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EXPLORATION OF LIFE IN DEEP SUBSEAFLOOR SEDIMENTS

BY DAVID C. SMITH AND STEVEN D'HONDT

The scientific ocean drilling community has been retrieving cores from hundreds of meters below the seafloor since the inception of the Deep Sea Drilling Project (DSDP) in 1968. While microbiological research was not part of the scientific impetus for the creation of DSDP, it became apparent to geochemists that some of the observations in pore water chemistry indicated microbiological activity at great depth (e.g., Claypool and Kaplan, 1974). Site after site yielded profiles of microbiologically relevant compounds (e.g., sulfate, methane) that indicated microbial activity.

Study of subseafloor life has increased

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steadily over the last 30 years. Although such studies were rare during DSDP, radiotracer experiments on the final DSDP expeditions aboard the *Glomar Challenger* (Legs 95 and 96) documented microbial activities in samples from multiple sites, taken from depths as great as 167 meters below the seafloor (mbsf) (Whelan et al., 1986; Tarafa et al., 1987). Exploration of subseafloor life gained considerable momentum during the Ocean Drilling Program (ODP; the international successor to DSDP). Studies began early in ODP with census of cells and activity profiles in subseafloor sediments of the Peru Margin (Cragg et al., 1990) and reached its current zenith with ODP Leg 201, the first scientific drilling expedition focused principally on study of subseafloor life (D'Hondt et al., 2003). Study of subseafloor life is now poised to advance much farther and faster during the Integrated Ocean Drilling Program (IODP), ODP's successor. Study of the

“Deep Biosphere and the Subseafloor Ocean” is one of the three principal themes of the IODP Initial Science Plan (Coffin, McKenzie et al., 2001). The multiplatform exploration capabilities of IODP will allow exploration of subsurface life in a wide range of previously inaccessible environments.

CONTAMINATION

Prior to subsampling cores, it is necessary to ensure that they are of sufficient quality to warrant the time and cost of subsequent microbiological analyses. The coring system on ODP's drillship *JOIDES Resolution* was not designed with the idea of collecting samples for microbiological studies. Surface seawater, which contains on the order of 10^9 bacteria per liter, is pumped through the drillstring to remove the tailings from the borehole so the bit can advance. Early in ODP, there were obvious concerns regarding contamination of cores



Figure 1. Subsampling (left) and adding tracer to drilling fluids (right).



with microorganisms entrained in the drilling fluid. Using a combination of a chemical tracer (perfluoro-methylcyclohexane) injected directly into the drilling fluid and 0.5- μm diameter fluorescent spheres delivered in the core catcher, it has been determined that the coring system employed can retrieve cores with little to no detectable signs of seawater intrusion (Figure 1) (Smith et al., 2000; House et al., 2003; Lever et al., in press). Although uncontaminated cores can be retrieved, not all cores are uncontaminated. The type of coring (advance piston coring versus rotary coring; see also Figure 2 in Thomas et al., this issue) and the lithology of the formation dictate the quality of the cores. Therefore, constant vigilance is required to ensure that the microbes recovered from a core are native to the cored formation.

ESTIMATING BIOMASS

Cell abundance determined with acridine orange direct counts (AODC) provide a broad overview of the distribution of microorganisms as a function of depth in the sediments (Parks et al., 2000). Because acridine orange (AO) fluoresces when bound to nucleic acid, DNA-bearing cells are visible in microscopic surveys of sediment stained with AO. Cell abundance is typically on the order of 10^8 – 10^9 cells cm^{-3} in sediments near the surface and declines exponentially with depth in the sediment column. However, there are significant departures from this trend at major geochemical interfaces, such as zones in Peru Shelf sediments where large fluxes of methane meet large fluxes of sulfate and sustain high rates of anaerobic methane oxidation (e.g., D'Hondt et al.,

2004; Parkes et al., 2005). Currently, the deepest samples that contained visible cells were collected at ~ 800 mbsf and contained 10^5 cells cm^{-3} . Many AODC samples from other depths, both shallower and deeper, do not contain microorganisms, although with a detection limit of $\sim 6 \times 10^4$ cells cm^{-3} , AODC is a relatively insensitive method. The application of newer, more sensitive, specific fluorochromes that stain DNA (e.g., SYBR green; Figure 2), may increase the sensitivity, but the limits of microscopy will remain.

Whitman et al. (1998) used these data to conclude that subsurface sediment contains the largest reservoir of Earth's biomass, an estimated 3.5×10^{30} cells. This provocative paper concluded that the mass of these cells, which contain an estimated 3×10^{17} gC, is equivalent

