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Microbiological observations in the anoxic basin Golfo Dulce, Costa Rica

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Abstract: Our basic microbiological studies of the water column and the sediment of Golfo Dulce, Costa Rica, were focused on aerobic and denitrifying sulfur-oxidizing bacteria and anaerobic sulfate-reducing bacteria. We observed no increasing numbers of total bacterial counts within the water column. Although no oxygen was present hydrogen sulfide was only detectable close to the sediment. The highest numbers of sulfate-reducing bacteria measured by Most-Probable-Number counts were found in or close to the sediment. In the anoxic bottom water sulfide-oxidizing bacteria typically containing large sulfur globules were observed microscopically. They were identified as free-swimming *Thiovulum* and *Thiospira* species. At one station large vacuolated forms of the filamentous colourless sulfur bacterium *Beggiatoa* were noted. Together with these sulfur containing bacteria there were long free swimming rods showing no sulfur inclusions of unknown character. The microscopic observations showed good correlation with Most-Probable-Number-counts and molecular biological techniques for sulfate-reducing bacteria.

Key words: Microbial diversity, 16S rRNA probes, *Thiovulum, Thiospira, Beggiatoa,* sulfate reduction, sulfide oxidation.

Golfo Dulce is a small, tropical bay on the Pacific coast of Costa Rica. In March 1969, the water column was studied by Richards and coworkers (1971). They found oxygen concentrations steeply declining across the pycnocline to less than 1 µg-atom kg^{-1} below 140 meters followed by decreasing nitrate concentrations.

On our cruise in January 1994, we expected similar conditions. How the situation looked like is described in an accompanying paper (Thamdrup *et al.*, 1996). Our main interest was to study the distribution of sulfate-reducing and sulfur-oxidizing bacteria in the stratified water column compared to the sediment. We used traditional microbiological techniques like phase microscopy of fresh and fixed samples and Most Probable Number counting in specific media suitable for the different bacteria and modern molecular biological approaches. The later used specific probes for sulfate-*reducing bacteria* and a technique known as DGGE (Denaturing Gradient Gel Electrophoresis) for describing the microbial diversity and the characterization of their members. From enrichment cultures grown on typical substrates we isolated some representatives of the microbial world of Golfo Dulce using agar dilution series with medium containing the same substrate.

Together with physical and chemical data (see Thamdrup *et al.*, 1996) these data should give a picture of what is going on in the water column of Golfo Dulce.

MATERIAL AND METHODS

Sampling of bacteria: We concentrated our efforts on station GD1 (description of the station is listed in Thamdrup *et al.*, 1996) as the station resembled the specific conditions of Golfo Dulce best. Water samples were obtained with a Niskin bottle at 20 m intervals and concentrated by filtering 500 ml of seawater through a 0.22 µm-pore-size filter (Durapore, Millipore, 25 mm in diameter) using a vacuum

pump. For molecular biological studies filters were directly frozen in liquid nitrogen and stored at -20 °C until processed. Samples for microscopic studies were prepared by rinsing the filter with 500 µl sterile seawater (concentration 1000x). These samples were directly used for microscopy or conserved by addition of formaldehyde before further use. For MPN and enrichment cultures water samples were directly taken from the Niskin bottles without further concentration by filtration. Because of the limited amount of tubes we used only samples from the surface, 100 m depth, 200 m depth, the sediment surface layer and the upper 6 cm of the sediment for these studies.

Sediment samples were taken from sliced sub cores mixed and stored as described above. For microscopic observation and MPN-enumeration sliced sediment was diluted in sterilised seawater or medium and directly used fur further work.

Cultivation of bacteria: The reduced and bicarbonate-buffered marine medium for isolation and MPN counts of sulfate-reducing bacteria was prepared according to Widdel and Bak (1992). The substrates used were acetate (20 mM) and lactate (20 mM). The medium with hydrogen was prepared in 20 ml tubes with a 10 ml gas phase containing a mixture of 90% hydrogen and 10% carbon dioxide (vol./vol.).

For isolation and counting of marine sulfur-oxidizing bacteria we used the medium described by Jannasch *et al.* (1991). The pH was adjusted to 7.5 and 20 mM sodium thiosulfate were added as electron donor. The incubation were done in 20 ml tubes containing a 10 ml gas phase. For counting of denitrifying sulfur-oxidizing bacteria 5 mM KNO_3 were added and the gas phase was exchanged against nitrogen gas.

