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DIVERSITY OF BIOGEOCHEMICAL CYCLING GENES FROM PUGET SOUND SEDIMENTS USING DNA MICROARRAYS

S.M. TIQUIA,[1]* S. GURCZYNSKI[1], A. ZHOLI[1] AND A. DEVOL[2]

*[1]115F Science Building, Department of Natural Sciences, The University of Michigan, Dearborn, MI 48128, USA
[1]Department of Natural Sciences, The University of Michigan, Dearborn, MI 48128, USA
[2]School of Oceanography, University of Washington, Seattle, WA 98295, USA

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ABSTRACT

Oligonucleotide-based microarray permits the simultaneous analysis of thousands of genes on a single chip, so that a better picture of the interactions among thousands of genes can be investigated at the same time. Our oligo microchips contained 763 50-mer probes that scan the region of different functional genes encoding *amo*A, *pmo*A, *nir*S, *nir*K, *nif*H, and *dsr*AB. These genes code for key enzymes in the ecosystem processes of nitrification, methane oxidation, denitrification, nitrogen fixation and sulfur reduction, respectively. We used these oligochips to characterize the distribution of the above genes from Puget Sound sediments at different depths. The composition and distribution of genes from shallower sediments (depths 0-0.5 cm, 2.0-2.5 cm, 5.0-5.5 cm, and 25.0-25.5 cm) were highly similar but were different from those collected at deeper depths (depths 50-50.5 cm and 84.0-84.5 cm). The deeper sediments present a different community structure with a markedly lower diversity than the shallower depths. Analysis of positive hybridization signals also revealed presence of genes common to all samples. The majority of these genes were similar to those retrieved from various environments (i.e. soils, groundwater, river water, strotomites, marine sediments, and estuarine sediments). Parallel coordinate display showed that the most dominant functional guilds are those that are involved in nitrogen cycling. Our results also indicated that this technology has potential as a tool in revealing a comprehensive "snapshot" of the functional gene composition in marine sediments, although more work is needed to understand the biological meaning of each detectable hybridization signal.

Keywords: DNA microarrays, microbial community, marine sediments

INTRODUCTION

Marine sediments constitute 70% of the total earth surface and play an important role in global cycling of C and nutrients [1]. Organic matter from primary production settles to the sea floor, where a major part is remineralized by microorganisms that colonize the sediments. In these areas, the rates of sedimentary oxygen consumption and denitrification are a function of the overlying water concentrations of oxygen and nitrate, and changes in those concentrations directly affect process rates, and thus global C and N dynamics. Nitrification, denitrification, nitrogen fixation, methane oxidation, and sulfate reduction are important environmental processes in biogeochemistry and global changes. It is clear that microbes play important roles in these processes. A great variety of benthic animals (macrofauna) disturb the texture and chemistry of coastal marine sediments. Bioturbation (mixing of surface sediments by the activity of macrofauna) is pervasive in marine environments. The resulting physical and chemical changes are known to enhance microbial activities and growth rates [2]. Our previous work suggested that the bioturbation of the sediments could be one of the key factors affecting the microbial community structure variation with depth [3-5]. We tested this hypothesis more systematically by examining the structure of the denitrifier community within the mixed zone (bioturbation zone) as well as the unmixed zone (below bioturbation zone) from Western Mexico (oxygen-deficient; tropical environment), Pacific Northwest (oxygenated; temperate environment), and Gulf of Mexico (oxygenated; tropical environment). The denitrifier community structure was highly similar down to 37 cm (mixed zone) in Puget Sound and Washington State continental margin sediments, respectively. On the other hand, deeper sediments, retrieved from the unmixed zone, presented a different community structure with a markedly lower diversity. At present,

patterns of microbial composition and diversity of several functional genes in a single margin ecosystem at the same time have not been systematically investigated. Therefore, it is uncertain whether our findings pertain only to the diversity of denitrifying bacteria or if they can be generalized to other microbes involved in biogeochemical cycling.

Several studies to understand the molecular diversity of the enzymes (amo, pmo, nir, nif, and dsr) involved in biogeochemical processes have been conducted in marine sediments using cloning and sequencing method [3, 6-8], fingerprinting techniques (i.e. terminal restriction fragment length polymorphisms, TRFLP) [9] and quantitative PCR [10]. While these molecular techniques remain vital to the studies of microbial community dynamics, they are slow, labor-intensive, insensitive, non-quantitative, and/or expensive. Microarray-based technology has the potential of overcoming the limitations of traditional molecular methods for studying microbial community structure as high-throughput, rapid, and parallel tools for analyzing microbial community structure and activities in marine sediments [11]. In the past several years, DNA microarray technology has played important roles in gene expression studies of individual organisms [12-14]. It is only recently, that this technology has been extended to the studies of microbial communities in the environment, especially those communities that are involved in the N, C, and S cycles [11, 15-17]. Microarrays are simply glass slides onto which fragments of genes have been bound in an array of small dots. Complementary DNA or RNA from a sample, which has been labeled with a fluorescent dye, hybridizes to matching sequences and binds to the dots. One type of array contains different functional genes. Microarrays containing functional gene sequence information are referred to as functional gene arrays (FGAs), as they are primarily used for functional analysis of microbial communities in the environment [11, 15-16].

Wu et al. [15] previously constructed DNA-based FGAs containing nitrite reductase (*nir*S and *nir*K) and ammonia monooxidase (*amo*A) genes. Taroncher-Oldenburg et al. [16] developed 70-mer FGAs containing 64 *nir*S probes. In this study, we used 50-mer FGAs to monitor the composition and population dynamics of many different functional genes in Puget Sound sediments [11]. The array contains 763 probes involved in nitrification (*amoA*), denitrification (*nirS* and *nirK*), nitrogen fixation (*nif*H), methane oxidation (*pmoA*), and sulfate reduction (*dsr*AB). The probes on these arrays are derived from sequences recovered from a variety of environments, and represent well known microbial population diversity involved in these biogeochemical processes.

MATERIALS AND METHODS

Study Site and Sampling

We analyzed sediment cores from Carr Inlet site in Puget Sound. Cores of at least 38 cm length were obtained from each site using a Soutar box corer. Subcores were collected from each principal core and later sectioned into different depth sediment samples (0-0.5 cm, 2.5-3.0 cm, 5-5.5 cm, 7.5-8 cm, 10-10.5 cm, 25-25.5 cm, 50-50.5 cm, and 84-84.5 cm). Deep samples were obtained using a gravity corer. Samples were placed in sterile polypropylene tubes, immediately dropped in liquid nitrogen, and then shipped on ice to the laboratory. The samples were stored at -80ºC until nucleic acid was extracted.

