APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 2005, p. 679–690 Vol. 71, No. 2
0099-2240/05/$08.00+0 doi:10.1128/AEM.71.2.679–690.2005

Structure of Sediment-Associated Microbial Communities along a Heavy-Metal Contamination Gradient in the Marine Environment

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Received 6 July 2004/Accepted 2 September 2004

Microbial community composition and structure were characterized in marine sediments contaminated for >80 years with cadmium, copper, lead, and zinc. Four sampling sites that encompass a wide range of sediment metal loads were compared in a Norwegian fjord (Sørfjord). HCl-extractable metals and organic matter constantly decreased from the most contaminated site (S1) to the control site (S4). All sampling sites presented low polychlorinated biphenyl (PCB) concentrations (Σ_7PCB < 7.0 ng g [dry weight]$^{-1}$). The biomass ranged from 4.3×10^8 to 13.4×10^8 cells g (dry weight) of sediments^{-1} and was not correlated to metal levels. Denaturing gradient gel electrophoresis indicated that diversity was not affected by the contamination. The majority of the partial 16S rRNA sequences obtained were classified in the γ- and δ-*Proteobacteria* and in the *Cytophaga-Flexibacter-Bacteroides* (CFB) bacteria. Some sequences were closely related to other sequences from polluted marine sediments. The abundances of seven phylogenetic groups were determined by using fluorescent in situ hybridization (FISH). FISH was impaired in S1 by high levels of autofluorescing particles. For S2 to S4, the results indicated that the HCl-extractable Cu, Pb, and Zn were negatively correlated with the abundance of γ-*Proteobacteria* and CFB bacteria. δ-*Proteobacteria* were not correlated with HCl-extractable metals. Bacteria of the *Desulfosarcina-Desulfococcus* group were detected in every site and represented 6 to 14% of the DAPI (4′,6′-diamidino-2-phenylindole) counts. Although factors other than metals may explain the distribution observed, the information presented here may be useful in predicting long-term effects of heavy-metal contamination in the marine environment.

Pollution of coastal zones by heavy metals, such as Cd, Pb, Hg, and Ni, is a major environmental problem in many parts of the world (13). Once in the marine environment, these problematic contaminants accumulate in sediments (12). It is generally agreed that bacteria, as the most abundant sediment organisms, have a major role in the fate of these contaminants (12, 25). Bacteria may volatilize or precipitate metals and transform them into toxic organic derivatives (12, 20, 25, 71). They can also produce anionic polymers that are able to complex metals (20). According to the type of physicochemical environment and of microbial metabolism, contaminants may be released from sediments into the water column. In such cases, marine sediments become a secondary source of pollution, leading to the possible contamination of benthic organisms living in contact with them and, finally, of all of the benthic food chain (12).

Understanding the microbial community structure in sediments from the continental shelf (i.e., the zone most exposed to pollutants) is essential in order to understand microbial processes underlying secondary pollution phenomena. In order to reliably characterize the community structure, quantitative methods, such as fluorescent in situ hybridization (FISH) or rRNA slot blot hybridization, are necessary. To date, the number of community structure studies that have been conducted in marine sediments from the continental shelf area using quantitative methods is limited (7, 38, 52, 54), and many studies are restricted to specific groups, such as sulfate-reducing bacteria (19, 49, 53, 58), or particular species (9). Very little is known about the diversity and structures of indigenous microbial populations within heavy-metal-contaminated coastal areas. The few reports that are available for polluted marine sediments deal with other contaminants, such as polyaromatic hydrocarbons (27, 32), hydrocarbons (40, 55, 56), and organic matter (OM) (45, 65). Some reports on the effects of heavy metals on bacterial diversity in marine sediments may be found in the literature (26, 29, 48, 50), but most of them lack important ecological information, such as that offered by 16S rRNA sequencing or in situ hybridization.

The aim of the present work was to study the sediment-associated microbial communities along a gradient of heavy-metal pollution in the marine environment. An approach utilizing environmental gradients may offer elements pivotal to an understanding of natural microbial communities (24). The area studied was the Sørfjord in southern Norway (Fig. 1). This fjord displays very high levels of heavy metals in the sediments, which are the result of the discharge of considerable amounts of Zn, Cd, and Pb (since 1920) by smelters located at the head of the fjord (15, 61, 68). Figures for 1980 indicated that 1,387 tons of Zn, 329 tons of Pb, and 14.6 tons of Cd were discharged into the fjord during that year alone (68). Although inputs were significantly reduced in 1986, heavy metals in sediments still show a remarkable gradient from high concentrations in the inner fjord (station S1) to background levels at the opening of the fjord (station S4) (Fig. 1) (15, 68). Mean metal concentrations determined in the total sediments of S1 in 2000 were,

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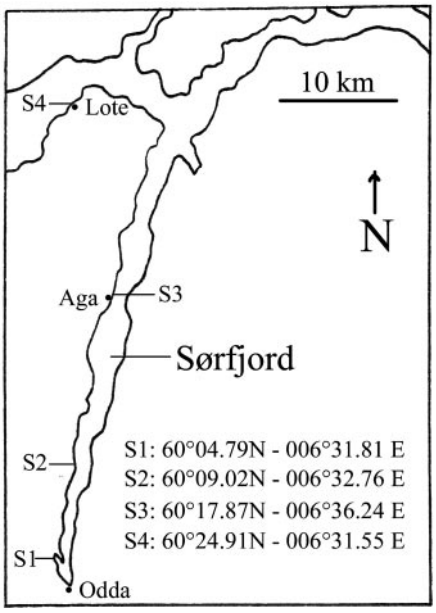

FIG. 1. Map of the Sørfjord in southern Norway showing the locations of the four sampling stations (S1, S2, S3, and S4). S1 is the most contaminated site and is located near the city of Odda. N, north.

in milligrams per kilogram (dry weight), 60 for Cd, 100 for Cu, 800 for Pb, and 100 for Zn (15). These concentrations are 10 to 30 times higher than those reported in sediments sampled in the most contaminated areas of the southern North Sea (14) and up to 60 times higher than the ecotoxicological assessment criterion of OSPAR (Oslo and Paris Commission for the Protection of the Marine Environment of the North-East Atlantic), which is defined as the concentration level above which concern is indicated (reports are available at www.ospar.org).

In the present work, the microbial communities of the Sørfjord were studied using direct DAPI (4′,6′-diamidino-2-phenylindole) counts, FISH, and denaturing gradient gel electrophoresis (DGGE), as well as cloning and sequencing. Levels of HCl-extractable metals, organic matter, and polychlorinated biphenyls (PCB) in the sediments were also determined. This is the first study in which microbial communities in shallow heavy-metal-contaminated marine sediments are quantified in situ and in which HCl-extractable metal concentrations are tentatively correlated with the structure of the microbial population.

MATERIALS AND METHODS

Study sites and sampling. The sampling stations (S1 to S4) were disposed as represented in Fig. 1 (in this study, station S3 is different from station S3 of previous studies [15]). S1 was located 100 m away from a zinc smelter and was the most heavily contaminated site (15, 68). S2 was located in front of a titanium smelter, with S3 12 km further north and S4 in the neighboring fjord in the direction of the open sea. In the present study, S4 was considered a control site. Sediments were collected in March 2003 by SCUBA divers at a depth of 15 m. The salinity of the seawater above the sediments was 31‰, and its temperature was 5°C. The four sites were located in the oxic zone of the fjord. The epibenthic macrofauna is principally composed of echinoderms, bivalves, tunicates, and flatfishes (15, 61, 68). The top 3-cm layer of the sediments was collected using acid-washed 500-ml polyethylene vials. Six replicate samples were collected at each site. Sediments from S1 and S2 were muddy, S3 was composed of fine sand, and S4 was coarse sand. The granulometries of the sediments, as determined by dry sieving and expressed in weight percentages, are presented in Table 1.

