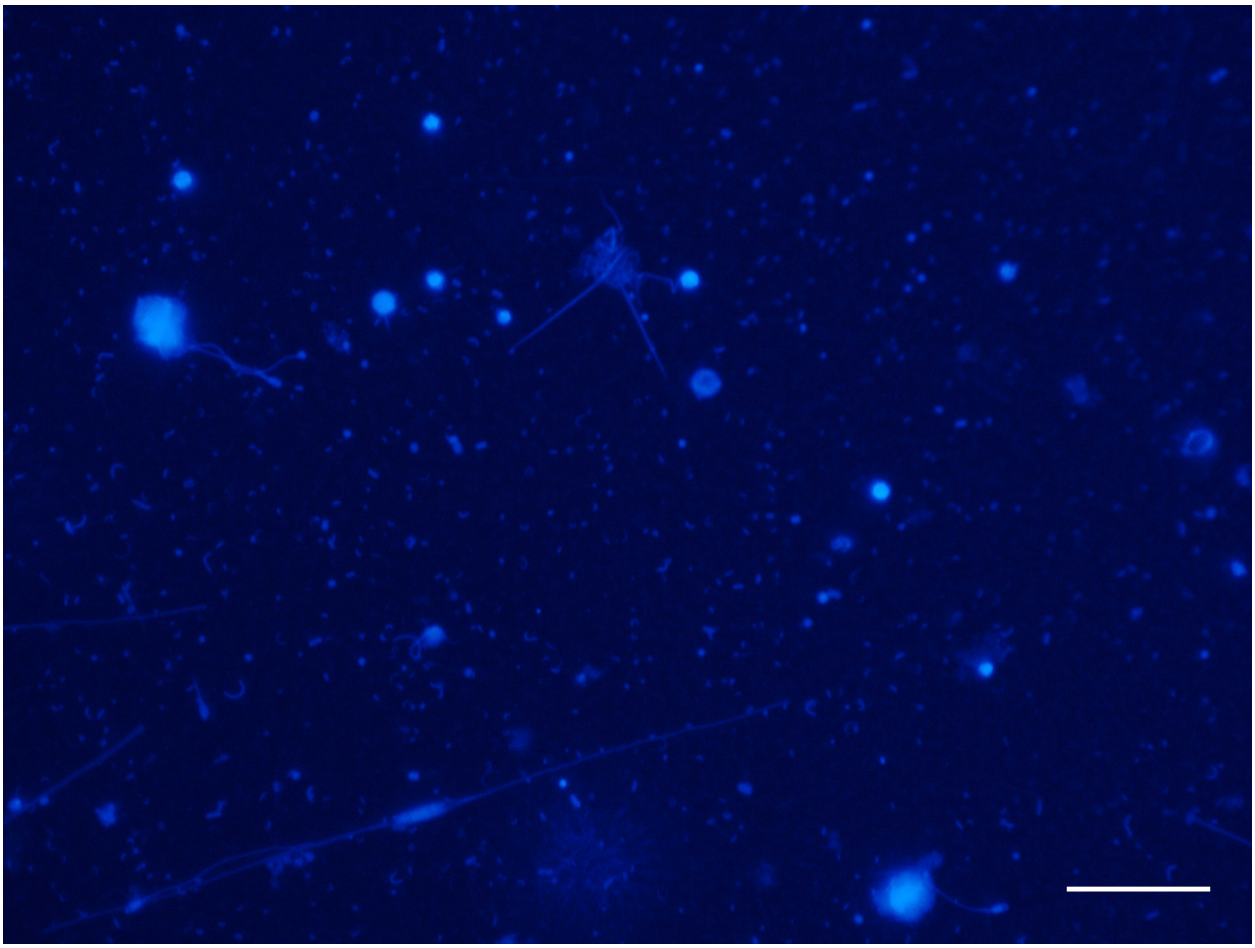

Figure 7.18 Micrograph of DAPI-stained deep seawater sample collected nearby Hole 1025C June 27, 2010. Scale bar is 10 µm.

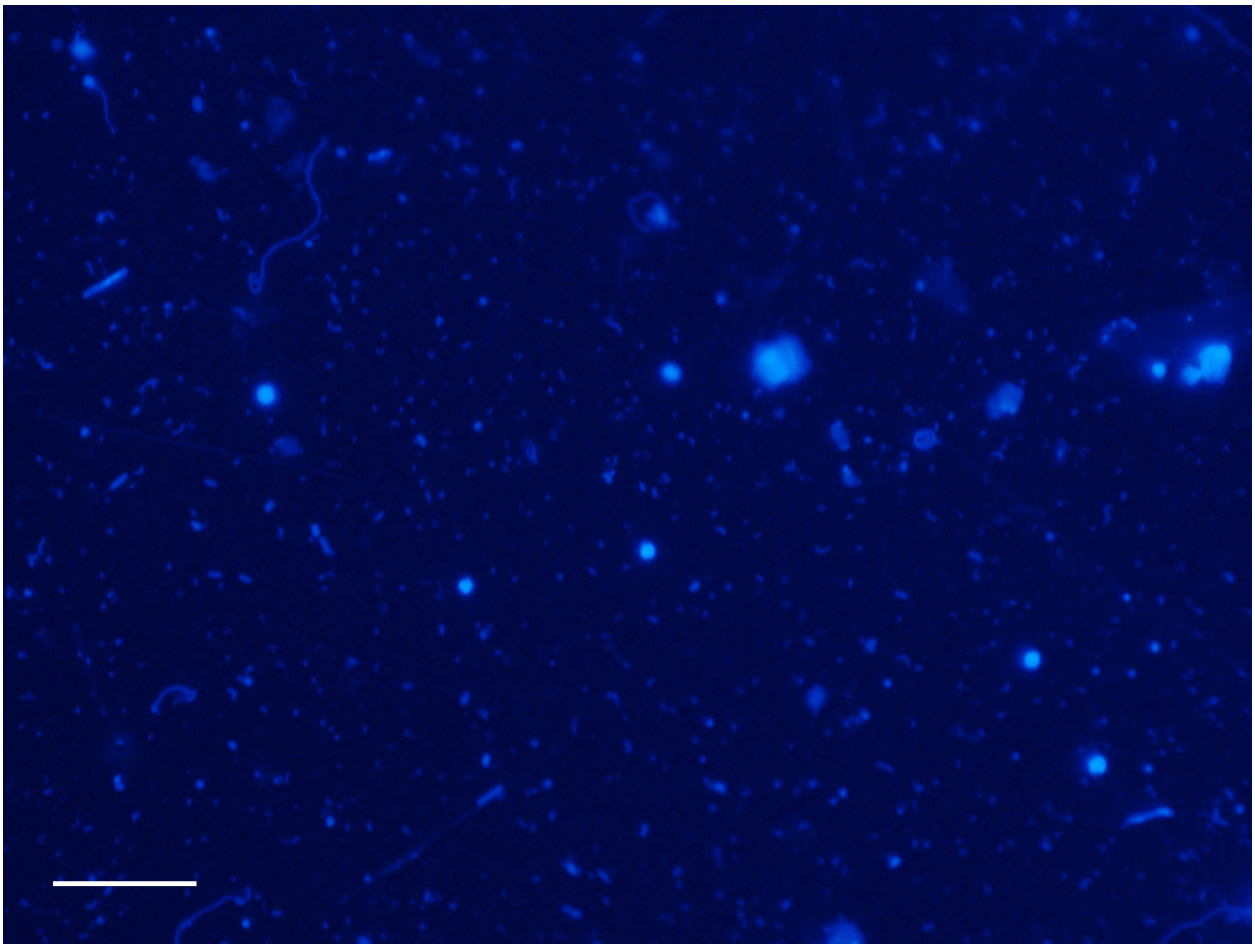

Figure 7.19 Micrograph of DAPI-stained deep seawater sample collected nearby Hole 1025C June 27, 2010. Scale bar is 10 µm.

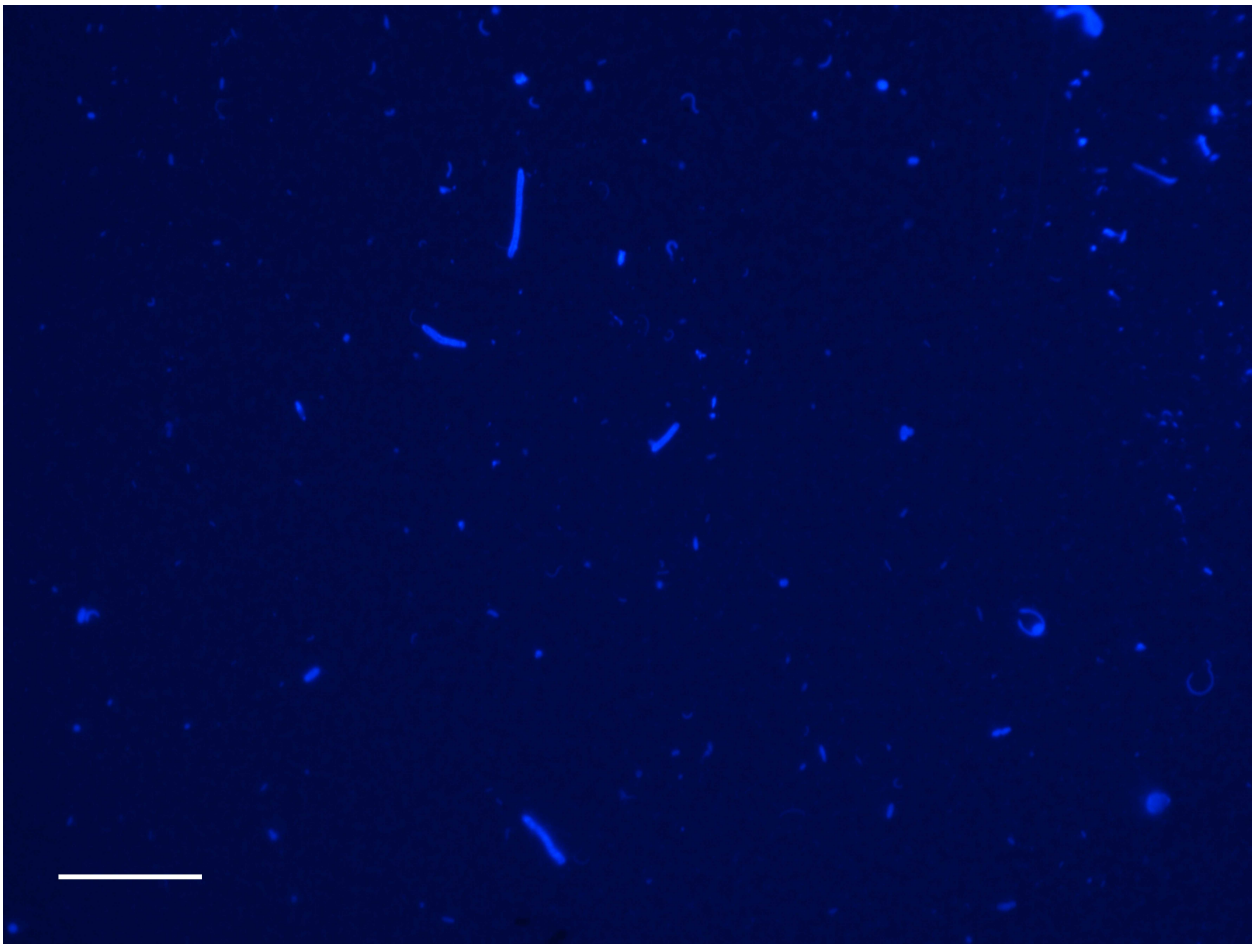

Figure 7.20 Micrograph of DAPI-stained deep seawater sample collected nearby Hole U1301A June 28, 2010. Scale bar is 10 µm.

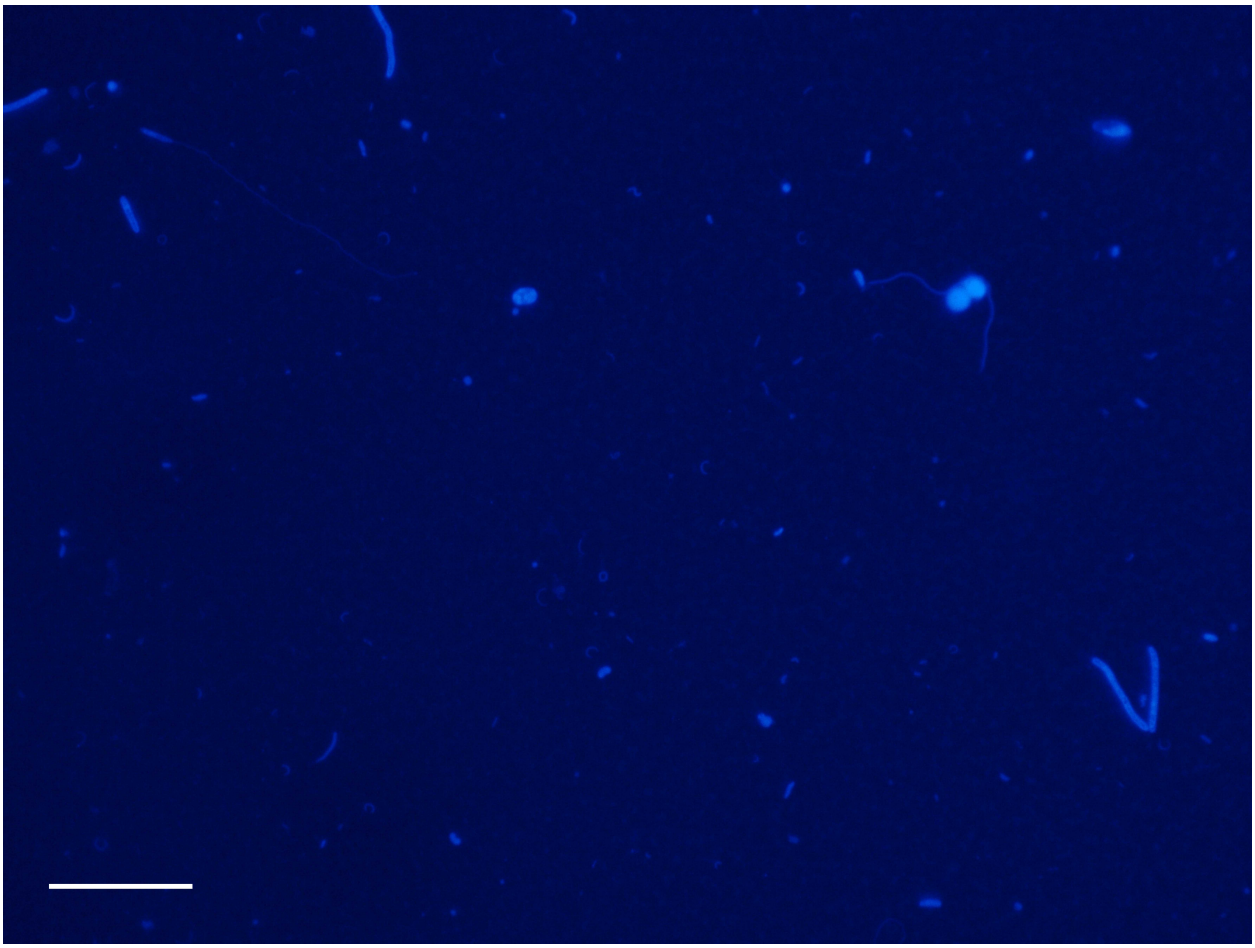

Figure 7.21 Micrograph of DAPI-stained deep seawater sample collected nearby Hole U1301A June 28, 2010. Scale bar is 10 µm.