Oxygen Profiles and Chemical Analysis of Sediment Samples

The overlying waters and porewaters were sampled with a whole core squeezing apparatus and analyzed for oxygen concentration with an inline polarographic electrode and for nitrate concentration by the cadmium reduction method [18]. Ammonium nitrogen (NH_4^+-N) content of the sediments was measured according to the methods of Strickland and Parsons [19]. The Fe^{2+} and Mn^{2+} concentrations in the sediments were measured using colorimetric methods [20-21], whereas SO_4^{-2} concentrations were measured using turbidimetric method [22]. Upon shipment to University of Michigan-Dearborn, sediment cores were stored at –80°C until used.

DNA Extraction, Purification and Quantitation

Community DNAs from marine sediments were isolated and purified using the method described by Wu et al. [23]. Two grams of marine sediment samples from frozen stocks were used for DNA extraction. The DNA was purified using Qiagen RNA/DNA purification system with Qiagen Tip 20 (Qiagen, Valencia, CA). The purification was performed according to the manufacturer's instructions. Extractions and purifications for each treatment were carried out in duplicates. DNA concentration was determined using a NanoDrop™ ND-1000 spectrophotometer (NanoDrop Technoplogies, Inc., Montchanin, DE) by taking measurements at 230, 260, and 280 nm.

50-mer Oligo Microarray Design and Construction

The 50-mer oligo array was constructed to deal with a diversity of functional genes (*amo*A, *pmo*A, *nir*S, *nir*K, *dsr*AB, and *nif*H) involved in biogeochemical cycling [11]. The probes were derived from sequences from diverse environments (i.e. soils, groundwater, river water, marine sediments, estuarine sediments, and lake mat). A total of 763 oligonucleotide probes consisting of 35 *amo*A, 3 *pmo*A, 93 *nirK*, 108 *nirS*, 218 *nifH*, and 306 *dsr*AB genes were designed [11]. Ten human genes were used as negative or quantitative. Positive control probes were also designed based on fifty 16S rRNA gene sequences common in environmental samples [11]. The oligos were synthesized by MWG Biotech, Inc. (High Point, NC) and were diluted to a final concentration of 50 pmoles μl^{-1} in 50% dimethyl sulfoxide (DMSO; Sigma, St

Louis, MO). The probes were arrayed with eight pins (Chipmaker 3; Telechem International, Inc., Sunnyvale, CA) at a spacing distance of 250 μm onto 25- by 75-mm Superamine glass slides (Telechem International, Inc., Sunnyvale, CA) using a PixSys 5500 printer (Cartesian technologies, Inc. Irvine, CA) at 45 % relative humidity. All of the 763 probes as well as negative and positive control probes were arranged in 9 rows x 16 columns, and printed on a slide in duplicates. The slides were left to dry for two hours, cross-linked, washed and then dried as described previously [11].

Labeling and Microarray Hybridization

Two μg of bulk community DNA from each sediment samples was used for labeling. The DNA was mixed with 1 μg random octamers, denatured by boiling for 2 min and immediately chilled on ice. The labeling reaction mixture contained the following in a total volume of 50 µl: denatured DNA; 5 mM dATP, dTTP, dGTP; 2.5 mM dCTP (New England Biolabs, Beverly, MA); 10 µM Cy3 dCTP (Amersham Pharmacia Biotech, Piscataway, NJ); and 40 U of Klenow fragment (Invitrogen, Carlsbad, CA). The reaction mixture was incubated at 37°C for 24 h, boiled in a heating block for 3 min and then chilled on ice. Labeled target was purified using a QIAquick PCR purification column (Qiagen, Valencia, CA), concentrated in a Speedvac at 40°C for 1.5 h, and then resuspended in an appropriate volume of dH_2O.

To improve the efficiency and reliability of microarray data, a two-color experiment was carried out. An internal reference DNA was used to co-hybridize with each of the Cy3 labeled sample on the same slide. The internal reference used in this study was aliquots of purified DNA from each sediment sample. The mixture was quantified and then adjusted to 200 ng μl^{-1} prior to labeling. The same amount of DNA (2 μg) was used for labeling. To quantify the target DNA, known concentrations of human DNA PCR products (gene AF126021, 1000 pg; gene X13988, 500 pg; gene M21812, 250 pg; X07868, 100 pg; and gene AK001779, 1pg) were mixed with the target DNA prior to labeling. The amount of each gene per 1 μg of bulk community DNA was quantified based on the standard curve.

All hybridizations were carried out in 8 replicates (four slides x 2 spots). The hybridization solution contained 3×SSC (1x SSC had 150 mM NaCl and 15 mM trisodium citrate), 1µg of unlabeled herring sperm DNA (Invitrogen, Carlsbad, CA), and 0.31% SDS in a total standard volume of 15 µl. To avoid bubbles, the hybridization solution was deposited directly onto the microarrays prior to placing a cover slip (6.25 mm × 8 mm). The microarray (array-side down) was placed on the cover slip and then into a self-contained flow cell (Telechem International). Fifteen µl of 3X SSC was dispensed into the hydration wells on each side of the microarray slide, and hybridization was carried out at 55°C for 12–15 h. Following hybridization, the arrays were washed at 37°C for 5 min and dried as described previously [11].

Image Processing and Data Analyses

The microarrays were scanned with ScanArray 5000 System (Packard Bioscience, Meriden, CT). The Cy3 and Cy5 images were saved separately as 16-bit TIFF files and analyzed by quantifying the pixel density (intensity) of each hybridization spot using the software of ImaGene™ version 5.0 (Biodiscovery, Inc., Los Angeles, CA). Local background measurements were subtracted automatically for each spot. The poor quality spots were flagged and removed from the data set for further analysis. Since the signal intensities of Cy3 and Cy5 hybridizations are generally not the same (Cy5 being 5 to 10 times higher than Cy3), the ratio between Cy3 and Cy5 hybridization signal intensities from known concentrations of human DNA was calculated to standardized Cy3 and Cy5 microarray data.

The signal-to-noise ratio (SNR) was also computed for each spot to discriminate true signals from noise [11, 24]. Spots that appeared to have SNR less than 3 were removed from the data set for further analysis. Thereafter, standard random error estimation and outlier detection and removal were carried out using ArrayStat™ (Imaging Research, Inc., Ontario, Canada). Outliers were detected by examining the standardized residuals. Residuals that fell well off the expected values (spots with either very high or very low residuals) indicated that the corresponding observations were not very reproducible and were removed from the data set.

