

14. INVESTIGATION OF MICROORGANISMS AND DNA FROM SUBSURFACE THERMAL WATER AND ROCK FROM THE EAST FLANK OF JUAN DE FUCA RIDGE¹

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ABSTRACT

During Leg 168 of the Ocean Drilling Program, basalts were recovered by drilling and subsurface water was collected with a water sampling tool at Hole 1026B on the east flank of Juan de Fuca Ridge. Microorganisms were found in both types of samples. The microorganisms in the basalt appear to have been in situ, but the origin of microorganisms in the water is not certain. Particles filtered from the formation water collected with the water sampling tool indicate that there were several potential sources of contamination including the drill string, sea water, and the water sampling tool. The number of microorganisms in the formation water (including those introduced through contamination) was probably less than 1000 per mL, and this low number of cells did not permit us to identify them. Improvements in sampling may provide suitable samples for identification and culturing of microbes from seafloor aquifers.

INTRODUCTION

Background

Abundant evidence exists for microbial life within the oceanic volcanic crust (Deming and Baross, 1993; Furnes et al., 1996; Giovannoni et al., 1996; Thorseth et al., 1995; Fisk et al., 1998). Two objectives of Leg 168 were to understand the alteration of the ocean crust and the formation of fluids in the relatively young ocean crust. The microbial communities that may exist in this environment could have significant effects on both the alteration of rocks and the chemistry of circulating fluids and so are relevant to the primary objectives of Leg 168. Fluids and rocks collected from Hole 1026B were examined to determine the presence and identity of the subsurface microbial community.

Hole Characteristics and Drilling History

Hole 1026B was drilled in the northeast Pacific Ocean (47°45.739'N, 127°45.552'W) on the east flank of the Juan de Fuca Ridge (Fig. 1). At this location there is a smooth abyssal plain at 2658 m and 250 m of sediment above volcanic basement. The hole was designed to be reentered, so it was cased through sediment to basement (256 mbsf) with 10.75-in. pipe. Drilling below the casing fractured the basalts, which fell into the hole and jammed the drill string. The hole was deepened to 295 mbsf, but rubble in the hole limited water sampling and temperature measurements to a maximum depth of 286 mbsf. The hole was left on July 22, 1996 to continue work on other holes, and when Hole 1026B was revisited on August 2, it had filled with 64°C water from a subsurface aquifer (formation water).

SAMPLES

Rock

About 39 m of basalt and basalt breccia were cored, but only 5% of that was recovered. The sample of interest for this study is from a

breccia in Core 168-1026B-3R-1 (fig. 14A in Shipboard Scientific Party, 1997). Glass in the breccia of Sample 168-1026B-3R-1, 16–27 cm, was altered to clay along fractures (Fig. 2A) in a manner similar to alteration that has been attributed to microbial activity (Fisk et al., 1998; Furnes et al., 1996; Giovannoni et al., 1996; Torsvik et al., 1998). Dark zones between alteration products and glass are the locations within this rock that are likely to house microbes. Close examination by electron microscope of freshly exposed surfaces of clay in these samples shows that objects are present with many of the physical characteristics of microbes (Fig. 2B). These “cells” are about 1.5 µm long and 1 µm across; they are clustered into a small colony and have depressions that commonly occur in cells that are not carefully preserved (i.e., rocks were allowed to dry in air), and one pair of cells may have recently divided. These observations with optical and electron microscopy indicate that extant life is present in the basalts Hole 1026B.

Water

The water sampling temperature and pressure tool (WSTP) was used to collect two water samples in situ from Hole 1026B. The tool (Barnes, 1988) collects water through a 1-µm filter that extends through the drill bit. The water passes through a stainless steel tube into a sample coil that is within a titanium overflow chamber. The coil in this case was copper tubing. The WSTP collected 10 mL of water in the copper tube and about 1000 mL in the overflow chamber. Water from the overflow chamber was filtered (100 mL at a time) through 0.45-µm sterile filters to remove particulate matter before the routine shipboard chemical analyses were conducted on the water (Davis, Fisher, Firth, et al., 1997). On the first run of the WSTP, three unopened filter cartridges were placed in 125-mL jars that contained 100% ethanol, and two filter cartridges were placed in a jar with sterile artificial sea water (SASW). The filtered water from the WSTP appeared to be 80% formation water and 20% sea water. Water from the second deployment of the WSTP was filtered as well. Four filters were placed in ethanol and four in SASW. The water from the second run appeared to be closer to the that of formation water. The filters were stored on board in the dark at 4°C and transported under refrigeration to Oregon State University.

Material on the filters from the first deployment of the WSTP was light brown, whereas that from the second was pale green. Most effort was expended in analyzing filters from the second water sample because initial analyses indicated that this sample contained less seawater than the first WSTP.

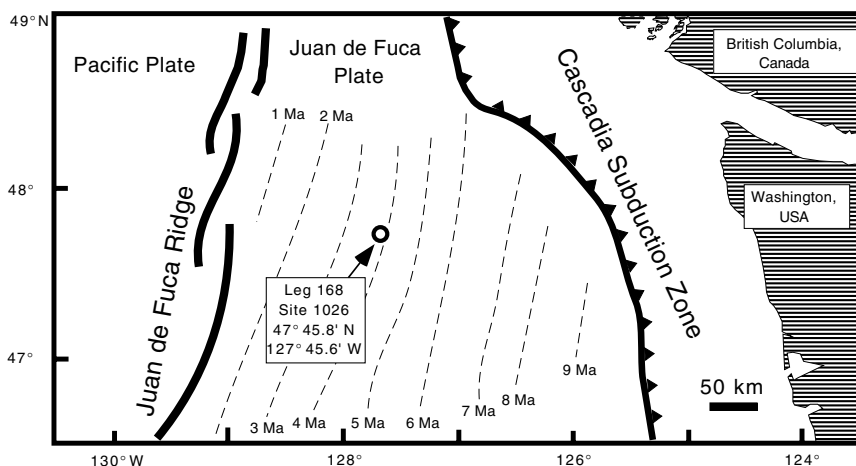
¹Fisher, A., Davis, E.E., and Escutia, C. (Eds.), 2000. *Proc. ODP, Sci. Results*, 168: College Station TX (Ocean Drilling Program).

