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Anaerobic Thiosulfate and Sulfur Oxidation/Disproportionation Mediated by Autotrophic Prokaryotes in the Cariaco Basin's Redoxcline

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Abstract of the Thesis

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Oxygen depletion below ~250 m within the Cariaco Basin results in a redoxcline between 200 and 400m which represents a perfect site to study autotrophic prokaryotic communities due to labile organic matter depletions and vertical distribution of inorganic electron donors and acceptors. Previous studies have revealed enriched concentrations of $S_2O_3^{2-}$, S^0 and SO_3^{2-} within the suboxic zone where O_2 and H_2S are both depleted. Enriched microbial communities within the redoxcline also coincide with peaks in dark

carbon fixation. Production of this biomass is believed to be coupled to chemoautotrophic activity fueled by availability of sulfur species of reduced and intermediated redox states.

Based on the most probable numbers technique (MPN), the abundances of sulfur and thiosulfate disproportionating bacteria are high within the redoxcline reaching numbers as high as $2.7x10^6$ cells L^{-1} for elemental sulfur oxidizers at 275m. This thesis research focused on the quantification and isolation of thiosulfate and elemental sulfur metabolizing bacteria from the Cariaco Basin.

In the laboratory, isolations of these physiotypes were pursued by using the dilution to extinction technique, and deep agar stabs in basal media containing thiosulfate or elemental sulfur as the sole energy source and by continuous cultivation with selective inorganic media enriching for thiosulfate disproportionaters under anoxic conditions. In theory, continuous cultivation seems to be the best approach for the reduction in the number of microorganisms growing in a community.

Terminal restriction fragment length polymorphism (T-RFLP) was the molecular approach used to determine the isolation status of my cultures and it showed the presence of multiple fragments shown as terminal peaks in electropherograms. This suggested the presence of mixed cultures and not pure isolates in any of the forty two putative isolates that were analyzed.

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I. Introduction

The Cariaco Basin is a tectonically-formed submarine depression located off the central north coast of Venezuela. The basin is 1400 m deep and is divided by a saddle at a depth of 900m creating eastern and western sub-basins. It is separated from the Caribbean Sea by a sill at depths of 90-150 m (Richards, 1975) (Fig.1). Lateral water exchange with Caribbean waters into the Basin is confined to the surface few hundred meters due to the Basin's geomorphology. Limited advective exchange and high productivity in surface waters result in oxygen depletion at shallow depths and totally anoxic waters below 300 m. These features result in the Basin being one of the largest permanently anoxic pelagic systems on Earth, second only to the Black Sea (Richards, 1975)

Trade Wind intensification induces seasonal upwelling events above the Basin, allowing vertical shoaling of nutrient-rich water masses (Richards, 1975; Muller-Karger et al., 2004). Intrusions of oxygenated water from the Caribbean Sea have also been reported on several occasions. These intrusions induce physical and geochemical stratification of this unusual water column (Scranton et al., 2001, Astor et al., 2003). Another peculiarity of the Basin's water column is its relatively constant warm temperature as well as uniform density below 200-250m (Richards, 1975; Taylor et al., 2001; Astor et al., 2003). The average temperature of deep waters in the Cariaco Basin is $>17^0$ C, 12-13^0 C warmer than most of the oceans' water at similar depths (Astor et al., 2003) (Fig. 2). The Cariaco Basin water column can be divided into four layers: photic zone (<100m), oxycline from 100 - 250 m, a suboxic zone in which oxygen concentrations are estimated to be below 5 μM and sulfide is <1μM, and an anoxic layer

below 300 m that contains increasing concentrations of H_2S, NH_4^+ and PO_4^{3-} with depth. (Richards, 1975; Scranton et al., 2001; Astor et al., 2003).

The Cariaco Basin's oxic-anoxic interface can be defined as the depth of first appearance of sulfide or the depth where oxygen is totally depleted and may vary from 250 to 350 m (Richards, 1975; Muller-Karger et al., 2001). These two depths often coincide, but a suboxic zone where sulfide and oxygen are both undetectable is also common. Inherent to such stratified systems is a redoxcline which is defined as the depth interval over which the transition from oxic to sulfidic state occurs and the chemical redox potential undergoes a large change from positive to negative values. In the Cariaco Basin, the redoxcline supports enriched inventories of prokaryotes, viruses and protozoa (Taylor et al., 2001, 2003, 2006).

Water columns with marked oxic-anoxic stratification such as the Black Sea, deep basins in the Baltic Sea, and Framvaren and Mariager Fjords have long been known to support oxygenic photosynthesis, anoxygenic photosynthesis, chemolithoautotrophy and heterotrophy. Previous investigations of oxic-anoxic transition zones (Jørgensen et al., 1991; Sorokin et al., 1995; McKee and Skei, 1999; Taylor et al., 2001) have demonstrated the importance of microbial communities to carbon cycling and other biogeochemical processes including the sulfur and nitrogen cycles (Taylor et al., 2001; Daffonchio et al., 2006). Using the 16S rRNA clone library approach, Madrid et al. (2001) found compositional differences between microbial communities occupying the Cariaco Basin's redoxcline and the anoxic zone. Observed variations in community composition in Cariaco suggested that physiological variations could be controlled by

depth-dependent gradients in electron acceptors and donors (Vetriani et al., 2003; Lin et al., 2008).