The Shannon diversity index was computed for each gene group to determine the diversity and dominance at different sediment depths. Analysis of variance (ANOVA) was calculated to determine the effects of sediment depths on the amount of bulk community DNA, number of genes present per microgram of community DNA, and Shannon diversity index. When ANOVA showed significant difference between sediment depths ($P = \leq 0.05$), means were separated using Bonferroni's t-test ($P = \leq 0.05$). Microarray experiments generate large data sets, and a major challenge in micro array experiments is to extract meaningful information out of the data. One of the key goals for microarray analysis is to identify genes that give statistically significant differences in signal intensities across treatments. For comparisons of microarray data, one approach is to use parallel coordinates technique. Parallel coordinates technique is a multivariate visualization technique that allows for efficient analysis and understanding of complex data [25]. In this study, parallel coordinates technique was used to cluster micro array data and determine the most dominant genes at different depths. This technique uses hierarchical clustering algorithm that aims at grouping items so that items in a cluster are similar as possible and as different as possible from data items in the other clusters. In order to determine the most dominant genes in a sample, microarray data with similar signal intensities were clustered. Parallel coordinates technique was then used to display the result. Principal components analysis (PCA) was performed to group or separate sediment samples, which are similar or different from each other based

on the relative abundance of genes from each depth. PCA for each gene group (*amo*a, *pmo*A, *dsr*AB, *nir*S, *nir*K, and *nif*H) was computed using SYSTAT (SYSTAT version 10.0; SPSS, Inc., Chicago, IL). Parallel coordinates and PCA were performed using SYSTAT computing package (SYSTAT version 9.0).

RESULTS

Biogeochemical Properties

Puget Sound is characterized by high C flux, due to their high surface productivity and shallow water depth, leading to a high sedimentary respiration rate. The sediment was clayey silt with few visible macro-benthic organisms in the upper 25 cm. The sediment below (50-84 cm) was darker brown and a smell of hydrogen sulphide was present. Vertical profiles of nitrate depended upon oxygen concentration. At the site, oxygen was depleted at about 1 cm depth and nitrate at about 1.5 cm. Ammonium and Fe^{2+} concentrations increased with increasing depth, while Mn^{2+} concentrations decreased. The concentrations of SO_4^+ were similar across samples (Table 1). Marine invertebrates were detected in these sediments down to at least 25 cm, indicating that this depth is still within the bioturbation zone. No invertebrates were observed in the deep samples analyzed (50-84.5 cm).

Diversity of Biogeochemical Cycling Genes

Significant differences were found among 8 different samples in terms of DNA content (Table 2). The DNA content of the sediments ranged from 1.69 to 18.10 μg, with highest yield from sediment samples collected at 0-0.5 cm. Within the mixed zone (0-25.5 cm depth), the DNA content ranged

Table 1. Biogeochemical properties of sediments at different depths.

Sediment depths (cm)	Biogeochemical properties					
	O_2 μM	NO_3^- μM	NH_4^+ μM	SO_4^+ μM	Fe(II) μM	Mn (II) μM
Mixed zone						
0-0.5	193.53	14.83	37.45	24.40	-0.92	245.55
2.5-3.0	0.00	0.00	37.33	24.40	30.40	243.98
5.0-5.5	0.00	0.00	51.51	24.00	41.09	160.16
7.5-8.0	0.00	0.00	66.37	24.40	52.88	181.06
10.0-10.5	0.00	0.00	91.75	23.80	34.77	123.75
25.0-25.5	ND	ND	ND	ND	ND	ND
Unmixed zone						
50.0-50.5	ND	ND	ND	ND	ND	ND
84.0-84.5	0	0	171.45	18.00	0.95	6.22

ND=not determined

Table 2. DNA amount and number of genes present at different depths.

Sediment depths (cm)	DNA amount (ug)†	Number of positive signals	Number/ percentage of unique genes	Number of genes present‡					
				*amo*A	*pmo*A	*dsr*AB	*nir*S	*nir*K	*nif*H
Mixed zone									
0-0.5	18.10 ± 1.77a	564	36 (6.40%)	31 ± 0a	3 ± 0a	239 ± 1a	54 ± 3a	70 ± 3a	167 ± 6a
2.5-3.0	9.83 ± 1.33ab	502	29 (5.80%)	31 ± 0a	3 ± 0a	209 ± 1b	44 ± 1ab	58 ± 0b	157 ± 2b
5.0-5.5	6.44 ± 1.38b	529	31 (5.90%)	23 ± 0b	3 ± 0a	227 ± 0a	47 ± 0a	57 ± 0b	172 ± 1a
7.5-8.0	6.39 ± 1.17b	378	28 (7.40%)	19 ± 0b	3 ± 0a	156 ± 1c	35 ± 1b	44 ± 0c	121 ± 1c
10.0-10.5	6.25 ± 1.71b	333	31 (9.30%)	17 ± 0b	3 ± 0a	165 ± 2c	37 ± 6b	32 ± 0d	87 ± 2d
25.0-25.5	5.23 ± 2.78b	153	20 (13.07%)	9 ± 0c	2 ± 0a	93 ± 0d	13 ± 0c	2 ± 0e	7 ± 0e
Unmixed zone									
50.0-50.5	3.56 ± 0.14c	128	23 (18.00%)	13 ± 0bc	2 ± 0a	95 ± 1d	9 ± 12cd	2 ± 0e	7 ± 0e
84.0-84.5	1.69 ± 0.12c	57	11 (19.30%)	3 ± 0d	0 ± 0b	36 ± 0e	4 ± 0d	1 ± 0e	7 ±0e

†Mean and standard deviation of two replicates are shown. Column values followed by different letters are significantly different based on ANOVA followed by Bonferronis' f-test at P ≤ 0.05 probability level.
‡ Mean and standard deviation of eight replicates (2 field replicates x 2 slides x 2 spots per slide) are shown. Column values followed by different letters are significantly different based on ANOVA followed by Bonferroni's *f*-test at P ≤ 0.05 probability level.