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Figure 1. Area of Site 1026 on the east flank Juan de Fuca Plate. The ridge and Cascadia Subduction Zone are shown schematically. Estimated ages of the seafloor are based on the magnetic reversal time scale. Modified from Davis et al. (1997).



Potential Contamination

The second water sample collected by the WSTP appeared to contain little seawater, but there were several potential sources of microbial contamination in addition to sea water. These were the steel hole casing, drill pipe left in the hole, drilling mud, and the WSTP itself. The WSTP had a pair of filters to remove particles from the water that entered the sample coil. The smaller filter had an opening that was nominally 1 μm , but it was evident that many particles in the water sample were larger than 1 μm . This implies that the 1- μm filter over the entrance port to the WSTP was compromised.

The green particles on the filter we infer to be caused by Cu as indicated by several types of analyses. The Cu could be derived from fluids or from the WSTP. We attribute high levels of Cu in the second run to corrosion of the WSTP copper coil in the sample container between deployments of the tool or to contamination of the filter element at the tip of the WSTP with an antiseize compound that contains Cu and was used on threaded junctions of the tool.

The filters used to separate particles from the WSTP water were 0.45- μm Sterile Acrodiscs from Gelman Sciences. These have a polysulfone membrane that is covered with a wetting agent. The composition of the membrane and wetting agent are proprietary, but Gelman Sciences did reveal that the filter contained about 2 ppm of copper.

METHODS

Filters were removed from their cartridges and examined with a light microscope, which revealed that they were covered with a thin film and with particles of various sizes, up to about 200 μm . The particles were red, various shades of green, and clear. Euhedral crystals on the filter appeared to have precipitated from the solution in the filter housing. Additional examination of the particles on the filter were undertaken with electron beam techniques (electron microscopy, electron microprobe microanalysis, and transmission electron microscopy) and laser confocal microscopy after staining with epifluorescent dyes. Extraction of nucleic acids for cloning for 16sRNA genes was also attempted.

Electron Microscope

The electron microscope at Oregon State University is an Amray 3300 field-emission, scanning electron microscope. Operating voltage typically was 20 kV, and the working distance varied from 8 to 25 mm. The nominal resolution at 30 kV is 15 Å. For the SEM observations at the University of Bergen JEOL a scanning microscope

(JSM-6400) connected to a Tracor Northern (TN 5600 Series II) energy dispersive spectrometer (EDS) system with a Z-MAX 30 (diamond window) detector was used. The observations (and X-ray analyses) were performed at an acceleration voltage of 20 kV. The working distance varied from 8 to 39 mm.

Transmission Electron Microscope

For the TEM observation a Philips CM 200 electron microscope connected to an EDAX DX-4 EDS system with a thin window detector was used at the University of Bergen. The accelerating voltage was 120 kV. X-ray microanalyses were performed in the STEM mode with a tilt angle of 8° and a counting time of 100 s. Sixteen elements ranging from carbon to barium were detected in energy dispersive analyses, but no quantitative measurements of their concentrations were attempted. The intensity of the elemental peaks was designated strong, intermediate, or weak depending on the peak height relative to the maximum peak height in a given analysis. Strong peaks were within 40% of the maximum, intermediate peaks were from 10% to 40% of the maximum, and weak peaks were less than 10% of the maximum.

Electron Microprobe

Preliminary energy dispersive and wavelength dispersive electron microprobe measurements were made of particles on the filters with a Cameca SX-50 microprobe at Oregon State University. The correction procedure for X-ray absorption and fluorescence assumes a flat analytical surface, and because the particles on the filters were not flat, the analyses should be considered qualitative. In addition the spectrometers were tuned to only 10 elements, and we did not attempt to analyze for carbon, sulfur, or copper, so the analysis totals are considerably less than 100%.

Laser Confocal Microscopy

A Leica DM IRBE laser confocal microscope was used for photographing fluorescence of particles on the filter with a 100 \times objective lens with oil immersion. This technique was used rather than epifluorescence microscopy because of the irregular nature of the filter, and the particles on it were more suitably imaged with the confocal microscope. The filter was cut and dried in air and then fixed in glutaraldehyde. The unstained filter was had a diffuse greenish yellow autofluorescence and rare spots of blue fluorescence. For this reason the dye chosen for this study was Syto-59 (a nucleic acid-specific stain from Molecular Probes), which fluoresces in the near infrared—a part of the spectrum where we did not see autofluorescence.