TABLE 1. Granulometries of sediments expressed in weight percentages[a]

Site	% with granulometry (μm) of:					
	≤63	63–125	125–250	250–500	500–1,000	≥1,000
S1	8.0	22.6	58.4	10.5	0.2	0.3
S2	4.1	18.4	73.0	3.6	0.5	0.4
S3	2.4	14.6	27.0	25.1	15.4	15.5
S4	1.2	6.4	22.5	17.7	17.1	35.1

[a] One sample of ca. 500 g of total sediments (dry weight) was passed through five sieves with decreasing pore sizes.

HCl-extractable metals. Fresh sediment subsamples were immediately frozen in liquid nitrogen and stored at −80°C. The frozen sediment samples ($n = 6$) were then oven dried (48 h; 60°C). Aliquots of the dried sediments (500 ± 3 mg) were placed in acid-washed Teflon vials with 10 ml of 0.5 M Suprapur HCl (Merck) (66). The sediments were then agitated at 200 rpm for 1 h at ambient temperature using an orbital shaker. After centrifugation (10 min at 700 × *g*), the supernatant was separated and the metal content was determined directly by atomic absorption spectrometry, as described elsewhere (14). A contamination index (CI) was calculated for each site using the HCl-extractable metals. This index is a measure of contamination relative to the metal content of the sediment at the most pristine site included in the study (S4) (24). The CI was calculated for each site as follows: $CI_n = Cd_n/Cd_{S4} + Cu_n/Cu_{S4} + Pb_n/Pb_{S4} + Zn_n/Zn_{S4}$, where n represents S1, S2, S3, or S4.

PCB analyses. Dried sediment samples from S1, S2, and S4 ($n = 4$; collected in June 2000) were homogenized, and a surrogate (PCB no. 103) was added in order to evaluate PCB losses during extraction. PCB were extracted using organic solvents and mercury as described elsewhere (17). PCB no. 155 was used as an internal standard. Concentrations of PCB congeners no. 28, 52, 101, 118, 153, 138, and 180 were then determined using a Finnigan GCQ GC/MS (17). The detection limits ranged between 0.01 and 0.1 ng g (dry weight)$^{-1}$, depending on the PCB congener. The congener numbers used in this work follow the International Union of Pure and Applied Chemistry nomenclature (http://www.epa.gov/toxteam/pcbid).

OM determinations. Frozen sediment samples were oven dried for 48 h at 60°C and sieved in order to remove large particles (mesh size, 4 mm). Aliquots of the dried sediments (ca. 50 g; $n = 3$) were then weighed using an analytical balance, transferred into a Carbolite CWF 11/23 furnace, and carbonized at 450°C for 4 h. After the sediments cooled to ambient temperature, samples were reweighed, and the organic-matter content was determined as the ash-free dry weight.

Direct counts. Sediment samples ($n = 4$) were fixed using 4% (wt/vol) paraformaldehyde for 3 h, centrifuged, rinsed in filtered seawater (FS; 0.2-μm-mesh-size filter), centrifuged, and stored in a 1:1 mixture of FS and 100% ethanol at −20°C (5 ml of sediment in 5 ml of FS-ethanol). On the day the analyses were performed, the tubes were vortexed and left untreated for 5 s (settling of the sediment), and then an aliquot of 75 μl of the suspension was sampled. This allowed selection of a single-size fraction of the sediment, namely, a grain size between 10 and 150 μm, as checked by microscopy. The aliquot was then diluted 10 times in FS-ethanol and treated by sonication (three times for 30 s each time using pulse mode) with a sterilized sonic probe (VibraCell 375 ultrasonic processor; output control, 3; 5-mm microtip; duty cycle, 20%) in order to detach bacteria from the particles (22). After sonication, the samples were left untreated for 3 min in order to precipitate large sediment particles that could otherwise darken the microscope field. A volume of 100 μl of sonicated bacteria was then combined with 10 ml of FS and filtered using an Isopore membrane filter (0.2-μm pore size; Millipore catalog no. GTTP02500) placed on a 0.45-μm-pore-size filter (Millipore catalog no. HAWP02500). The filters were stained with DAPI (1 μg ml^{-1}) for 3 min, rinsed briefly with distilled water and 70% ethanol, air dried, and mounted in Vectashield (Vector Laboratories, Burlingame, Calif.). The filters were observed under a Leitz Diaplan microscope fitted for epifluorescence microscopy with a 50-W mercury high-pressure bulb and a filter set for DAPI. A total of 14 pictures (1.536 by 2.048 pixels) were acquired for each filter at ×100 objective magnification with a Peltier-cooled high-resolution charge-coupled device camera (QImaging MicroPublisher; 3.3 megapixels) controlled by QCapture software version 1.1.8 (exposure time, 5 s). The 14 pictures were taken randomly along two transects, at right angles to each other, that crossed in the

TABLE 2. HCl-extractable metals, percent OM, and CI of the Sorfjord sediments[a]

Site	Cd	Cu	Pb	Zn	% OM	CI
S1	3.76 ± 2.22α	43.83 ± 7.43α	259.71 ± 17.45α	333.37 ± 87.1α	3.8 ± 0.1α	73.9 ± 15.2α
S2	0.23 ± 0.02β	20.98 ± 1.11β	158.32 ± 3.56β	124.0 ± 5.89β	1.9 ± 0.01β	31.3 ± 1.1β
S3	0.22 ± 0.02β	3.90 ± 0.13γ	12.18 ± 1.75γ	24.39 ± 5.25γ	1.1 ± 0.03γ	5.4 ± 0.4γ
S4	0.40 ± 0.06β	1.32 ± 0.49γ	19.19 ± 2.14γ	18.72 ± 1.92γ	1.0 ± 0.04γ	4.0 ± 0.5γ

[a] For metals, values are mean concentrations (μg g^{-1} [dry weight] ± SD) ($n = 6$); for OM, values are percentages of dry weight ± SD ($n = 3$). α, β, and γ refer to comparisons between sites; different symbols indicate significant differences (Tukey's test; $\alpha = 0.05$).

center of the filter (the filter edges and the filter center were included in the counts) (2). Each picture was then viewed with the public-domain NIH Image program, version 1.6.3 (U.S. National Institutes of Health; available at http://rsb.info.nih.gov/nih-image/), the image was converted to black and white by using the program's threshold option (the threshold is automatically set based on an analysis of the entire image) and autofluorescing mineral particles were removed. The bacteria were then counted automatically using the Analyze Particles command of the software (minimum particle size, 7 by 7 pixels, i.e., ±0.25 μm in diameter). The bacteria in the 14 pictures (total area observed, 5.39×10^{-8} m^2) were summed, and the number obtained was compared to the effective filtration area (1.77×10^{-4} m^2). Four filters were counted for each site ($n = 4$). The mean number of bacteria counted per filter was 570 ± 66 (mean ± standard deviation [SD]). The total number of bacteria was expressed per gram (dry weight) of sediment (the weight of particles in the aliquot of 75 μl was evaluated by centrifugation and drying of a 750-μl aliquot; the values were then divided by 10).

Cloning and sequencing of 16S rRNA genes. DNA was extracted from 500 mg of sediments by the in situ lysis method described previously (29, 59). Briefly, this method uses lysozyme, proteinase K, sodium dodecyl sulfate (SDS), and heat shock to lyse the cells. Proteins are then removed using phenol-chloroform-isoamyl alcohol, and DNA is precipitated using ethanol. DNA samples were then purified using a DNA cleaning kit (QIAquick; QIAGEN, Hilden, Germany) and eluted in 50 μl of PCR water. Relative amounts of DNA were estimated visually after agarose electrophoresis through comparison with a molecular mass ladder (Gibco-BRL). The DNA yield was estimated as 10 μg of DNA per g of wet sediment. The DNA was diluted 20 times before the PCR.