between 5.23 and 18.10 μg, whereas it was between 1.69 and 3.56 μg in the unmixed zone (50-84.5 cm depth). The hybridization image indicated that the microarrays hybridized reasonably with DNA's from Puget Sound marine sediments (Figure 1). Strong signals were obtained with some nitrogenases (*nif*H) dissimilatory sulfate reductase (*dsr*AB), ammonia monooxygenase (*amo*A), methane monooxygenase (*pmo*A), and nitrite reductase (*nir*K/S). Microarray analysis indicated difference in the number of positive hybridization signals and signal intensity between sediments from shallow (bioturbation zone) and deeper (below bioturbation zone) depths. After hybridization on the arrays containing 763 probes, each sample yielded 57-564 positive hybridization signals (Table 2). Sediment samples collected at 0-0.5 cm gave the highest number of positive hybridization signals (564) consisting of 31 *amo*A, 3 *pmo*A, 206 *dsr*Ab, 54 *nir*S, 70 *nir*K, and 167 *nif*H. These numbers were significantly different with other sediment samples. The number of positive hybridization signals decreased with depth, but a dramatic decline was observed in the unmixed zone, where the numbers of positive hybridization signals are 5 to 10 times lower than that from depth 0-0.5 cm (Table 2). To monitor the dynamics of the microbial communities, unique genes from each of the 8 samples were identified. On the surface sediments (depth 0-0.5 cm), 36 unique genes were found (Table 3). These genes were not detected in any of the 7 sediment samples. The number of unique genes decreased with increasing depth. Within the bioturbation zone (depth 0-25 cm), 20 to 36 unique genes were found, whereas there were only 11 to 23 unique genes observed below the bioturbation zone (depth 50-84 cm). However, when the percentage of unique genes (number of unique genes/number of positive signals x 100) was calculated, it appears to increase with increasing depth (Table 2). The percentage of unique genes increased from 6.4% (shallowest depth; depth 0-0.5 cm) to 19.30% (deepest depth; depth 84-84.5 cm).

Analysis of positive hybridization signals also revealed presence of genes common to all samples. Thirty-four genes comprising of 14 *dsr*AB, 6 *nir*K, 6 *nir*S, and 8 *nif*H genes were consistently present in all samples (Table 4). The majority of these genes were similar to those retrieved from various environments (i.e. soils, groundwater, river water, strotomites, marine sediments, and estuarine sediments). Among known cultivated microorganisms found were the sulfate-reducing bacteria *Desulfovibrio vulgaris* subsp. *oximicus* and *Desulfotomaculum nigrificans*; the denitrifying bacteria *Azobacter* sp. and *Rhodobacter sphaeroides*; and the nitrogen-fixing bacteria *Treponema* sp. and *Calothrix* sp.

There were significant differences among samples in terms of the Shannon-diversity and equitability indices (Table 5). These diversity statistics were calculated based on the number and relative abundance of the gene (ng gene μg^{-1} bulk community DNA) for each gene group. In general, the Shannon diversity index values were similar within the mixed

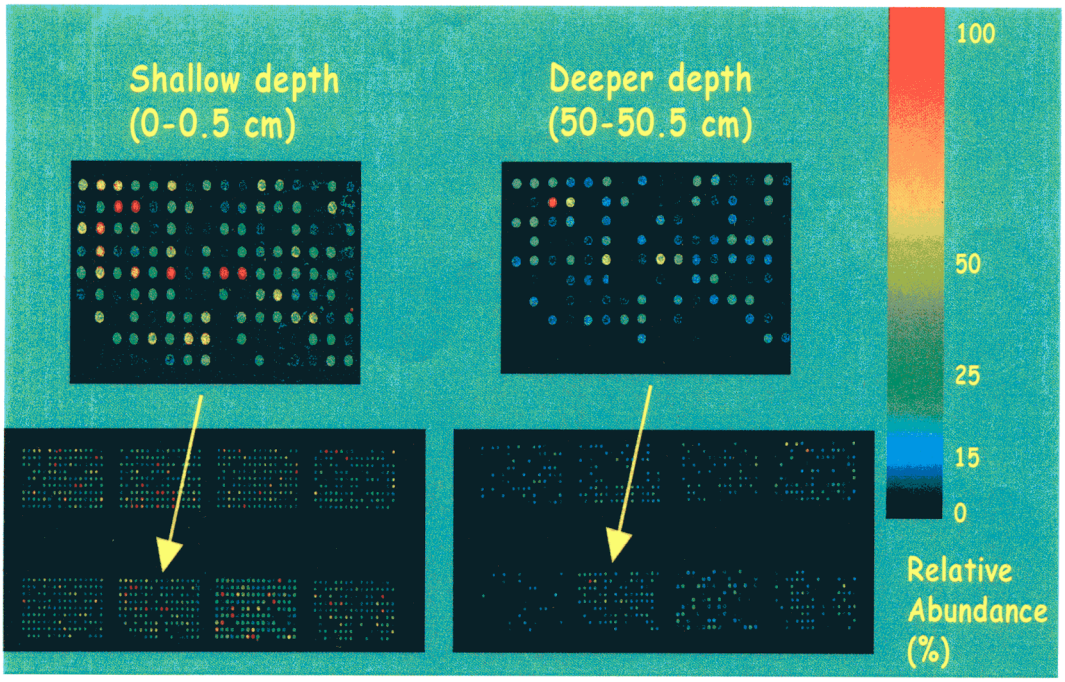

Figure 1. Hybridization images showing the profiles of different *dsr*AB, *nir*S, *nir*K, *nif*H, *amo*A and *pmo*A genes.

Table 3. Genes that are unique to each sample.