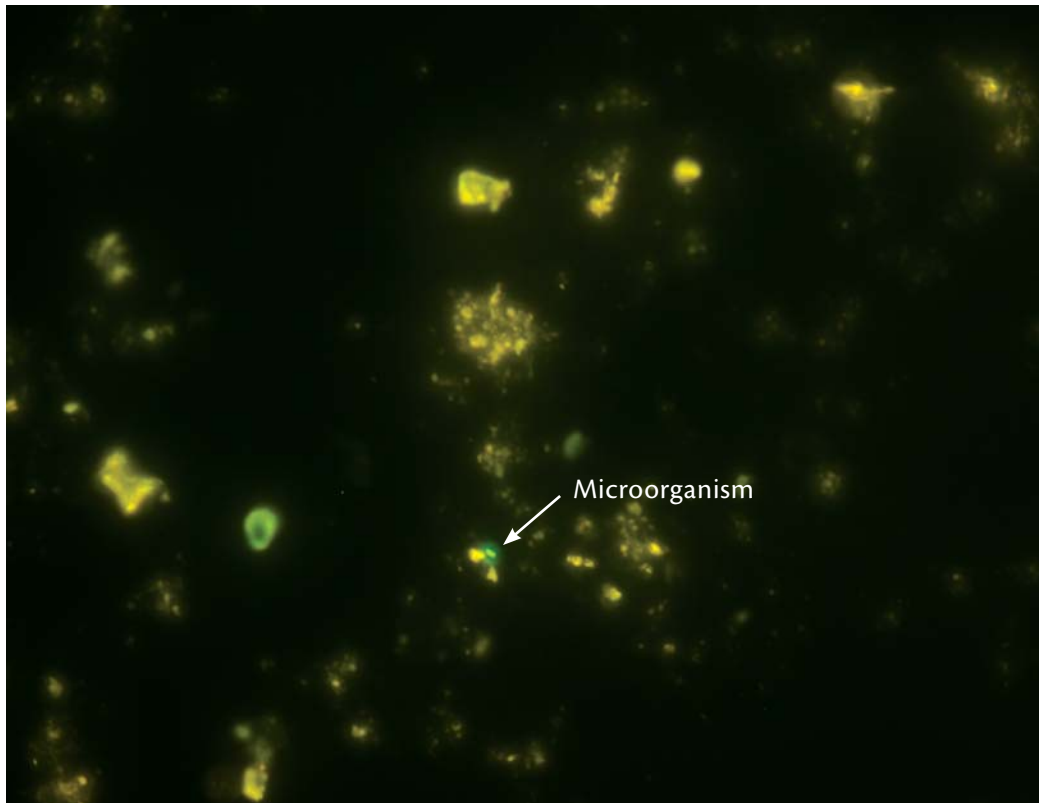


Figure 2. A lone microorganism in deeply buried marine sediments as viewed with epifluorescence microscopy. The sample was stained with the DNA-specific fluorochrome SYBR-green. The sediment was cored on the Lomonosov Ridge in the central Arctic Ocean during IODP Arctic Coring Expedition (see Moran et al., this issue).

to ~ 30 percent of the total biomass on Earth. A slightly more recent estimate suggested that cells in subseafloor sediments constitute 10 percent of Earth's total biomass (Parkes et al., 2000). Both of these estimates were based on the average concentrations of counted cells in previously examined sites. More recent data from ODP Leg 201 suggest that the sites used for these estimates are not truly representative of the full range of subseafloor sediments; cell concentrations in Peru Margin sediments were close to these historic averages, but cell concentrations in the open-ocean sites with the lowest levels of microbial activity were

far lower (ODP Sites 1225 and 1231; Figure 3). If similar results are found in future studies of open-ocean sites, the estimate of subseafloor biomass will need to be revised downward.

These biomass estimates implicitly assume that all of the cells observed by AODC are alive and active. However, many AODC-enumerated cells may be dead or inactive. Distinction of living and active cells from dead or inactive cells is crucial for accurate biomass estimates. It is also critical for considering the fluxes of energy required to maintain subseafloor biomass, particularly given the quiescent nature of this environ-

ment. This distinction is a major issue in some areas of microbial ecology and is not unique to the subsurface. Several methods, relying on some basics of cell biology, have been developed to determine the viability of cells. Some of the methods examine the entire community by quantifying compounds (e.g., Adenosine 5'-triphosphate [ATP], phospholipids, ribonucleic acid [RNA]) in bulk samples while other methods focus on examining individual cells (e.g., 5-cyano-2,3-ditolyl tetrazolium chloride [CTC] and catalyzed reporter deposition-fluorescence *in situ* hybridization [CARD-FISH]). Despite their differenc-