All cultures were incubated at room temperature on the ship and later at 30°C in the laboratory.

Nucleic acid extraction: Nucleic acids were extracted by the method of Oelmüller *et al.* (1990), adapted as follows: The filters or 0.5 g of the sediment samples were thawed and washed twice with 1 ml cold AE-buffer and treated with 6 ml of hot (60 °C) phenol-chloroform-isoamylalcohol (25:24:1) and 60 µl 25% SDS. After 5 minutes of incubation at 60 °C the mixture was cooled on ice and centrifuged at 5000 rpm in an Eppendorf Centrifuge 5416. After addition of 250 µl 2 M sodium acetate (pH 5.2) the aqueous phase was repeatedly extracted with phenol-chloroform-isoamylalcohol, until no protein could be detected in the interphase. Nucleic acids were precipitated with 2.5 volumes of 96% ethanol for 3 hours at -20°C and pelleted for 1 hour at 5000 rpm in an Eppendorf centrifuge. The pellet was washed with 75% ethanol, dried under vacuum and resuspended in 50 µl water. These nucleic acid extractions were used for amplification of 16 S ribosomal DNA-fragments. Nucleic acid preparations were tested in 2% agarose gels using MOPS-buffer as described by Sambrook *et al.* (1989).

PCR amplification: A 550 bp region of the eubacterial 16 S ribosomal RNA gene was amplified for DGGE analysis using primers GM5-GC-clamp and DS907 (Muyzer *et al.*, 1995). 100 µl-reactions were performed using 2 µl of the nucleic acid preparations as starting material. PCR products were tested in a 2% agarose gel using TBE-buffer stained with Ethidium bromide.

DGGE: The PCR products were analyzed by denaturing gradient gel electrophoresis (DGGE) as described by Muyzer *et al.* (1993). PCR products were prepared as follows: 300 µl of PCR product was precipitated with 0.1 volume of 3 M sodium acetate (pH 5.5) and 2.5 volume ethanol for 10 minutes at room temperature. After centrifugation the pellet was washed with 70% ethanol, dried under vacuum and dissolved in 25 µl water. A denaturing gradient gel, ranging from 20 to 70% denaturants, was cast and run for 4 hours at 60 °C. After ethidium bromide staining and visualization of the band pattern under UV-light, the DNA was electroblotted onto a nylon membrane for one hour at 400 mA using a semidry-transfer cell from Biorad.

Hybridization: The 16 S rDNA region amplified by primers GM5 and DS907 contains several target sites for general and genus specific oligonucleotide probes for sulfate reducing bacteria. Hybidization was carried out with DIG-labelled probe 385 (Amman *et al.*, 1990),

specific for sulfate-reducing bacteria and other members of the *delta* proteobacteria and probe 804 (Devereux *et al.*, 1992), specific for the acetate-oxidizing genera *Desulfobacter* and *Desulfobacterium* and the species *Desulfosarcina variabilis*, *Desulfococcus multivorans* and *Desulfobotulus sapovorans*, as described by Teske *et al.* (1995).

RESULTS

Microscopic observations

Phytoplankton: In several samples of the water column *Nitzschia* -like organisms were present. Living phytoplankton is abundant at the surface and downwards to a depth of at least 40 m, although the numbers are low compared to the surface. In samples below 40 m phytoplankton is scarcely observed and resembles in most cases dead algae cells. Residues of *Chaetoceros* sp. could be found in the water column, sometimes attached to aggregates of unknown character and in higher numbers at the bottom close to or in the sediment.

Plant detritus and amorphous particles: Plant detritus with conserved plant cell structures of terrestrial origin (mangroves and coconuts) constituted a substantial fraction of the particulate organic matter in the upper part of the sediment. In some cases plant detritus is covered by bacteria and often stained black due to FeS. Plant detritus is rare in the water column.

Reddish-brownish amorphous particles (10-50 µm), often assembled in larger aggregates, dominated the zone from 80 to 120 m. The amorphous material might consist of Fe(III)-oxides or other minerals together with condensed organic material.