The complete 16S rRNA gene was amplified using the bacterial primers 8F and 1492R (10). The PCR amplification procedure was performed with an Eppendorf Mastercycler using the PCR kit Red'y'StarMix (Eurogentec). A 50-μl reaction tube of Red'y'StarMix contains HotGoldStar DNA polymerase, 200 μM (each) deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, Tris-HCl (pH 8.0 at 25°C), KCl, and red dye loading buffer. The final concentration of primers was 1 μM. Three microliters of diluted DNA was used in the PCR. The tubes were first incubated for 10 min at 95°C to activate the HotGoldStar DNA polymerase. The PCR was performed by using 30 cycles consisting of denaturation at 94°C for 1 min, annealing at 46°C for 1 min, and primer extension at 72°C for 1 min. Annealing at 46°C (instead of 40.0°C [10]) was chosen to ensure higher specificity. For the last extension step, the tubes were incubated for 10 min at 72°C.

The PCR products were then purified with QIAquick columns and cloned into TOP10 chemically competent *Escherichia coli* cells using the TOPO TA Cloning kit (Invitrogen). Clones containing the complete 16S rRNA gene, as revealed by PCR with primer M13, were selected for plasmid isolation with the QIA prep spin miniprep kit (QIAGEN). Plasmids were partially sequenced with the bacterial primer 518F (10) on an ABI Prism 3100 genetic analyzer.

Phylogenetic analysis. Sequences generated in this study (500 to 700 bp long) were submitted to BLAST (41) to identify the closest relatives and download their 16S rRNA sequences. All sequences were manually aligned and analyzed using SeqPup version 0.6f (28). Distance, parsimony, and maximum-likelihood trees were generated with the Phylip program package, version 3.6 (23). Distance trees were generated with Dnadist using Jukes-Cantor distances and neighbor joining, parsimony trees were generated with Dnapars using unweighted ordinary parsimony, and maximum-likelihood trees were generated with Dnaml (Ti/Tv = 2.0; empirical base frequencies; one category of sites with a constant rate of variation). The statistical significance of the phylogenetic groups within the trees was tested by using bootstrap analysis with the Phylip programs Seqboot and Consense (100 bootstrap replicates). Trees were created using the program Treeview (version 1.6.6).

DGGE. DNA was extracted by the in situ lysis method described above and amplified with the bacterial primers GM5F-GC-clamp and 518R (29, 30). PCR products (ca. 230 bp long) were then quantified on 1.5% agarose gels by densitometry using Quantity One software, version 4.2. (Bio-Rad), and Gel Doc 2000 (Bio-Rad). The quantities of PCR products did not differ significantly among the sites, except for S3, where it was significantly lower. A volume of 40 μl of PCR products was then loaded on two DGGE gels (gel 1, S1-S2; gel 2, S3-S4; $n = 6$). Some samples were loaded on both gels to allow for comparison among the gels. DGGE was performed with a Bio-Rad DCode system as described previously (29, 30). The denaturing gradients contained 25 to 75% denaturants (100% denaturants corresponded to 7 M urea and 40% [vol/vol] formamide). After DGGE, the individual band patterns were compared to each other by using the pairwise similarity coefficient of Dice (S), which was determined as follows: $S = 2j/(a + b)$, where a was the number of DGGE bands in pattern 1, b was the number of DGGE bands in pattern 2, and j was the number of common DGGE bands (30).

FISH. The tested oligonucleotide probes were EUB338 for eubacteria (1), ARC915 for archaebacteria (64), GAM42a for γ-*Proteobacteria* (43), CF319a for the *Cytophaga-Flexibacter-Bacteroides* (CFB) bacteria (42), HGC69a for *Actinobacteria* (57), PLA886 for planctomycetes (46), and DSS658 for δ-*Proteobacteria* of the *Desulfosarcina-Desulfococcus* group (44). NON338 (73) was used as a negative control. Sediments were fixed, and cells were placed on filters as described above. Filter sections were placed into small 0.2-ml tubes (one filter section per tube) with 150 μl of hybridization solution (0.9 M NaCl; 20 mM Tris-HCl, pH 7.5; 0.01% SDS; 750 ng of probe; formamide, 10% for probes EUB338 and NON338, 35% for probes ARC915, GAM42a, CF319a, and HGC69a, and 60% for probe DSS658) (43). The competitor probes cBET42a (43) and cPLA886 (46) were used for hybridization with probes GAM42a and PLA886, respectively. The tubes were incubated for 1.5 h at 46°C in a water bath. The probes were purchased from QIAGEN (high-performance liquid chromatography-purified oligonucleotides labeled with Cy3 at the 5′ end). The filters were then placed in 5 ml of washing solution at 48°C for 20 min. The washing solution consisted of 20 mM Tris-HCl, pH 7.5; 0.01% SDS; NaCl, 450 mM (EUB338 and NON338), 70 mM (ARC915, GAM42a, CF319a, and HGC69a), or 4 mM (DSS658); and EDTA, 5 mM (no EDTA was added for probes EUB338 and NON338) (43). The filter sections were then rinsed briefly with distilled water, air dried, stained with DAPI, placed under an epifluorescence microscope, and photographed as described above (a filter set for Cy3 was added). Image pairs (one with DAPI, one with Cy3), acquired at ×100 objective magnification, were then superimposed using Photoshop version 6.0 software. DAPI- and Cy3-stained cells were then manually enumerated. All the Cy3-stained cells that were counted presented a DAPI signal. Three filters were counted for each site ($n = 3$). The number of DAPI-stained cells that were counted for each probe at each site was 1,385 ± 388 cells (mean ± SD). The signal obtained with probe NON338 at each sampling site was subtracted from all the counts (this signal was 0.5% of the DAPI counts for each station).

Statistical analyses. For HCl-extractable metals, organic matter, direct counts, and FISH data, the four sampling sites were compared by one-way analysis of variance ($\alpha = 0.05$). Significant differences were determined by Tukey's HSD test (Systat version 9.0). The arcsine transformation was used for percentages ($x' = \arcsin \sqrt{x}$). For correlations, the Pearson correlation coefficient was used (Systat version 9.0). The Bartlett chi-square test was used to test the significance of all correlations, and then the Bonferroni-adjusted probabilities associated with each correlation coefficient were determined.

Nucleotide sequence accession numbers. The 61 sequences obtained in this study have been deposited in the GenBank database under accession no. AY665403 to AY665463.

RESULTS

HCl-extractable metals and OM. The metal concentrations in S1 were always significantly higher than those determined in

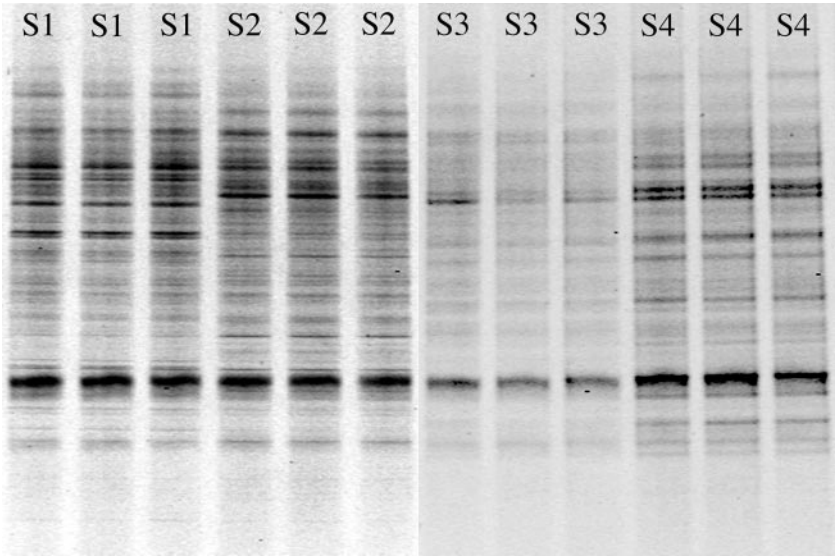

FIG. 2. DGGE banding patterns of the sediment-associated microbial communities of the Sørfjord. Three replicates are shown for each sampling site.