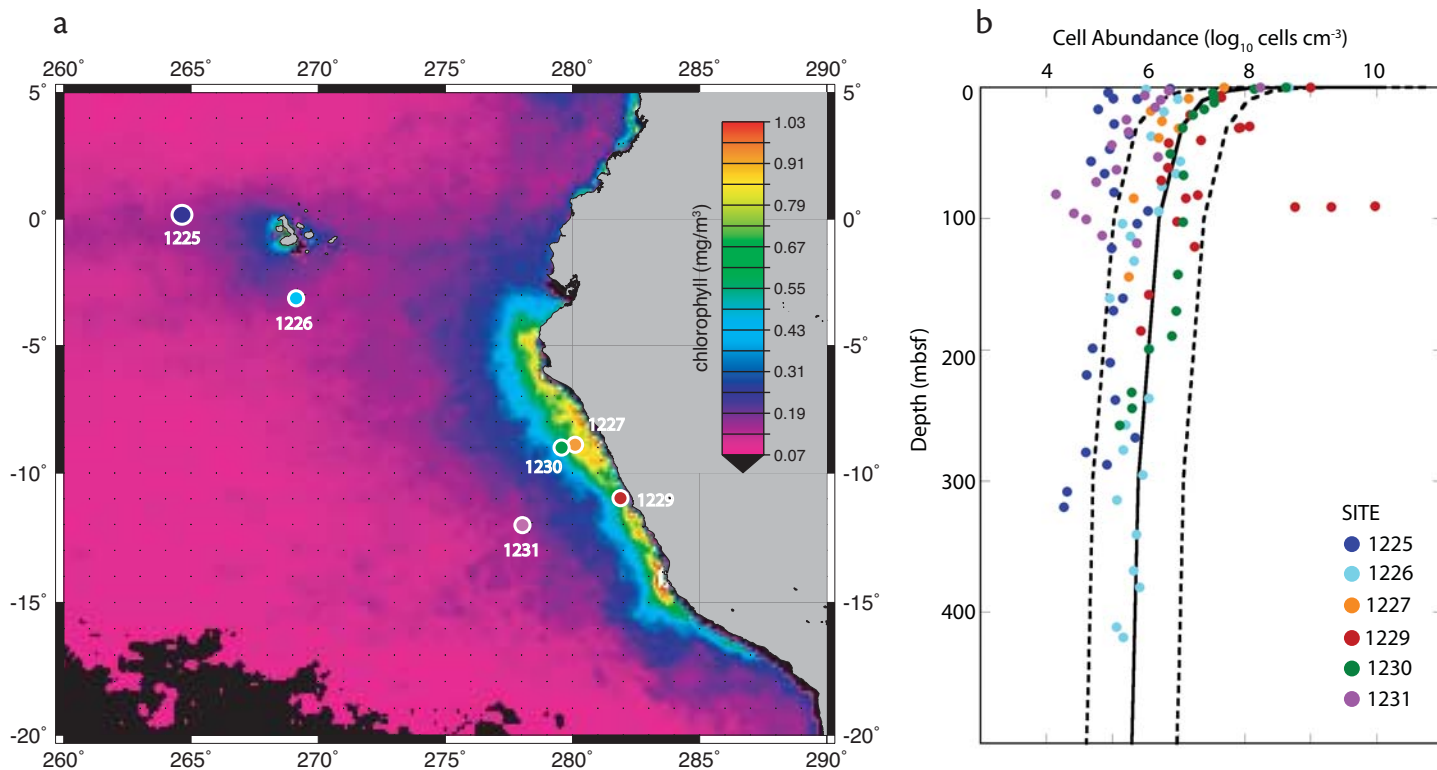


Figure 3. (a) Map of location of sites cored in the Equatorial Pacific and along the Peru Margin during ODP Leg 201. Sites 1225 and 1226 are located under the oligotrophic (nutrient-poor) areas of the equatorial Pacific. Sites 1227–1230 lay under the more productive waters of the Peru Margin and Site 1231 is in the oligotrophic Peru Basin. Satellite-derived mean chlorophyll data are from the Coastal Zone Color Scanner (1978–1986). (b) Microbial biomass estimates based on microscopic examination of sediment samples collected during Leg 201. The solid line is the global average with 95% confidence interval (Parkes et al., 2000). The biomass in the sediments reflects the productivity of the overlying waters (and subsequent organic matter flux to the sediment). The low-activity sites (1225, 1226, and 1231) are 10–100 fold lower than global average while biomass peaks at sulfate-methane transitions zones in the margin.

es, all of these methods share a common goal in targeting labile molecules that do not persist long after cell death.

ATP is the common currency for biologically available energy and has been used as a proxy for biomass in microbial ecology for many years (reviewed in Karl, 1980). This approach was used to calculate biomass in the upper 60 m of the sediment column at Blake Ridge off the coast of Georgia, USA (ODP Leg 164) (Egeberg, 2000). Converting ATP concentrations to biomass assumes a constant ATP per cell as well as a carbon-to-ATP ratio. Both properties are known to vary considerably (Karl, 1980).

Although there is very limited direct comparison to AODC data from this site, ATP-based cell abundance was ~ 50-fold less than the AODC estimates.

RNA has been the subject of many studies that address microbial metabolic states. One method that targets ribosomal RNA using oligonucleotide probes is the FISH method. This method has been modified to use an enzyme to amplify the signal in order to increase the sensitivity (CARD-FISH). These are cell-specific assays where cells containing sufficient amounts of rRNA that can be visualized microscopically are operationally defined as being metabolically ac-

tive. Because the oligonucleotide probes can be targeted at different phylogenetic levels, the metabolic activity can be associated with specific groups of microorganisms. Three recent studies employed either FISH or CARD-FISH assays to sediments recovered from the Peru Margin during ODP Leg 201. Although the studies did not all examine the same samples, the general geologic setting of the Peru Margin from which they were collected provides an opportunity to compare the findings.

Mauclaire et al. (2004) used CARD-FISH with domain-specific probes (Archaea and Bacteria) to analyze nine sam-

ples from 1.0–110.0 mbsf at Site 1229 (Leg 201) at the Peru Margin. They determined that Bacteria outnumbered Archaea in all but two of the samples and, in general, the Archaea-to-Bacteria ratio increased with depth. In addition, they compare their CARD-FISH positive cells to total cell counts (which relied on the generic DNA fluorochrome 4′6–diamidino-2-phenyl-indole [DAPI]). Mauclaire et al. (2004) concluded that 10 to 55 percent of the total cells observed contained sufficient rRNA to be detected with CARD-FISH. Schippers et al. (2005), a second study, presented data from Sites 1227 and 1230 (Peru Margin) using the same domain-specific oligonucleotide probes and concluded that CARD-FISH-positive cell abundance (Figure 4) showed no decrease with depth and ac-

counted for ≤ 10 percent of the total cell abundance, as determined by AODC. No Archaea were detected using the CARD-FISH approach in this study, suggesting Archaea were not metabolically active. Finally, Biddle et al. (2006) used FISH analysis with the same domain-specific probes and concluded that Archaea are dominant in the sediment column (35 to 98 percent of FISH positive cells are Archaea) at Sites 1227, 1229, and 1230. In the 19 samples they analyzed, collected from a depth of 0.7–121.4 mbsf, Archaea comprised an average of 73 percent of the total microbial community recognized by the FISH probes.

In short, results of these studies agreed in operationally identifying a relatively large fraction of the total cells as alive and active. However, they disagreed

considerably in the relative proportions of Archaea and Bacteria identified as alive and active. This discrepancy may point to problems with the application of some techniques to marine sediments. The sediment matrix makes it difficult to routinely address these types of questions. The total concentration of rRNA in bulk sediment samples can also be used as an indicator of the metabolic activity of the *in situ* microbial community. By using reverse transcriptase followed by the reverse transcription-polymerase chain reaction (RT-PCR), the rRNA and, therefore, the implied metabolic activity, can be assigned to specific phylogenetic groups based on the specificity of the primers used. Sørensen and Teske (2006) used this approach to analyze nine samples from 7.35 to 49.85 mbsf