Dark inorganic carbon assimilation is a prominent feature of the Cariaco Basin's redoxcline. A large portion of this process takes place at depths where no O_2 is detected (Fig.3), implying that terminal electron acceptors other than O_2 are important (Taylor et al., 2001). Within the redoxcline, sulfide is diffusively and advectively transported from deep waters and reacts with oxidized compounds (O_2, Fe^{3+}, Mn^{4+}, NO_3^-) either under biotic or abiotic control. These reactions produce intermediate oxidation states of sulfur, such as thiosulfate ($S_2O_3^{2-}$), tetrathionate ($S_4O_6^{2-}$), sulfite (SO_3^{2-}) and elemental sulfur (S^0), among others. Considering these facts, I hypothesize that the redoxcline is inhabited by obligate or facultative chemoautotrophic organisms that are adapted to utilizing these sulfur intermediate species as an energy source.

Elevated concentrations of elemental sulfur, sulfite and thiosulfate within the redoxcline imply that these sulfur species also may be available as oxidants below (Hasting and Emerson, 1988; Li et al., 2007). Depths enriched in elemental sulfur have previously been observed to correspond to mid-water peaks in chemoautotrophic activity (Hayes et al., 2006; Li et al., 2008) (Fig.3), leading to the hypothesis that metabolism of the microorganisms growing in the Cariaco's redoxcline relies on anaerobic oxidation or disproportionation of intermediate sulfur compounds. The disproportionation process involves the inorganic fermentation of a chemical compound with an intermediate oxidation state in which the element (sulfur in this case), can serve as an electron donor and acceptor at the same time in an energy-generating process (Finster et al., 1998).

Disproportionation of elemental sulfur has been described for *Desulfocapsa spp*., *Desulforhopalus spp.* and *Desulfofustis spp.* isolated from anoxic sediments (Lovley and Phillips, 1994; Isaksen and Teske, 1996; Friedrich et all., 1996; Janssen et al., 1996). However, these groups of bacteria could only be grown in cultures in the presence of iron or manganese oxides as sulfide scavengers (Lovley and Phillips, 1994). Even though a lot of research has been done on these particular bacteria, little is still known about their physiology, the detailed reactions they mediate, or where these microorganisms are in the Cariaco Basin.

To date, bacteria capable of anaerobic oxidation or disproportionation of S^0, and S_2O_3 have not been described for the Cariaco Basin. In order to understand processes central to biological productivity and chemical transformations in the Basin's redoxcline, we need to explore whether these physiotypes are present and common to the Cariaco's redoxcline. A deeper understanding of these microbial communities and their physiological constraints will facilitate refinement of elemental budgets and improve our comprehension of the linkages between redoxcline microbial communities and the rest of the ecosystem. This information will provide better insight into carbon, sulfur, and metal cycling across interfaces and underlying anoxic waters in the Cariaco specifically and other similar stratified systems.

Although cultivation-dependent methods for environmental microbes are laborious and success is not guaranteed, they are still essential if we want to understand microorganisms with novel types of metabolism and truly comprehend their role in biogeochemical cycles. The role of prokaryotes in biogeochemical processes can be

deduced from *in situ* measurements. However, their activities in elemental cycles, as well as their abundances and physiology, cannot be determined using culture-independent approaches exclusively. Progress in understanding the role of microorganisms in biogeochemical processes has been hampered by limited understanding of the microbial community structure and population dynamics in a given geochemical context. Chemolithotrophic thiosulfate disproportionating bacteria such as those described by Finster et al. (1998) from oxidized marine sediments containing ferrihydrite could play an important role in the Cariaco Basin's redoxcline.

Recently, combinations of molecular techniques, *in situ* measurements and culture-dependent methods have helped elucidate the ecology and evolution of free-living microorganisms (Strous et al., 2002; Giovannoni et al, 2007). Molecular approaches, such as phylotyping, metagenomics, fluorescent *in situ* hybridization (FISH), denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (TRFLP), have provided tremendous new insights into uncultured environmental microorganisms (Giovannoni et al., 2007). However, linking phylogenetic information to ecological function is often difficult if not impossible, because many identified lineages have no cultivated relatives with known physiologies due to difficulty in cultivability of environmental microorganisms (DeLong, 1992). This is particularly true for anoxic environments because of the technological difficulties associated with continuously maintaining environmental redox conditions.

Nowadays continuous cultures are being used in some labs as a tool to isolate microorganisms. This approach maintains microorganisms in exponential growth phase

by constantly adding nutrient media at a fixed rate that determines the growth rate (James, 1961; Strous et al., 2002). Continuous cultures, also known as chemostats, select organisms from mixed communities that are most fit to grow in the selective media at a specific dilution rate. Microbes not belonging to the groups of interest either grow slower than the media turnover rate or starve. If either of these two happens, then they are removed from the system by washout from the growth reactor through time. However, in practice, cohabiting syntrophic organisms and contaminants may remain in growth reactors if they attach to other microorganisms or even the reactor walls.