S2 (16-fold for Cd, 2-fold for Cu, 1.6-fold for Pb, and 2.7-fold for Zn) (Table 2). Sediments from S4 were found to be the least contaminated (9.4-fold less than in S1 for Cd, 33-fold for Cu, 13.5-fold for Pb, and 17.8-fold for Zn). Sediments from S3 displayed metal levels very similar to those found in S4. The values of the CI constantly decreased from S1 to S4 (Table 2). Similarly, the OM content of the sediment decreased from S1 to S4 (Table 2). Stations S3 and S4 did not differ significantly for the CI and the percentage of OM.

PCB analyses. PCB levels were determined in the total sediments of the Sørfjord, except for sampling station S3. When considering individual congeners, the PCB concentrations measured in sediments were always <1.57 ng g (dry weight)$^{-1}$. There was no significant difference among the PCB levels in the sediments of the three sampling stations, except for congener no. 52, which was significantly higher in S2 than in S4. When considering the sum of PCBs (Σ_7PCB), no significant difference was found between the levels determined in the sediments of the three stations (S1, 4.75 $\pm$ 1.55 ng g [dry weight]$^{-1}$; S2, 7.01 $\pm$ 1.45 ng g [dry weight]$^{-1}$; S4, 1.21 $\pm$ 0.70 ng g [dry weight]$^{-1}$) (Tukey test; $P < 0.05$).

Direct counts (DAPI). The numbers of cells observed in the selected sediment fractions ($\leq$150 μm) were $4.3 \times 10^8 \pm 0.7 \times 10^8$ (S1), $5.3 \times 10^8 \pm 1.1 \times 10^8$ (S2), $7.6 \times 10^8 \pm 0.9 \times 10^8$ (S3), and $13.4 \times 10^8 \pm 3.6 \times 10^8$ (S4) cells g (dry weight) of sediments^{-1}. Using the direct counts, no significant differences were found among S1, S2, and S3 (Tukey test; $P < 0.05$). The cell density observed in S4 differed significantly from that observed in the sediments of the other sites. As revealed by microscopy, this was not an artifact of differences in the observed size fractions, as no significant differences in granulometry were found: for the four sampling sites, 53.2% of the particles observed in the selected fractions were <10 μm in diameter, 27.5% were 10 to 20 μm, 8.1% were 20 to 30 μm, 3.6% were 30 to 40 μm, 2.1% were 40 to 50 μm, and 1.8% were 50 to 60 μm. For all sampling sites, large grains (60 to 150 μm) represented 3.7% of the particles.

DGGE analysis. A total of 36 band positions were observed after DGGE (Fig. 2). The maximum number of DGGE bands in one sample was 27, and the minimum was 10. Nine DGGE band positions were detected in all of the profiles. Except for S3, some DGGE band positions were unique in each sampling site (bands 5, 11 and 19 for S1; bands 8, 12, and 21 for S2; bands 23, 25, and 36 for S4). The DGGE patterns were compared to one another by using the similarity coefficient of Dice (*S*). The *S* values ranged from 0.432 to 1.000, and the mean *S* value was 0.67 $\pm$ 0.16. The mean intrasite *S* values were high ($>$0.91) and always significantly higher than the mean intersite *S* values (Tukey test; $P < 0.001$) (Table 3). This suggests that DGGE was highly reproducible and that the microbial communities were different in each site.

Phylogenetic analysis. Four 16S rRNA clone libraries were constructed (one for each sampling station), and the clones were partly sequenced with the primer 518F. After sequences that were suspected of being chimeric, diatom sequences, and sequences that were of poor quality were discarded, 61 sequences of ~620 nucleotides remained (19 for S1, 20 for S2, 6 for S3, and 16 for S4). The most abundant sequences belonged to three eubacterial groups: the γ-*Proteobacteria* (17 sequences), the δ-*Proteobacteria* (17 sequences), and the CFB bacteria (18 sequences). Some sequences grouped with the planctomycetes, the *Actinobacteria*, the *Nitrospira* group, and

TABLE 3. Mean similarity values (Dice coefficient) calculated with the DGGE band patterns

Site	Mean similarity value[a]			
	S1	S2	S3	S4
S1	**0.97 ± 0.02**			
S2	0.71 ± 0.05	**0.91 ± 0.06**		
S3	0.47 ± 0.03	0.54 ± 0.04	**0.91 ± 0.06**	
S4	0.59 ± 0.03	0.65 ± 0.04	0.68 ± 0.06	**0.95 ± 0.03**

[a] The boldface values are the mean intrasite similarity values.

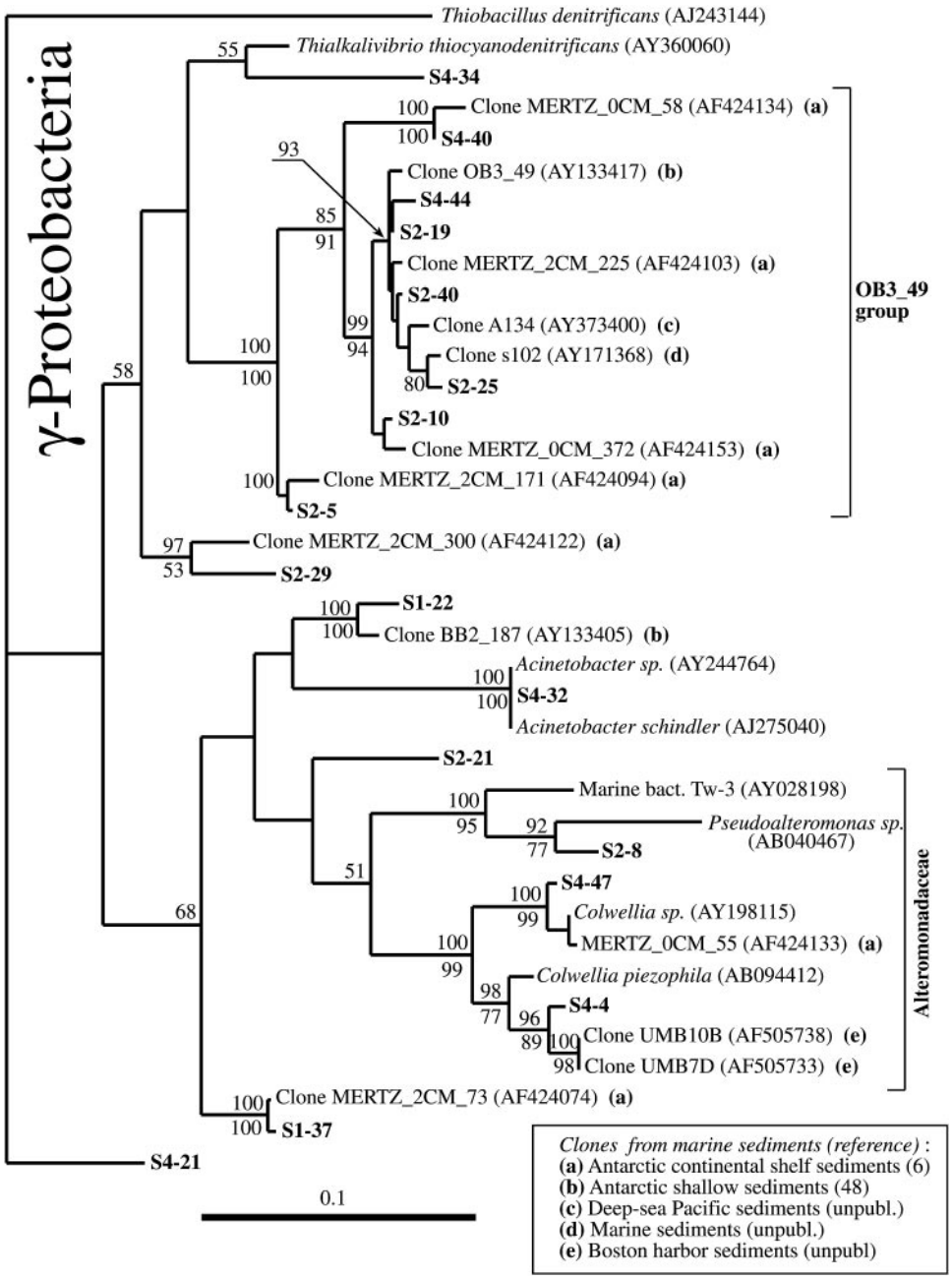

FIG. 3. Maximum-likelihood tree showing the relationships among the 16S rRNA clones of the Sørfjord (in boldface) that belong to the γ-*Proteobacteria*. *Thiobacillus denitrificans* (β-*Proteobacteria*) served as the outgroup. A matrix of 631 nucleotides was used. GenBank accession numbers are listed for the close relatives of the clones. Bootstrap values of >50% (obtained with 100 resamplings) are shown, with upper and lower values representing those from distance and parsimony, respectively. The bar represents the expected numbers of substitutions per 10 nucleotides.