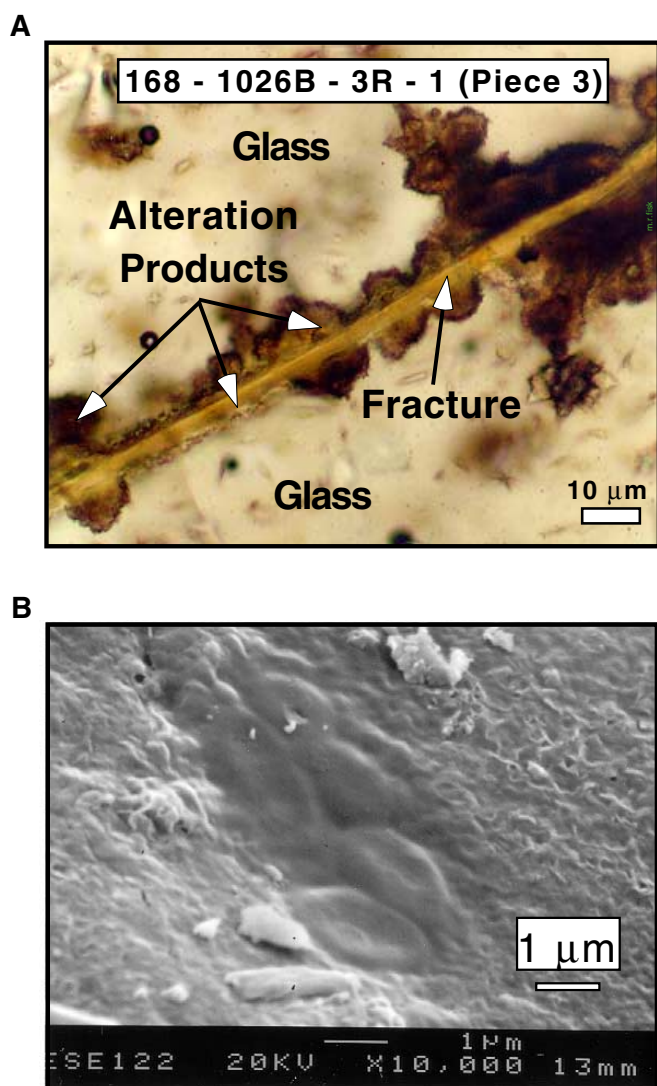


Figure 2. **A.** Petrographic thin section of glass from the basalt breccia, Sample 168-1026B-3R-1, 16–24 cm. A 10- μ m-wide fracture filled with yellow clay runs diagonally through the photo. Volcanic glass on either side of the fracture contains microlites. The fracture is surrounded by regions of alteration that extend 10–50 μ m into the glass. The boundary between the alteration and the glass is marked by dark zones about 2 μ m thick. A color version of this figure is available in PDF format on the volume CD-ROM. **B.** Scanning electron micrograph of clay surface in Sample 168-1026B-3R-1, 16–24 cm. Cells are clustered on the surface of clay from an interior surface of the sample. The flattened, dimpled shapes of the cells are due to air drying.

DNA Isolation and Amplification

Preliminary observation of the filters suggested that the amount of bacteria collected was quite small. For this reason, considerable care was taken in our attempts to limit contamination so that we might have success at identifying the small amounts of DNA that might be present. The techniques used here are not routine, and for this reason they are described in detail.

Preliminary Optimization of DNA Extraction Method

The bulk of materials on the sample filters observed by SEM appear to be clay particles, with microbes present as thin encrustations.

There is therefore relatively little DNA to be extracted from these samples. Under these conditions it is extremely important to minimize contamination with exogenously added DNA, as even small amounts of such contaminants can dominate the final extract and predominate among clones in the final library. Buffers and reagents (except oligonucleotides and enzymes) were UV irradiated, and pipetors and plasticware were cleaned with 10% sodium hypochlorite and/or UV irradiated to destroy contaminating DNA. Aerosol-barrier pipette tips were used. In addition, our protocol has been designed to include the smallest number of added reagents to minimize potential routes of sample contamination.

Preliminary tests were performed to identify a high-yield DNA extraction method compatible with the commercial filter units used to collect the Leg 168 samples. These 0.45- μ m pore-size Acrodisc units (Gelman) are sealed, disposable units designed for removing particles from liquid solutions, not to enable particle recovery. We developed a “surgical” technique for aseptically breaking open and excising filters from these units and used this technique to prepare filters for preliminary control experiments, as well as for our analysis of the Leg 168 particulate sample. Using blank filters and mock sample filters spotted with *Escherichia coli* hosting a 16S rRNA plasmid clone, we tested two DNA extraction protocols: a low-loss alkaline extraction (Urbach et al., 1998) and a guanidinium thiocyanate protocol (Pitcher et al., 1989), for sensitivity and compatibility with Acrodisc filters. In the past, the alkaline extraction protocol has been successfully used to amplify single-copy protein-encoding genes from environmental samples containing $\approx 10^5$ cells (Urbach and Chisholm, 1998) and 16S rRNA genes from 10^3 cultured *E. coli* spotted onto polycarbonate filters (E. Urbach, unpubl. data). The guanidinium thiocyanate protocol has been shown to lyse microorganisms belonging to diverse taxa (Pitcher et al., 1989) and is highly efficient (Boom et al., 1990).

Cultured *E. coli* hosting a 16S rRNA gene cloned into a high copy-number plasmid ($\approx 10^6$ plasmid copies cell⁻¹, pCRII, Invitrogen) was used to test DNA recovery from the mock sample filters. This protocol effectively separates the assay for cell lysis from tests for sensitivity of the PCR assay and allows reliable detection of DNA released from small samples using a standard, moderately sensitive PCR protocol. DNA in the extracts was assayed by PCR amplification of 16S rRNA genes (35 rounds of 1 min 94°C, 1 min 55°C, and 2 min 72°C, in the presence of bacterial-specific primers 27F and 1492R; Lane, 1991), combined with agarose gel electrophoresis. Because the number of gene copies released by lysis of even a small number of cells is above the detection limit of the PCR assay, the absence of PCR product in an extraction assay is firm evidence either that DNA was not released from the cells or that a PCR-inhibitory substance was co-extracted with the DNA.