Gene group	Gene ID	Source	Gene group	Gene ID	Source
Depth 0-0.5 cm			Depth 5-5.5 cm		
*dsr*AB	dsr_AF360651	Estuarine sediment (Denmark)	*dsr*AB	dsr_AF388254	Marine sediment (Denmark)
	dsr_AF388268	Marine sediment (Denmark)		dsr_AY015504	Groundwater (uranium mill tailings site)
	dsr_AF388277	Marine sediment (Denmark)		dsr_AY015508	Groundwater (uranium mill tailings site)
	dsr_AY015596	Groundwater (uranium mill tailings site)		dsr_AY015614	*Desulfotomaculum aeronauticum*
	dsr_FW003305A	Groundwater (FRC site, ORNL)		dsr_FW005251B	Groundwater (FRC site, ORNL)
	dsr_FW005251A	Groundwater (FRC site, ORNL)		dsr_FW010103A	Groundwater (FRC site, ORNL)
	dsr_FW01280A	Groundwater (FRC site, ORNL)		dsr_FW015318B	Groundwater (FRC site, ORNL)
	dsr_FW010280B	Groundwater (FRC site, ORNL)		dsr_FW300015A	Groundwater (FRC site, ORNL)
	dsr_FW015084B	Groundwater (FRC site, ORNL)		dsr_FW300181A	Groundwater (FRC site, ORNL)
	dsr_FW300151A	Groundwater (FRC site, ORNL)		dsr_M300099A	Marine sediment (Mexico)
	dsr_M400128B	Marine sediment (Mexico)		dsr_M300270B	Marine sediment (Mexico)
	dsr_M300214B	Marine sediment (Mexico)		dsr_M300308A	Marine sediment (Mexico)
	dsr_M300307A	Marine sediment (Mexico)		dsr_TPB16142B	Groundwater (FRC site, ORNL)
	dsr_M300374B	Groundwater (FRC site, ORNL)		dsr_TPB16288B	Groundwater (FRC site, ORNL)
	dsr_M306048B	Marine sediment (Mexico)		dsr_TPB16340A	Groundwater (FRC site, ORNL)
	dsr_M306118B	Marine sediment (Mexico)	*nif*H	nifh_10863112	*Phormidium* sp.
	dsr_TPB160090A	Groundwater (FRC site, ORNL)		nifh_10863114	Marine stromatolite cyanobacterium
*nif*H	nifh_10863148	Marine sediment (Mexico)		nifh_1255473	Intestinal microflora (termite *Reticulitermes speratus*)
	nifh_10863150	Marine sediment (Mexico)		nifh_1255479	Intestinal microflora (termite *Reticulitermes speratus*)
	nifh_13936674	Lake water (Lake Michigan)		nifh_1698861	*Myxosarcina* sp.
	nifh_3157703	Termite gut		nifh_3372187	Ocean water (Atlantic ocean)
	nifh_6277246	Termite gut		nifh_7339968	Rhizosphere (*Spartina alterniflora*)
	nifh_7339862	Rhizosphere (*Spartina alterniflora*)		nifh_862322	*Azospirillum lipoferum*
	nifh_7339866	Rhizosphere (*Spartina alterniflora*)	*nir*K	nirK_AJ224908	*Rhodobacter sphaeroides*
	nirK_M318015	Marine sediment (Mexico)		nirK_NKTT19	Soil (Virginia Csite)
*nir*K	nirK_NKFF27	*Soil (Virginia Csite)*		nirK_NKTT71	Soil (Virginia Csite)
	nirK_NKFF29	Soil (Virginia Csite)		nirK_PA50	Marine sediment Puget Sound)
	nirK_NKFF34	Soil (Virginia Csite)		nirK_PSEUDO	Pseudomonas aeruginosa
	nirK_NKFF35	Soil (Virginia Csite)		nirK_RHU65658	*Rhizobium hedysari*
	nirK_NKTT72	Soil (Virginia Csite)	*nir*S	nirS_F14	Soil (Michigan State University)
	nirK_S57	Soil (Michigan State University)		nirS_M312B20	Marine sediment (Mexico)
	nirK_U62291	*Rhodobacter sphaeroides*		nirS_M318A21	Marine sediment (Mexico)
*nir*S	nirS_M312A38	*Mexico marine sediment*			
	nirS_M318A36	Marine sediment (Mexico)			
	nirS_M318B12	Marine sediment (Mexico)			
	nirS_S14	Soil (Michigan State University)			
	Depth 2.5-3.0 cm			**Depth 25-25.5 cm**	
*dsr*AB	dsr_AF360664	*Estuarine sediment (Denmark)*	*dsr*AB	dsr_AY015542	Groundwater (uranium mill tailings site)
	dsr_AF388278	*Marine sediment (Denmark)*		dsr_AY015597	Groundwater (uranium mill tailings site)
	dsr_AF388295	*Marine sediment (Denmark)*		dsr_AY015599	Groundwater (uranium mill tailings site)
	dsr_AY015600	*Groundwater (uranium mill tailings site)*		dsr_FW010084A	Groundwater (FRC site, ORNL)
	dsr_FW005121A	Groundwater (FRC site, ORNL)		dsr_M300206A	Groundwater (FRC site, ORNL)
	dsr_FW010031A	Groundwater (FRC site, ORNL)		dsr_M300278B	Mexico marine sediment

table 3 continues/……..

Gene group	Gene ID	Source	Gene group	Gene ID	Source
	dsr_FW300038B	Groundwater (FRC site, ORNL)		dsr_TPB16318B	Groundwater (FRC site, ORNL)
	dsr_FW300058A	Groundwater (FRC site, ORNL)		dsr_TPB16340A	Groundwater (FRC site, ORNL)
	dsr_FW300246A	Groundwater (FRC site, ORNL)	*nif*H	nifh_10863132	Marine stromatolite (Bahamas)
	dsr_M300206B	Marine sediment (Mexico)		nifh_1255485	Marine stromatolite eubacterium
	dsr_M300308B	Marine sediment (Mexico)		nifh_13173328	Intestinal microflora (termite *Reticulitermes speratus*)
	dsr_M306080A	Marine sediment (Mexico)		nifh_15187010	Aboveground biomass of *Spartina alterniflora*
	dsr_TPB16340A	*Groundwater (FRC site, ORNL)*		nifh_3157531	Termite gut
*nif*H	nifh_10863120	*Marine stromatolite (Bahamas)*		nifh_3157589	Termite gut
	nifh_10863130	*Marine stromatolite (Bahamas)*		nifh_3157605	Termite gut
	nifh_10863140	Marine stromatolite (Bahamas)		nifh_3157673	Termite gut
	nifh_10863142	Marine stromatolite (Bahamas)		nifh_3157677	Termite gut
	nifh_12659179	*Spirochaeta stenostrepta*		nifh_7339854	Rhizosphere (*Spartina alterniflora*)
	nifh_12659181	*Spirochaeta zuelzerae*		nifh_7339864	Rhizosphere (*Spartina alterniflora*)
	nifh_3157541	Termite gut	*nir*K	nirK_S33	Soil (Michigan State University)
	nifh_7339906	Rhizosphere (*Spartina alterniflora*)			
*nir*K	nifK_NKFF01	Soil (Virginia Csite)			
	nirK_NKFF12	Soil (Virginia Csite)			
	nirK_NKFF32	Soil (Virginia Csite)			
	nirK_X91394	*Ralstonia eutropha*			
*nir*S	nirS_M305027	Marine sediment (Mexico)			
	nirS_M306B04	Marine sediment (Mexico)			
	nirS_M306B38	Marine sediment (Mexico)			
	nirS_M312A19	Marine sediment (Mexico)			
	nirS_RSP224908	*Rhodobacter sphaeroides*			
	Depth 50-.50.5 cm			**Depth 84-84.5 cm**	
*dsr*AB	dsr_AY015598	Groundwater (uranium mill tailings site)	*dsr*AB	dsr_M300293A	Marine sediment (Mexico)
	dsr_FW010274	Groundwater (FRC site, ORNL)		dsr_DMU58127	*Desulfococcus multivorans*
	dsr_M300206A	Marine sediment (Mexico)		dsr_DSU58121	*Desulfobotulus sapovorans*
	dsr_TPB16318B	*Desulfosporosinus orientis*	*nir*S	dsr_AY015615	Groundwater (uranium mill tailings site)
*nif*H	nifh_10863146	Marine stromatolite (Bahamas)		nirS_S45	Soil (Michigan State University)
	nifh_1255507	Intestinal microflora (termite *Reticulitermes speratus*)		nirS_S36	Soil (Michigan State University)
	nifh_1698869	Unidentified cyanobacterium Dinitrogenase reductase		nirS_F14	Soil (Michigan State University)
	nifh_3165373	Termite gut		nirS_M312B20	*Marine sediment (Mexico)*
	nifh_4529865	*Desulfobacter curvatus*		nirS_M318A21	Marine sediment (Mexico)
	nifh_497879	Rhizosphere (*Oryza sativa*)	*nif*H	nifh_10863108	Marine stromatolite (Bahamas)
	nifh_507853	Rhizosphere (*Oryza sativa*)		nifh_7339854	Rhizosphere (*Spartina alterniflora*)
*nir*K	nirK_E04-15-31	Groundwater (FRC site, ORNL)			
	nirK_E08-16-129	Groundwater (FRC site, ORNL)			
	nirK_PA50	Marine sediment (Puget Sound)			
*nir*S	nirS_D09-300-67	Groundwater (FRC site, ORNL)			
	nirS_G06-05-160	Groundwater (FRC site, ORNL)			
	nirS_G08-05-349	Groundwater (FRC site, ORNL)			
	nirS_G10-16-153	Groundwater (FRC site, ORNL)			
	nirS_PA63	Marine sediment (Puget Sound)			
	nirS_S32	Soil (Michigan State University)			
	nirS_S4	Soil (Michigan State University)			
	nirS_44	Soil (Michigan State University)			
	nirS_Y32S	Marine sediment (Washington)			