the α and β-*Proteobacteria* (nine sequences). A large proportion of the Sørfjord sequences (65%) were closely related to other clones found in cold marine sediments (shallow, continental-shelf, or deep-sea sediments), such as those from the Antarctic, the Arctic, the Pacific, the Atlantic, and the Black Sea (see Fig. 5 to 8) (6, 36, 37, 48, 49, 54, 70). A few sequences grouped with clones found in hydrothermal sediments (39, 69) or sediments above the gas hydrate (35).

Two main clusters of γ-*Proteobacteria* were detected in this study (Fig. 3). The first cluster (the OB3_49 group) was exclusively composed of clones obtained from cold marine sediments. Some sequences within this group were ≥98.5% similar. For example, S2-19, S2-40, and S4-44 were 98.9 to 99.4% similar, and clone S2-25 and clone S102 were 99.0% similar; these sequences might belong to the same phylotype. The other main cluster of γ-*Proteobacteria* that was detected in the Sørfjord was the *Alteromonadaceae*.

Four main clusters were detected in the δ-*Proteobacteria* (Fig. 4). The first cluster, the large *Desulfosarcina-Desulfococcus* group, included seven Sørfjord clones. All of these clones

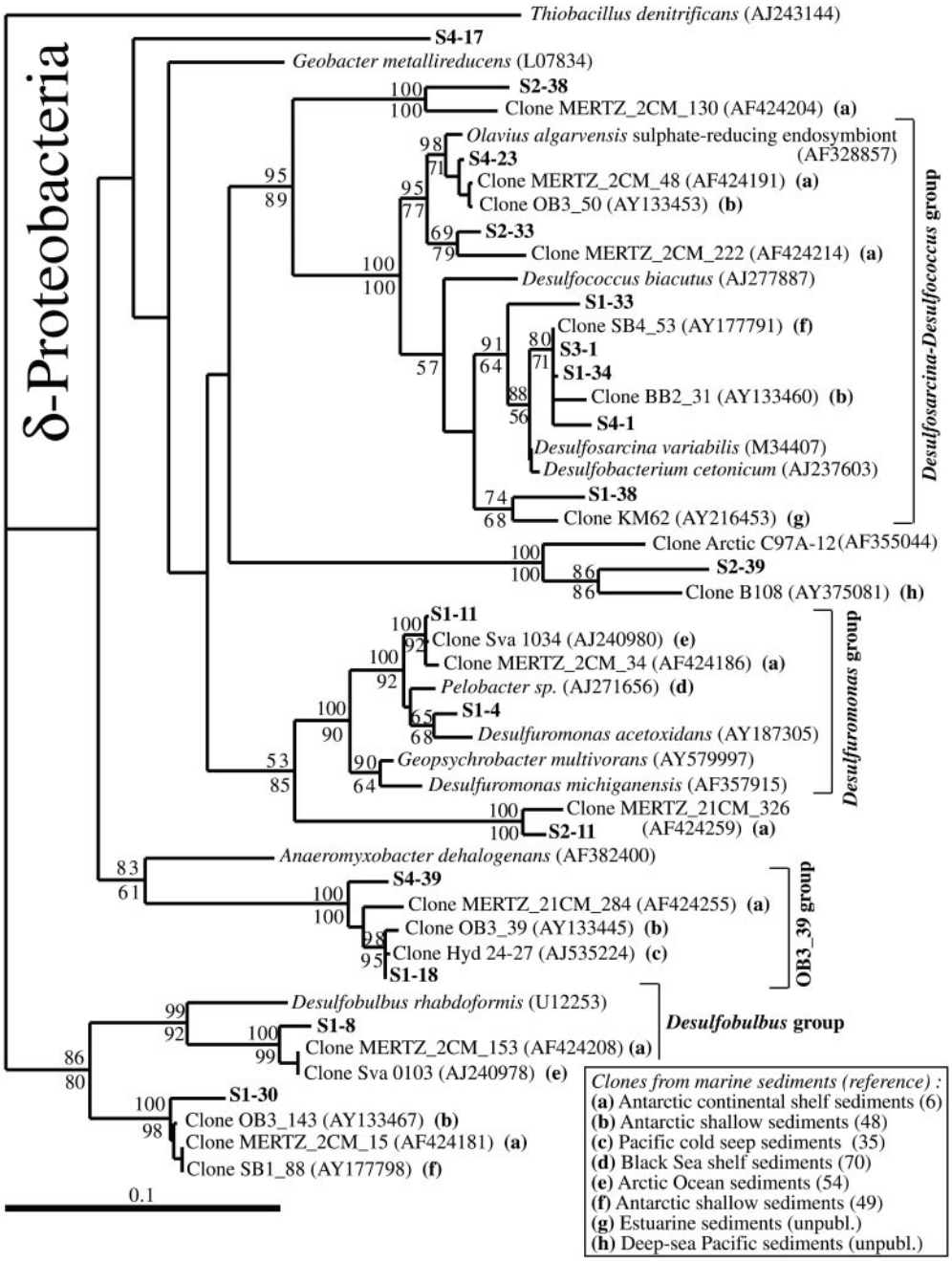

FIG. 4. Maximum-likelihood tree showing the relationships among the 16S rRNA clones of the Sørfjord (in boldface) that belong to the δ-*Proteobacteria*. *Thiobacillus denitrificans* (β-*Proteobacteria*) served as the outgroup. A matrix of 615 nucleotides was used. See the legend to Fig. 3 for other information.

(except S1-38) featured the characteristic DSS658 probe sequence between positions 658 and 685 (*E. coli* numbering) of the 16S rRNA (53). Surprisingly, the most similar clones in that cluster (S1-34 and S3-1; $S = 99.7\%$) were found in two different stations and grouped with clone BB2_31 from heavy-metal-polluted marine sediments (48). The second cluster was the *Desulfuromonas* group (two clones). In that cluster, clone S1-11 was 99.7% similar to clone Sva 1034 from Arctic Ocean sediments (54). The third and fourth clusters were the *Desulfobulbus* group (two clones) and the OB_39 group (two clones). In the latter group, clone S1-18 was 99.8% similar to clone Hyd 24-27 from Pacific sediments above the gas hydrate (35).