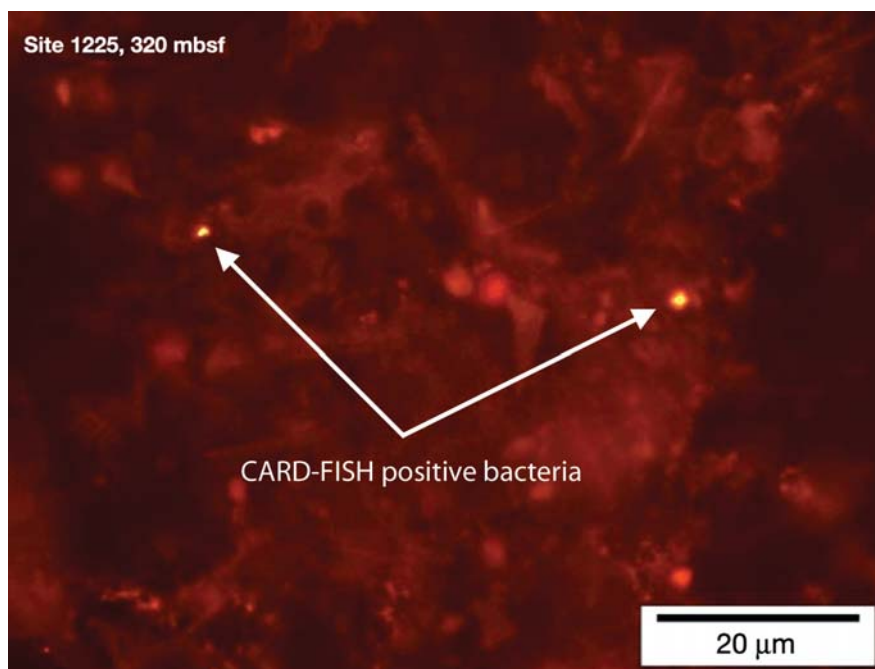


Figure 4. Bacteria detected using catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH) from a sediment sample recovered at Equatorial Pacific (Site 1225) at a depth of 320 meters below seafloor (mbsf) (Schippers et al., 2005). This method targets messenger RNA, and therefore, cells detected in this manner are presumed to be active.

at Site 1227. Their results show that archaeal rRNA concentrations vary greatly through the sediment column, and reflect changes in the level of activity associated with the sulfate-methane transition zone. These results support the idea that Archaea make up an active component of the subsurface microbial community (Figure 5).

Quantitative polymerase chain reaction (Q-PCR) has also been used to estimate biomass in the marine subsurface. By targeting ribosomal DNA (rDNA), this method also allows biomass estimates to be partitioned into phylogenetic groups based on the specificity of the oligonucleotide primers used.

Schippers et al. (2005) used this approach with domain-specific primers on samples from the Peru Margin

(Sites 1227 and 1230). The trends were consistent with the CARD-FISH data from the same samples in that Bacteria dominated the Q-PCR signal over Archaea. Converting the Q-PCR data to cell counts, assuming 3.6 gene copies per cell, suggests that AODC overestimates biomass. The dominance of Bacteria at these sites determined by Q-PCR is supported by Inagaki et al. (2006).

Another class of labile molecules that has great potential for estimating biomass in the marine subsurface is lipids. Phospholipid molecules are structural components of cell membranes. They have been used as proxies for microbial biomass in many environmental settings, particularly soil environments (White et al., 1979). Intact phospholipids degrade upon cell death, making them useful in

this application. The use of HPLC-MS coupled with electrospray ionization (HPLC-ESI-MS) has been applied to the marine subsurface in Nankai Trough (off Japan), Hydrate Ridge (Oregon, USA continental margin) (Zink et al., 2003), and the Peru Margin (Sturt et al., 2004; Biddle et al., 2006). Although this method is very useful for indicating viable microorganisms in the subsurface, due to the lack of standards for calibration, this method remains semi-quantitative. As with the nucleic-acid-based methods, intact phospholipid analysis allows assignment of biomass to phylogenetic groups based on structural differences. Analysis of the relative abundances of intact phospholipids from the sulfate methane transition zone of Site 1229 (Sturt et al., 2004; Biddle et al., 2006)

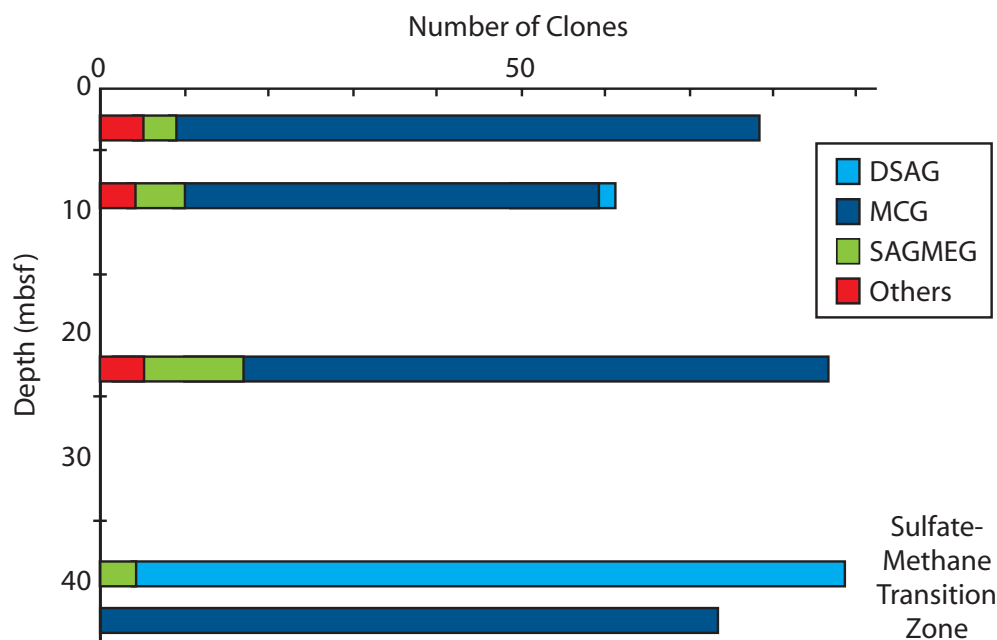


Figure 5. Phylogenetic composition of Archaeal community at a Peru Margin site (1227) as determined by reverse transcription-polymerase chain reaction (RT-PCR). This method amplifies ribosomal RNA and therefore is considered to reflect the active members of the microbial community. Changes in the community composition reflect changes in the chemical environment. Here, the deep-sea archaeal group (DSAG) dominate at the sulfate-methane transition zone (Sørensen and Teske, 2006).