In preliminary experiments the alkaline extraction protocol proved incompatible with the Acrodisc filters, but the guanidinium thiocyanate technique was highly sensitive and compatible with the filters. No PCR amplification products were obtained from alkaline extracts of Acrodisc filters spotted with 10^6 16S rRNA clone-containing *E. coli* cells. This result indicates either that the alkaline lysis protocol was unable to lyse *E. coli* in the presence of Acrodisc filters, or that an inhibitory substance was extracted from the filter along with the DNA. Better results were obtained with the guanidinium protocol, which yielded amplification products from clone-containing *E. coli* spotted filters $\approx 75\%$ as strong as from the same cells extracted in the absence of filters. The guanidinium extraction protocol was used to prepare DNA from Leg 168 filters.

Design of a New, Archaea-Specific PCR Primer

Both bacterial and archaeal microbes are likely to be present in the submarine thermal fluids sampled during Leg 168. We scanned the rapidly growing 16S rRNA sequence database to learn whether our

PCR primers would successfully amplify genes from currently known microbial organisms. Although this survey did not identify serious problems with our bacteria-specific primer 27F or our universal primer 1492R, we discovered that our archaea-specific primer Arch 21F would fail to amplify 16S rRNA genes from a number of potentially important archaeal lineages. This is because new sequences added to the 16S rRNA database (Barns et al., 1996) have considerably broadened the diversity of known archaea. We therefore designed a new archaea-specific PCR primer Arch 25F, a degenerate oligonucleotide that should amplify small subunit rRNA sequences from almost all known archaea. We successfully tested PCR amplification by Arch 25F and universal primer 1492R using a marine environmental fosmid clone containing an archaeal 16S rRNA gene (Stein et al., 1996) as template.

PCR Analysis of Leg 168 Particulate Samples

A filter containing Leg 168 particulate samples was removed from its Acrodisk unit and subjected to the guanidinium extraction procedure. Processed in parallel were three blank filters, three extraction blanks without filters, and one positive control filter spotted with 10^6 plasmid-containing *E. coli*. Half of each DNA fraction was subjected to 35 cycles of PCR amplification using bacteria-specific 16S rRNA gene primer pairs, and the other half was similarly amplified with archaeal-specific primers. Samples yielding no PCR products were subjected to a second round of amplification in which 1/50 of the first-round reaction products were transferred to tubes with fresh reagents and amplified for 40 additional cycles.

RESULTS

Scanning Electron Microscope

Examination of the filters with the electron microscope revealed that the filter fibers were peppered with particles that were less than $1\ \mu\text{m}$ (Fig. 3A). Rod-shaped particles that were less than $0.5\ \mu\text{m}$ were typical, as were spheres (Fig. 3A). These particles have the morphology of cells, and in several cases appear to be joined end-to-end. However, they are possibly too small to be bacteria. Alternatively, they could be minerals entrained in the subsurface water or that precipitated from the water after it was collected. Particles larger than $1\ \mu\text{m}$ were also present on the filter. These could have been already present in the WSTP when it was deployed or they could have passed through the $1\text{-}\mu\text{m}$ filter on the WSTP. A clay particle on the filter is shown in Figure 3B. This clay particle does not have the coating of small particles that were seen in Figure 3A. The clay particle is resting on unidentified material (which we named "matte") that also was collected on the filter (Fig. 3B). Matte may be polysaccharide, which appears to be abundant in the waters of Hole 1026B (S. Giovannoni, unpubl. data). In addition to mineral grains, there are a few objects that appear to be of biological origin (Fig. 3C). These are similar to what has been described as microbial floc (Juniper et al., 1995) from the Juan de Fuca Ridge, which were 20%–25% Fe.

Particles on the filter were analyzed with an energy dispersive spectrometer attached to the SEM using a standardless technique. The analyses were normalized to 100% and should be considered qualitative (Table 1). In addition to those elements listed in Table 1, analysis of phosphorus was attempted, but it was not detected. The particles were irregular, which also affected X-ray absorption and fluorescence. Cu was detected in all of the analyses, and in some of them Cu is the major component. This is not an artifact of the analytical technique, because Cu was detected with the other microbeam analyses. Cu is present in the $0.45\text{-}\mu\text{m}$ Acrodisk filters, but at a level of only 2 ppm, so this is not the source of the Cu signal.