Zooplankton: Zooplankton has not been encountered often enough for making a rigid statement apart from the fact that at all depth accounted only for a minor fraction of the organisms observed.

Bacteria: In the upper samples, reaching from 1 m to 40 m depth, of station GD1 and GD2 several bacteria, many of them motile, could be observed. Below 40 m depth the number of bacteria dropped dramatically and at 100 m only very few fairly small bacteria were found. At 140 m, 160 m, 180 m and 200 m the bacterial population was clearly different from the population encountered above these depths. The predominant microorganism was a thin filament of up to 20 µm length and a diameter of 1 µm. It was motile but sometimes it showed only a bending movement. Near the bottom at 200 m the bacterial numbers were raising and many vibrioid and ovoid morphologies were found.

In the bottom water directly above the sediment the number of bacterial cells was higher than in samples from the water column above. Here a distinctive qualitative shift in the bacterial population was found, marked by the presence of several morphological types of bacteria with bright granules. The bright granules resembled elemental sulfur and were also observed outside of cells, probably released by lysis of cells due to the rough treatment of the water filtration. Most spectacular were large spirilloids (20-30 µm) motile by flagella or flagella tuft. The sulfur granules were located in a chain and consisted of up to five granules. These bacteria were identified as *Aquaspirillum bipunctata* or a close relative to this species (Dubinina *et al.*, 1993). In older literature the name *Thiospira* was used. The second interes-ting organism was coccoid to ovoid with a diameter of up to 20 µm. It was extremely motile. The sulfur was deposited in one part of the cell. All these features were identical with *Thiovulum majus* (Hinze 1913, Wirsen and Jannasch 1978, Garcia-Pichel 1989, La Reviere and Schmidt 1992, Fenchel 1994).

At station GD160 at a depth of 160 m the situation was different to all other stations observed. Here several white tufts consisting of single filaments were found directly attached to the sediment. They could be seen without magnification and reached sometimes a length of 1.5 cm. Under the microscope we found varying diameters of filaments containing vacuoles and sulfur globules (see figure 2a and 2b). The diameter of the filaments reached from 10 µm for the small ones to nearly 80 µm for the large ones. They all showed large central vacuoles and moved by gliding. These filaments were identified as *Beggiatoa* sp. (Nelson *et al.* 1989, Larkin and Henk 1989).

Isolation of bacteria and MPN counting: Vibrio formed sulfate-reducing bacteria resembling *Desulfovibrio* sp. were isolated with lactate or hydrogen as only substrates, whereas acetate enriched for *Desulfobacter*-like organisms. The production of sulfide was determined as CuS, qualitatively (Cord-Ruwisch 1985). It was very interesting that some of the bottles with sulfate-reducing bacteria turned brown or green after they were exposed to light indicating the presence of green and brown *Chlorobium* sp. Even samples from 200 m showed this phenomenon. Although no light is penetrating to that depth, these bacteria seemed to survive these conditions.

We were able to isolate some aerobic sulfur-oxidizing bacteria showing a rod- or vibrio-like morphology. All these isolates were able to grow under autotrophic conditions utilizing sulfide, sulfur, thiosulfate and tetrathionate as electron donor. No growth occurred under anaerobic conditions with nitrate as electron acceptor. These isolates might resemble *Thiobacilli* and *Thiomicrospira* sp.

The MPN results for sulfate-reducing bacteria are summarized in figure 1. The counts for sulfur-oxidizing bacteria were not conclusive. They varied between 10^2 to 10^5 bacteria per ml Golfo Dulce water showing no significant trend. The numbers for denitrifying bacteria were low and did not exceed 10^2.

It is important to note that attempts to enrich or cultivate the sulfur-containing bacteria mentioned above were not successful. They only survived for a week at a temperature of 15°C after arrival in the laboratory. In a sediment-water sample from station GD1 a white layer close to the water surface developed. This layer showed several thin filament- and spirilloid-like structures containing bright coloured inclusions and forming a matlike structure. These inclusions represented not the sulfur granules observed in the micro-organism mentioned before. The microscopic picture of these matlike structure fits to the description of *Thiodendron*, as it is given by Dubinina and coworkers (1993a 1993b).