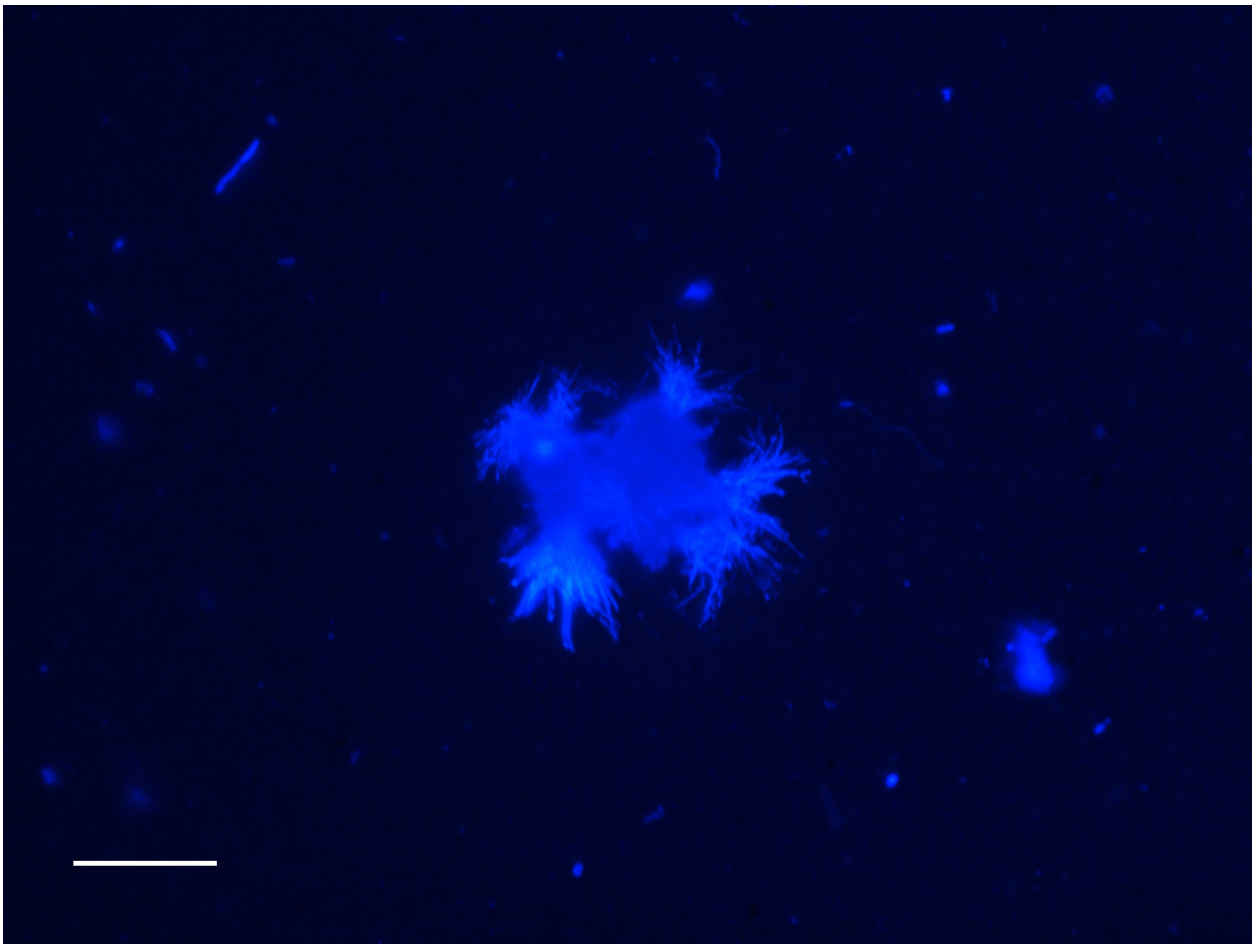

Figure 7.22 Micrograph of DAPI-stained borehole U1301A sample from July 4, 2011. Scale bar is 10 µm.

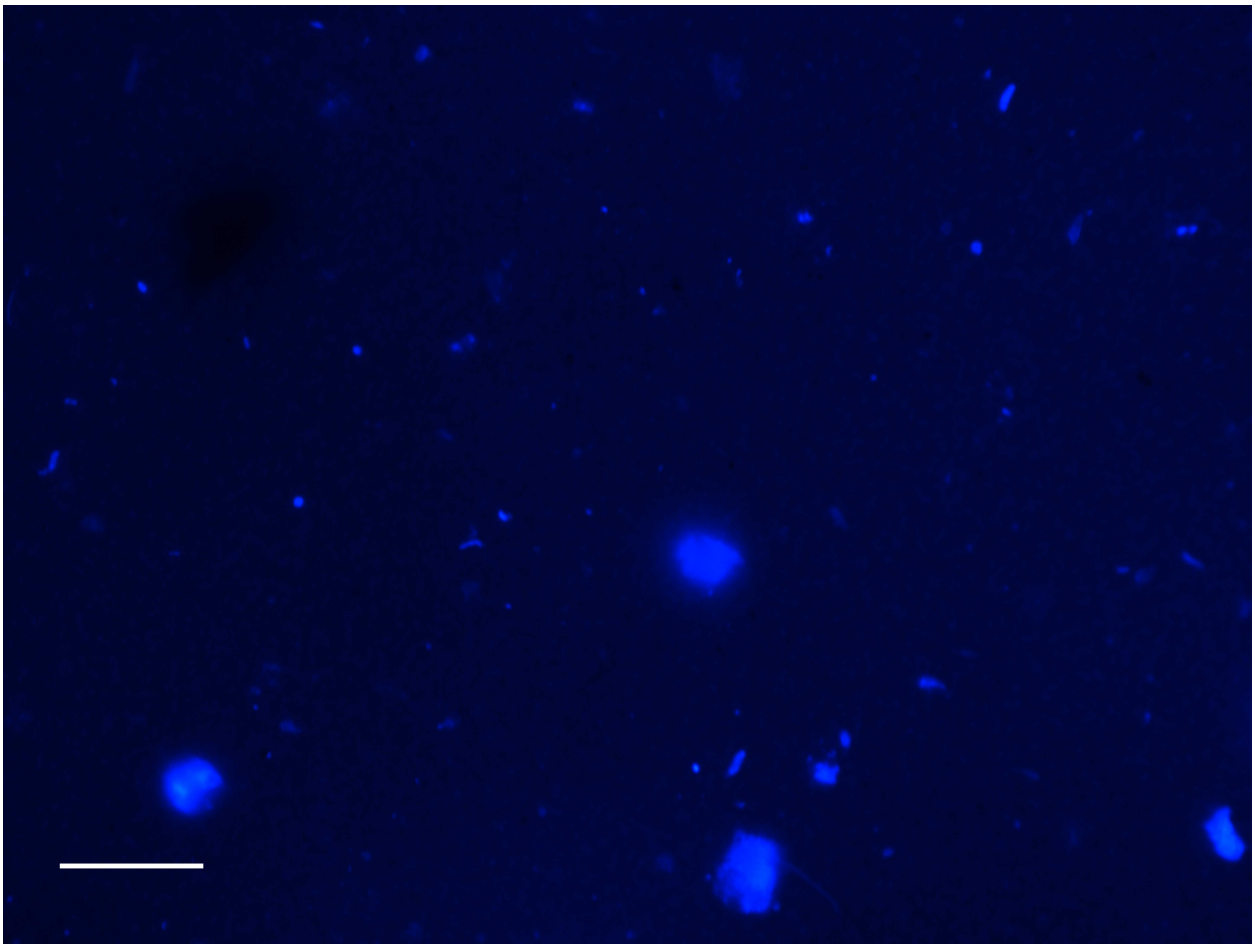

Figure 7.23 Micrograph of DAPI-stained borehole U1301A sample from July 4, 2011. Scale bar is 10 µm.

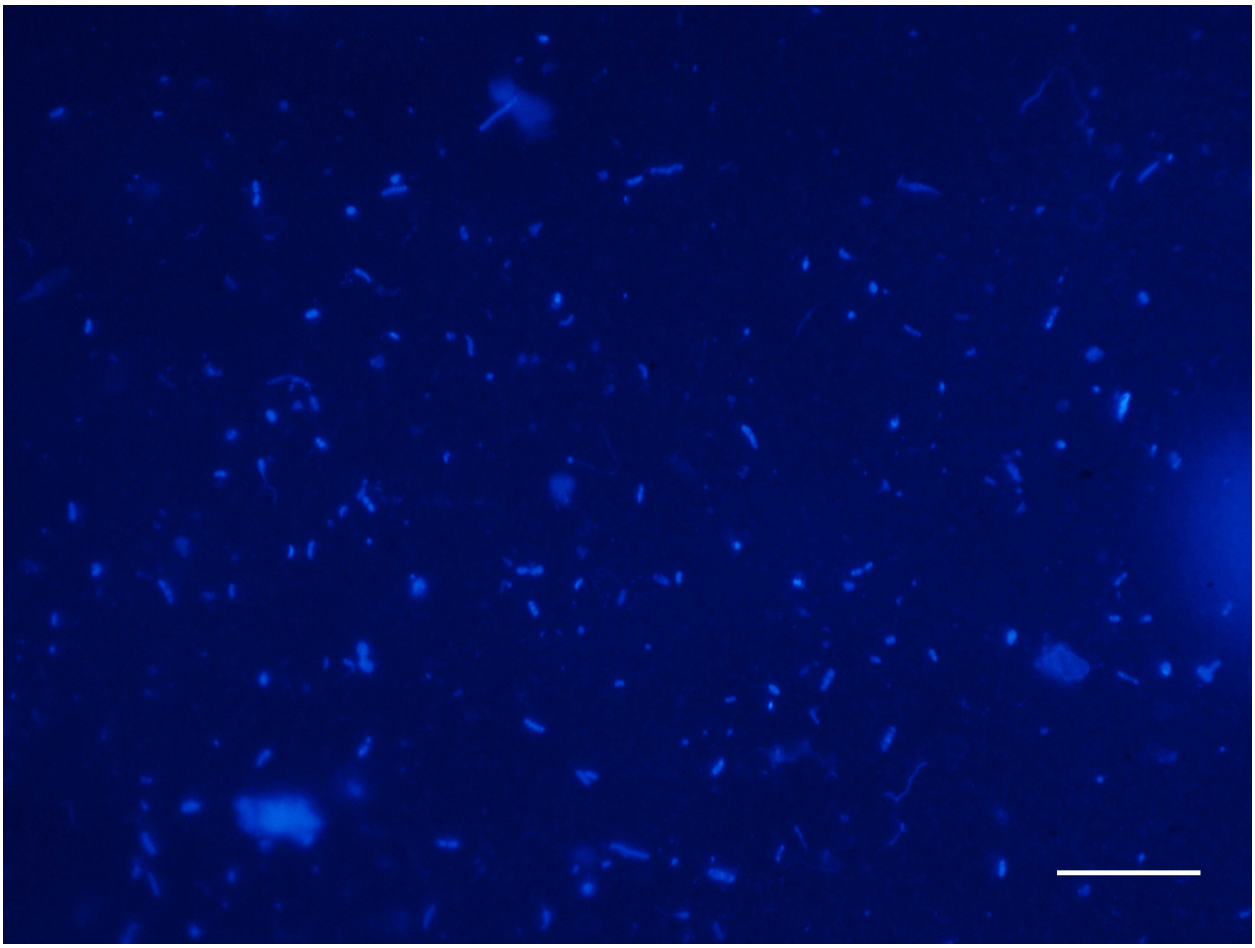

Figure 7.24 Micrograph of DAPI-stained borehole U1301A sample from July 4, 2011. Scale bar is 10 µm.

