Microbial communities from core intervals, JAPEX/JNOC/GSC Mallik 2L-38 gas hydrate research well

F.S. Colwell[1], M.E. Delwiche[1], D. Blackwelder[1], M.S. Wilson[2], R.M. Lehman[1], and T. Uchida[3]

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Abstract: Microbial cell distribution in sediment core samples from the JAPEX/JNOC/GSC Mallik 2L-38 gas hydrate research well on the Mackenzie Delta (Canada) was studied using acridine orange direct counts of stained cells for total cell estimation, and by most probable number statistical enumeration for culturable methanogens. The purpose was to characterize the microbial communities in gas-hydrate-bearing sediments. Results indicated that the total cell count values were in the range of $1.1x10^5$ cells/g to $2.8x10^6$ cells/g with culturable methanogens present at $1x10^{-4}$% to 1.0% of those values. These results also indicated that culturable methanogens may be more numerous in the porous sandy strata of the Mackenzie Bay Sequence than in clay and silt units of the Kugmallit Sequence. These data expand the known distribution of methanogens in deep sediments and establish the presence of microbial communities in subpermafrost environments.

Résumé : On a étudié la distribution de cellules microbiennes dans les carottes de sédiments prélevées dans le puits de recherche sur les hydrates de gaz JAPEX/JNOC/GSC Mallik 2L-38 foré dans le delta du Mackenzie, au Canada. Pour ce faire, on a évalué le nombre total de cellules par comptage direct de cellules souillées par l'orange d'acridine et le nombre de bactéries méthanogènes susceptibles d'être cultivées à l'aide de la méthode statistique du nombre le plus probable. Le but de l'étude était de caractériser les communautés microbiennes contenues dans les sédiments renfermant des hydrates de gaz. Les résultats ont donné un nombre total de cellules allant de $1,1 \times 10^5$ à $2,8 \times 10^6$ cellules/g; le nombre de bactéries méthanogènes susceptibles d'être cultivées s'échelonnait de 1×10^{-4} à 1,0 % de la totalité des cellules présentes. Ces résultats indiquent en outre que les bactéries méthanogènes susceptibles d'être cultivées pourraient être plus nombreuses dans les strates de sable poreux de la Séquence de Mackenzie Bay que dans les unités d'argile et de silt de la Séquence de Kugmallit. Ces données permettent d'étendre la distribution connue des bactéries méthanogènes aux sédiments profonds et confirment la présence de communautés microbiennes dans le subpergélisol.

[1] Biotechnologies Department, Idaho National Engineering and Environmental Laboratory, P.O. Box 1625, Idaho Falls, Idaho 83415-2203, U.S.A.
[2] Department of Biology, Humboldt State University, 1 Harpst Street, Arcata, California 95521, U.S.A.
[3] JAPEX Research Center, Japan Petroleum Exploration Company, Ltd., 1-2-1 Hamada, Mihama-ku, Chiba 261-0025, Japan

INTRODUCTION

We undertook investigations of the microbial communities in sediment samples from the JAPEX/JNOC/GSC Mallik 2L-38 gas hydrate research well to describe the distribution of microbial communities that can contribute to the methane present in gas hydrate and thus the distribution and origin of gas hydrate in circumpolar regions. The total bacterial cell numbers, numbers of methanogenic micro-organisms, and numbers of nonmethanogenic aerobic heterotrophs were determined in samples from different depths and the abiological factors controlling the distribution of total cells and methanogens in these sediments were explored.

The possibility of gas hydrate becoming a source of energy is currently under consideration. However, such considerations of gas hydrate as a source of fuel or solely as a quasi-stable structural phenomenon requires a more thorough understanding of the processes that govern their distribution, formation, and concentration in deep sediments. Stable isotope ratios of the carbon in the methane found in most gas hydrate deposits suggests that the methane therein is largely biogenic (e.g. Kennicutt et al., 1993). While it is probable that most of the methane in gas hydrate has been produced by methanogenic micro-organisms, little is known of these microbial communities including the rates at which they produce methane, their probable natural substrates, or even their distribution with respect to the gas hydrate deposits. Field studies involving the collection of cores from gas-hydrate-rich sediments constitutes a key method of initiating the studies that will address these issues (Griffin et al., 1997) and the ensuing laboratory investigations can yield model microbial isolates that allow specific questions to be answered on the nature of gas hydrate formation.