Molecular biological methods

Nucleic acid extraction: 1/10 of the nucleic acid preparations from the different water layers of GD1 were analyzed in an agarose gel. RNA was not detectable in all samples and genomic DNA was only visible (ca. 50 ng) in the samples from the surface and 20 m depth.

PCR amplification: Nevertheless, PCR amplification of the eubacterial 16 S rDNA fragments was successful indicating that eubacterial 16 S rDNA is present in all samples tested.

DGGE and hybridization analysis: Further separation of the PCR products using DGGE showed a pattern derived from the bacterial population representing the species and strain diversity in the water column. Different bands in the pattern correspond to different PCR-amplified 16 S rDNA fragments, obtained from the different bacterial species or strains (see figure 2a). Hybridization of the DGGE pattern with probe 385 gives positive results at all depth with stronger signals in 60 m and 80 m depth (pycnocline) and at the sediment surface (see figure 2b). In contrast, hybridization with the more specific probe 804 (mainly complete oxidizing sulfate-reducing bacteria) gives no positive signal in samples from the water column and only an extremely faint signal in the sediment sample (see figure 2c). Controls done with *Desulfobacterium vacuolatum* which should react with both probes were positive (see figure 2b and 2c).

DISCUSSION

Water column: Our preliminary study showed that there are two areas of high microbial activity present in the water column of the bay of Golfo Dulce. The first was the upper layer reaching from the surface to 40 m containing high numbers of bacteria and planktonic organisms. This area represented the photic zone. It is characterized by a high oxygen concentration which will decrease rapidly below this area (see Thamdrup *et al.* 1996).

The presence of anaerobic sulfate-reducing bacteria indicated by figure 1 and the positive hybidization signals obtained with probe 385 in the upper layer can be explained by micro niches occupied by these bacteria or the ability to tolerate temporarily oxygen. On the other hand probe 385 is not specific for sulfate-reducing bacteria alone. It will also react with other members of the *delta*-subdivision of the pro-

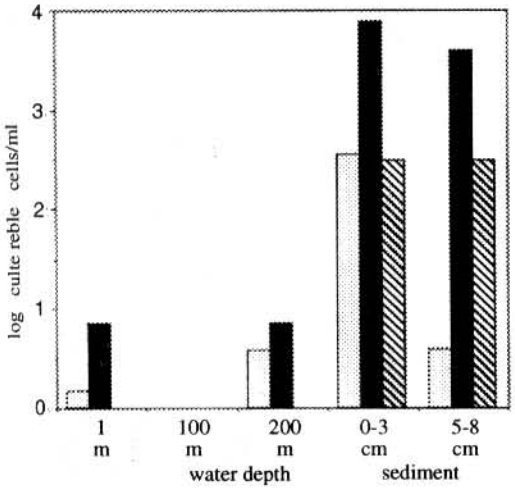


Fig. 1. Distribution of sulfate-reducing bacteria estimated by Most-Probable -Number counts in the water column and the sediment of Golfo Dulce, Costa Rica. (Substrates used were: column 1: lactate; column 2: hydrogen, column 3: acetate).

teobacteria as well as with the aerobic marine nitrifying bacterium *Nitrospira gracilis.*

The second area of high microbial activity was the bottom water and the sediment. The high numbers of sulfate-reducing bacteria as shown in figure 1 indicates that sulfate reduction must be an important process in the sediment resulting in a release of hydrogen sulfide or other reduced sulfur species. In the water column there was no positive signal with the more specific probe 804 for complete-oxidizing sulfate-reducing bacteria at all, meaning they are not dominant in the water column. This is interesting because a major fraction of the water column showed no oxygen. It might be that these bacteria are only present in the sediment and found in general in low numbers. The difficult interpretation of the results with probe 385 has already been addressed above.

That sulfide must be important as an electron donor in the bottom water was indicated by the presence of different types of colorless sulfur bacteria. These bacteria are known to oxidize sulfide to elemental sulfur, which is stored as sulfur granules inside the cells. In general they are characterized as autotrophic, often catalase negative and obligate aerobic or microaerophilic. Many of them are not in pure culture and their physiology is not completely clear. Several organisms containing sulfur granules were found in the bottom water of station GD1 and others. The situation in the bottom water perfectly fits the conditions needed by these organism. A low oxygen concentration will meet a low sulfide concentration. The finding of sulfur bacteria depending on oxygen and probably nitrate as electron acceptor stands in good agreement with the model from Richards and coworkers (1971). As suggested by them oxygen and nitrate are probably continuously replenished by a temporary input of more dense sea water entering the bay over the sill. This denser water flowed into the bay, sinking as it moved. Because sulfide as the final product of the decomposition of organic material by sulfate-reducing bacteria was only found in the sediment or close to the bottom (Richards *et al.* 1971; Thamdrup *et al.* 1996) these sulfur-globules containing bacteria are limited to this zone.