The largest cluster of clones in the CFB bacteria of the Sørfjord was the *Cellulophaga-Gelidibacter* group (six clones) (Fig. 5). In that cluster, three clones from three different sampling stations grouped together: S1-21 and S2-12 (99.8% similar) and S4-35 (99.2% similar to S1-21). Interestingly, these clones grouped with clone BB2_71 from heavy-metal-polluted marine sediments (48). Other examples of high similarity be-

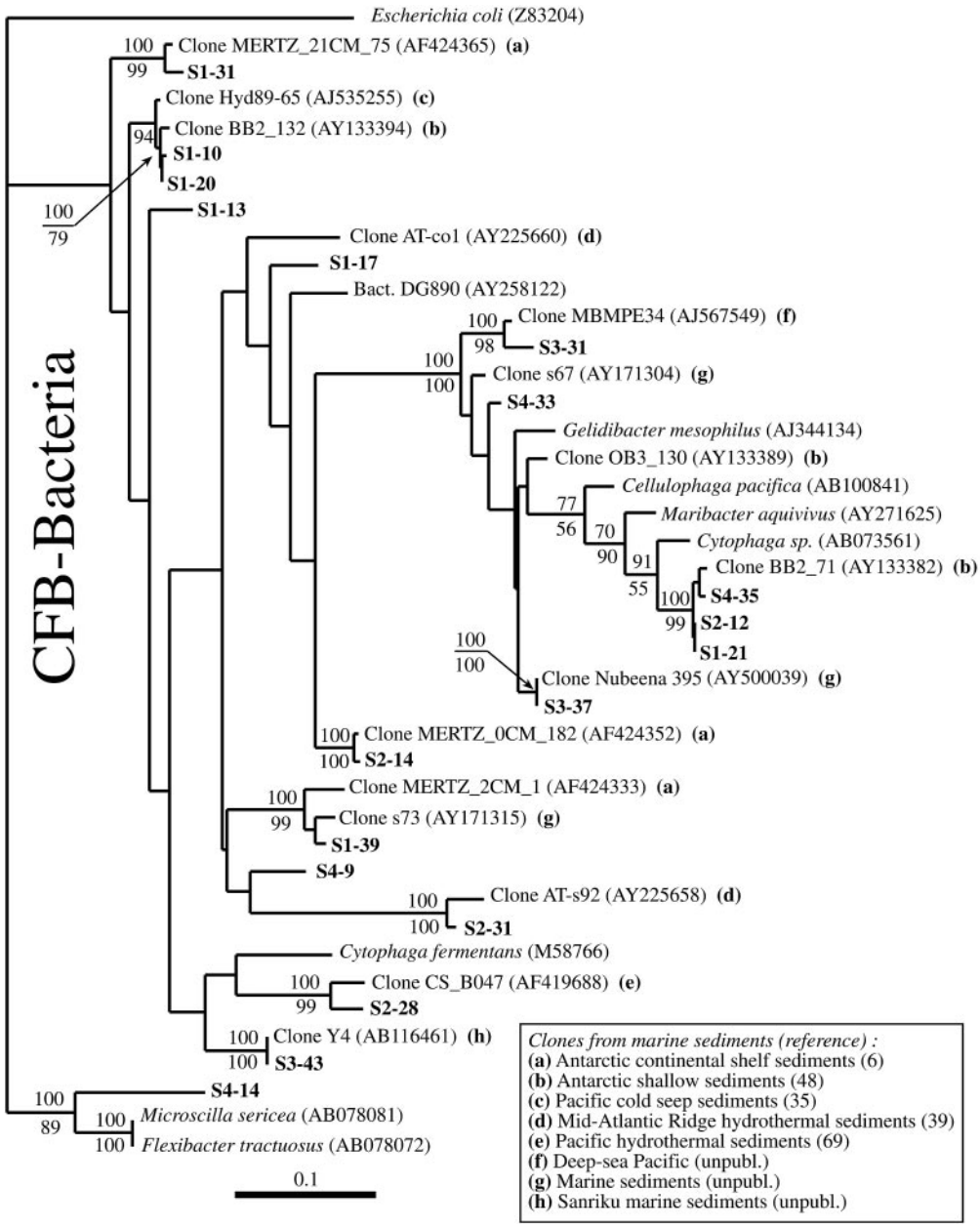

FIG. 5. Maximum-likelihood tree showing the relationships between the 16S rRNA clones of the Sørfjord (in boldface) that belong to the CFB group. *E. coli* (γ-*Proteobacteria*) served as the outgroup. A matrix of 643 nucleotides was used. See the legend to Fig. 3 for other information.

tween Sørfjord clones and CFB clones from heavy-metal-polluted marine sediments are S1-10, S1-20, and clone BB2_132 (48). Clone S3-43 from the Sørfjord and clone Y4 from Sanriku marine sediments off northeast Japan (unpublished results) differed by only 1 nucleotide (over a total of 635 nucleotides).

Nine clones were not affiliated with the previous eubacterial groups (Fig. 6). Among these, clone S4-25 was closely related to the β-proteobacterium *Janthinobacterium lividum* (S = 99.8%). This clone was also related to clone HTC018, which was found in deep-sea sediments off Japan (67).

FISH. The most contaminated station (S1) displayed the lowest percentage of eubacterial cells (4.9% of DAPI counts) in comparison to the other stations, in which values reached 65% of the DAPI counts (Fig. 7). The low percentages of eubacterial cells in S1 might be a counting artifact related to the high proportion of autofluorescing mineral particles in this site. These autofluorescing particles were not removed when the sonication time was reduced. The quantities of autofluorescing particles were very low at the other sites. Archaebacteria were below the detection limit in S1, S2, and S4; in station S3, they represented 1.4% of the DAPI counts. Planctomycetes were below the detection limit in S1; they represented 3.5% of the DAPI counts in S2, 6.0% in S3, and 5.0% in S4 (Fig. 7). None of the DAPI-stained cells were labeled with probes GAM42a, CF319a, DSS658, and HGC69a in station S1 (Fig. 8). For these probes, the FISH signal significantly increased