The ubiquity of viable microbial communities in deep terrestrial and marine sediments is now widely accepted (Parkes et al., 1994; Fredrickson and Onstott, 1996). Most of these deep microbiology study sites are continental or offshore marine and of the few investigations conducted on the microbiology of gas hydrate, all have focused on marine sediments. Methanogenic and other microbial communities including sulphate reducers have been detected in these samples (Parkes et al., 1994; Cragg et al., 1996). To date, there have been no studies conducted on the microbiology of subpermafrost gas hydrate zones, yet estimates indicate large quantities of methane in these deposits (Collett and Kuuskraa, 1998). The Mackenzie River Delta is a high-energy depositional environment distinct from offshore marine depositional sediments. Deltaic sediments are more likely than marine sediments to contain a range of geological materials that yield stratigraphic differences that dictate the physical and chemical characteristics of the sediments such as porosity, permeability, and total organic carbon. Furthermore, different types and different concentrations of organic matter are likely to be associated with a deltaic environment than an offshore marine system (Tissot and Welte, 1984). These factors are likely to be significant in determining the distribution of micro-organisms and their activities (Murphy et al., 1992).

This paper considers the total numbers of microbial cells and the numbers of culturable methanogens in gas-hydrate-rich sediments acquired from the Mallik 2L-38 research well. We consider these values relative to 1) the total numbers of cells found in prior microbiological studies of the terrestrial and marine subsurface, 2) the presence of overlying permafrost, and 3) the textural and gas hydrate characteristics of the sediments from which the samples came.

METHODS OF INVESTIGATION

Site description and sample locations

Conventional, mud-rotary cores were obtained (February and March, 1998) from the Mallik 2L-38 core hole located on the Mackenzie Delta, Northwest Territories, Canada. These cores were from nearshore deltaic sediments of Oligocene age beneath the base of the permafrost which is located at 640 m (all depths were measured from kelly bushing [8.31 m above sea level]). The Mackenzie Bay Sequence (346–926.5 m), consisting of sand, weakly cemented sandstone, and minor silt or shale interbeds, and the Kugmallit Sequence (> 926.5 m) consisting of interbedded sand and silt (Dallimore et al., 1999; Jenner et al., 1999) were sampled for microbiological analysis. Gas hydrate occurs primarily in the coarse-grained strata between 897 m and 1110 m and gas hydrate concentrations were low to nonexistent in the silty sediments that were interbedded with the sand. Seven samples were obtained from depths of 900–955 m: four from the Mackenzie Bay Sequence and three from the Kugmallit Sequence. Two Pliocene permafrost samples were obtained from the Iperk Sequence (0–346 m) at 114 m and 123 m.

Method of sample collection

Procedures were used to reduce contamination of cores by nonindigenous micro-organisms and to assess the extent of potential contamination (Griffin et al., 1997). Rapid processing of cores was undertaken to minimize intrusion of drilling fluids. Following extrusion of cores from the liner, external mud was wiped from the core and a segment of the frozen core was excised and transferred to an anaerobic glovebox on site. Cores were aseptically pared to remove the outer contaminated portions. On-site liquid enrichments for methanogens were immediately started using pared subcore material to assess the effect of sample holding time and transit conditions on subsequent culturability of this functional group at the laboratory (media conditions described under 'Microbiological cultivation'). Subcores were then placed in nitrogen-flushed air-tight containers, provided with two Gas Pak® packets (available from BBL Inc., Franklin Lakes, New Jersey) to ensure anaerobic conditions and either refrigerated or frozen depending upon analyses to be performed. At the completion of drilling, all samples were shipped frozen on dry ice or refrigerated on blue ice packs by overnight courier to the Idaho National Engineering and Environmental Laboratory (INEEL). On arrival at the laboratory, nonfrozen samples were immediately transferred to a large anaerobe jar (15 L), flushed with inert gas, provided with two Gas Pak® packets

and placed at 4°C until they were processed (3 days later). Frozen samples were placed immediately in a -70°C freezer. Additional frozen samples were acquired two months after coring from the core libraries of the Geological Survey of Canada (Ottawa, Ontario). These archived cores have been frozen continuously since the time of acquisition.