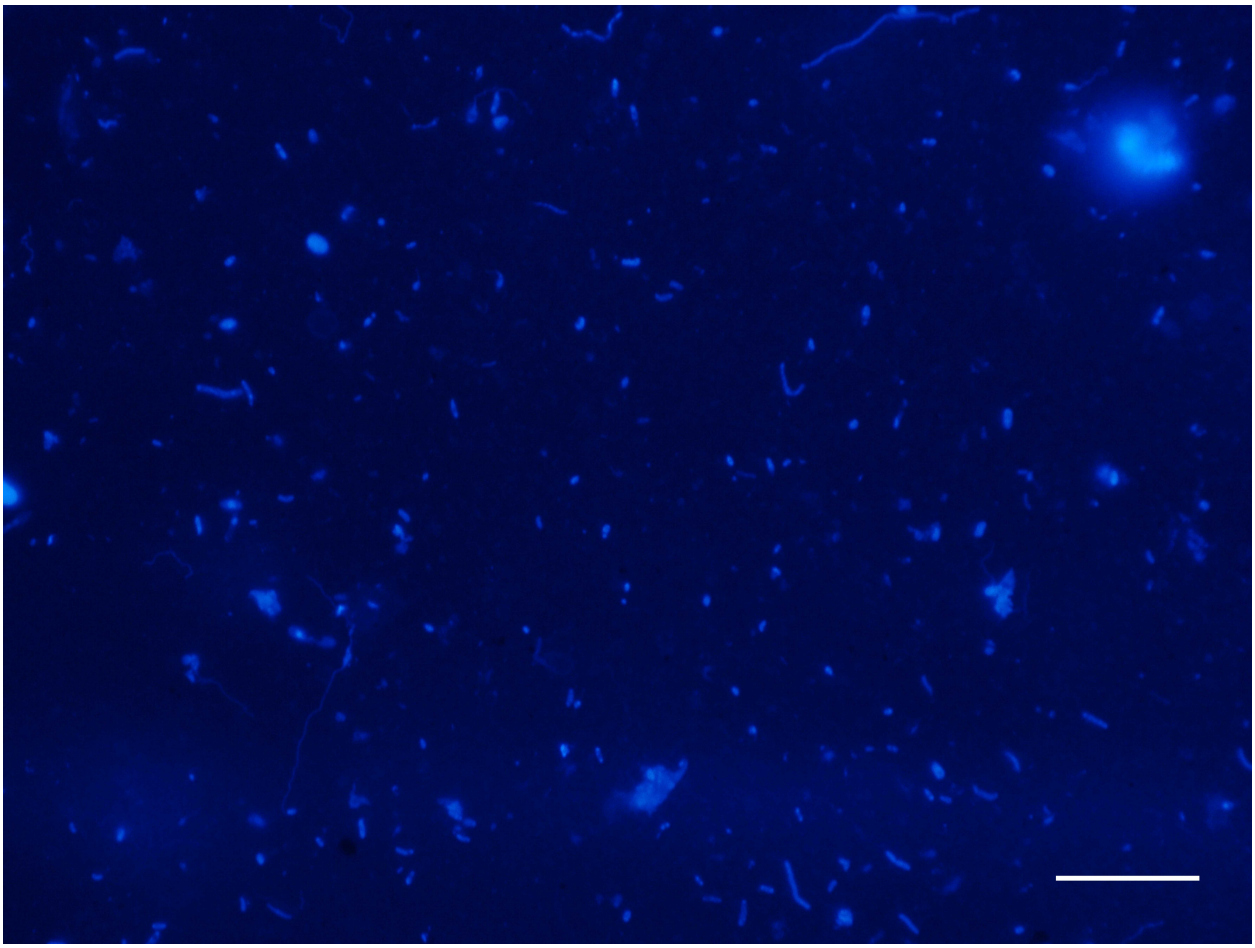

Figure 7.25 Micrograph of DAPI-stained borehole U1301A sample from July 4, 2011. Scale bar is 10 µm.

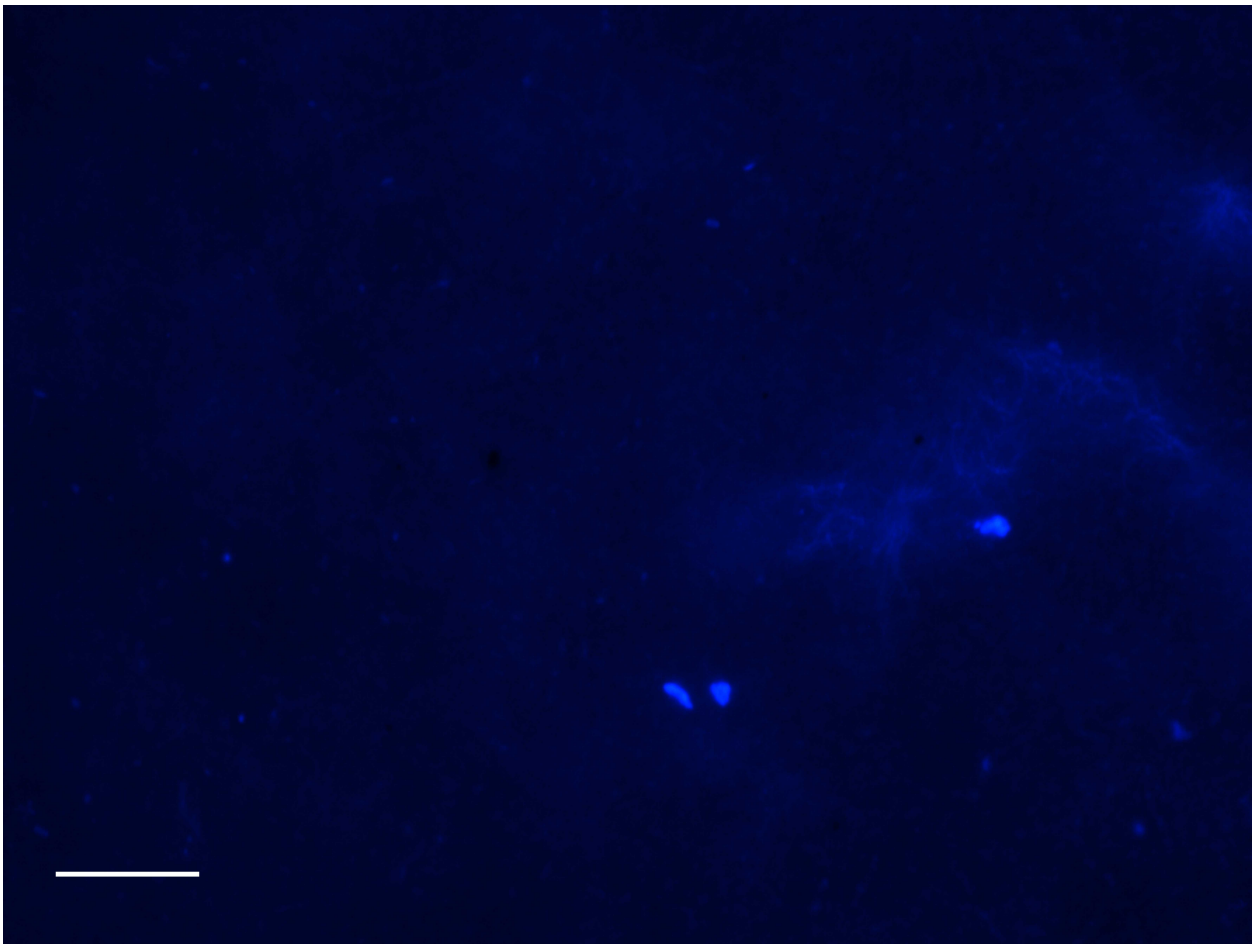

Figure 7.26 Micrograph of DAPI-stained borehole U1362A sample from July 12, 2011. Scale bar is 10 µm.

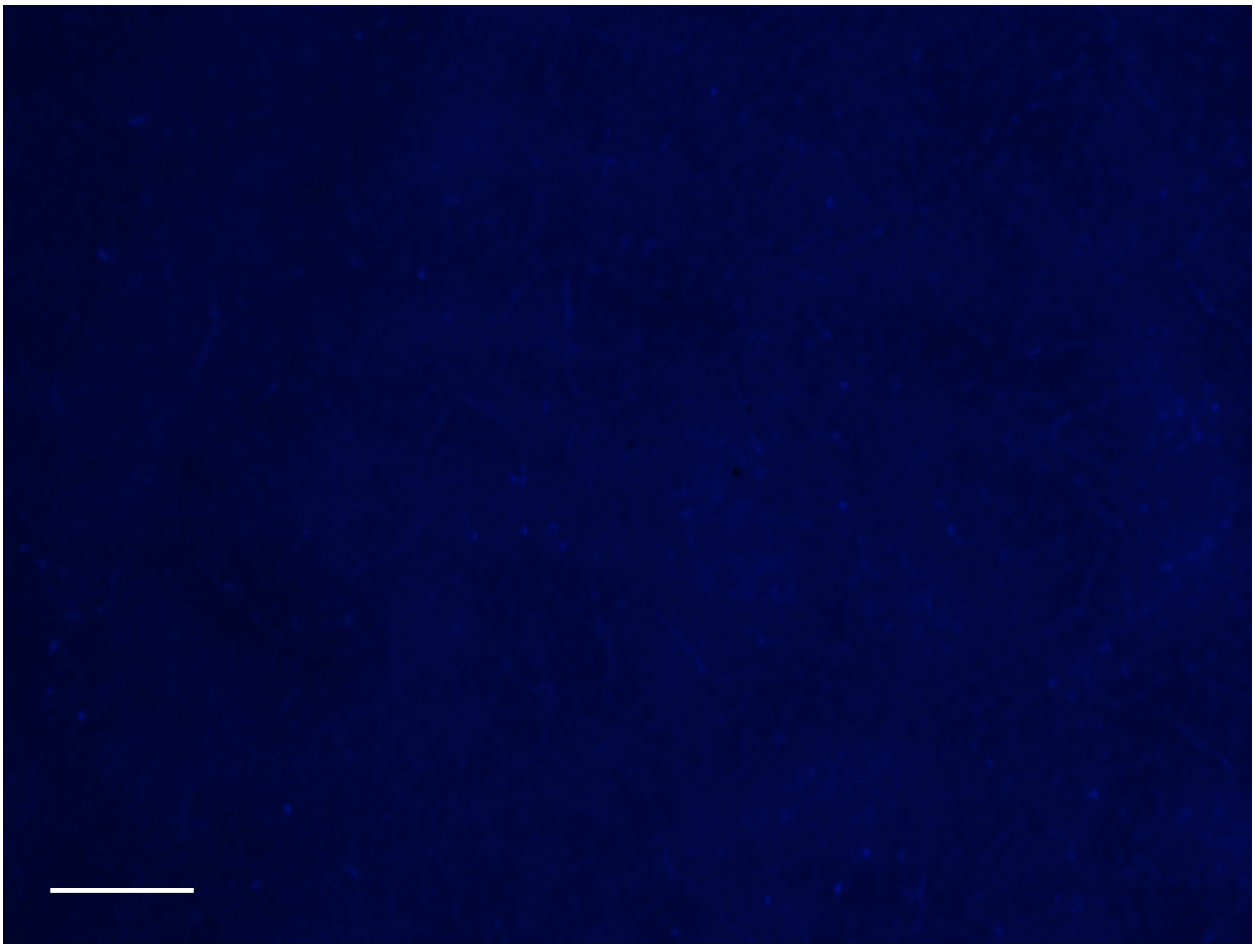

Figure 7.27 Micrograph of DAPI-stained borehole U1362A sample from July 12, 2011. Scale bar is 10 µm.

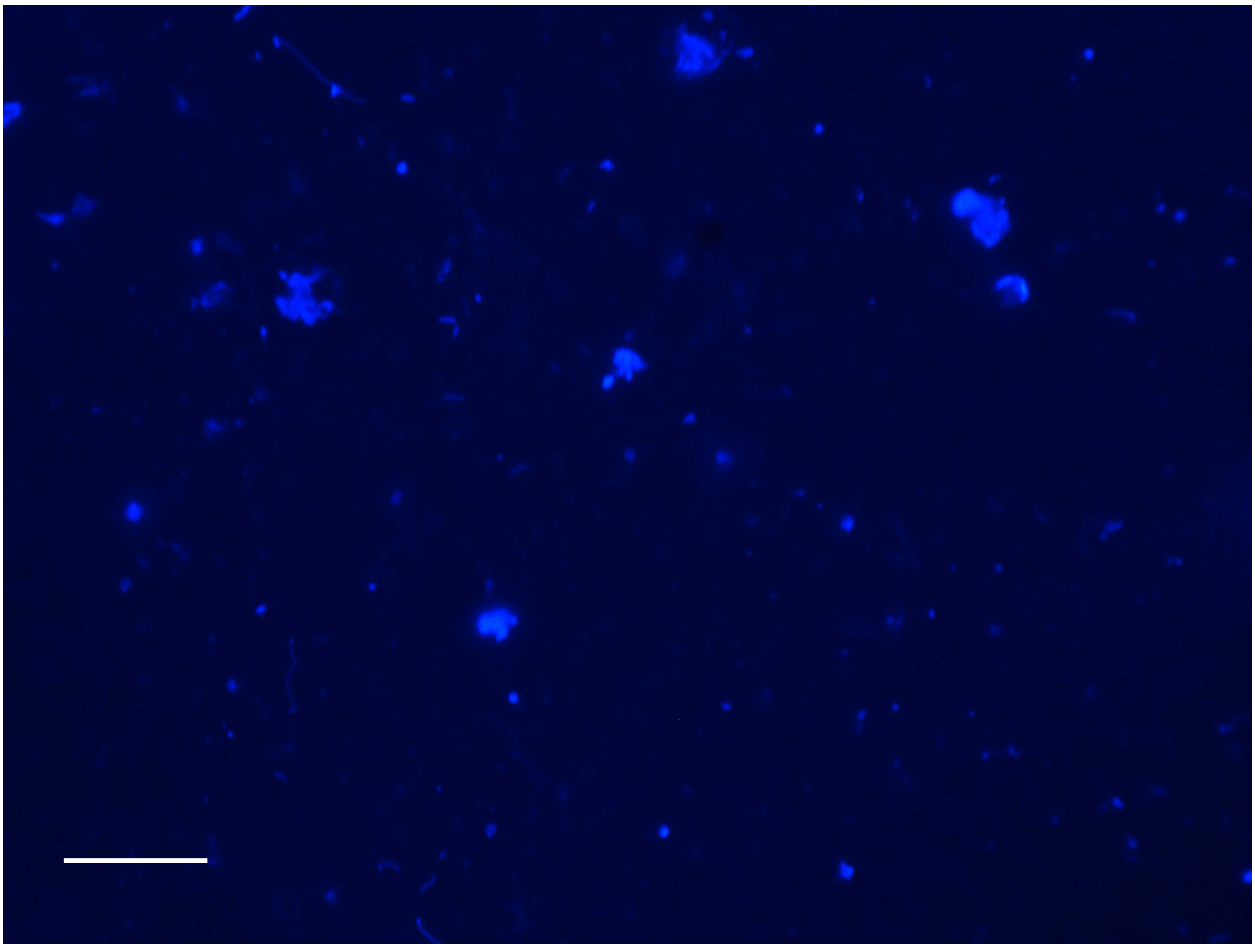

Figure 7.28 Micrograph of DAPI-stained borehole U1362B sample from July 10, 2011. Scale bar is 10 µm.