and Sites 1227 and 1230 (Biddle et al., 2006) revealed the prominence of tetraethers, intact phospholipids associated with Archaea. This result supports the idea that Archaea are active in the marine subsurface.

On a global scale, the biomass in this subseafloor habitat is enormous. Unfortunately, many challenges come from estimating biomass in individual samples where the biomass in a cubic centimeter is miniscule and embedded in a matrix that often confounds our attempts to quantify it. Despite the differences in some of the major conclusions, the studies cited above all point to a large, active biomass in the marine subseafloor environment. The use of multiple methods will eventually lead to greater confidence in biomass estimates. Better estimates, coupled with the ability to assign portions of the biomass to specific phylogenetic groups, will greatly improve understanding of factors that influence the distribution and activity of microorganisms in the marine subsurface.

ACTIVITY

Fluxes of metabolic reactants and metabolic products from one subseafloor depth to another clearly demonstrate that diverse microbial activities occur in anoxic deep marine sediments (D'Hondt et al., 2004). These activities include reduction of SO_4^{2-} , Fe(III), Mn(IV), NO_3^- , and O_2 ; carbon oxidation; and the production and destruction of ammonia, acetate, lactate, formate, hydrogen, methane, ethane, and propane (D'Hondt et al., 2003; Hinrichs et al., 2006). Many of the reductive processes compete with each other for electron donors and have been assumed to competitively ex-

clude each (Lovley and Chapelle, 1995). However, pore water chemical distributions (D'Hondt et al., 2002; D'Hondt et al., 2004) and radiotracer experiments (Parkes et al., 2005) demonstrate that at least some of these reductive processes consistently co-occur in deep subseafloor sediments (e.g., sulfate reduction and methanogenesis).

Experiments with radiotracers demonstrate that potential rates of many microbial activities, such as sulfate reduction and methanogenesis, are often highest at very shallow depths in marine sediments (Parkes et al., 2000). However, rates of at least some activities, such as sulfate reduction, can exceed near-surface rates in deep subseafloor sediments where chemical transport brings electron donors and acceptors into contact at high rates. For example, sulfate-reducing methane oxidation is sustained at very high rates by diffusion of sulfate and methane that are in contact with each other in discrete zones deep beneath the seafloor of the Peru Margin (D'Hondt et al., 2004, Parkes et al., 2005).

Rates of activities integrated over entire drilled sediment columns demonstrate that predominant activities and total rates of activities (as well as cell abundances) vary predictably from ocean margins to open-ocean anoxic sediments (D'Hondt et al., 2002; 2004). Net redox activity is dominated by sulfate reduction in the anoxic sediments of ocean margins, where total activity and cell abundance are highest (D'Hondt et al., 2004) (Table 1). In anoxic sediments of open-ocean sites, metal reduction and nitrate reduction become increasingly important as total activity and cell abundance decline (Table 1).

Potential sources of food (electron donors) in deep subseafloor sediments include: (1) burial of organic matter from the surface photosynthetic world, (2) cleaving of reductants (e.g., H_2) from water by radioactive bombardment from the surrounding mineral grains, (3) burial of reduced minerals (e.g., minerals with reduced iron, manganese), and (4) thermogenesis of reduced organic compounds.

In the anoxic subseafloor sediments that have been studied to date, photosynthesized organic matter appears to be the principal food source (D'Hondt et al., 2004). Thermogenesis may be a spectacular source of electron donors in some marine environments (e.g., the Lost City hydrothermal system, Mid-Atlantic Ridge; Kelley et al., 2005). However, it is not a significant source of electron donors in open-ocean sediments, where *in situ* temperatures are typically low (less than 30°C) and reduced compounds diffuse from the microbially active sediments into the basement below (D'Hondt et al., 2004). Burial of reduced minerals is not likely to be a significant source of electron donors in anoxic deep marine sediments where SO_4^{2-} is the predominant electron acceptor, because SO_4^{2-} cannot be used to oxidize reduced metals. However, it could be a source of electron donors in very low-activity subseafloor environments where O_2 and NO_3^- diffuse down from the overlying ocean or up from the seawater that circulates through the underlying basement. Radiolysis (the breakup of chemicals into smaller components [e.g., water into H and O] by ionizing radiation) of water may be a significant source of electron donors in the least active sub-

Table 1. Modeled Electron Acceptance Rates in Sediment Columns Along the Peru Margin and Equatorial Pacific

Location	ODP Site	Water Depth (mbsl)	Electron Acceptance Rates (mol cm ⁻² yr ⁻¹)			
			Net NO ₃ ⁻ Reduction	Estimated Mn(IV) Reduction	Estimated Fe(III) Reduction	SO ₄ ⁴⁻ Reduction
Peru Margin						
Slope	1230	5086	ND	2.8 x 10 ⁻¹⁰	2.5 x 10 ⁻⁷	2.0 x 10 ⁻⁵
Shelf	1227	427	BLD	4.4 x 10 ⁻¹¹	1.0 x 10 ⁻⁷	7.2 x 10 ⁻⁶
Open Pacific						
Equatorial	1225	3297	ND	1.2 x 10 ⁻⁸	7.2 x 10 ⁻⁸	1.1 x 10 ⁻⁶
	1226	3760	6.4 x 10 ⁻⁹	6.0 x 10 ⁻⁸	1.0 x 10 ⁻⁸	1.5 x 10 ⁻⁷
Basin	1231	4813	4.0 x 10 ⁻⁸	1.2 x 10 ⁻⁷	4.0 x 10 ⁻⁸	BDL

Notes: ND = not determined; BDL = below detection limit; mbsl = meters below sea level
Source: D'Hondt et al. (2004).

seafloor sediments, where very little organic carbon has been buried. This hypothesis can be tested in the region of the South Pacific Gyre, where sedimentation is slower and water-column productivity lower than in any other region of the world's open oceans (D'Hondt et al., 2006).