A typical example is represented by *Thiovulum* sp. which was common at that depth. It is known to grow autotrophically at low oxygen concentrations by oxidation of sulfide to sulfur followed by a oxidation to sulfate (Wirsen and Jannasch 1978, La Reviere and Schmidt, 1992). There is no evidence that nitrate as electron acceptor can be used. It is the fastest known bacterium (Garcia-Pichel 1989, Fenchel 1994). Its high motility will allow a rapid response to variations of the sulfide and oxygen level.

Another organism found, was *Aquaspirillum* sp. which was originally described as *Thiospira* (Dubinina *et al.* 1993). In contrast to *Thiovulum* , this organism is known to grow by oxidation of organic compound. The storage of sulfur inside the cells was described as a mechanism to eliminate toxic hydrogen peroxide. Sulfide will react with hydrogen peroxide to form water and sulfur which is deposited in the cell (Dubinina *et al.* 1993). This mechanism represents a double detoxification, because sulfide is transformed, too. If sulfur can be oxidized further to sulfate coupled to the formation of ATP is not known, but might be likely.

Thiobacilli and *Thiomicrosphaera* sp. seemed to be present throughout the whole water column. Similar to the organisms mentioned above they prefer microaerophilic conditions. Beside our isolated strains, which represents autotrophic members, other mixotrophic *Thiobacilli* or *Thiomicrospira* sp. might occur. The presence of nitrate in the water column (see Thamdrup *et al.* 1996) would imply