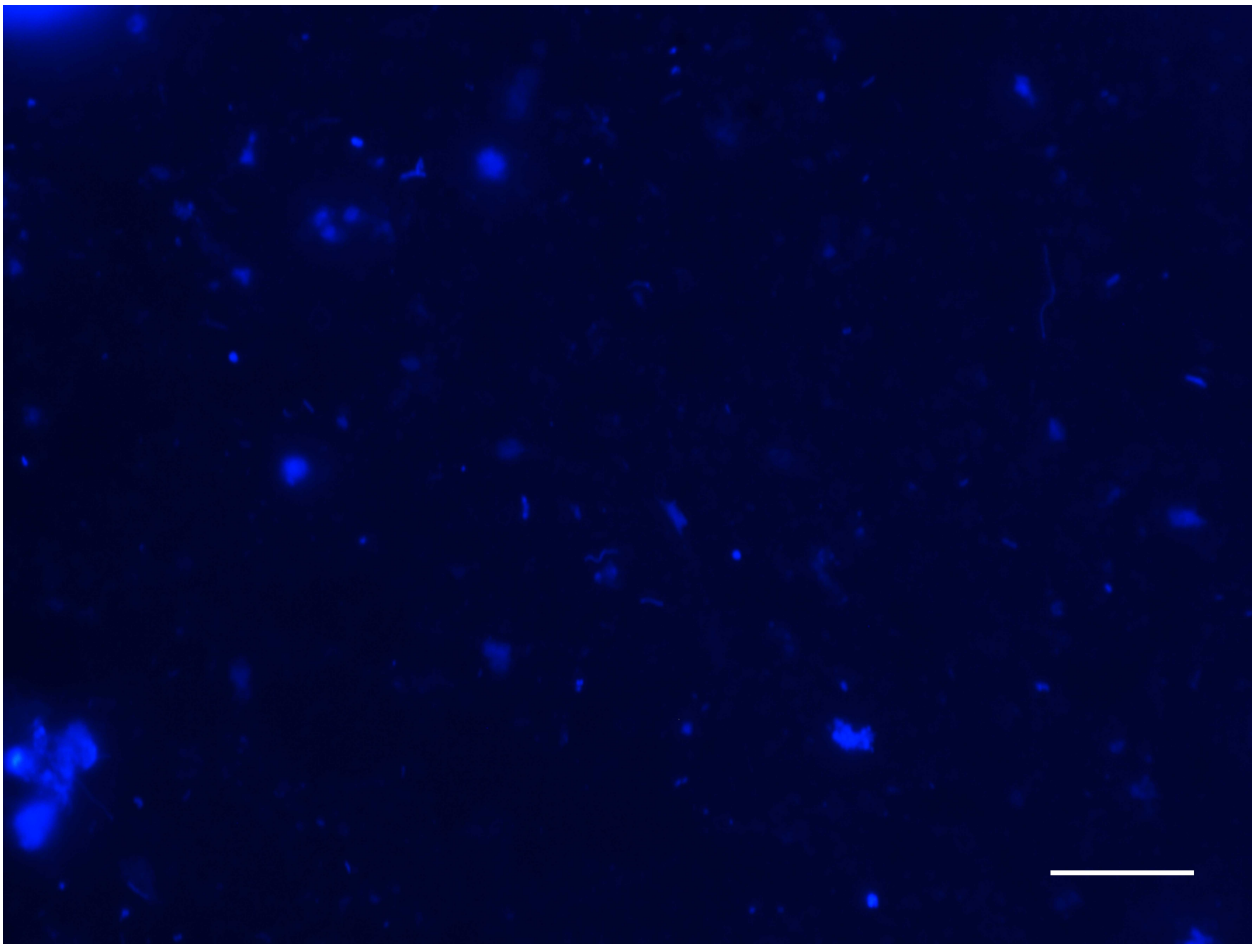

Figure 7.29 Micrograph of DAPI-stained borehole U1362B sample from July 10, 2011. Scale bar is 10 µm.

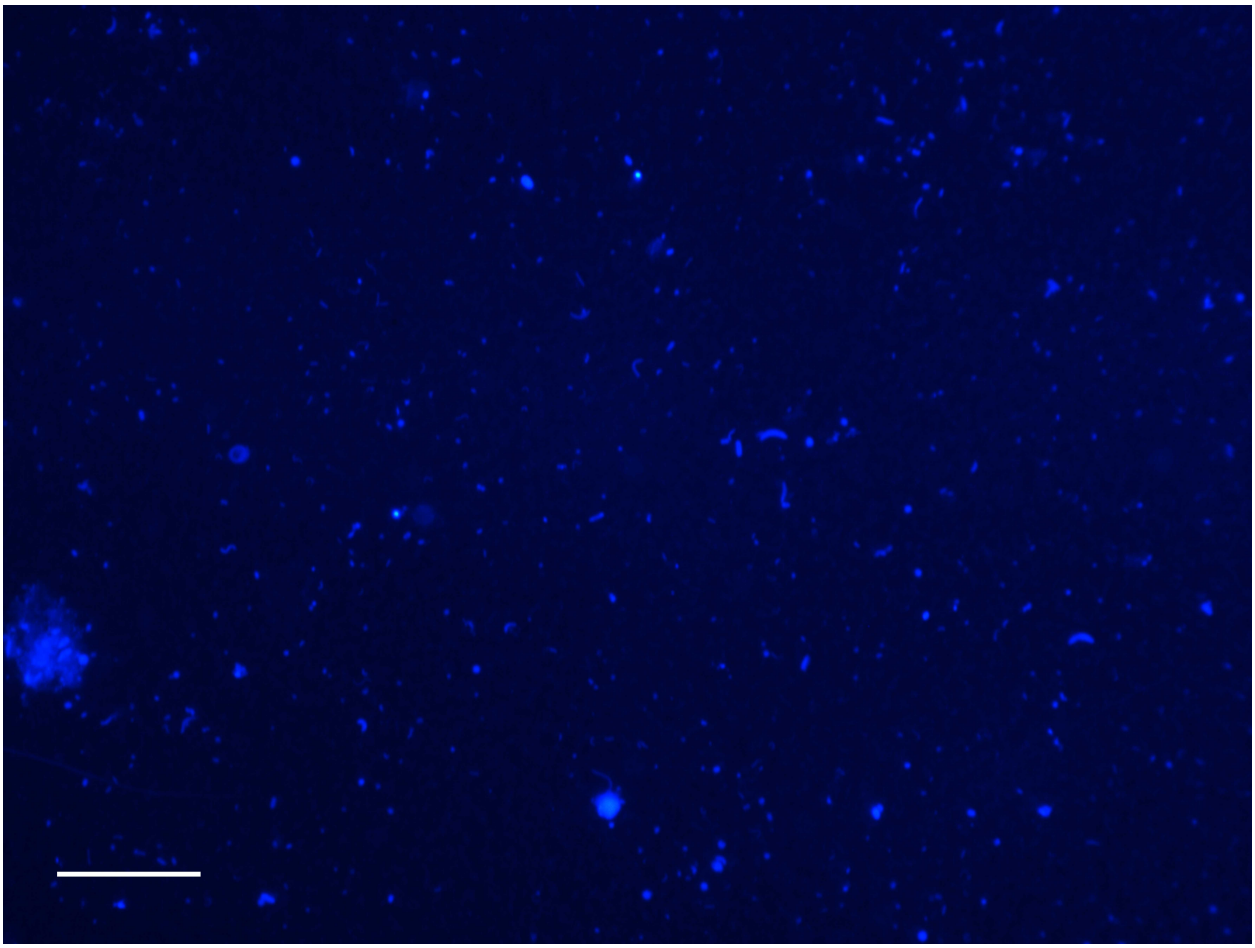

Figure 7.30 Micrograph of DAPI-stained deep seawater sample collected nearby Hole U1301A July 4, 2010. Scale bar is 10 µm.

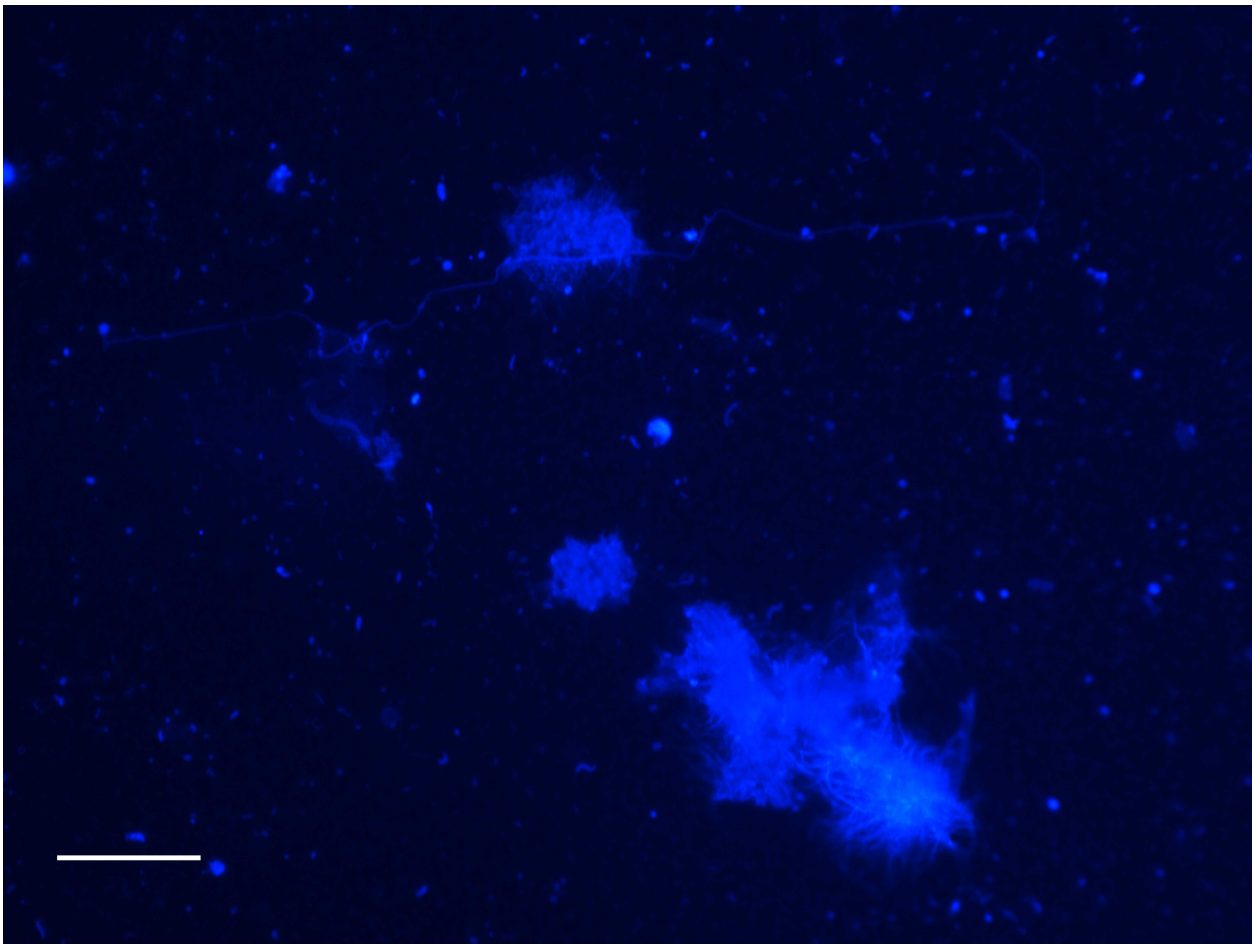

Figure 7.31 Micrograph of DAPI-stained deep seawater sample collected nearby Hole U1301A July 4, 2010. Scale bar is 10 µm.

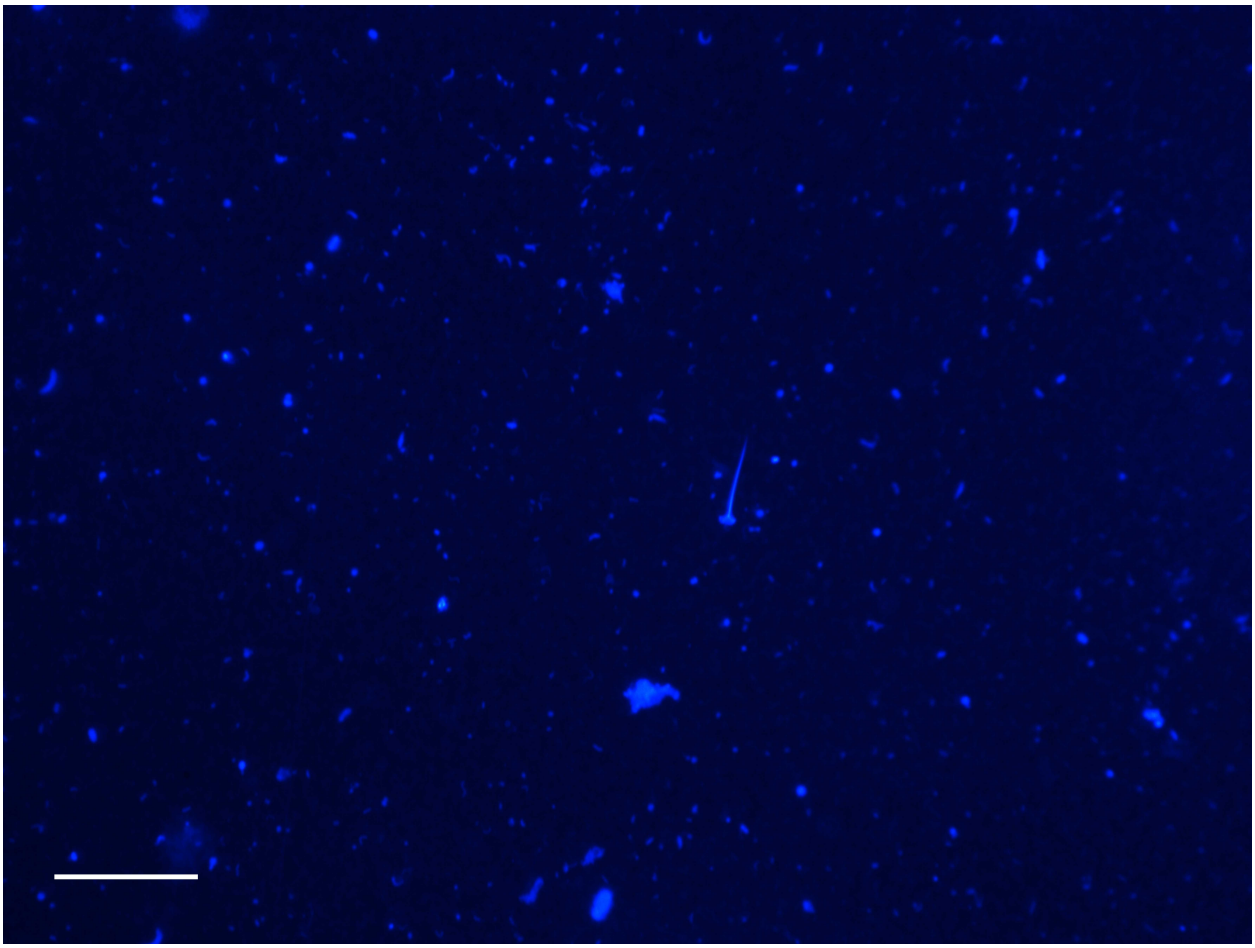

Figure 7.32 Micrograph of DAPI-stained deep seawater sample collected nearby Hole U1301A July 4, 2010. Scale bar is 10 µm.