Sample quality

Indigenous microbial properties of the subcores, core parings, and the potential sources of core contamination (i.e. drilling muds and surface sediments) were evaluated to estimate sample quality (Lehman et al., 1995; Colwell et al., 1997). These tracers were used because it was not possible to add tracers to the drilling fluid or the coring tool. Indigenous microbial properties included the microbial community-level physiological profiles, which distinguish microbial communities in different samples based on their use of organic carbon (Lehman et al., 1995); the total number of aerobic heterotrophic bacteria by agar plate count; and the colony morphology of aerobic heterotrophic bacteria. Since potassium chloride was added to drilling fluids as a freezing point depressor, concentrations of soluble potassium were used as a fortuitous tracer for a liberal estimate of drilling fluid intrusion (Colwell et al., 1997). To analyze cores and drilling fluids for potassium ion, 0.5 g of sediment was diluted in 50 mL of deionized water and then potassium ion was determined by atomic absorption spectroscopy. The detection limit after adjustment for the dilution factor was 100 mg/L.

Direct microscopic counts of micro-organisms

Total cells in sediments were enumerated by direct microscopic counts (procedure modified from Ghiorse and Balkwill (1984)) using a Nikon Eclipse E800^{TM} confocal microscope (available from Nikon USA, New York) and referred to as acridine orange direct counts (AODC). Triplicate 2.5 g subsamples from frozen cores were shaken in 22.5 mL of 0.1% sodium pyrophosphate. Duplicate sediment subsamples of 5 µL were smeared in 1.1 cm diameter circles on glass slides, dried, stained for 2 min with 20 µL of 0.01% acridine orange, and then washed with sterile filtered aqueous 1 M NaCl solution. The stained sample was covered with 10 µL of a 1:9 dilution (with water) of Dabco^{TM} fluorescent mountant (available from Sigma Chemical, St. Louis, Missouri) and then covered with a cover slip. The mercury vapor lamp provided with the microscope and suitable filters were used to visualize fluorescing acridine orange-stained cells at 1000x total magnification. For each sample preparation, the cells in 60 microscopic fields were enumerated and corrected for the area of the microscopic field and the dilution of the original sample to determine a mean cell concentration in the original sample. Argon and helium-neon laser light sources with suitable filters were used for higher resolution to establish identity in cases of questionable morphology. Negative control slides were prepared using only reagents to evaluate the microbiological purity of the reagents and the sterility of the handling process. Positive controls which consisted of an active microbial culture were prepared to verify the effectiveness of the staining procedure. These controls were prepared and observed microscopically with each of the samples.

Microbiological cultivation

Culture-based methods were used to determine the relative number of methane producers in samples from different core intervals. These techniques included most probable number (MPN) and plating on solid media to look for both methane producers and other types of anaerobic organisms that grow on short chain fatty acids. Most probable number is a method of statistical enumeration which employs the replicate, serial transfer of samples in vials containing liquid medium until the micro-organisms of interest have been diluted to extinction.

All sample processing was conducted under strict anaerobic conditions in a glove bag (available from Coy Products, Grass Lake, Michigan) containing an atmosphere of 5% H_2:5% CO_2:90% N_2. Surface layers of individual core samples were removed using sterilized tools and the newly exposed facies were aseptically subcored with sterilized plastic syringes from which the ends had been removed (Griffin et al., 1997). For positive controls, freshwater marsh sediments from Market Lake Wildlife Management Area, Roberts, Idaho were inoculated into MPN vials and spread on plates. Negative controls consisted of media tubes inoculated with sediment that had been combusted at 550°C for 18 h. Approximately 10 g of subcored sediment from each core was placed in serum vials which contained 90 mL of carbonate-buffered minimal salts (MS) medium (medium modified from Boone et al. (1989)). This medium was amended with 0.5 g/L each of yeast extract and peptone. Acetate, formate (10 mM sodium salts), and H_2 (1 atm) were supplied as electron donors for methanogen MPNs and secondary enrichments. Carbonate buffer in the medium provided carbon for autotrophic growth with H_2. The same ingredients were provided for methanogenic growth on plates with the exception that H_2 was present at 5% by volume in the incubation chamber.

For MPN analyses, 9 mL of MS medium were dispensed into 20 mL Hungate tubes and stoppered with butyl rubber stoppers (available from Geomicrobial Associates, Ochelata, Oklahoma). For each core sample, vials containing $1x10^{-1}$ dilutions of the sample were shaken vigorously for 10 s and then 1 mL was added to successive tubes in triplicate for final dilutions of $1x10^{-2}$,$1x10^{-3}$, and $1x10^{-4}$. After one month incubation, headspace in MPN vials from core intervals whose plates showed the most growth were analyzed by gas chromatography with an atomic emission detector (GC-AED) (available from Hewlett-Packard, Avondale, Pennsylvania). Thereafter, headspace in selected vials was analyzed on a regular basis and all vials were tested after 10 weeks incubation. All incubations were conducted at 21°C.