FRC=Field Research Center; ORNL=Oak Ridge National Laboratory

Table 4. Genes consistent to all samples.

Gene group	Gene ID	Source
*amo*A	None	-
*pmo*A	None	-
*dsr*AB	dsr_AB061543	*Desulfovibrio vulgaris* subsp. *oxamicus*
	dsr_AF179326	Microbial mat (Solar Lake, Egypt)
	dsr_AF360669	Estuarine sediments (Kysing Fjord, Denmark)
	dsr_AF388249	Marine sediment (Aarhus Bay, Denmark.)
	dsr_AY015499	*Desulfotomaculum nigrificans*
	dsr_AY015507	Groundwater (uranium mill tailings disposal site, Shiprock, New Mexico)
	dsr_AY015588	Groundwater (uranium mill tailings disposal site, Shiprock, New Mexico)
	dsr_FW003032B	Groundwater (FRC site, ORNL)
	dsr-FW005196A	Groundwater (FRC site, ORNL)
	dsr_FW005721A	Groundwater (FRC site, ORNL)
	dsr_FW010029B	Groundwater (FRC site, ORNL)
	dsr_FW015243B	Groundwater (FRC site, ORNL)
	dsr_M300244A	Marine sediment (Western Mexico continental margin)
	dsr_M306157	Marine sediment (Western Mexico continental margin)
*nir*K	nirk_AZOV	*Azobacter* sp.
	nirk__F04-15-43	Groundwater (FRC site, ORNL)
	nirk_G01-03-134	Groundwater (FRC site, ORNL)
	nirk_NKFE04	Soil (Virginia Csite)
	nirk_NKTT53	Soil (Virginia Csite)
	nirk_S12	Soil (Michigan State University)
*nir*S	nirS_D07-05-221	Groundwater (FRC site, ORNL)
	nirS_E02-03-85	Groundwater (FRC site, ORNL)
	nirS_M306B38	Marine sediment (Western Mexico continental margin)
	nirS_RSP224908	*Rhodobacter sphaeroides*
	nirS_S23	Soil (Michigan State University)
	nirS_S8	Soil (Michigan State University)
*nif*H	nifh-10863110	Marine strotomite (Bahamas)
	nifh_12001863	River water, Neuse, River Estuary, North Carolina
	nifh_12001899	River water, Neuse, River Estuary, North Carolina
	nifh_1255497	Intestinal microflora (termite *Reticulitermes speratus*)
	nifh_12659197	*Treponema* sp.
	nifh_1698855	*Calothrix* sp.
	nifh_3157679	*Termite gut*
	nifh_7339912	*Rhizosphere* (Spartina alterniflora)

FRC=Field Research Center; ORNL=Oak Ridge National Laboratory

Table 5. Diversity estimates of *amo*A *pmo*A, *dsr*AB, *nir*S, *nir*K and nifH genes of Puget Sound sediments collected at different depths.

Sediment depths (cm)	Shannon diversity index					
	*amo*A	*pmo*A	*dsr*AB	*nir*S	*nir*K	*nif*H
Mixed zone						
0-0.5	3.28 ± 0.07a	1.40 ± 0.05a	4.44 ± 0.07a	4.18 ± 0.05a	4.28 ± 0.05a	4.13 ± 0.05b
2.5-3.0	3.20 ± 0.00a	1.31 ± 0.06ab	4.13 ± 0.05b	4.18 ± 0.05a	4.10 ± 0.00bc	4.09 ± 0.04b
5.0-5.5	3.21 ± 0.04a	1.35 ± 0.05ab	4.16 ± 0.05b	4.20 ± 0.05a	4.16 ± 0.05bc	4.14 ± 0.05b
7.5-8.0	3.06 ± 0.07b	1.30 ± 0.08b	4.16 ± 0.07b	4.25 ± 0.05a	4.18 ± 0.05b	4.18 ± 0.05b
10.0-10.5	3.06 ± 0.09b	1.29 ± 0.08b	4.11 ± 0.06b	4.15 ± 0.09a	4.08 ± 0.09c	4.14 ± 0.05b
25.0-25.5	2.95 ± 0.05c	1.05 ± 0.05c	4.20 ± 0.00b	4.24 ± 0.05a	3.80 ± 0.00d	4.20 ± 0.00a
Unmixed zone						
50.0-50.5	2.43 ± 0.05d	1.09 ± 0.04c	4.15 ± 0.09b	4.18 ± 0.20a	3.59 ± 0.08e	4.20 ± 0.00a
84.0-84.5	0.99 ± 0.04e	0.00 ± 0.00d	3.30 ± 0.00c	3.59 ± 0.04b	2.96 ± 0.07f	2.34 ± 0.05c

Mean and standard deviation of eight replicates (2 field replicates x 2 slides x 2 spots per slide) are shown. Column values followed by different letters are significantly different based on ANOVA followed by Bonferroni's *t*-test at $P \leq 0.05$ probability level.