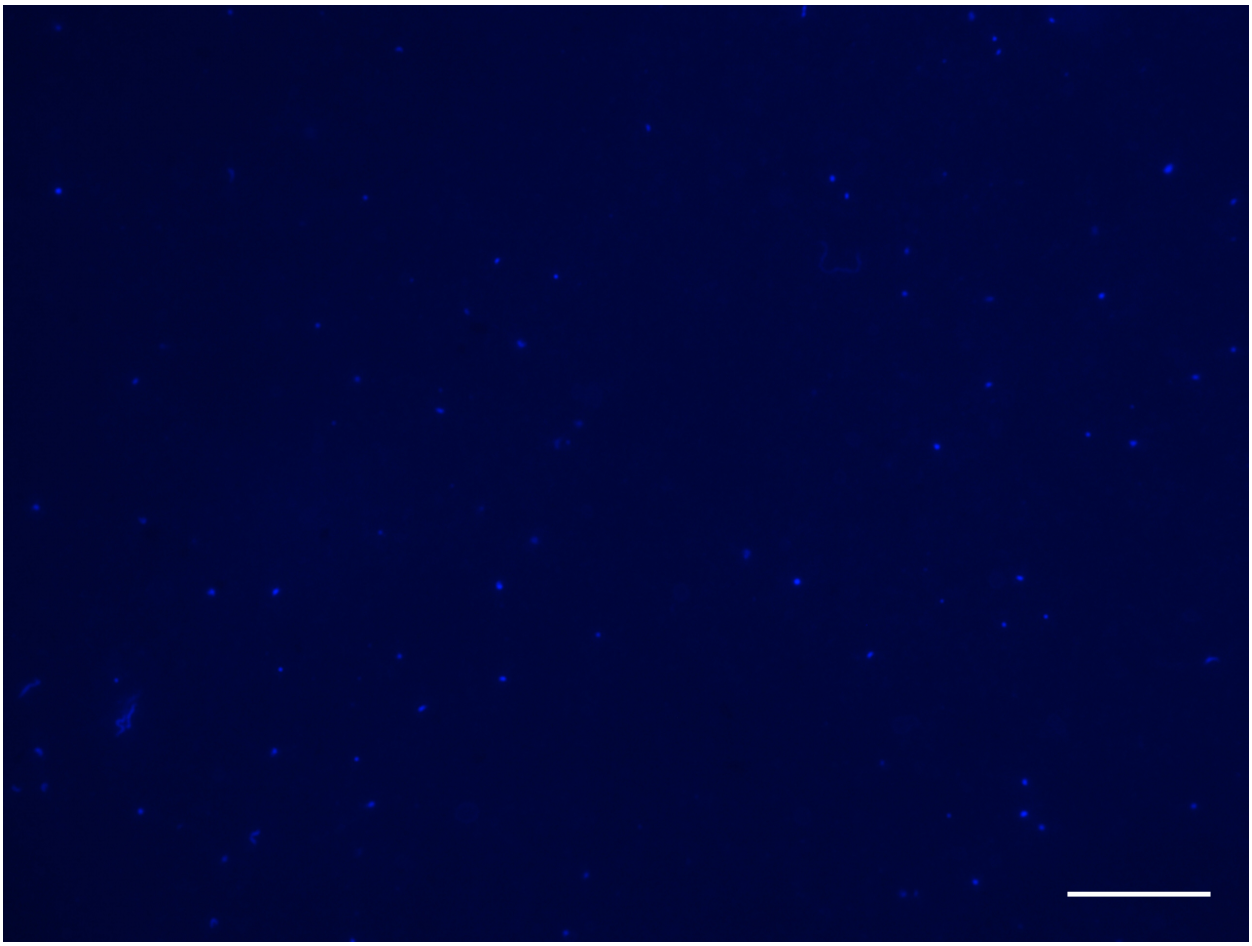

Figure 7.33 Micrograph of DAPI-stained borehole U1301A sample from July 22, 2013. Scale bar is 10 µm.

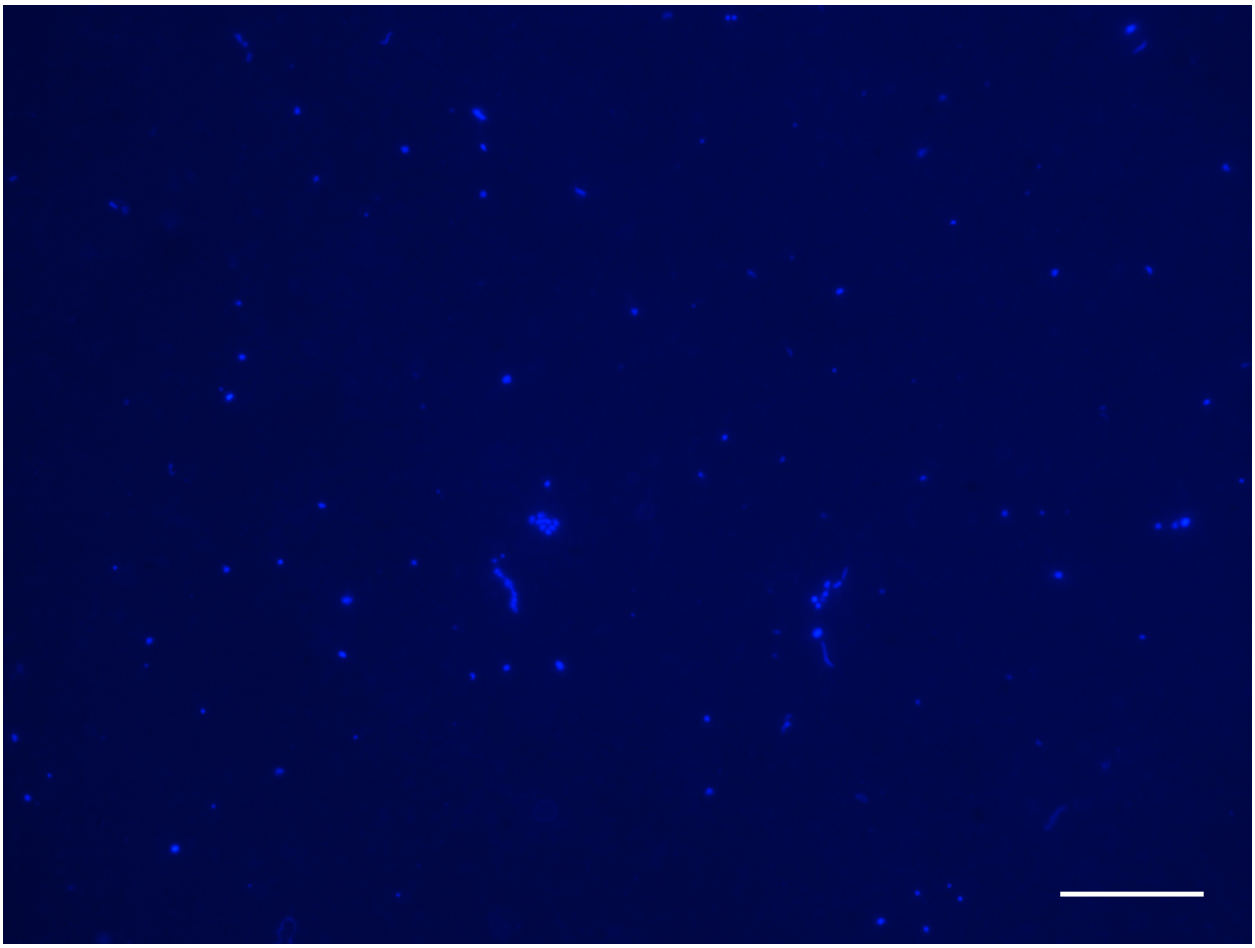

Figure 7.34 Micrograph of DAPI-stained borehole U1301A sample from July 22, 2013. Scale bar is 10 µm.

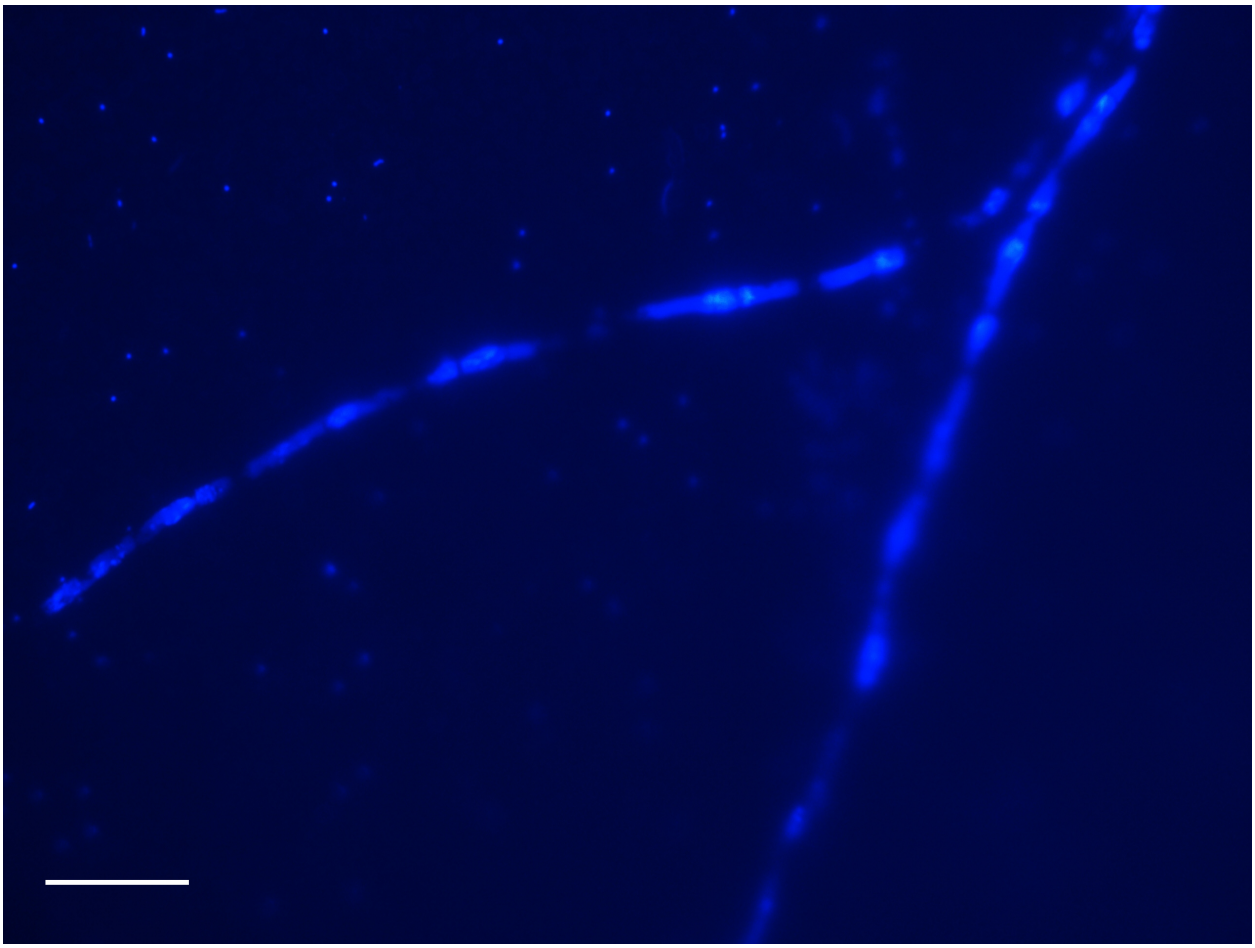

Figure 7.35 Micrograph of DAPI-stained borehole U1362A sample collected from Bioline and deep horizon on July 16, 2013. Scale bar is 10 µm.