Plate counts were done on MS medium solidified with 12 g/L agar. Medium was prepared anaerobically, autoclaved in sealed flasks to prevent loss of buffer, and the plates poured in an anaerobic chamber. Plates were spread with 0.1 mL of liquid from the $1x10^{-1}$ serum vials for a final dilution on the

plate of $1x10^{-2}$. After inoculation, plates were packaged and allowed to incubate in the anaerobic chamber (for methanogens) or in a sealed autoclave container purged and pressurized with 1 atm of deoxygenated nitrogen (for nonmethanogen anaerobic heterotrophs). Nonmethanogenic anaerobic heterotroph plates contained acetate, pyruvate, and succinate (10 mM sodium salts of each). Plates were monitored for growth, colony numbers, and morphologies. Representative colony types were picked from the surface and inoculated into MS medium.

RESULTS

Samples from the interior of the cores (subcores) appeared to be unique relative to the drilling fluids, soils, and core parings when comparing microbial communities or potassium ion concentration (Table 1). Samples from the Mackenzie Bay Sequence (904.14–920.1 m) contained numbers of culturable aerobic heterotrophs comparable to those noted in the drilling fluids. However, the colony types noted were often distinct when subcores were compared to drilling fluids (data not shown). Samples from the Kugmallit Sequence (928.1–951.15 m) exhibited a decrease in aerobic heterotroph numbers equivalent to three and four orders of magnitude relative to core parings and drilling fluids, respectively. Comparisons of subcores and parings based on the community-level physiological profiles showed a general decrease in the ability of the respective communities to use different organic carbon substrates; however, the data were inconclusive (data not shown). Potassium ion concentrations in the subcores ranged between 14% and 60% of the values in the core parings showing a distinct decrease between the inside and the outside of the cores. Differences between the subcores and the drilling fluids were even more pronounced.

Table 1. Comparison of microbial and chemical properties of drilling fluids, surface soils, core parings, and subcores from the Mallik 2L-38 well.

Sample depth or source (m)	Numbers of aerobic heterotrophs (mean log cells/g or cells/mL)		K^+ concentration (mg/L)	
	subcore	core parings	subcore	core parings
904.14	4	ND	550	1000
912.87	3.7	ND	300	1000
920.1	4	ND	< 100	700
928.1	BD	3.2	1300	2150
936.23	BD	3.7	1150	2850
951.15	1	3.3	1000	2150
drilling mud*	4		3650	
surface soil*	2.9		< 100	

* n = 3; the designations "subcore" and "core parings" are not relevant for drilling fluids and soils
BD = below detection. BD for aerobic heterotrophs was less than 0.3 culturable cells/mL or cells/g
ND = not determined