Division of electron acceptor fluxes by cell abundances in subseafloor sediments indicates that the average metabolic activity of subseafloor cells varies considerably from site to site, but is often orders of magnitude lower than the average per-cell activity in shallow sediments or laboratory cultivation experiments (D'Hondt et al., 2002). This result can be explained in one of three ways: (1) the vast majority of subseafloor cells are inactive or dead, (2) fluxes of electron acceptors (e.g., SO₄²⁻) and their reduced products (e.g., dissolved sulfide, Fe(II),

and Mn(III)) underestimate total sub-seafloor activity by orders of magnitude, or (3) many subseafloor communities are sustained at rates of total activity that are far below traditional estimates of required maintenance energies (Price and Sowers, 2004). The first of these explanations is extremely difficult to reconcile with (CARD)-FISH studies that suggest a large fraction (up to 55 percent) of the cells in subseafloor sediments are active (Mauclaire et al., 2004; Schippers et al., 2005; Biddle et al., 2006). The second explanation is extremely difficult to reconcile with the finding of ODP Leg 201 that the fluxes of electron acceptors and their reduced products are in approximate balance with estimates of organic matter burial rates at the same sites (D'Hondt et al., 2004). This explanation is being tested further by quantification of dissolved inorganic carbon produc-

tion (Wang et al., 2006) and estimation of radiolytic hydrogen production (Blair et al., 2006) at the same sites. If these (CARD)-FISH and mass-balance results are taken at face value, many deep sub-seafloor microbial communities truly exist at rates of total activity that are orders of magnitude lower than traditional estimates of the rates required to maintain living communities.

COMMUNITY COMPOSITION

Most of our current knowledge regarding the composition of the microbial communities inhabiting subseafloor sediments is derived from 16S rRNA gene sequence data. Clone libraries provide insight into the total diversity and phylogenetic makeup of the *in situ* communities. (Clone libraries are constructed by introducing individual fragments of DNA extracted from the

environment into plasmids of bacteria. After growth of the bacteria, the plasmids are extracted and the DNA insert is sequenced.) This non-culture-based approach has been widely used for microbial communities in many environments and has led to the discovery of many microbial lineages. Despite the relatively small number of samples examined from the marine subsurface, some patterns in microbial community composition are beginning to emerge. In some cases, similarities in communities residing in large, geographically diverse settings are apparent while large differences are seen on much smaller vertical scales that appear to be driven by fluxes of chemicals from above and below.

Archaeal Diversity

Several major Archaeal lineages are commonly found in subseafloor sediments. Sequences assigned to the Deep-Sea Archaeal Group (DSAG), Miscellaneous Crenarchaeotal Group (MCG), Marine Crenarchaeotic Group I (MG-I), Marine Benthic Group (MBG), and South African Gold Mine Euryarchaeotic Group (SAGMEG) often dominate clone libraries constructed from environmental DNA extracted from these samples. Samples retrieved during ODP Leg 201 have been examined extensively by several research groups and collectively serve as an opportunity to determine if there is a consensus of the structure of the microbial community at these sites. Two research groups analyzed separate clone libraries from organic-rich sediments along the Peru Margin (Site 1229). Both sulfate-methane transition zones at this site (D'Hondt et al., 2004) were dominated by MCG sequences (Parkes

et al., 2005; Biddle et al., 2006). At another site along the Peru Margin (1227), the dominance shifted from MCG to SAGMEG clones in the upper 50 meters of the sediment stack (Inagaki et al., 2006). Although a few clones belonging to SAGMEG appeared in the Biddle et al. (2006) clone library from Site 1227, MBG-B clones dominated. The last multiple comparison from this expedition came from the organic-rich sediment of Site 1230. DSAG clones dominated the upper 50 meters and transitioned to MG-I clones with increasing depth (Inagaki et al., 2006). In separate samples from Site 1230, the only clone reported belonged to MBG-B (Biddle et al., 2006). The organic-poor sediments of Site 1231 contained sequences belonging to MGI, SAGMEG, and DSAG (Sørensen et al., 2004).

Sequences related to thermophilic Archaea are commonly seen in clone libraries from subseafloor sediments (Kormas et al., 2003; Sørensen et al., 2004; Inagaki et al., 2006). These are unexpected due to the relatively low-temperature of the sediment most commonly sampled. It is not clear whether these sequences are there as a result of the long-term survival of thermophilic Archaea in cold sediments or whether our assumptions regarding these organisms are biased by the inclusion of a large number of thermophilic organisms in the database. Either way, this example points out one of the weaknesses in using the clone-library approach. Thermophilic bacteria have been isolated from the relatively cool sediments ($\leq 12^{\circ}\text{C}$) along the Peru Margin (Lee et al., 2005) supporting the former hypothesis. In addition to any existing extraction and PCR biases, the clones

do not yield information regarding the active participation of these phylotypes in biogeochemical transformations in the subsurface.

Bacterial Diversity

As with Archaea, some Bacterial lineages are well represented in clone libraries constructed with environmental DNA retrieved from the marine subsurface. Green non-sulfur bacteria, Proteobacteria, Bacteroidetes, Planctomycetes, Chloroflexi, and the candidate division JS1 (unclassified Bacteria, originally observed in the Japan Sea) are all well represented.