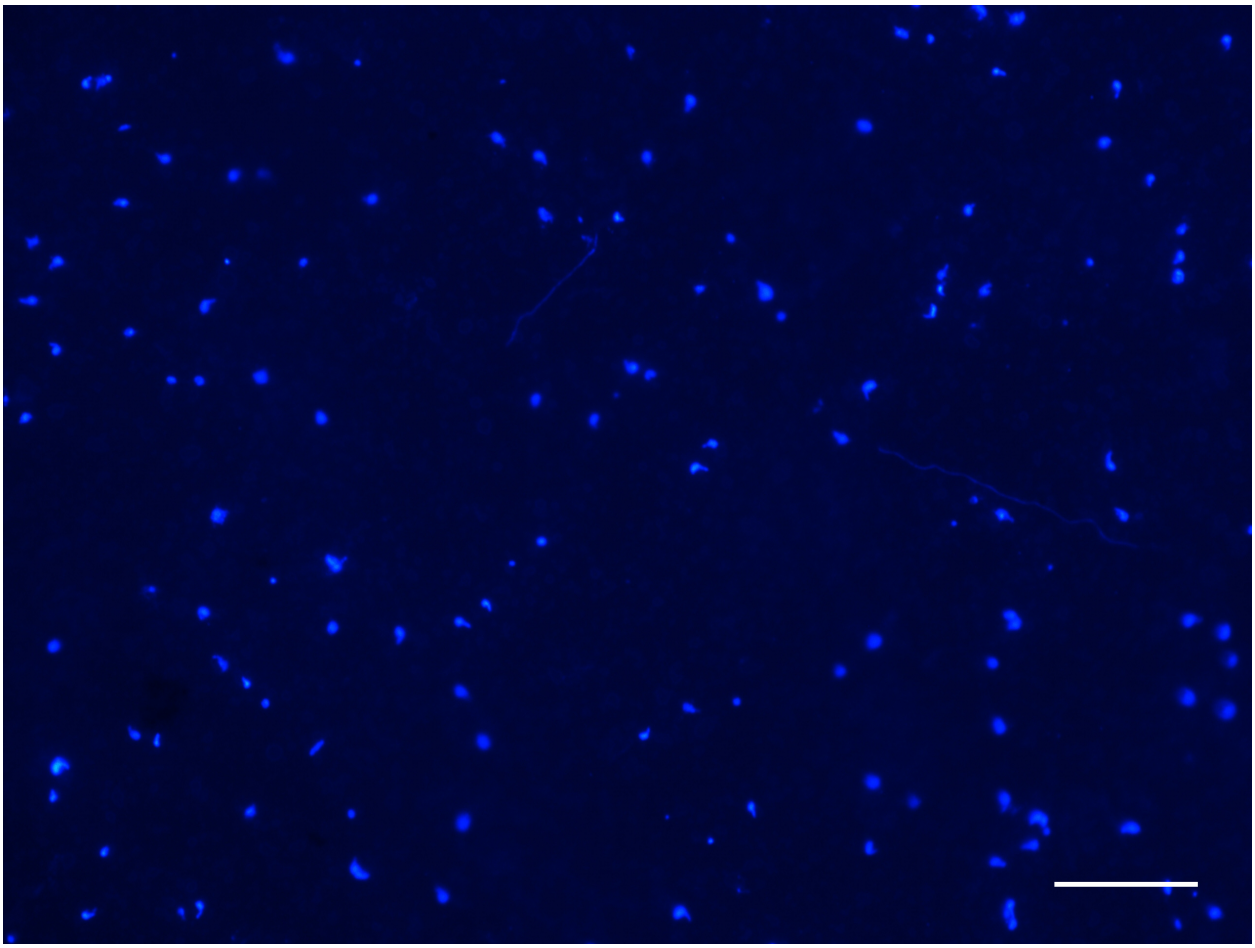

Figure 7.36 Micrograph of DAPI-stained borehole U1362B sample collected from Bioline on July 14, 2013. Scale bar is 10 µm.

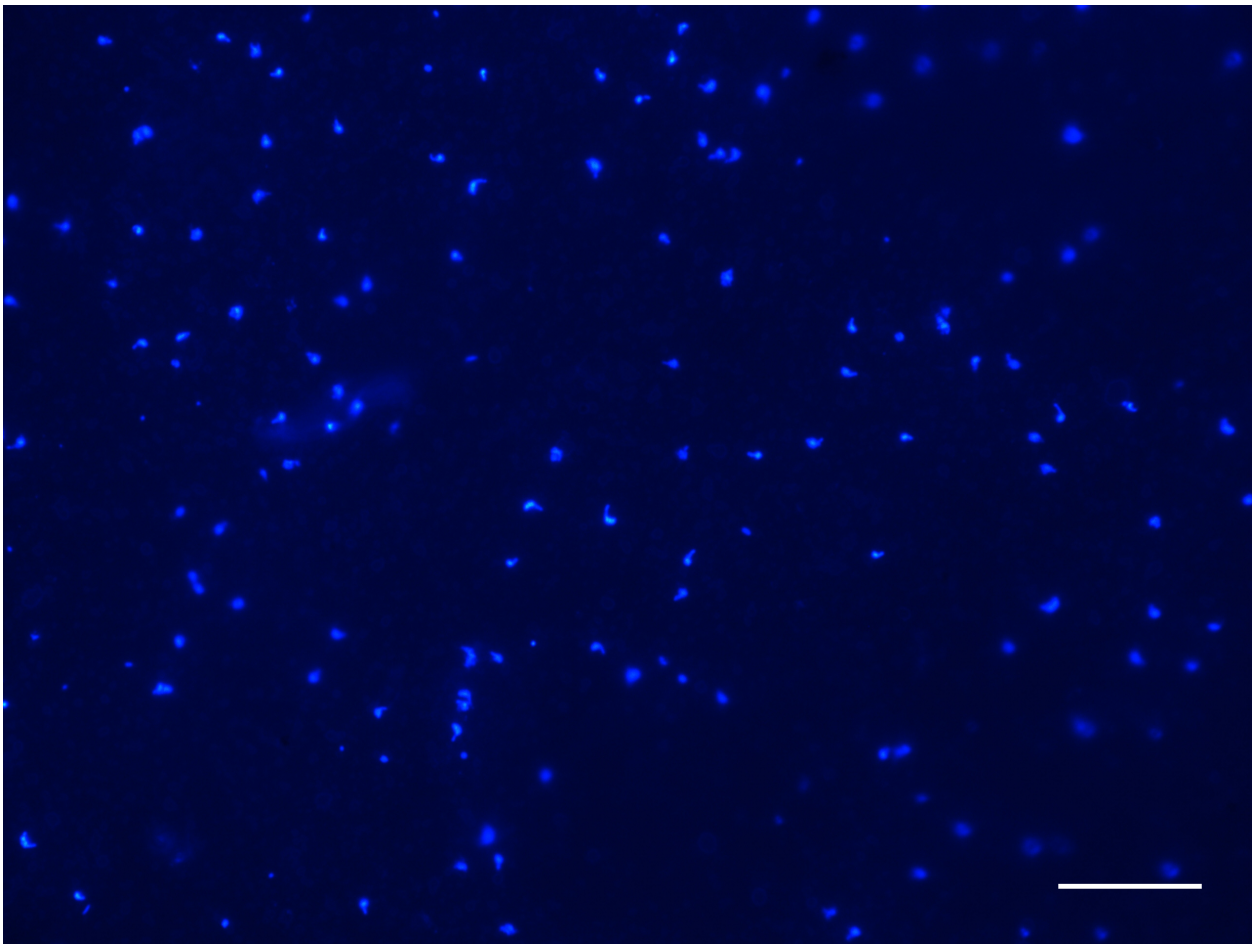

Figure 7.37 Micrograph of DAPI-stained borehole U1362B sample collected from Bioline on July 14, 2013. Scale bar is 10 µm.

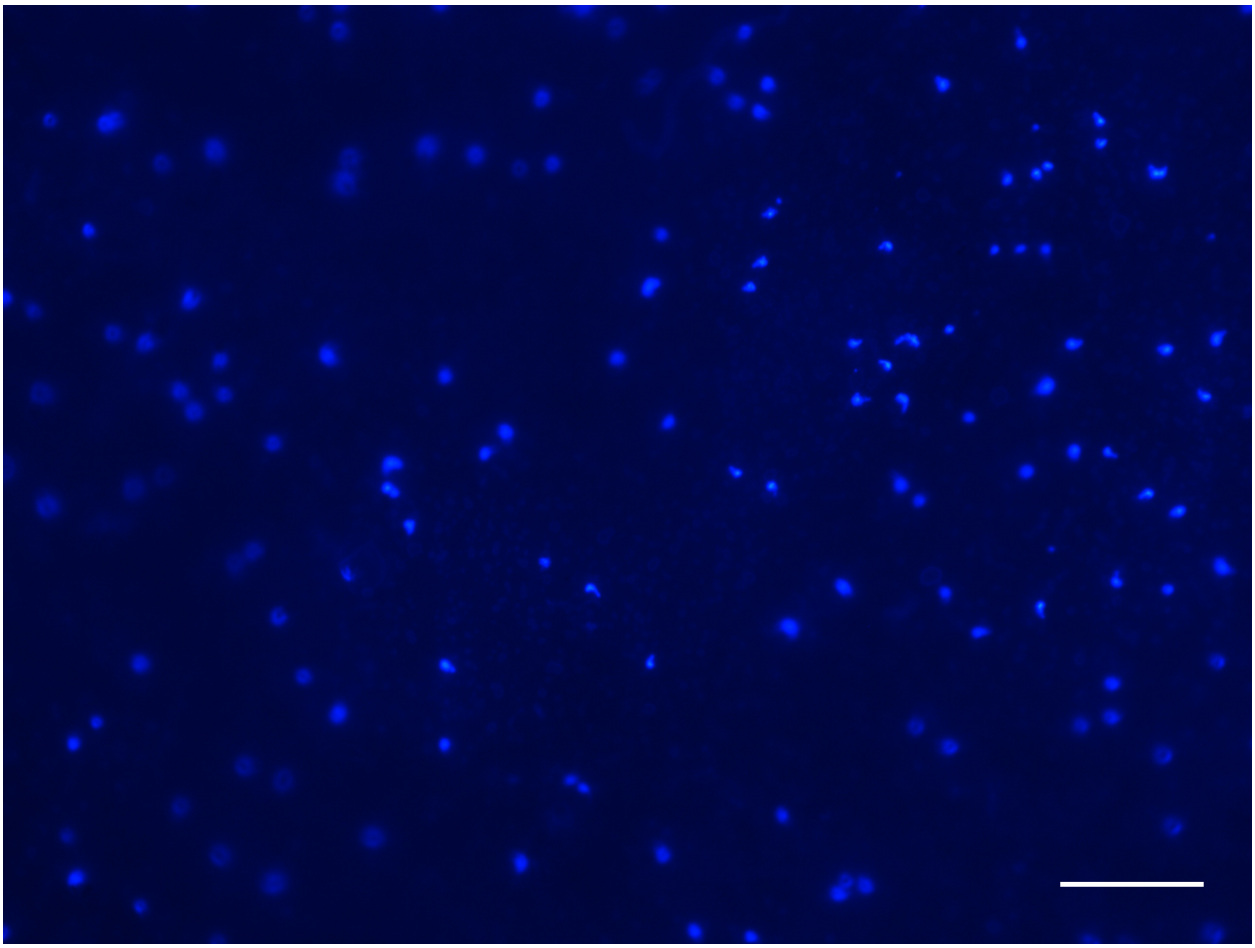

Figure 7.38 Micrograph of DAPI-stained borehole U1362B sample collected from Bioline on July 14, 2013. Scale bar is 10 µm.

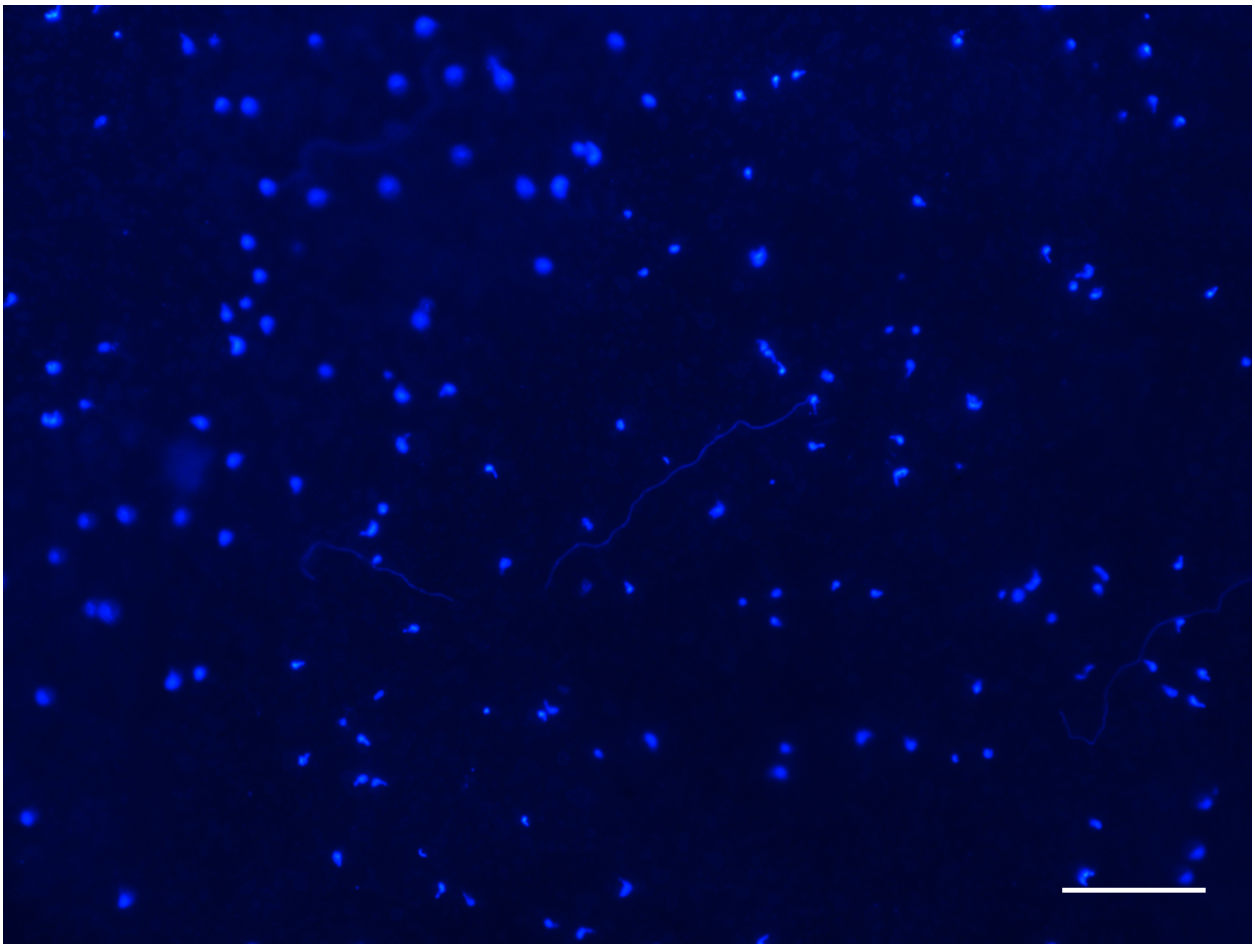

Figure 7.39 Micrograph of DAPI-stained borehole U1362B sample collected from Bioline on July 14, 2013. Scale bar is 10 µm.