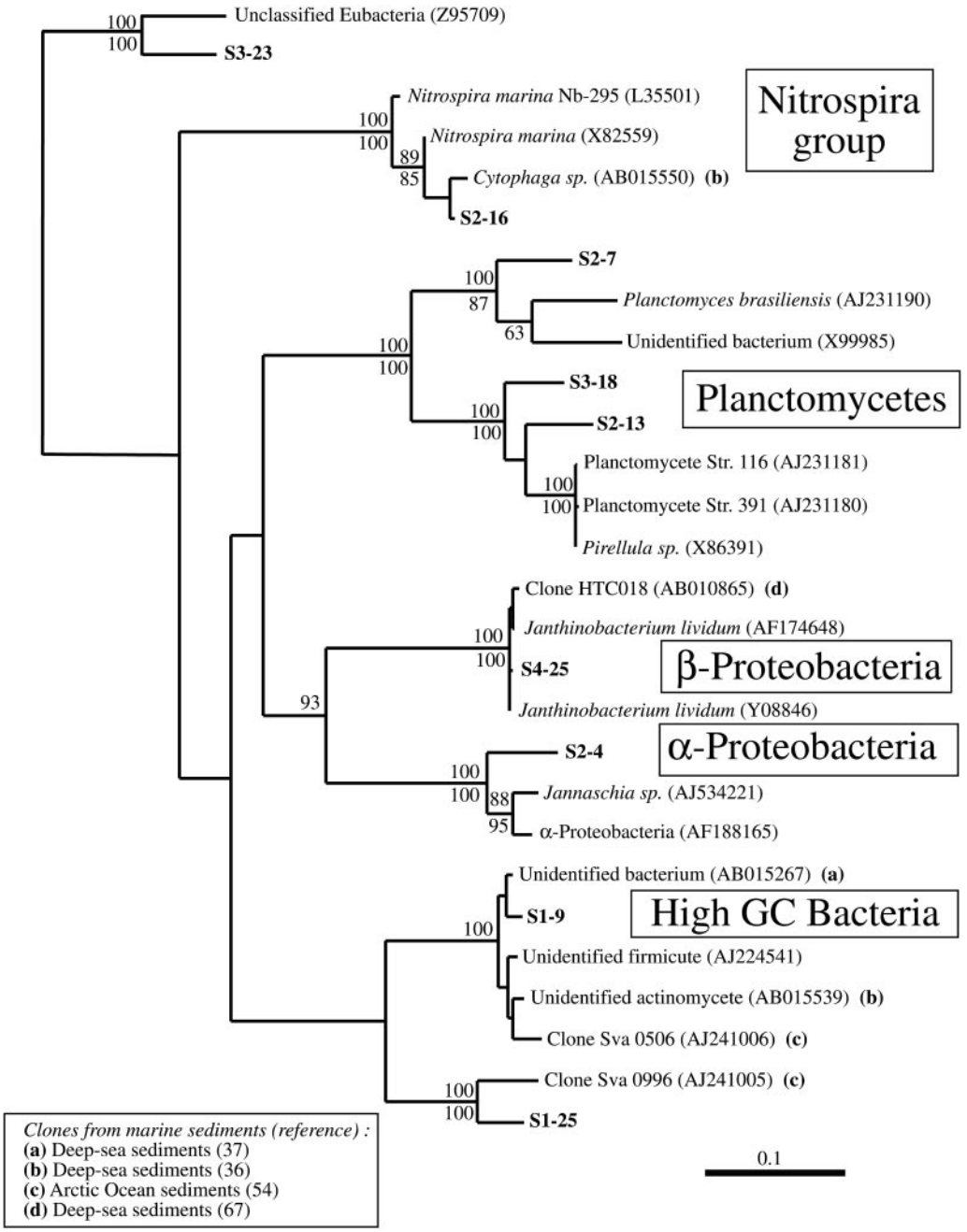

FIG. 6. Maximum-likelihood tree showing the relationships between the 16S rRNA clones of the Sørfjord (in boldface) that belong to the *Nitrospira* group, the α-*Proteobacteria*, the β-*Proteobacteria*, the *Planctomycetales*, and the high-G+C bacteria. A matrix of 635 nucleotides was used. See the legend to Fig. 3 for other explanations.

from 1 to 6% of the DAPI counts in S2 to 8 to 14% of the DAPI counts in S4 (except for probe HGC69a, where the increase was not significant) (Fig. 8).

Data correlations. All HCl-extractable metals were positively correlated with organic-matter levels (Table 4). Two bacterial groups were significantly and negatively correlated to extractable Cu and Zn: the γ-*Proteobacteria* (probe GAM42a) and the CFB bacteria (probe CF319a) (FISH data obtained in S1 were not used for the correlations). The CFB bacteria were also negatively correlated with extractable Pb. No significant correlations were found for Cd or between PCB levels and HCl-extractable metals (data not shown).

DISCUSSION

Metal analyses performed in this work demonstrated that a clear decreasing gradient of metals is present in sediments along the Sørfjord. Estimation of bioavailability using HCl-extractable metals is important, because the majority of metals in sediments are included in various mineral phases or che-

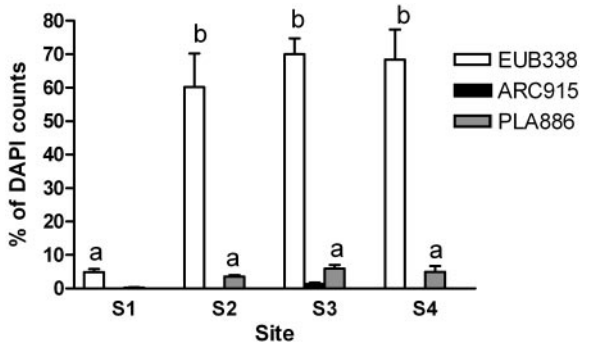

FIG. 7. Community structure of the Sørfjord sediment-associated microbial communities as determined by FISH with rRNA-targeted oligonucleotide probes. Data are given as percentages of DAPI-stained cells (mean plus SD; $n = 3$). Probes used: EUB338 for eubacteria; ARC915 for archaebacteria; PLA886 for planctomycetes. Histogram bars sharing the same superscript did not differ significantly (Tukey's HSD test; $\alpha = 0.05$).

lated to organic matter and are consequently less bioavailable (12, 66). For instance, a previous study of marine sediments estimated that only 0 to 3.7% of the total heavy metals were bioavailable (47). Results obtained here show that microbial communities of the Sørfjord have to cope with high metal concentrations in their surrounding environment. Few studies have used the 0.5 M HCl extraction technique in marine sediments, making direct comparisons uncertain. It was estimated, however, using the same HCl extraction method, that the quantity of extractable Cu was 0.15 $\mu g\ g^{-1}$ in a North Sea coastal area showing background metal concentrations (29). Values in S1 sediments were ~292-fold higher.

The amounts of PCBs in the sediments of the Sørfjord were comparable to and even lower than the background values observed in the coastal zones of the southern North Sea (14, 17). In addition, as no differences were observed between stations (except between stations S2 and S4 for PCB no. 52), and as no correlations were found between PCB levels and microbiological data, we can suggest that PCBs have limited effects

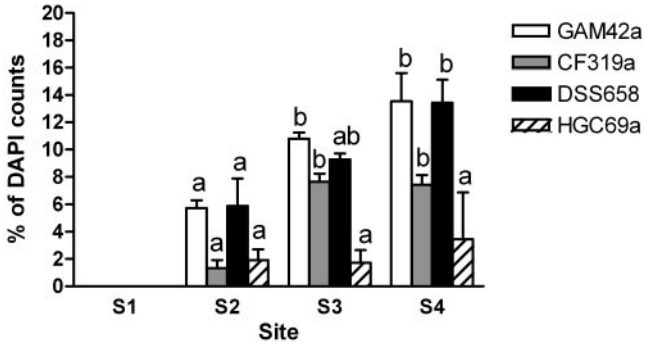

FIG. 8. Community structure of the Sørfjord sediment-associated microbial communities as determined by FISH with rRNA-targeted oligonucleotide probes. Data are given as percentages of DAPI-stained cells (mean plus SD; $n = 3$). Probes used: GAM42a for γ-*Proteobacteria*; CF319a for CFB bacteria; DSS658 for δ-*Proteobacteria* (*Desulfosarcina-Desulfococcus* group); HGC69a for high-G+C bacteria. Histogram bars sharing the same superscript did not differ significantly (Tukey's HSD test; $\alpha = 0.05$).

TABLE 4. Correlation coefficients between HCl-extractable metals and microbial characteristics

Characteristic	Correlation[a]			
	Cd	Cu	Pb	Zn
DAPI counts	−0.432	−0.723	−0.706	−0.642
% OM	**0.785**	**0.985**	**0.968**	**0.977**
GAM42a	0.732	**−0.871**	−0.838	**−0.872**
CF319a	0.380	**−0.950**	**−0.957**	**−0.939**
DSS658	0.639	−0.752	−0.680	−0.690

[a] S1 values are not included for GAM42a, CF319a, and DSS658 data. Significant correlations are in boldface ($P < 0.05$).

on the structure of the microbial communities in the Sørfjord. Surprisingly, higher PCB concentrations (2.3-fold) were reported in S1 during 1992 (61). Although PCBs are relatively inert, it is possible that some PCBs were degraded by microorganisms. Several bacteria capable of PCB degradation have been described in the literature (8). However, if this were true, the PCB component in the sediments would have been depleted of readily degradable congeners (such as PCB no. 28 and PCB no. 52) in comparison to the 1992 data (61). This was not the case, as the concentrations were equally reduced for all congeners. Other factors, such as reduced pollution since 1992 and/or analytical differences, can explain the discrepancies found.

As shown by DGGE analysis, the bacterial diversities in S1 and S2 were comparable to the bacterial diversity of the uncontaminated site, S4. It may be suggested that the diversity of microbial communities in the Sørfjord were initially affected by the increasing levels of heavy metals, as was found in other microbial communities (5, 51, 62), but that after 80 years of heavy-metal contamination, the microbial communities have become as diverse as those living in uncontaminated places, such as S4. Previous workers, addressing Hg contamination of soils, suggested that recovery of lost genetic diversity is fast and begins immediately after contamination (51). As suggested here by DGGE analysis, each sampling station now has its own type of microbial community. Apparently, the recovery of the genetic diversity in S1 and S2 was not due to a reversion to the preexposure community but mainly to the appearance of new dominant species. Microbial diversity is thus a bad indicator of long-term heavy-metal pollution. Similar findings have been described in a heavy-metal-contaminated soil with a long history of contamination (21).