Shannon index ($\hat{H}$) = $C/N\ (N \log_{10} N - \sum n_i \log_{10} n_i)$ where $C = 2.3$; N= sum of the relative abundance for gene group; n_i = relative abundance of each gene i; and i = number of gene for each gene group. Higher number represents more diversity

zone (depths of 0-25.5 cm) and were significantly lower compared to the unmixed zone (depths of 50-84.5 cm), supporting the conclusion that the mixed zone had higher diversity than the unmixed zone. The surface sediments (depth, 0-0.5 cm) had the highest diversity of all sites studied, while the deepest (depth 84-84.5 cm) had the lowest. Diversity changes within the mixed zone were more subtle, with the presence of different *amo*A, *pmo*A, *dsr*AB, *nir*S, *nir*K, and *nif*H genes gradually decreasing with depth. Diversity changes were more evident between the mixed zone and the unmixed zone, with surface sediments (depth 0-0.5 cm) demonstrating highest diversity, and deepest sediment (depth 84-84.5 cm) showing the lowest diversity.

Parallel Coordinates Analysis

Parallel coordinates are a more robust and accurate method for grouping large data sets. In this analysis, microarray data were plotted such that data points that showed similar signal intensities are clustered together. Using the hierarchical rule induction method, the test generated five layers of hierarchy corresponding to five signal intensity groups (1×10^4 , 2×10^4, 3×10^4, 4×10^4, and 5×10^4) (Figure 2). The eight most abundant functional genes (signal intensities between 3×10^4 and 5×10^4) are similar to those found in groundwater, terminate gut, sediments and known cultivable bacteria. Some of these genes were dominant in all samples collected at different depths, while others are abundant only within the bioturbation zone or below the bioturbation. For example, the nitrite reductase gene similar to nirK_NKTH17 was found to be dominant across all samples; the nitrogenase gene similar to that isolated from termite gut (nifH_3157499), the nitrogenase-containing bacterium *Pseudanabaena* sp. (nifH_1698867), and the ammonia monooxidase gene similar to *amo*A_E03A16280 were only detected within the bioturbation zone; and nitrite reductase gene similar to nirK_WA20 was dominant only below the bioturbation zone.

PCA analysis

Principal components analysis (PCA) of the microarray data, which represented 89% of the total variance, revealed consistent difference between sediments within and below the bioturbation zones (Figure 3). Sediments collected at depths 0-0.5 cm, 2.5-3.0 cm, 5.0-5.5, cm and 25.0-25.5 cm (bioturbation zone) grouped together, and were separated from sediments collected at depths 50-50.5 cm and 84-84.5 cm (below bioturbation zone). The overall patterns showed that sediments within the bioturbation zone were similar but were different from sediments below the bioturbation zone.

DISCUSSION

Microarrays are one high-throughput approach that promised to introduce higher resolution study of the environment. Prior testing and evaluation of microarrays used in this study demonstrated that the developed arrays are sensitive and specific, and that it can discriminate biogeochemical cycling microbes at species-level [11]. It has been known that microarray hybridization and washing conditions greatly affect microarray sensitivity and specificity

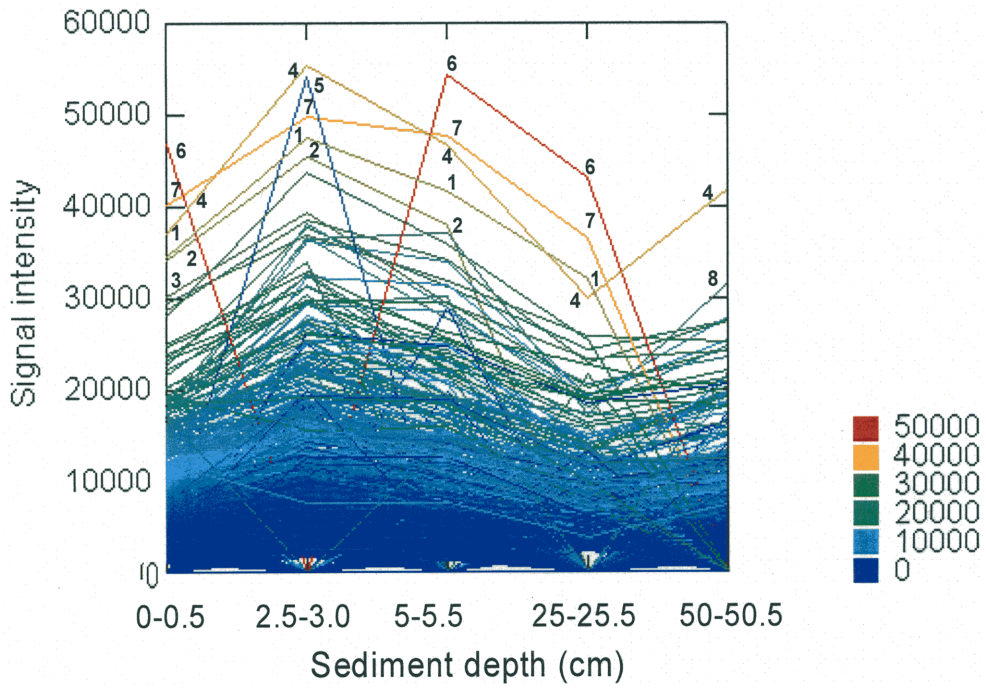

Figure 2. Parallel coordinate display showing the eight most abundant genes. (1) *amo*A_E03A16280 (isolated from ground water); (2) *nir*S_M13A42 (marine sediment; (3) *dsr*A_M300270A (isolated from marine sediment); (4) *nir*K_NKTF17 (isolated from soil); (5) *nif*H_296359 (isolated from *Frankia* sp.); (6) *nif*H_3157499(isolated from termite gut); (7) *nif*H_1698867 (isolated from *Pseudanabaena* sp.); (8) *nir*K_WA20 (isolated from marine sediment).