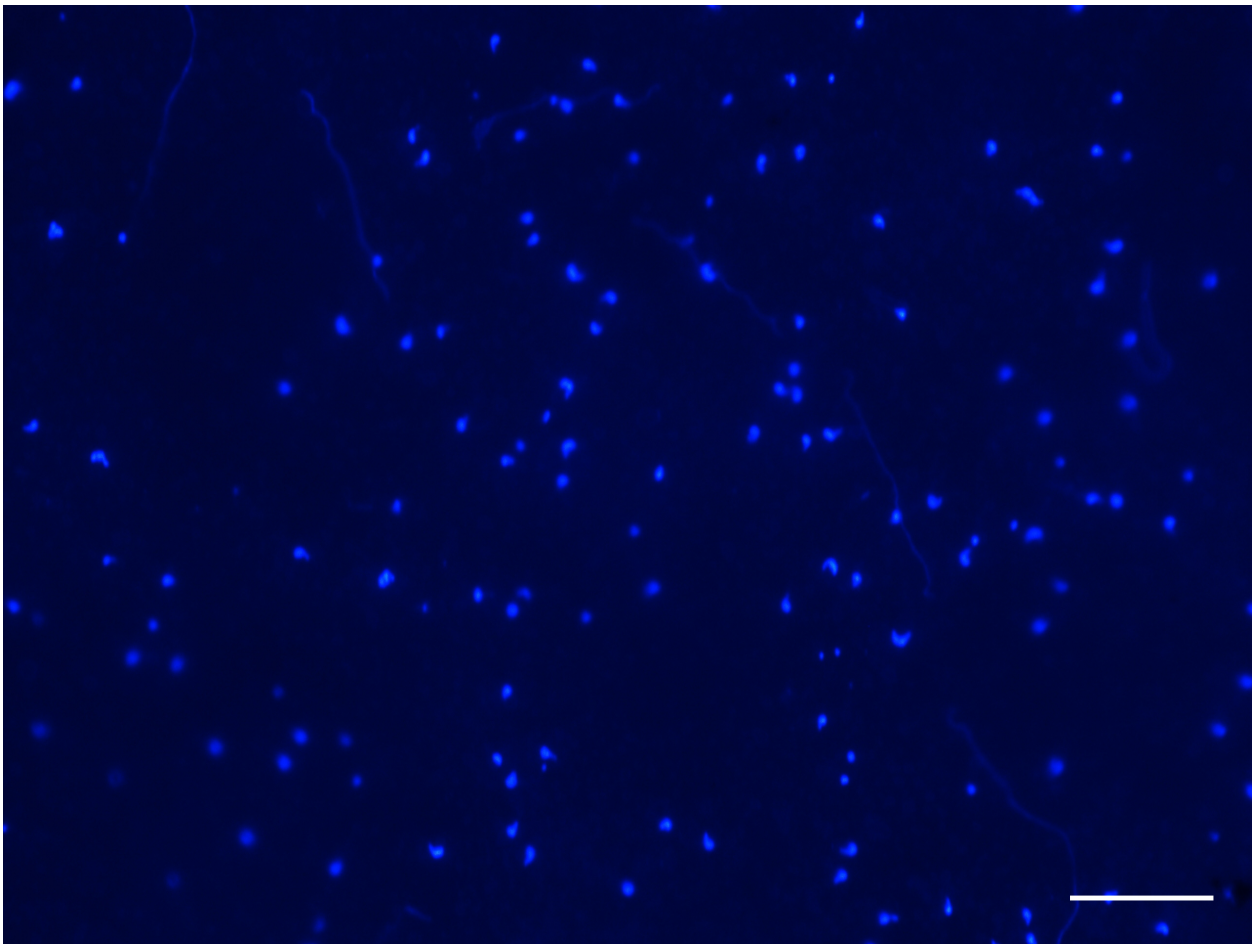

Figure 7.40 Micrograph of DAPI-stained borehole U1362B sample collected from Bioline on July 14, 2013. Scale bar is 10 µm.

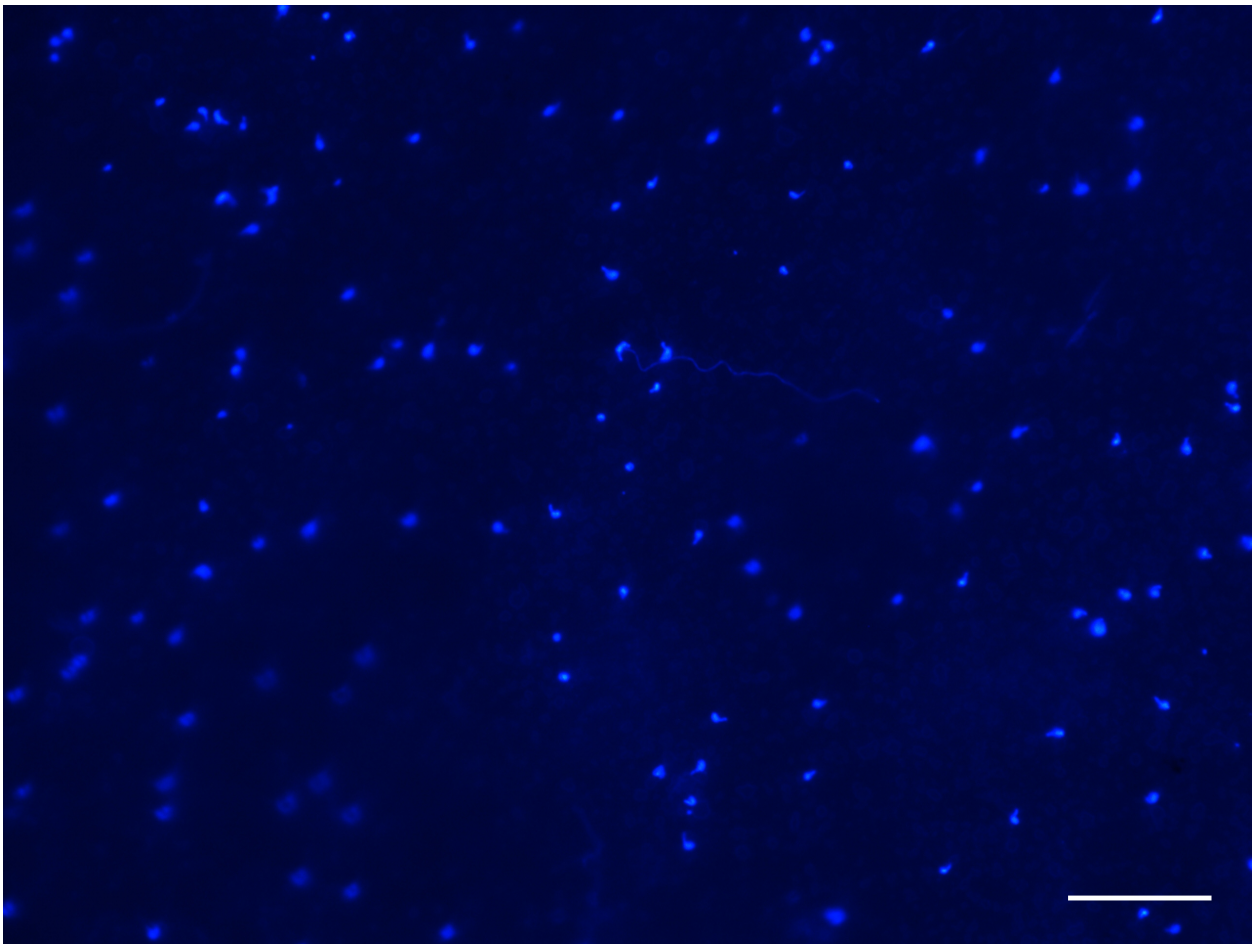

Figure 7.41 Micrograph of DAPI-stained borehole U1362B sample collected from Bioline on July 14, 2013. Scale bar is 10 µm.

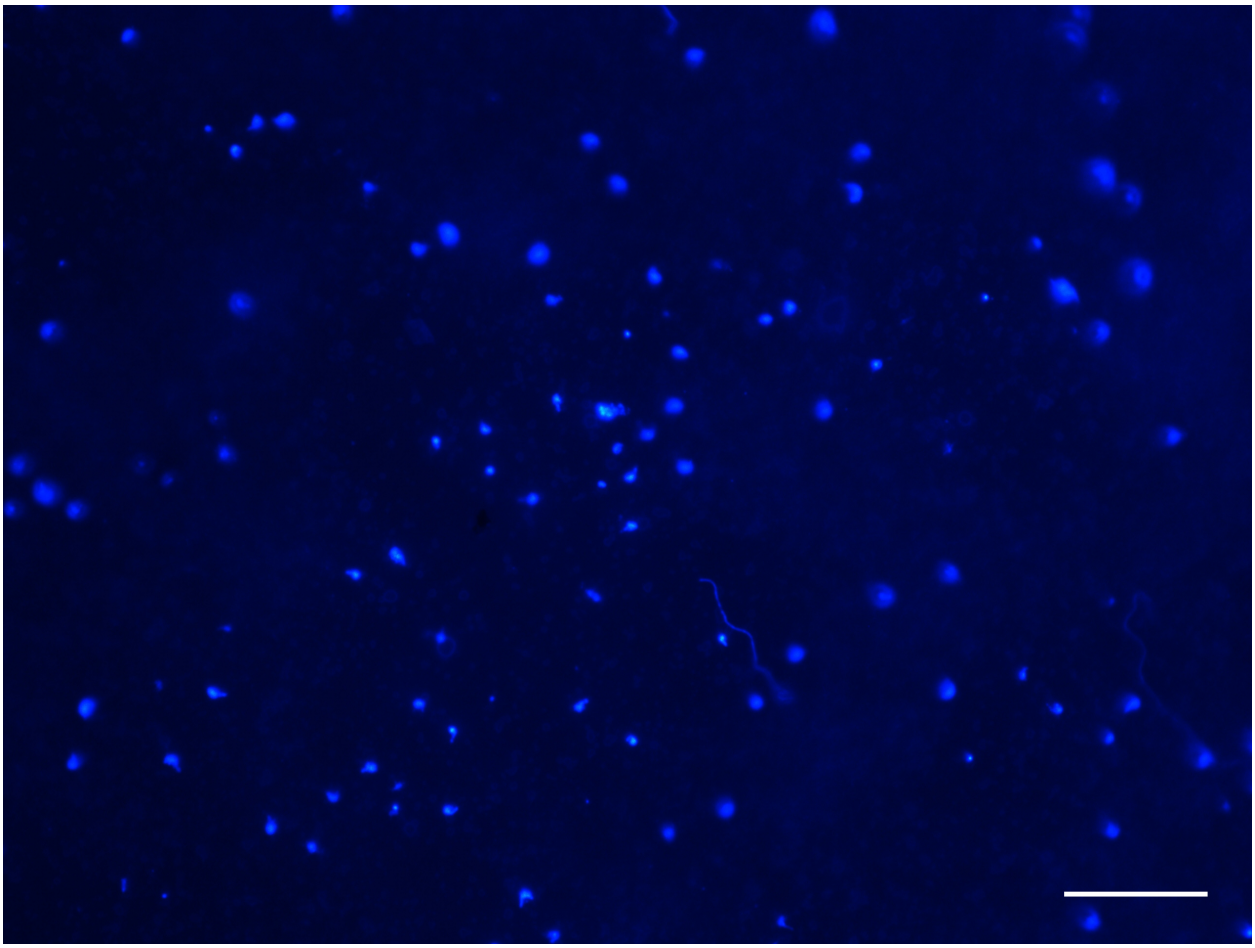

Figure 7.42 Micrograph of DAPI-stained borehole U1362B sample collected from Bioline on July 14, 2013. Scale bar is 10 µm.