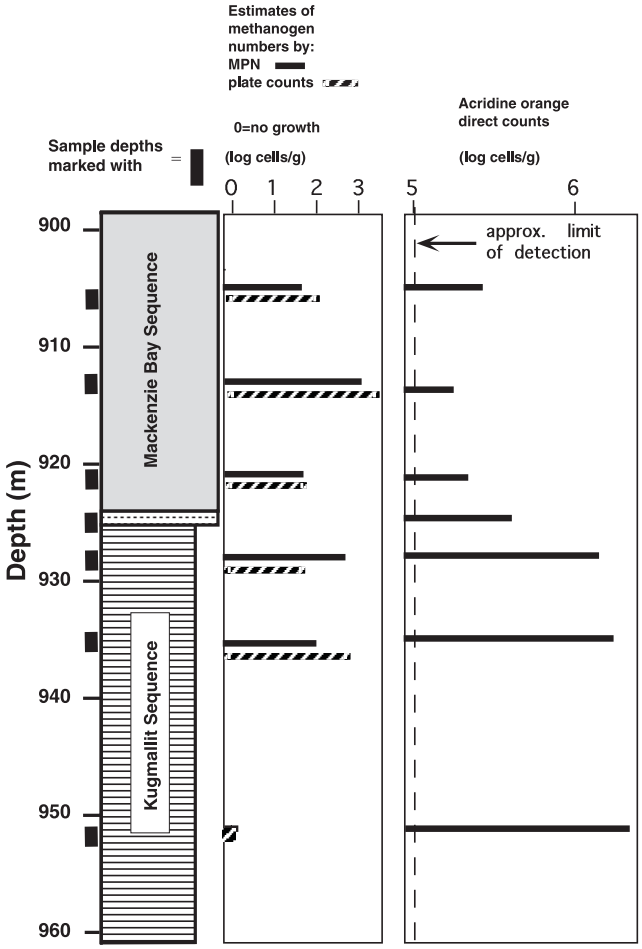

Figure 1. *Generalized stratigraphy of microbial sampling zones from the Mallik 2L-38 gas hydrate well on the Mackenzie Delta, and the results of the enumerations of total cells by AODC and of methanogens by MPN and plate count methods.*

All of the samples analyzed from the Mallik 2L-38 well contained cells visible by AODC, a method that has a detection limit of approximately $1x10^5$ cells/g. Total cells enumerated using this method ranged from $1x10^5$ to more than $1x10^6$ cells/g (Fig. 1). Over the intervals that were sampled, from 905 m to 951 m, there was a general increase in total numbers of cells. This increase was most evident when comparing samples from the Mackenzie Bay Sequence which yielded the lowest values for AODC to those from the Kugmallit Sequence which yielded the highest values for AODC.

Most probable number and plate-count methods allow enumeration of culturable or viable methanogens based on serial dilution of these micro-organisms from the original sample and their ability to grow in liquid or on solid laboratory media, respectively. These methods indicated that all of the Mallik 2L-38 gas hydrate well samples contained culturable methanogens except the core from 951 m. Estimates ranged from approximately 1x10 to more than $1x10^3$ methanogens/g (Fig. 1). In general, MPN enumerations yielded lower values than plate-count enumerations; however, the

latter tend to overestimate the number of methanogens present because some of the colonies seen in plate counts may not be methanogens. In contrast with the AODC results, enumeration of culturable methanogens suggested that there may be an inverse relationship with depth over the intervals that were sampled. In samples showing the greatest contrast, the numbers of culturable methanogens (by MPN) were as much as 1000 times higher in sediments from the Mackenzie Bay Sequence than in sediments from the Kugmallit Sequence. The relative number of viable methanogens (by MPN) compared to AODC values is low in both sequences. In the Mackenzie Bay Sequence as many as 1.0% of the cells seen in acridine orange direct counts for the same depth are viable methanogens as measured by MPN. In the Kugmallit Sequence this value decreases to between 0.02% and 0.0001%. Methanogens were not detected in any of the negative controls by MPN. With one exception (sample 912.87 m), the numbers of nonmethanogenic anaerobic heterotrophs were at the limit of detection (data not shown).

DISCUSSION AND CONCLUSIONS

The differences in biomass, types of micro-organisms, and potassium ion concentration suggest that minimal infiltration of drilling fluids into the interior of the cores (subcores) occurred. The significant decrease in potassium ion concentration measured between the exterior and the interior of the cores conveys the strongest evidence that the subcores were protected from the drilling fluids. The ability of a soluble ion such as potassium to penetrate the core is likely to be high relative to a larger microbial particle. Although it is impossible to discount the chance of some microbial contamination from the drilling fluids, our preliminary microbiological characterization indicated that in most cases the microbial properties measured in the subcores were distinct from that of the exterior of the cores and the drilling fluids. This distinction suggests that the subcores can yield microbiological values that are representative of the authentic microbial communities in these sediments.

The microscopic observation of cells in core samples from more than 900 m in Mallik 2L-38 well appears to be unique when compared to prior research conducted in deep subsurface environments. The ability to visualize intact cells in these samples suggests that the micro-organisms are more abundant in these sediments than in other samples from comparable depths. Furthermore, these data reinforce the conclusion that depth should not be considered alone as the most important determinant of microbial survival in the subsurface. These data extend the depths of cores from which cells have been observed using AODC beyond that reported for marine environments (Parkes et al., 1994). Core samples from such depths are infrequently recovered for microbial analysis but samples from depths comparable to those at Mallik 2L-38 well were acquired from the Piceance Basin in western Colorado, U.S.A. Microbial communities at 860 m in the Piceance Basin were sparse (Colwell et al., 1997) and were below the limits of detection using AODC methods (T. Kieft, pers. comm., 1995). This environment consists of sandstone with low permeabilities (less than 1 mDarcy), porosities below 10%, and perhaps most important, a prolonged thermal maxima of 120°C which only moderated in the last five million years. In contrast to the high temperatures of the Piceance Basin, which almost certainly limited the survival of most if not all cells, the temperatures at the sampling depth in Mallik 2L-38 well were less than 10°C (Dallimore et al., 1999). Permafrost is likely one of the most stable natural environments for conferring microbial survival (Varobyova et al., 1997). If it can be assumed that the sediments sampled in the Mallik 2L-38 well have always been less than 10°C, if not frozen, then these deltaic sediments present an environment more conducive to survival of intact cells than many other comparably deep strata.