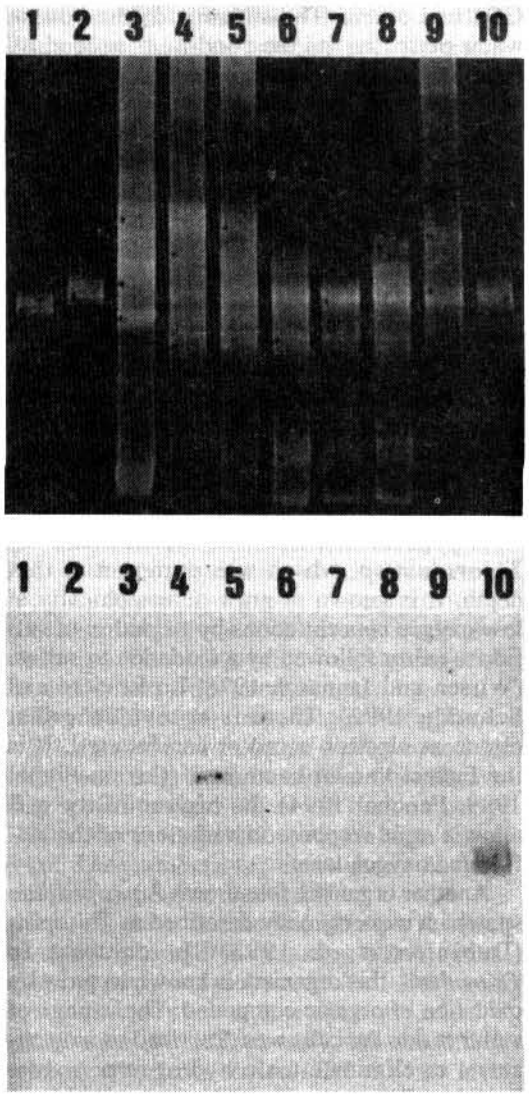
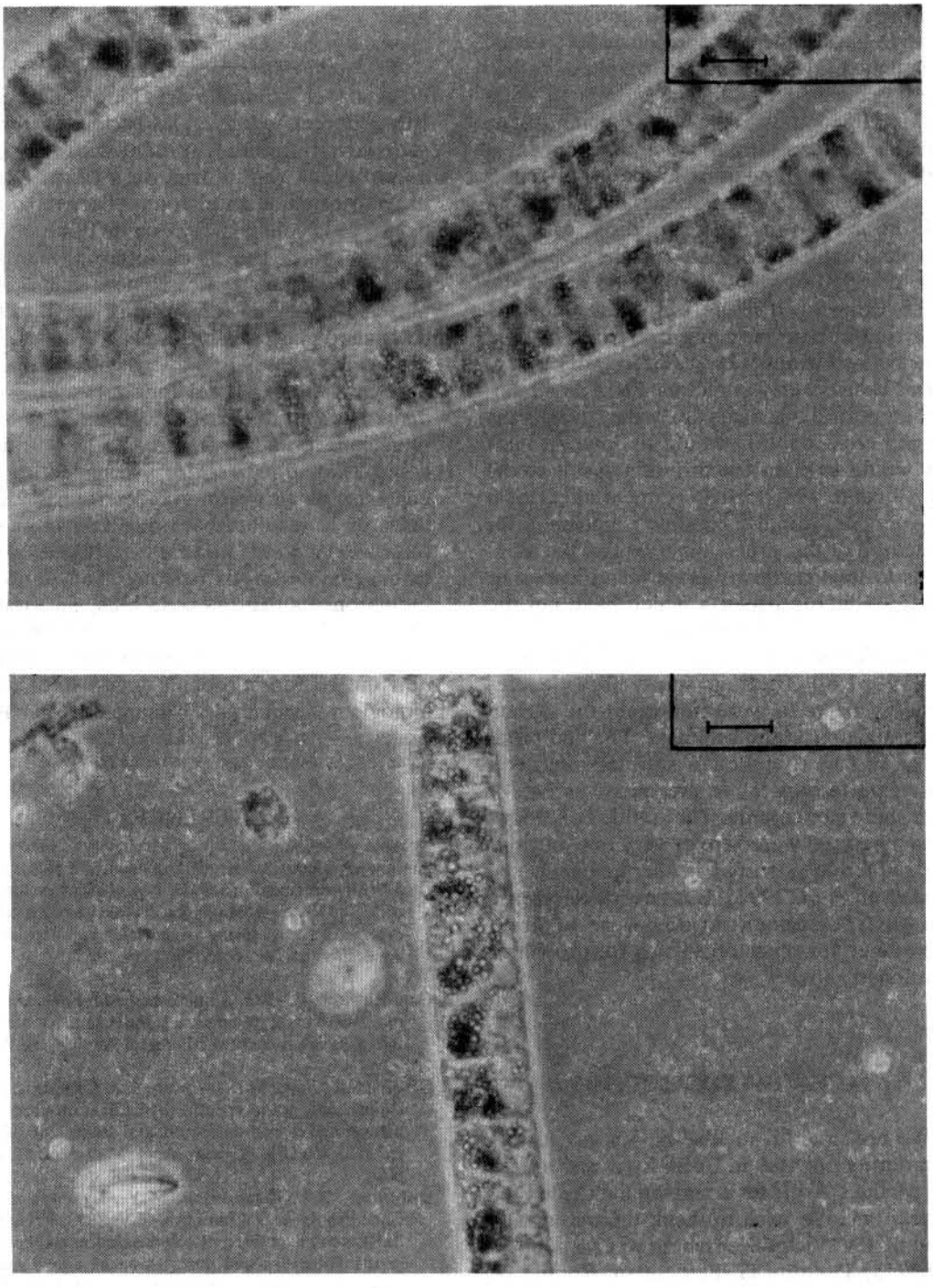


Fig. 2. Distribution of sulfate-reducing bacteria in the water column and the upper sediment of Golfo Dulce, Costa Rica, analyzed by DGGE followed by hybridazation with specific 16S rRNA-gene oligonucleotide probes. a) DGGE analysis (Lanes 1: *Escherichia coli*, 2: *Alcaligenes eutrophus*, 3: 20 m depth, 4: 60 m depth, 5: 80 m depth, 6: 120 m depth, 7: 160 m depth, 8: bottom water, 9: sediment 0-0.5 cm, 10: *Desulfobacterium vavuolatum*). b) Hybridazation with probe 385, a general probe for sulfate-reducing bacteria and other members of the delta proteobacteria. c) Hybridization with probe 804, a specific probe for the acetate-oxidizing genera *Desulfobacter, Desulfobacterium and the species Desulfosarcina variabilis Desulfococcus multivorans and Desulfobotulus sapovorans.*