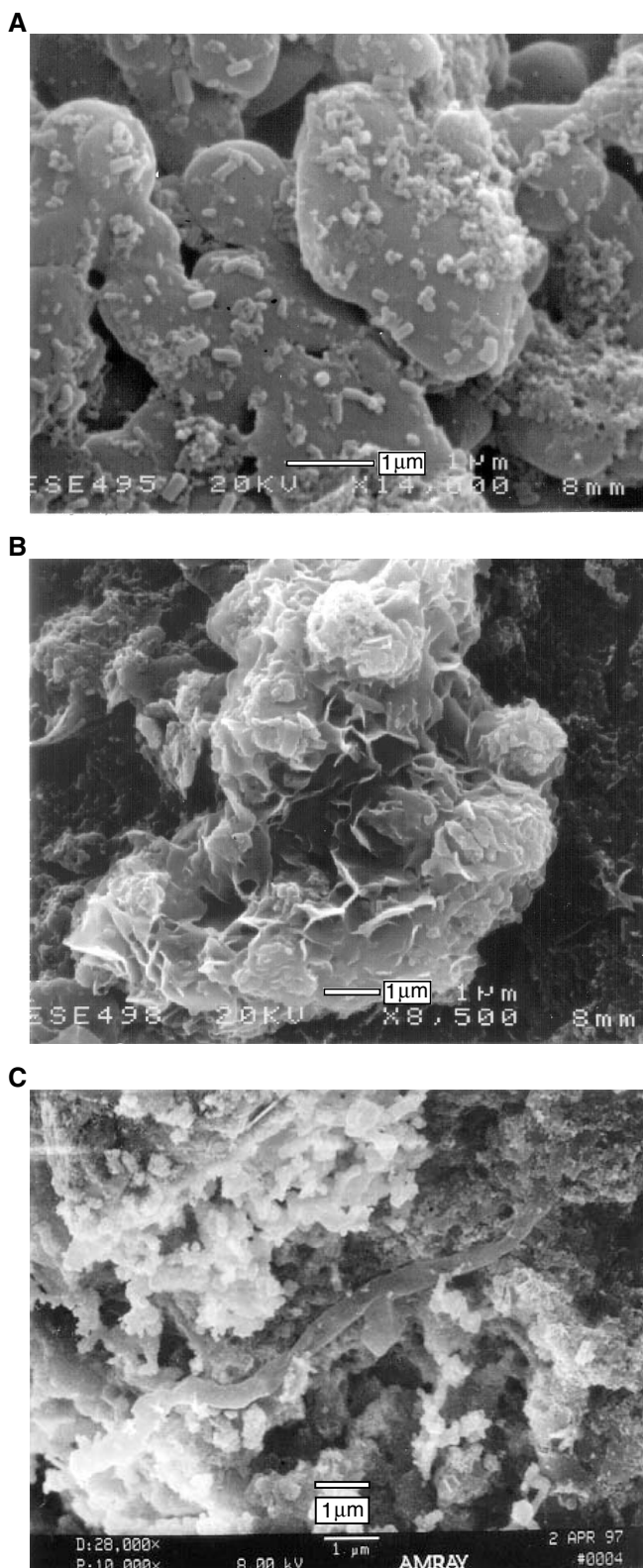


Figure 3. Scanning electron microscope image of (A) the Acrodisk filter, (B) a clay particle on the filter, and (C) what appears to be microbial floc. Operating conditions were 20 kV and a working distance of 8 mm for (A) and (B). For (C) conditions were 8 kV. Scale bars are $1\ \mu\text{m}$.

Table 1. Energy dispersive chemical analyses of particles removed from formation water of Hole 1026B.

Analysis	Description	SiO ₂	Al ₂ O ₃	MgO	CaO	Na ₂ O	K ₂ O	TiO ₂	Cr ₂ O ₃	MnO	FeO	NiO	CuO	S	Cl
C	Bright green	12	7.0	4.4	0.8	—	—	—	1.4	0	6.2	0.7	59	—	9
A	Dull green	40	23	1.8	—	—	—	4.9	—	1.7	2.8	—	21	0	4.1
B	Dull green	30	15	2.9	1.0	—	0.4	1.2	2.9	—	5.3	2.5	34	1.4	4.0
B	Dull green	30	13	2.0	2.9	—	0.7	1.4	3.4	—	20	—	26	—	2.4
Ti	Brown	2	1	0.2	0.2	—	—	92.1	—	—	1.1	0.7	3	—	—
D	Dull green	40	21	1.9	0.9	—	0.6	2.9	—	—	6.3	—	24	—	2.3
E	Dull green	30	15	2.0	1.8	—	—	—	—	—	1.6	—	47	—	2.5
E org	Dull green	41	20	3.2	2.4	—	—	2.3	—	—	5.0	—	25	—	0.8
F	Dull green	36	24	2.6	0.4	—	0.4	1.9	1.2	1.0	3.9	—	27	—	2.1
G	Brown	8	3	0.9	0.5	—	—	—	5.9	—	71	—	9	—	1.8
H		23	8	5.7	2.1	—	0.4	—	1.0	—	2.2	—	47	8.2	2.8
I	Brown	4	—	1.0	1.2	3.4	0.6	—	8.7	0.4	69	1.2	9	—	2.1
J		26	9	4.8	2.8	—	—	—	—	—	4.6	—	55	6.3	2.9
P4B-2		29	10	3.5	0.9	—	0.6	—	—	—	4.6	—	43	6.3	2.9
P4B-3		30	11	4.0	3.0	—	0.4	—	1.0	1.3	6.4	—	40	—	2.9
K	Unknown	50	39	0.6	0.4	—	0.1	3.3	0.8	—	1.7	0.4	3	—	—
L	Organism?	9	3	1.7	0.5	—	—	—	1.2	—	61	1.0	20	—	3.0

Notes: Analyses by JEOL electron microscope, Bergen. Analyses are normalized to 100%. — = not detected.

Cu is inversely correlated with silicon and aluminum; Si and Al have a roughly constant ratio (Fig. 4A). This suggests that the analyses are a mixture of clay and a copper compound. The makeup of the copper compound is not obvious from the analyses. Mg and Cu appear to be strongly correlated (Fig. 4B), but Cu is not strongly correlated with any other metals, sulfur, or chlorine (Fig. 4C). Fe, Cr, and Ni were elevated in some analyses, suggesting that rusted steel from the drill pipe could have contaminated the sample.

Electron Microprobe

Additional EDS and wavelength dispersive analyses of particles on a filter from Run 2 of the WSTP appear to be clays, oxides, and sulfates (Table 2). Euhedral crystals of calcium sulfate appear to have formed on the filter when they were dried. Cu was observed in the EDS spectra of particles on the filter as was found with EDS analyses in Bergen.

Transmission Electron Microscope (TEM)

A TEM image of the filter is shown in Figure 5. In addition to the particles shown in the figure, qualitative chemical identification (using the energy spectra from the energy dispersive detector) was made of particles elsewhere on the filter (Table 3). TEM spectral analyses always had CuK_α, CuK_β, CuL_α, Ck_α, and SK_α peaks. The filter and background were also measured. As in the SEM EDS analyses of the particles (Table 1), Cu is ubiquitous. The filter fiber showed the presence of S, Cu, and trace amounts of Si. Particles trapped on the filter included matte, aluminosilicates (presumably clay), steel, titanium-rich grains, sulfur-silica-iron-rich particles, and barite. A single analysis of what we have called a cell (Fig. 5) did not appear to be significantly different from the matte, except that the cell had less iron. The matte was interesting in that it was rich in C, O, Si, Fe, and Cu, as well as P. One Ti-rich particle was analyzed as well as one barite. Anatase (TiO₂) has been reported as an extreme weathering product of basalt (Howard and Fisk, 1988) and could be carried in the subsurface fluids; however, it could also be derived from the titanium pressure casing of the overflow chamber.