Comparisons from similar geochemical settings separated by large distances may prove to be very useful in determining the critical factors that dictate the composition of the *in situ* microbial community. This approach was recently utilized to compare microbial communities in sediments that contain methane hydrates with nearby sediments that lack hydrates, but contain abundant methane. Inagaki et al. (2006) used principal-component analysis to evaluate 2,819 clones constructed from hydrate-bearing and non-hydrate-bearing sediments along the Peru and Cascadia Margins. This study indicated that the hydrate sites, regardless of location, were dominated by DSAG in the Archaeal clone libraries, and JS1, Planctomycetes, and Chloroflexi in the Bacterial clone libraries. Sulfate-reducing bacteria have been the subject of many detailed studies, given the obvious importance of sulfate as an electron acceptor in marine sediments. The two sulfate-methane transition zones at ODP Site 1229 contained different relative proportions of acetate oxidizers and non-acetate oxidizers (Mauclaire et

al., 2004). The upper sulfate-methane transition zone, fed by the sulfate diffusing from the overlying water column, contained a higher proportion of acetate oxidizers (*Desulfobacteriaceae*) while the lower zone, fed by sulfate diffusing from a brine from below, had a higher percentage of non-acetate oxidizers (*Desulfovibrionaceae*). Both groups were distributed through the entire sediment column, including the sulfate-free zone between the transition zones. Because these cells were detected with CARD-FISH (and, therefore, presumably active), the high abundance of sulfate-reducing bacteria in sediments devoid of sulfate was unexpected.

Surveys of rDNA genes in subsurface sediments have provided a broad view of *in situ* communities, but not without some difficulties (Webster et al., 2003). Although these data are informative, a focus on functional genes that code for enzymes involved in metabolic activity relevant to the subsurface (e.g., methyl coenzymeM-reductase and dissimilatory sulfite reductase) are becoming more common (Dhillon et al., 2003; Parkes et al., 2005). These studies target the potential to perform a function rather than targeting the rDNA gene in which function is implied by virtue of belonging to a group where members are known to have the ability to perform a function. This is a significant step forward in determining the genetic potential of subsurface microbial communities. A potential revolution in the characterization of microbial communities in general is application of metagenomic analysis (whereby scientists extract DNA from organisms in the system being studied and insert it into a model organism;

the model organism then expresses this DNA and is studied using standard laboratory techniques). This type of analysis has been approached in some communities (e.g., Acinas et al. 2004; Tyson et al., 2004) and will surely be applied to the marine subsurface (see Box 1).

While it is true that studies of microbial diversity have been revolutionized by the introduction of molecular techniques, isolating microorganisms into culture provides insights into how these organisms have adapted to this environment. As with all methods used to study microorganisms in the deep subsurface, culturing techniques are being adapted to the unique challenges presented by this environment (see Box 2).

IODP CAPABILITIES

The IODP uses multiple drilling platforms, including the drillships *Chikyu* and *JOIDES Resolution*. The latter will soon undergo a major refit for IODP operations. Both of these ships will contain extensive microbiology and biogeochemistry facilities, complete with radioisotope isolation vans. These laboratories will include the capabilities to aseptically sub-sample cores in an anaerobic environment, manipulate subsamples on board for cultivation and biomass studies, properly store subsamples for shore-based molecular work, do radiotracer studies of microbial activities on site, and undertake a very wide range of biogeochemical studies. Most importantly, the capability to conduct microbiological research on these new drilling platforms was incorporated at the initial design phase. This will ensure the opportunity for microbiologists to pursue research in the marine subsurface in a truly unprecedented manner.

CONCLUDING STATEMENT

Although we have advanced knowledge of life in the seafloor over the past two decades, as is the case with all scientific endeavors, we are left with more questions. The toolbox of techniques that has been employed in studying microbes in the water column and surficial sediments has been taxed to the limit. New methods that have been recently developed in the field of molecular biology, while extremely powerful, are often difficult to translate for use in this challenging environment. Therefore, we need to continually work on optimizing these techniques for use in the subsurface or develop new methods specifically to address questions regarding life in the subsurface (e.g., Soffientino et al., 2006).

The expansion of the drilling program to incorporate three platforms in IODP will give scientists unprecedented access to the marine subsurface. Our understanding of the microbes in this environment will certainly change after we have had the chance to examine sediments collected from a wider variety of environments. Much of what we know is influenced by the fact that the majority of sites that have been cored for microbiological research are along coastal margins. The subsurface microbial activity at these sites is mainly driven by organic matter that has been exported from the overlying productive waters. The microorganisms, in terms of total biomass, community composition, and metabolic activities will be much different in open-ocean sediments that receive far less organic matter. The vast areas of sediments underlying the unproductive gyres of the major ocean basins may look vastly different from what we have

BOX 1: IODP MICROBIOLOGY THROUGH MASSIVELY PARALLEL DNA SEQUENCING

By Christopher H. House and Stephen C. Schuster

Over the last few years, increasing numbers of studies have attempted to apply shotgun sequencing (an approach used to decode an organism's genome by breaking it into many small pieces, sequencing the pieces, and reassembling the fragments) to study an environment's "metagenome" (Venter et al., 2004; Hallam et al., 2004; Treusch et al., 2004; Leininger et al., 2006), or have "prospected" for useful genes from vast metagenomic libraries (Rondon et al., 2000). In some cases, these efforts have been aimed at low-diversity ecosystems where nearly complete genome sequences for the microbes in the population can be obtained (Tyson et al., 2004). In other cases, genomic fragments are assembled or the data are used as a searchable genetic database for that environment. In either case, shotgun sequencing of environmental samples is becoming an accepted and useful approach.