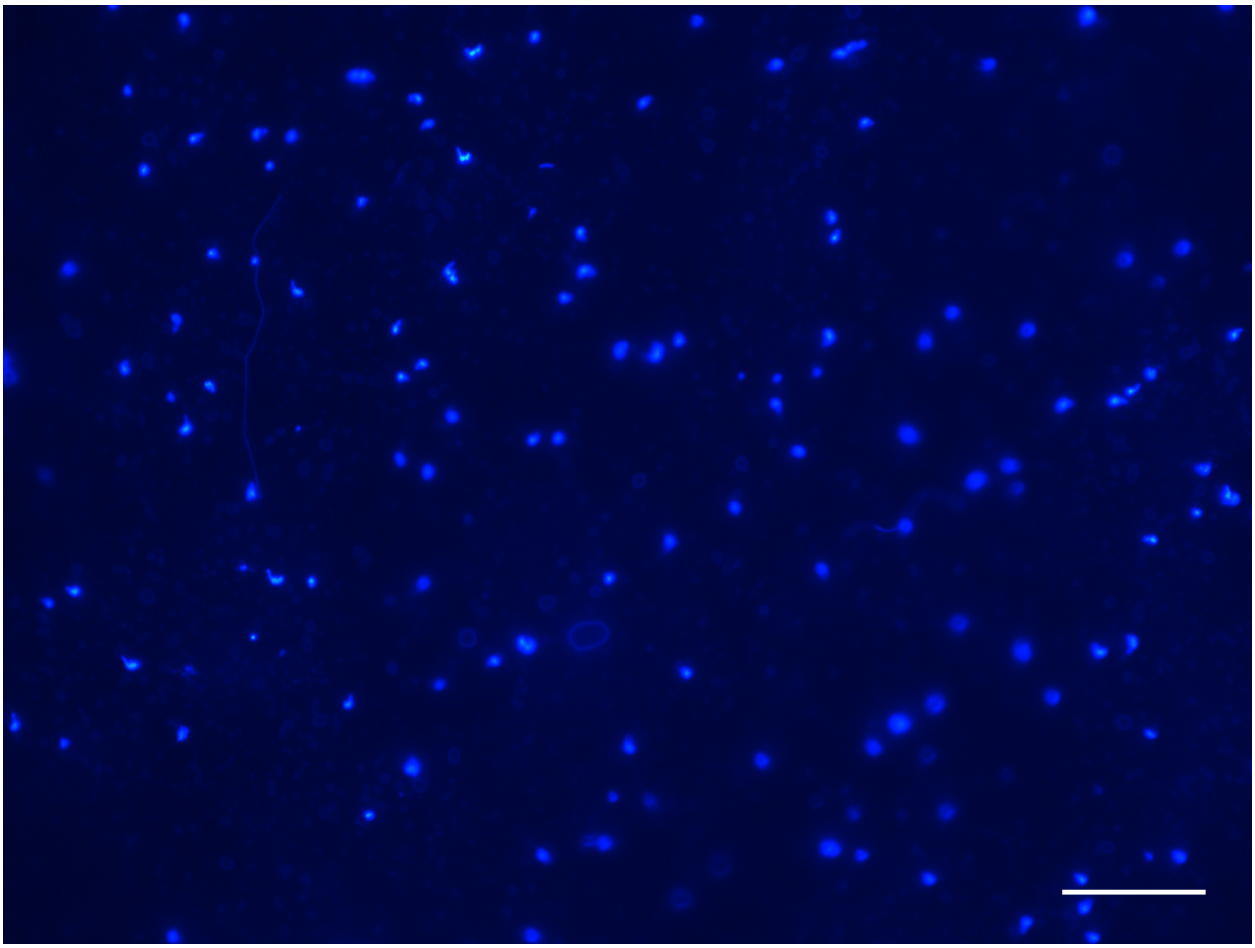

Figure 7.43 Micrograph of DAPI-stained borehole U1362B sample collected from Bioline on July 14, 2013. Scale bar is 10 µm.

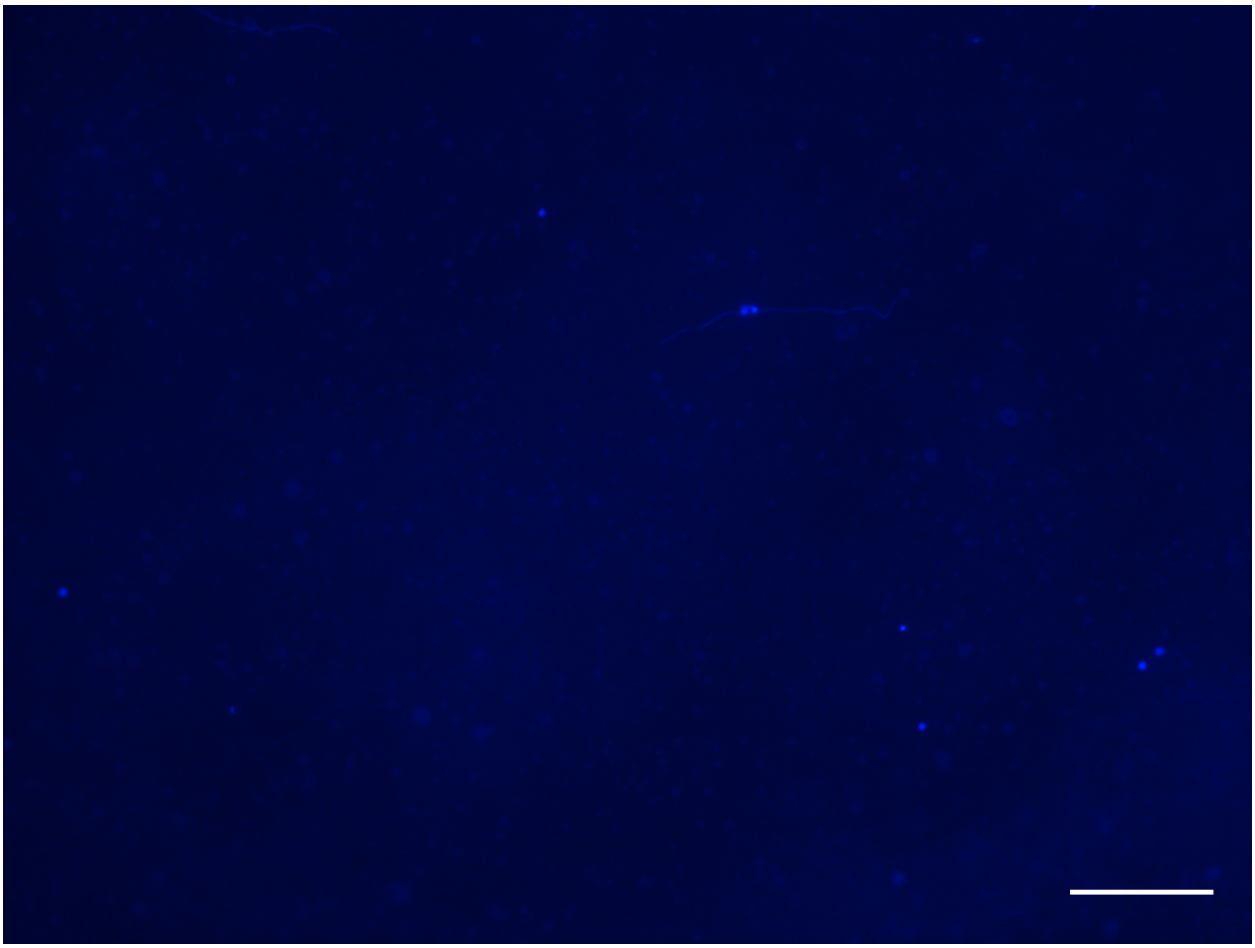

Figure 7.44 Micrograph of DAPI-stained borehole U1362B sample collected from Bioline on July 21 2013. Scale bar is 10 µm.

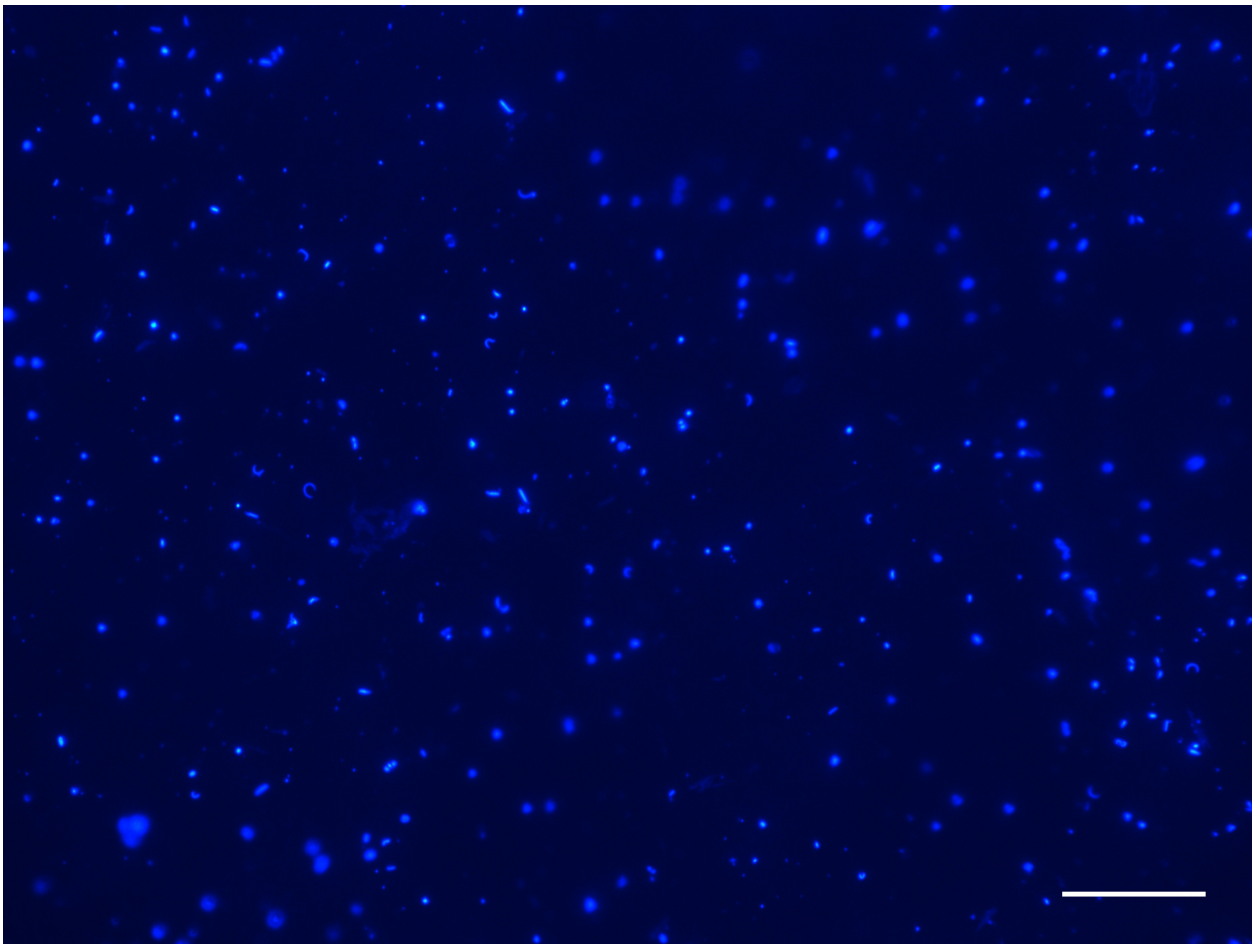

Figure 7.45 Micrograph of DAPI-stained deep seawater sample collected nearby Hole U1362B July 14, 2013. Scale bar is 10 µm.

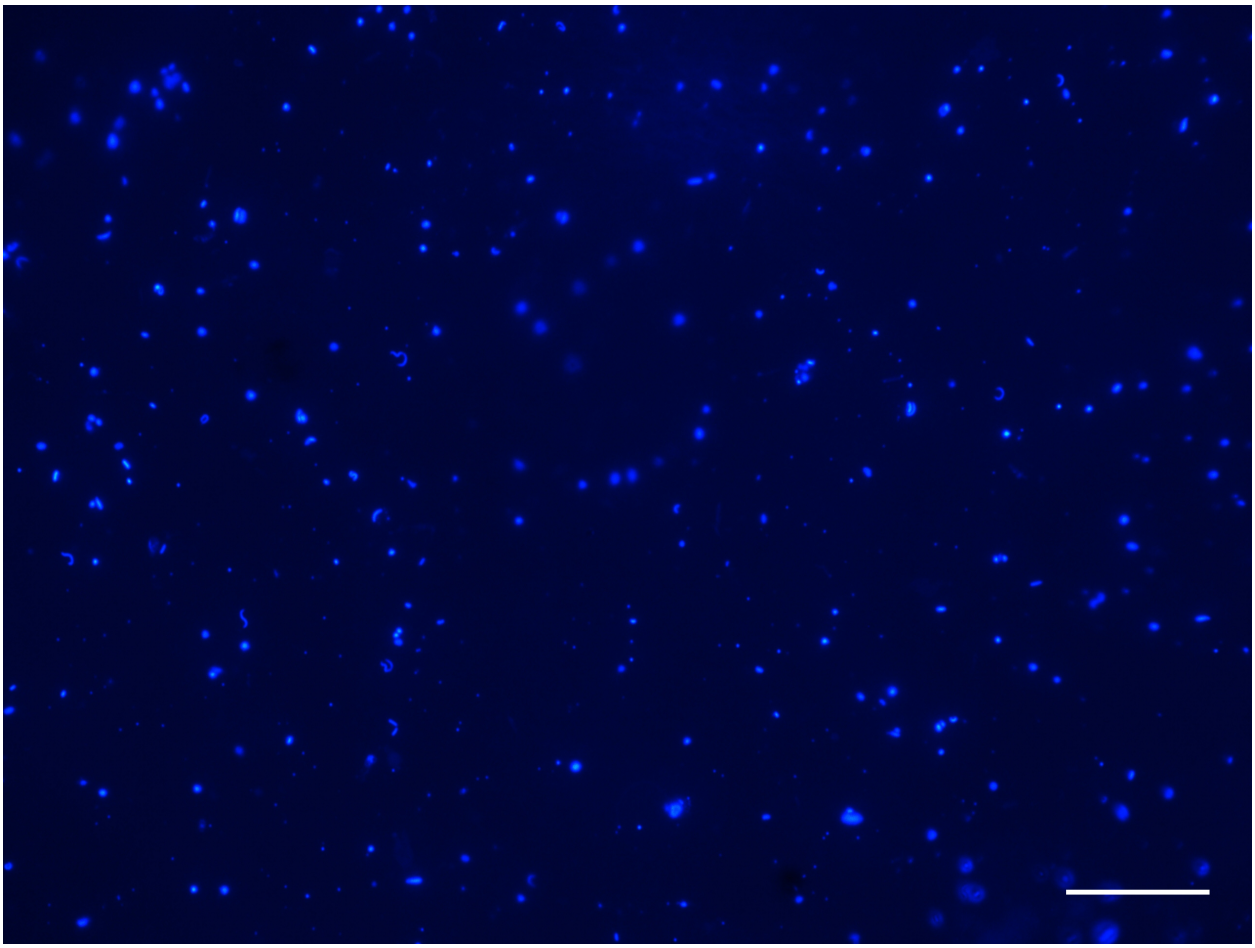

Figure 7.46 Micrograph of DAPI-stained deep seawater sample collected nearby Hole U1362B July 14, 2013. Scale bar is 10 µm.