Laser Confocal Microscopy

Fluorescence caused by the dye Syto-59 was localized on particles on the filter (Fig. 6). Reflection from the particles are seen in green and the fluorescence is indicated by red. Nucleic acids on the particles are indicated by red isolated pixels that are typically located in 1-μm spots throughout the particles.

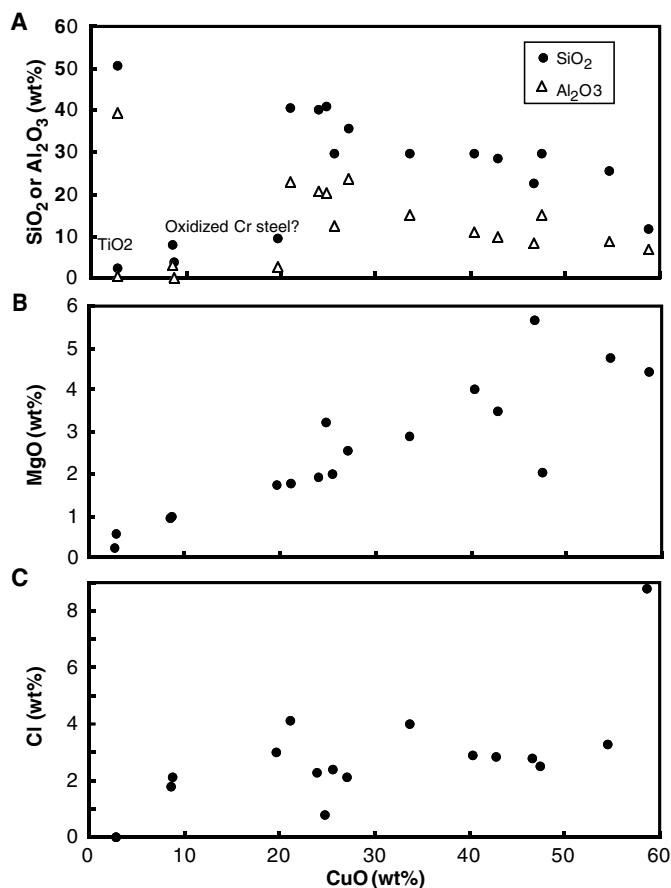


Figure 4. EDS chemical analyses of particles collected on the filters from Table 1. A. SiO₂, Al₂O₃, and CuO. B. MgO and CuO. C. Cl and CuO.

Results of Leg 168 PCR Analysis

Amplification with domain-specific 16S rRNA PCR primers failed to detect either bacterial or archaeal sequences in DNA fractions from a Leg 168 particulate sample. Amplification products were detected only in positive control samples containing DNA extracted from *E. coli* spotted onto filters and in positive controls containing *E. coli* DNA extracted by standard protocols (a faint band was

Table 2. Wavelength dispersive analyses.

Analysis	Description	SiO ₂	Al ₂ O ₃	MgO	CaO	Na ₂ O	K ₂ O	TiO ₂	MnO	FeO	P ₂ O ₅	Sum
20	Clear euhedral crystal	0.5	0.16	0.21	23	—	—	—	—	0.17	0.3	24
21	Clear euhedral crystal	4.0	0.43	0.20	29	—	0.1	—	0.1	0.73	0.2	35
22	Clear euhedral crystal	3.7	0.36	0.17	31	0.1	0.1	—	—	0.61	0.2	36
23	Clear euhedral crystal	3.4	0.38	0.15	38	0.1	0.1	—	—	0.59	0.1	43
25	Reddish brown particle	3.3	0.11	0.04	26	—	—	—	—	4.64	0.1	34
26	Clay	1.2	6.3	0.24	2	0.1	0.3	1.8	—	2.66	0.2	26
27-1	Nucleation site for CaSO ₄	3.2	0.26	0.13	17	—	—	—	—	2.89	0.5	24
27-2	Nucleation site for CaSO ₄	4.4	0.10	0.08	37	—	—	—	—	1.90	0.2	44
27-3	Nucleation site for CaSO ₄	1.9	0.07	0.01	44	—	—	—	—	0.42	0.1	46
28	Filter	6.4	0.26	0.15	2	0.1	0.1	—	—	0.80	0.6	10
29	Calcium sulfate	3.2	0.12	0.06	42	0.1	—	—	—	1.34	0.2	47
30	Reddish brown mineral	5.4	0.07	0.07	2	0.2	0.1	—	0.1	4.7	0.1	55

Notes: Analyzed by Cameca microprobe, Oregon State University. — = not detected.