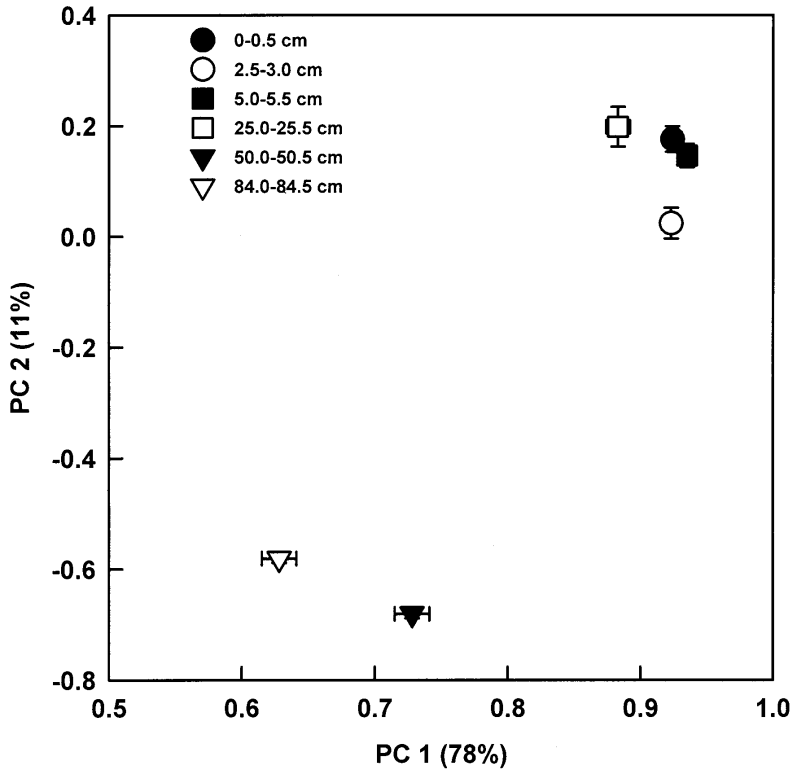

Figure 3. Ordinate plots from principal component analysis based on the amount of *amo*A/pmoa, *dsr*AB, *nir*S, *nir*K, and *nif*H genes at different sediment depths. Values in parentheses indicate percent of total variances of PCA derived from the amount of individual functional gene group data and the combined data.

[15]. Increased sensitivity can be achieved by destroying stable secondary structures of probes and enhancing duplex formation [26]. On the other hand, specificity can be significantly improved by preventing interactions between probes and non-complementary targets or by preventing non-specific binding [27]. Our preliminary evaluation of the arrays indicated that the specificity could be improved by increasing hybridization temperatures [11]. In all our experiments, hybridizations were performed at 55°C in the presence of 50% formamide, and washing at 37°C for 5 min. These hybridization and washing conditions differentiated genes possessing <85% sequence identities. Many prior microarray experiments have utilized PCR amplification for detecting genes from the environment [16-17, 28]. These developed arrays were criticized due to bias in terms of template to product ratio, which results in failure to quantitatively reflect community composition. Hybridization of marine sediment samples showed that DNA from bacteria could be detected by extraction and direct hybridization to the DNA microarray without the need for PCR amplification. The hybridization image (Figure 2) indicated that the 50-mer oligonucleotide arrays hybridized reasonably well with 2 μg of community DNA extracted from Puget Sound sediments. The most abundant genes included those genes encoding nitrogenase (*nif*H), dissimilatory sulfite reductase (*dsr*AB) and nitrite reductase (*nir*K and *nir*S). These results demonstrate the usefulness and potential of this high-throughput approach in monitoring the composition and structure of microbial populations involved in biogeochemical processes.

Analysis of microarray data reveals shifts in microbial community composition with increasing depth. Hybridization patterns indicate shifts in the relative abundance of specific genes and overall microbial diversity among samples collected at different depths. More probes hybridized to sediment samples within the bioturbation zone (depth 0-25 cm) in comparison to samples below the bioturbation zone (depth 50-84 cm). The difference was reflected not only by Shannon diversity measurements, but also by PCA analysis. The higher diversity in the bioturbation zone (depth 0-25 cm) may have active decomposition and transformation of deposited organic matter, which is encouraged by faunal bioturbation and current mixing of sediment, replenishing nutrients and energy resources [29]. On the other hand, the reduced diversity observed below the bioturbation zone (depth 50-84 cm) may be a result of lower nutrient availability, and thus a greater reliance on low-energy yielding metabolic processes as only poorer quality substances are available [5]. Community structure difference was most stark between surface (depth 0-0.5 cm) and deepest (depth 84-84.5 cm) sediments, due to variations in environmental conditions and nutrient availability. Environmental conditions and energy resources available tend to dictate microbial community composition and species richness [5], but also constrain the physiological characteristics of the community members. The apparent correlation in community composition and diversity with carbon (dissolved organic carbon) and nutrients (inorganic nitrogen) could be caused by selection or by sources transport of microbes in the sediments [30]. Although the functional gene profile of the deeper sediments (depths 50-84 cm) was different from that of the shallower sediments (depths 0-25 cm), many of the genes found in the deeper sediments were also found in the shallower sediments. The number of genes in the deepest sediment was least diverse compared to those in the surface sediments although the percentage of unique genes in this sediment was higher. The high level of diversity in the surface sediment (depth, 0-0.5 cm) is partly a result of the fact that oxygen is present at this zone. It is also expected that the surface layer has a ready supply of labile carbon and energy resources, thus increasing diversity. Microarray diversity results correlated with our previous studies on denitrifier community in marine sediments using TRFLP [9] and cloning sequencing techniques [5]. In the present study, the difference in diversity was not only reflected in denitrifier communities in the sediments, but all other functional guilds (nitrogen-fixing bacteria, sulfate-reducing bacteria, methane-oxidizing bacteria and ammonium-oxidizing bacteria) tested. Parallel coordinate display showed that the most dominant functional guilds are those that are involved in nitrogen cycling. The ammonium-oxidizing and nitrogen-fixing bacteria were dominant within the bioturbation zone, whereas the denitrifying prevailed across all depths. These result shows that DNA microarrays have great potential for mapping spatial variability of functional gene diversity in marine sediments.

Microarray technology has potential as a tool in revealing a comprehensive picture of the functional gene composition in marine sediments. With the arrays now in hand, it is now possible to probe sediments with sufficient spatial resolution, which is helpful if one wants to determine the sources and fates of specific microbial populations in the sediments. During the past decade, there has been considerable interest in the understanding of the influence of biogenic activities on physical, chemical, and biological conditions in marine sediments. Biogenic particle mixing such that occurs in the sediments can be important for the redistribution of reactive organic matter. One area worth exploring is the temporal and spatial variations of microbial processes, and fluxes of nutrients associated with biogenic structures and faecal pellets of infaunal animals. DNA microarrays may be useful to precisely determine microbial activities in the sediments and investigate to which extent biogeochemical dynamics relies upon diversity within functional microbial groups. To date, FGAs have been limited to detection of genes and inference about processes, but the hope is to be able to detect mRNA to provide direct information about which groups of microbes are active in the sample and to detect the dynamic distribution and expression levels of target genes. The low concentration of mRNA in environmental samples limits this application. Hence, systematic amplification of mRNAs in environmental samples might be required if microbial activities or gene expression

are to be analyzed using microarrays.

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