The ability to grow viable methanogens from these samples suggests that these micro-organisms comprise an important portion of the living cells within cores from the Mallik 2L-38 well. Microbiologists are often unable to grow any micro-organisms from some deep subsurface samples even if they use a broad range of growth conditions (Colwell et al., 1997). Although relatively few of the Mallik 2L-38 well samples were analyzed for methanogens, the results further suggest that these organisms are more readily cultured and may be more numerous in the Mackenzie Bay Sequence than in the Kugmallit Sequence. These geological strata are distinct from one another in terms of sedimentology and gas hydrate content (Dallimore et al., 1999; Jenner et al., 1999). The Mackenzie Bay Sequence is described as consisting of sand and interbedded gravel with high concentrations of gas hydrate in the pore space. In contrast, the Kugmallit Sequence consists of bioturbated clay and silt with thin interbeds of coal and silty sand and contains only little or minor quantities of gas hydrate. Previous studies of subsurface micro-organisms have demonstrated that higher numbers of viable micro-organisms and higher microbial activities are evident when cells are associated with sandy sediments rather than with sediments that are rich in clay (Balkwill, 1989; Fredrickson et al., 1989; Sinclair and Ghiorse, 1989).

Evidence of more culturable methanogens in samples acquired from the sandy Mackenzie Bay Sequence is consistent with other investigations; however, the way in which the presence of gas hydrate factors into methanogen viability is more difficult to understand. Viable methanogens can be enriched from Mallik 2L-38 well sediments, even though the pore space is largely filled with gas hydrate which predominantly consist of methane (Uchida et al., 1999). Methane is a waste product of these micro-organisms and thus, in high concentrations should inhibit their activity and possibly their viability. The fact that methanogens constitute up to 1% of the total cells observed by AODC is important given the common rule that only less than 1–10% of the cells visible by AODC can be cultured (Atlas and Bartha, 1993; Amann et al., 1995). This phenomenon of 'low culturability' of cells in environmental samples occurs partly because many of the cells seen in AODC are actually dead but still visible. In addition, microbiologists can rarely grow all of the living cells in a sample because it is impossible to provide the proper growth conditions for the large diversity of the micro-organisms present. Given this, our data indicate that methanogens may be a remarkably large fraction of the viable micro-organisms

in these samples. Clearly, a significant methanogenic community exists in some proximity to high concentrations of methane which is present in gas hydrate form.

It is also interesting to note that in the Mackenzie Bay Sequence the stable carbon isotopic signature of the methane suggests a thermogenic source for the gas (Lorenson et al., 1999). This indicates that even though methanogens are present in these strata they are not solely responsible for the methane present in the co-existing gas hydrate. The methanogenic communities that were enriched from the shallower permafrost samples in Mallik 2L-38 well at 114 m and 123 m (data not shown) may play a greater role in the production of methane as that methane is inferred to be of biogenic origin (Lorenson et al., 1999).

These microbiological data contribute important new information to another remote subsurface environment on Earth. Relative to other subsurface investigations, these deltaic sediments contain not only microbial communities that may be remarkably well preserved, as indicated by high direct counts, but also high numbers of culturable or viable microorganisms of a single physiological group. Some subsurface habitats that are not nearly so deep or so disconnected with the surface biosphere have far fewer numbers of microorganisms that can be grown in culture or seen microscopically (Colwell et al., 1992). This modest sampling and analytical effort has yielded new data on the relationship between microbial numbers and sediment properties as well as the ability of methanogens to survive in relatively close proximity to large quantities of methane in the form of gas hydrate. To our knowledge, the microbial characteristics of subpermafrost sediments have rarely, if ever, been studied. Yet, because of the importance of these strata in terms of worldwide methane content, it is essential to understand more fully both the abiotic and the biotic characteristics therein.

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