There are several competing technologies that aim to reduce reagent costs, sample size, and technical difficulty of sequencing. These technologies are driven by a commercial desire to ultimately provide medical instruments that can sequence a patient's genome quickly and cheaply. Industry projections suggest that the cost of sequencing a human genome will approach \$1000 within the next ten years (Zimmerman, 2004). This astonishing projection is based on recently developed sequencing-by-synthesis technologies that are radically different from Sanger-based sequencing currently used.

The first DNA sequencer (Roche Genome Sequencer 20) commercializing the new technology comes from 454 Life Sciences (www.454.com) and is distributed by Roche Applied Sciences. The 454 technology has reduced the reaction volume for sequencing by about five orders of magnitude by sequencing a high number of DNA segments in parallel. These

goals are accomplished by sequencing the different DNA segments in a PicoTiterPlate™, which holds up to 1.6 million (44 µm diameter) wells of 75 picoliter volumes. At Penn State, using this technology (e.g., Poinar et al., 2006), more than 20 Mb of metagenomic data is obtained for less than \$10,000 (yielding 200,000 or more sequences).

For microbiologists, these developments mean that shotgun sequencing will become relatively inexpensive and that such metagenome studies (Poinar et al., 2006; Edwards et al., 2006) will gain importance and become routine over the next decade. Currently, we have been able to consistently and reliably obtain metagenomes for subsurface samples from ODP Leg 201 after applying a genome amplification step (Gonzales et al., 2005; Holbrook et al., 2005). For the IODP, these new sequencing techniques mean that metagenomics should be applicable to DNA-poor subsurface samples. The cost of such studies has decreased, placing this type of research into the realm of "single-PI" grants. The difficulty of such work has also decreased. Considering these ongoing and projected developments, in the next decade, metagenomics has the potential to become a routine IODP shipboard analysis technique. Even with current technology, one can envision obtaining a metagenomics "snapshot" of a recovered core in sufficient time to influence the expedition's drilling and sampling decisions.

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BOX 2: DIVERSITY OF BACTERIA ISOLATED FROM THE DEEP SUBSEAFLOOR BIOSPHERE

By Heribert Cypionka

Although chemical gradients, activity measurements, and molecular studies prove the existence of active seafloor microbial communities, it remains a challenge to cultivate the members of the deep biosphere, in order to study their properties, limits, requirements, and performance in the laboratory. Only a very small percentage of the prokaryotes counted in deep sediments grow in traditional media (Süß et al., 2004). Most of the microorganisms are classified as “unculturable” although they have developed high population densities in their natural environments. New cultivation approaches involve the use of non-specific media, which allow a large variety of microorganisms to grow. The media contain mixtures of monomers, polymers, low concentrations of refractile substrates (100 μ M or less), or extracts from marine sediment. Particles (e.g., FeS precipitates) are added to promote adhesion and act as a reducing agent (see Figure B-1). Syntrophic interactions between populations are promoted by the addition of background bacteria. The use of more sensitive techniques to measure growth (e.g., formation of 35 S-sulfide from radiolabeled sulfate or fluorescent DNA stains to quantify increases in cell abundance) has allowed for the detection of growth in microbes that exhibit extremely slow growth rates. Interestingly, many of the strains isolated from anoxic environments in this manner are in fact facultative aerobes (organisms that are normally anaerobic but can also grow in the

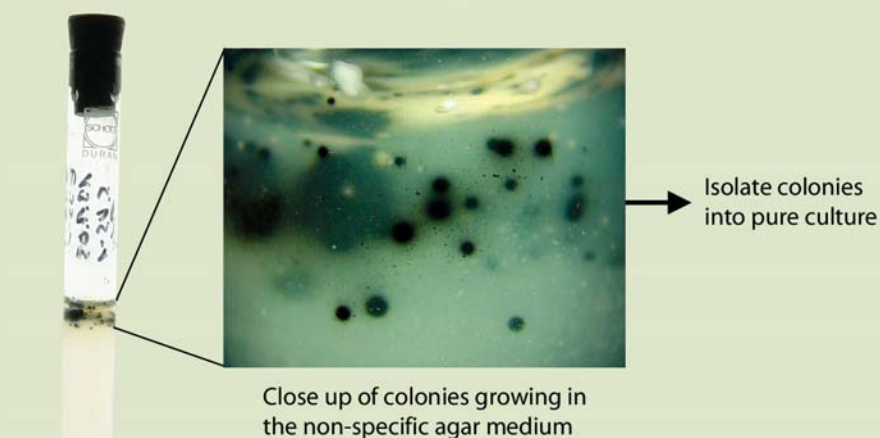


Figure B-1. Bacterial isolation scheme using non-specific media in agar tubes. The use of novel growth media that incorporates components of the environment (sediment extracts) that promotes the growth of a wider range of subsurface microorganisms. Individual colonies of bacteria are shown in the closeup. Images courtesy of Anja Batzke.

presence of oxygen). Analyses of the 16S rRNA genes from ODP cultures reveal that the isolates represent a broad spectrum within the bacterial kingdom and belong to the α -, β -, and γ -Proteobacteria, the Firmicutes, Actinobacteria, and Bacteroidetes. Several ODP strains can be assigned to spore-forming genera or are related to known *Rhizobium* species (Batzke et al., in press). Their authentic origin from the deep biosphere was confirmed by contamination assessments during the sampling procedure, by means of quantification of their DNA in the sediments, and by fingerprinting techniques that demonstrated the presence of different subpopulations (Batzke et al., in press).

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learned from the work conducted on the margins. These new investigations will surely result in a downward revision of global biomass estimates. Of course, working in sediments with low organic carbon, and presumably, low microbial biomass, will further push the limits of

currently employed techniques. These low-energy environments will create new challenges for the development of extremely sensitive techniques that will have great implications for this habitat and may be applicable to other environments, including extraterrestrial targets.

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