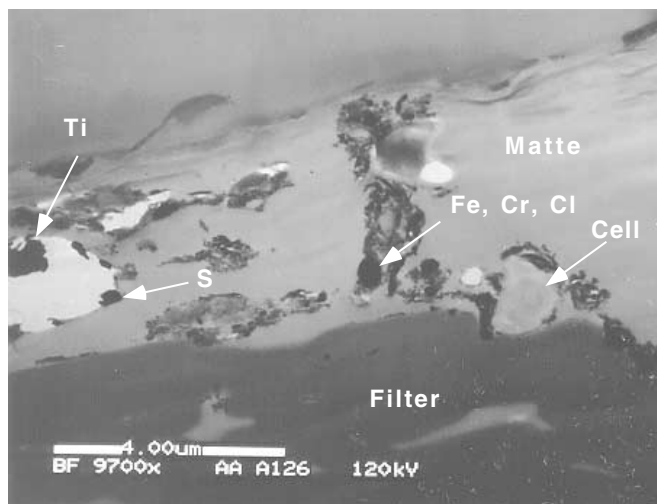


Figure 5. Transmission electron microscope image of a section of the Acro-disk filter. The filter is dark region at the bottom of the image. Particles on the filter are identified by major chemical elements. One cell (identified by internal structures) is located on the filter. The gray matter on top of the filter “matte” may be polysaccharide.

also present in one of the negative control filter extract as well). The most likely explanation for the failure of this effort is that there were too few cells present on the filter to be detected by PCR. However, another possible explanation for our inability to detect 16S rRNA genes from the Leg 168 sample is that a PCR-inhibitory substance was extracted from mineral particles present on the sample filter. Although we could test for this possibility by adding purified, positive control template DNA to Leg 168 filter extracts and assaying by PCR, we have too few Leg 168 samples to troubleshoot a purification protocol and eliminate such an inhibitory substance.

DISCUSSION

A subsurface microbial environment in the igneous rocks of the ocean crust has been inferred from the microbiology of vent waters. Huber et al. (1990) reported a hyperthermophilic, anaerobic Archaea above an eruption of Macdonald Seamount in the south Pacific that must have been washed from the interior of the volcano. Haymon et al. (1993) and Juniper et al., (1995) described a floc of probable microbial origin on the East Pacific Rise and Juan de Fuca Ridge, respectively. On the Juan de Fuca Ridge, ~1000 kg of floc covered 2.1×10^5 m² of the ridge axis. This floc was silica and iron in the shape of microbial filaments, coccoids, and rods (Juniper et al., 1995) and microbially produced filamentous sulfur (Taylor and Wirsén, 1997).

The floc was attributed to a massive subsurface community that either was dislodged by the eruption or possibly bloomed transiently at the subsurface confluence of oxidized sea water and reduced hydrothermal fluids. The number of microorganisms that appear to inhabit oceanic basalts is quite low (Fisk et al., 1998) and is not sufficient to produce the “snowblower” vents that produce the microbial floc. However, the subsurface organisms may undergo prolific growth when volcanic intrusions crack the crust and increase permeability and at the same time supply heat to drive convective fluid flow, which provides chemical growth substrates and nutrients. Presently, we are not able to evaluate these alternatives because we do not know the relationship between free floating and endolithic organisms at Site 1026.

The particles in the fluid appear to be a mixture of contaminants and material that was present in the formation water. Some particles are clearly contaminants such as the steel rust. Other particles could be derived from the subsurface rocks or from contaminants, such as the barite, TiO₂, floc, and clay. Contaminants could be derived from drilling mud, sediment from the upper part of the hole, and the WSTP tool. The origin of the high Cu on the filter and particles is not known, but we suspect that it is from the WSTP. Some of the particles were high in sulfur, which suggests that they could be microbial floc; however, these particles were rare. Rare filaments (Fig. 3C) could be the equivalent to the floc found at hydrothermal vents. Also particles with relatively high silica and iron (Table 3) could be microbial products. The matte found on the filter (Fig. 5) could also be a microbial product (polysaccharide).

Particles on the filters had sparsely distributed fluorescence from the dye Syto-59, which we attribute to microbes attached to the filters. Either these microbes could not be extracted from the filters, or they were present in such small numbers that the molecular techniques used to amplify them were not sufficient.

Our inability to extract and PCR amplify 16S rRNA genes from the Leg 168 particulate sample DNA extraction and PCR effort is not inconsistent with the existence of bacteria in submarine hydrothermal fluids. In earlier experiments using a fluorescent DNA stain, we detected approximately 10^5 microbial cells per filter in one Leg 168 sample. One round of PCR by our protocol is capable of detecting 10^3 plasmid-free *E. coli* cells, which implies that we should have detected amplification products from two rounds of amplification of extracts from the Leg 168 sample. However, it is likely that the potentially slow-growing microbes present in the Leg 168 samples contain both fewer 16S rRNA copies per genome and fewer chromosome complements than the fast-growing *E. coli* cells used to estimate our theoretical detection limit (Schmidt, 1997). Thus our detection limit for environmental microbes may be higher than our detection limit for *E. coli*. Although disappointing, it is therefore not too surprising that we were unable to detect 16S rRNA gene sequences from the small number of cells in the Leg 168 particulate sample.

Characterization of contamination of subsurface water samples is important for understanding the biology of the ocean crust. For this

Table 3. Qualitative analyses of filter and particles from the second WSTP water sample.

Sample	Element:	C	O	F	Mg	Al	Si	P	S	Cl	K	Ca	Ti	Cr	Fe	Cu	Ba
Area 1	Filter	s	w				w		i								i
	Background	s	w				w		w								i
	Crystal 1	i	s	i			w		w			w		i	s		s
	Crystal 2	i	i			w	i		w			w	s	w	w		i
	Crystal 3	s	s			s	s		w	w			w				s
	Crystal 4	s	w			w	w		w	w							s
	Matte 1	s	s			w	i	w	w			w		w	s		s
	Matte 2	s	s		w	w	s	i	w	w		w		w	i	s	
Area 2	Crystal 21	s	s			s	s	w			w				w		i
	Matte 21	s	o				w							w	i		i
Area 3	Background	s	w														s
	Filter	s	w						i								i
	Barite	i	s						s								
	Silicate	s	s			w	s	s	s						s		s
	Silicate	i	s			s	s				w				w		i
	Fe-oxide	s	s				i	w	w						s		s
	Matte	s	s				s	i	w						s		s
	Cell	s	i			w	w	i	w						w		i
	Ti-oxide	w	s			w	w						s	i	w		w
	Particle	i	s				i		w	i		w		i	s		i

Notes: s = strong, within 40% of the maximum intensity; i = intermediate, between 10% and 40% of the maximum intensity; w = weak, less than 10% of maximum intensity. Blank = no peak observed.