the possibility for a coupling of denitrification and sulfide oxidation catalysed by bacteria. Our MPN-counts with nitrate as electron acceptor were not conclusive, but station GD160 showed large vacuolated *Beggiatoa* sp. at the sediment surface. These bacteria are similar to the one found at the Guaymas basin and the Gulf of Mexico, where they form dense white mats (Nelson *et al.* 1989, Larkin and Henk, 1989). If these organisms can use nitrate as electron acceptor, is not known. In *Thioploca* sp., which can only be distinguished from *Beggiatoa* because the filaments are surrounded by a common sheath, high nitrate concentrations (up to 0.5 M) were found inside the cells (Fossing *et al.* 1995). In the sea water along the coast of Chile the nitrate concentration is low (< 20 µm) as in Golfo Dulce water. The organism is accumulating nitrate and the vacuoles might be the place for storing nitrate inside the cell. This might indicate that the vacuolated filaments we observed in the bay of Golfo Dulce could be able to use nitrate as alternative electron acceptor to oxygen.

Other microbial processes which might play a role in the water column and probably in the sediment are the oxidation of organic material or reduced sulfur compounds coupled to the reduction of Fe^{3+} or Mn^{4+} ions. The data represented by Thamdrup *et al.* (1996) allow this possibility.



Figs. 3a and 3b: Filamentous sulfur bacterium resembling *Beggiatoa*, note the vacuoles and the sulfur globules (bar represents 20 µm).

Sediment: A lot of organic material will fall down to the sediment, accompanied by a partial degradation. The final mineralization of carbon might occur in the sediment and not in water column. In contrast to the water column sulfate reducing bacteria were found in the sediment at high numbers. In contrast to the MPN-counts showing the presence of acetate oxidizing bacteria no acetate-oxidizing bacteria, or better complete oxidizing sulfate-reducing bacteria, were detected by the more specific probe 804 in the sediment. It might well be that acetate-oxidizing sulfate-reducing bacteria will only grow below the upper layer (0.5 cm) of the sediment, because they could not stand an temporary input of oxygenated sea water, even if the oxygen concentration would be low. At least this would explain the different result to the MPN-counts which used sediment slices of 3 cm intervals. Another possible explanation for the failure of this probe might be that the number is to low to allow a good identification or sulfate-reducing bacteria which have not the right target sequence were present.

Together with the data from Thamdrup *et al.* (1996) our findings imply that sulfate-reducing bacteria were important for carbon mineralization in the sediment. The end product of sulfate reduction is sulfide which will react with ferrous ion to precipitate as FeS or enter the water column. The sulfide concentrations measured were zero or very low, but sulfate reduction occurred in the sediment (see Thamdrup *et al.* 1996). But nevertheless even low sulfide concentrations were of great importance for other organisms for growth as mentioned above.

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RESUMEN

Se estudio microbiológicamente la columna de agua y el sedimento del Golfo Dulce, Costa Rica, concentrándose en las bacterias aeróbicas y desnitrificadoras oxidantes de azufre y anaeróbicas reductoras de sulfuro. No se observó cambios en el conteo bacteriano total dentro de la columna. A pesar de la ausencia de oxígeno solo se detectó el sulfuro de hidrógeno cerca del sedimento. Los conteos más altos de bacterias reductoras de sulfuros por el método de número más probable estaban dentro o cerca del sedimento. Microscópicamente se observó, en el agua anóxica del fondo, bacterias oxidantes de sulfuro, que típicamente contienen grandes esférulas de azufre. Se les identificó como *Thiovulum* y *Thiospira* nadadoras. En una estación se vieron grandes formas vacuoladas de la incolora bacteria filamentosa sulfurosa *Beggiatoa* . Junto a estas bacterias sulfurosas había largos bastones nadadores sin inclusiones sulfurosas visibles, de caracter desconocido. Las observaciones al microscopio mostraron buenas correlaciones con los conteos de Número Más Probable y con técnicas de biología molecular para bacterias reductoras de azufre.

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