Good correlations between metals and organic matter were observed in the Sørfjord. Although such correlations are not proof of the complexation of the metals by the organic matter, it has been observed many times in various aquatic environments (11). The high levels of organic matter in S1 may be explained by high inputs due to the proximity of the city of Odda or to a high sedimentation rate. It might also be explained by an inhibition of the activity of microorganisms due to the toxic effects of heavy metals, as reported in other environments (3, 31). However, as biomass (i.e., DAPI counts) is not reduced in S1, it is more probable that S1 microorganisms are well adapted to grow in this contaminated environment. This is supported by other studies, which have shown that metal stress does not always reduce productivity (60, 75) or

biomass (4, 24, 34, 60). We may provisionally conclude, until suitable productivity measurements are done, that cell density, and possibly productivity, is able to recover in marine sediments contaminated by heavy metals. As for diversity, these types of measurements cannot be considered suitable indicators of heavy-metal pollution for marine environments having a long history of contamination.

Three groups of bacteria were dominant in the clone libraries: the γ- and δ-*Proteobacteria* and the CFB bacteria. This is in agreement with previous results for continental-shelf sediments (7, 38, 54). Interestingly, some Sørfjord clones grouped with marine bacteria found in polluted nearshore Antarctic sediments featuring elevated levels of Cu, Pb, Zn, and hydrocarbons (48). These Sørfjord clones formed two "bushes" of closely related sequences in the *Desulfosarcina-Desulfococcus* group of the δ-*Proteobacteria* (S1-34, S3-1, and S4-1) and in the CFB bacteria (S1-21, S2-12, and S4-35) (Fig. 6 and 7). As bushes of closely related clones can result from PCR and cloning artifacts (63), it is possible that they represent two very common phylotypes of bacteria all along the Sørfjord. δ-*Proteobacteria* of the *Desulfosarcina-Desulfococcus* group are a very diverse group of sulfate-reducing bacteria able to degrade a variety of complex organic matter. This group was important in other marine environments as well (7, 53, 58). Some strains in this group have been isolated from contaminated sites, such as oil reservoirs, PCB-dechlorinating cultures, and petroleum-contaminated estuarine sediments (53). The association of some Sørfjord clones with clones from other polluted sediments suggests that some bacterial species in the Sørfjord are related to the metal pollution. If this is true, these bacterial species can be more abundant in S1 and S2 than in S3 and S4. However, the FISH signals obtained in this study were all negatively correlated to the metals. This discrepancy may nevertheless be explained if the positively correlated species are not dominant (the signal of the few positively correlated species might have been diluted by the signal of the species that were not correlated to metals). The abundance of these positively correlated species can only be determined by the design and use of specific oligonucleotide probes.

Although the biomasses were similar in S1 and S2, the quantities of eubacteria determined by FISH with probe EUB338 were completely different (5% of the DAPI counts in S1 and 60 to 70% of the DAPI counts in S2 to S4). Other microbial groups, such as archaebacteria and *Planctomycetales* (which are not targeted by probe EUB338) (16), were not detected in S1. The *Verrucomicrobiales* probe (16) was not used here because these bacteria were not detected by cloning. As suggested in Results, low EUB338 counts in S1 are probably a counting artifact due to high numbers of autofluorescing mineral particles. These particles might have originated from the mining industry at this site. Although low EUB338 counts in S1 may also result from a low ribosomal content due to metal toxicity (33), that seems not to be the case, as DGGE patterns were as complex as in S2, the biomass was not affected, and many 16S rRNA sequences were obtained by cloning.

The percentages of eubacteria (EUB338 signal) were not significantly different in stations S2, S3, and S4 and were comparable to those in other studies (38, 52). It may be calculated that 17.2 to 41.6% of the eubacteria were not affiliated with known eubacterial groups. The "black box" of unidentified eubacteria in the Sørfjord was comparable to that obtained in other studies of marine sediments (38, 52). The black box would probably not be composed of β-*Proteobacteria*, α-*Proteobacteria*, or *Nitrospira*-like bacteria, because only one sequence of these organisms was retrieved by cloning. Further FISH studies are needed to confirm this hypothesis. Other eubacterial groups frequently retrieved from marine sediments, such as *Arcobacter* spp. and other ε-*Proteobacteria* (38, 70), were not detected in this study. The black box of unidentified eubacteria may temporarily be explained by the limited coverage of the present probe set (52).

FISH analysis revealed a clearly increasing abundance of γ-*Proteobacteria*, δ-*Proteobacteria*, and CFB bacteria from station S2 to station S4. These microbiological gradients might be the result of the metal gradient or of other environmental gradients. Metal effects are supported by highly significant correlation values (only for γ-*Proteobacteria* and CFB bacteria). A previous report from the freshwater environment suggested that γ-*Proteobacteria* were positively correlated to total metal levels (24). However, in the present study, γ-*Proteobacteria* and CFB bacteria were negatively correlated to HCl-extractable metals (except Cd). This discrepancy can be explained by differences in the microbial composition between freshwater and marine proteobacterial communities. Nevertheless, two observations suggested that species positively correlated to metals might be present in the Sørfjord: (i) as discussed above, some sequences grouped with clones from other heavy-metal-polluted sediments; (ii) some DGGE bands were detected in S1-S2 and not in S3-S4.

In addition to metals, other environmental gradients may explain the distribution of the eubacterial groups revealed by FISH. It was shown, for example, that the abundance of viruses (74), the protistan grazing pressure (72), and nematode grazing (18) may all influence the structure of microbial communities. As a counterpoint to these arguments, it can be said that protists and nematodes are probably not able to differentiate between bacterial groups such as γ- and δ-*Proteobacteria*. Consequently, an increased grazing pressure would lead to differences in DAPI counts, a situation that is not observed in S1 to S3. Since viruses are very abundant in aquatic systems (74), and stations S1 to S3 are located in the same fjord, significant differences in the viral pressure between these stations would probably not be observed. Physicochemical factors (other than metals) may also explain the distribution of microorganisms. For instance, differences in the granulometries of the sediments may lead to differences in oxygen penetration and consequently to differences in microbial communities. Nutrients and the quality of organic matter may also influence microbial communities (31). In addition to metals and PCBs, other contaminants might also be considered (polyaromatic hydrocarbons, dioxins, furans, etc.). Many environmental variables are thus implicated in the Sørfjord, and it is difficult to take all these variables into account. This situation is inherent in all field studies. Microcosm studies, in which only one variable is changed, are thus absolutely required to complement the field studies (29).

Although FISH was unsuccessful in S1, it was shown in this work that microorganisms at this site have to cope with metal concentrations that are much more elevated than those observed at S4 or in other polluted marine environments (14, 48). It was

also shown that microorganisms in sediments of the most contaminated sites are as abundant and diverse as microorganisms in uncontaminated sediments. In addition, care should be taken when the structures of microbial populations in marine sediments are related to metal gradients. Even when metal gradients are extreme, many other environmental factors may still be implicated. The Sørfjord might be a good example of a long-term adaptation of microbial communities to high concentrations of heavy metals in the marine environment, but many questions remain to be answered. Future studies will focus on the design and use of species-specific oligonucleotide probes, on metal tolerance, on productivity measurements, and on the use of biosensors to evaluate the in situ bioavailability of metals.

ACKNOWLEDGMENTS

D.C.G. and P.D. are, respectively, Senior Research Assistant and Research Associate of the Belgian National Fund for Scientific Research (NSFR). B.D. was the recipient of a FRIA doctoral grant. The research was supported by a Belgian federal research program (SSTC, contract EV/11/23A) and by contributions from the Centre Interuniversitaire de Biologie Marine (CIBIM) and from the National Bank of Belgium (BNB).

Many thanks are due to the reviewers, who clearly improved the manuscript.

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