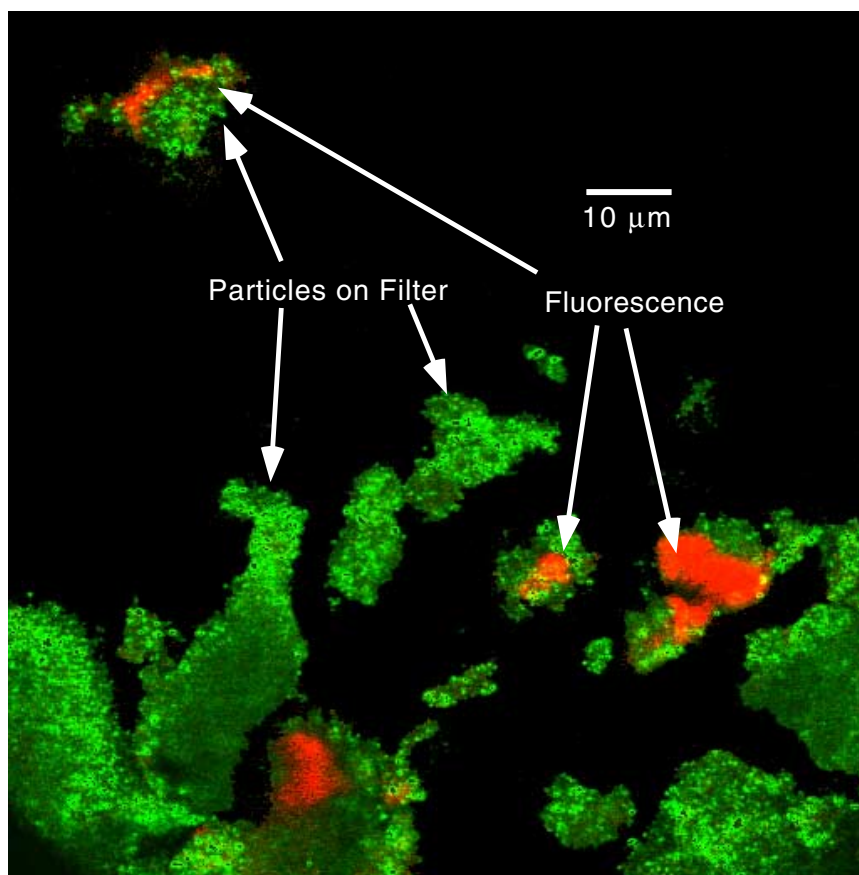


Figure 6. Laser confocal image of particles on a filter. The particles viewed in incident light are indicated in dark gray with light gray speckles. (Color image available in PDF format on the volume CD-ROM shows these particles as green.) Areas of fluorescence due to Syto-59 are indicated by arrows and appear here as an intermediate gray. (Color image available on the volume CD-ROM shows these areas in red.) Some particles have areas of intense fluorescence which are likely to be caused by groups of bacteria. Most particles also have regions of dispersed fluorescence where each fluorescing spot is 1–2 μm.

reason it is important that an aliquot of formation water be filtered and examined by electron microscope and electron microprobe so that the sources of contamination may be determined. In addition to this characterization of particles on filters, we have four recommendations for improving the water sampling technique. First, the filters that remove clay particles from the entry port of the WSTP should have an opening of at least 2 μm, and the filter should be kept free of shipboard contaminants with a shroud that is removed just prior to the lowering of the tool. Second, the water in the WSTP should be cap-

tured in stainless steel or titanium coils and the length of the coils increased so that larger samples can be collected. Third, a method of flushing the tool with sterile, 0.2-μm, filtered water should be devised, and the tool should be flushed immediately before deployment. And fourth, if cells are to be cultured from the water sample, the pressure case should be flushed with nitrogen as it is being closed so that organisms are not exposed to oxygen in the WSTP. Maintaining in situ pressure will also be important during subsampling water and culturing microbes if we wish to study barophilic microorganisms.

CONCLUSION

Several lines of evidence indicate that subocean aquifers and rocks are inhabited by microorganisms. The rocks from Hole 1026B have textural features that indicate microbes have been active in these basalts. Cells were identified by electron microscopy of clay on fractured surfaces of the rock. In the breccia studied here, clay is commonly an alteration product of glass. The fluids collected from Hole 1026B were some of the first samples from a subsurface aquifer to be examined for the presence of microorganisms. Evidence for microbes in the formation water that filled Hole 1026B come from fluorescent stains that show microbe-sized particles and TEM images of the particles filtered from the formation water. Based on laser confocal imagery and the sensitivity of our amplification techniques, the abundance of cells in this water appears to be low, possibly less than 1000 cells/mL. The small number of cells prevented us from identifying the types of microbes in the formation water. Improvements in techniques for extraction of nucleic acids, for reducing the nucleic acids in blanks, and in amplification could make it possible to identify the types of microbes in deep ocean aquifers.

The low cell numbers in this subseafloor aquifer appears contrary to cell abundance inferred from "snowblower" springs on ocean ridges. At least in the case of Hole 1026B, there is no large standing crop of microorganisms, so the hypothesis that snowblowers are the consequence of the prolific microbial growth triggered by magma injection in the crust appears to be supported.

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