In this thesis, I used classic enrichment cultivation, most probable number (MPN), dilution to extinction and continuous culture techniques to attempt to isolate bacteria with novel forms of sulfur metabolism from the Cariaco Basin's redoxcline. Then I screened putative isolates with modern molecular techniques to assess culture purity and to describe the different ribotypes that are involved in the biogeochemistry of sulfur and its species.

II. Objectives

1. The objectives of this thesis were to determine the abundance of elemental sulfur and thiosulfate oxidizing or disproportionating microorganisms residing in the Cariaco Basin's redoxcline using the most probable number (MPN) technique. I utilized two types of media; S^0 oxidizing media with Fe as the oxidant and S_2O_3 disproportionating media.
2. Determine if a mixture of phylogenetic lineages arise in the sulfur oxidizing and disproportionating chemoautotrophic enrichment cultures and if single species can be isolated.

III. Hypotheses

- Depth-dependent enhancements in chemoautotrophy in the Cariaco's redoxcline are attributed to the utilization of $S_2O_3^{2-}$ and S^0 by autotrophic microorganisms
- Elemental sulfur and thiosulfate-utilizing microorganisms within the Cariaco Basin's redoxcline obtain energy by either the anoxic oxidation or disproportionation of elemental sulfur or thiosulfate.

IV. Methods

IV.1 Sampling

Sampling was carried out twice a year, during high and low productivity seasons, in 2007 and 2008 for a total of four cruises. The cruises included CAR-132 (11-12 April, 2007), CAR-139 (30 Nov- 01 Dec. 2007), CAR-145 (21-22 May, 2008) and CAR-153 (19-20 Jan, 2009). Cruises were performed aboard R/V Hermano Gines, operated by Estacion de Investigaciones Marinas (EDIMAR), Fundacion la Salle de Ciencias Naturales, Margarita Island, Venezuela.

All samples were obtained from the CARIACO time series site, station A (Fig.1), located at 10°30' N, 64°40' W. Water samples were collected in 8-liter Teflon-lined Niskin bottles mounted in a SeaBird rosette. Collection depths and number of samples were adjusted according to the location of the redoxcline during each cruise. The depth of the transition zone was determined from the position of a light scattering layer using a Sea Tec c-beam transmissometer (beam attenuation at 660 nm) and by the first detection of hydrogen sulfide (by smell). Subsamples from Niskin bottles were withdrawn under a N_2 headspace provided by a gas line to the air vent. Continuous dissolved oxygen profiles were obtained from the rosette's oxygen probe. Discrete water samples for O_2, HS^-, $S_2O_3^{2-}$, S^0, heterotrophic, and chemoautotrophic production were also taken from the Niskin bottles. Protocols for all these methods have been previously described elsewhere (Taylor et al., 2001; Scranton et al., 2006; Percy et al., 2008). Samples for MPN and enrichments were collected every 10-20 m within this layer (3-5 depths total). Additionally, single reference samples were collected above and below the redoxcline.

Subsamples were dispensed into incubation vessels through a N_2-pressurized bottle to avoid atmospheric exposure.

IV.2 Abundances of elemental sulfur and thiosulfate-oxidizing and disproportionating microorganisms

The most probable number (MPN) technique is a widely used technique for estimating abundances of targeted microbial populations in waters and soils. The assay consists of serial decimal dilutions into selective liquid media in which 1 ml environmental subsamples reached a final dilution of 10^{-8}. Dilutions are scored as positive or negative, with positives appearing turbid or discolored. Results are used to derive most probable numbers based on the calculations originally presented by Halvorson and Ziegler (1933). Because decimal dilutions were employed, results from this technique are accurate only within an order of magnitude. Greater accuracy is possible if finer scale dilutions are performed, but this was not logistically feasible in the present study.

Inoculations into sulfur-oxidizing and thiosulfate disproportionating media and MPN dilutions were conducted onboard immediately after sample collection. Filtered Cariaco seawater from previous cruises was used for the media preparation to make a highly selective low organic media (Appendix I). Bicarbonate was used as the primary carbon source probably along with some residual (but minimal) organic matter from the Cariaco sea water was. The energy source was either elemental sulfur (S^0) or thiosulfate ($S_2O_3^{2-}$). $FeSO_4$ and $FeCl_3$ salts were added as electron acceptors as well as indicators of FeS

production (formation of black precipitate). Sulfide can be produced by disproportionation of S^0 or by disproportionation of thiosulfate (equations 1-4).

- **Disproportionation of S^0, with Fe^{2+} as S^{2-} sink**

$$4H_2O + 4S^0 + 3Fe^{2+} \longleftrightarrow 3FeS + SO_4^{2-} + 8H^+ \qquad \textbf{(eq. 1)}$$

$\Delta G^{0'} \approx -70.2$ kJ mol sulfur $^{-1}$

- **Oxidation of S^0 with Fe^{3+}**

$$12H_2O + 5S^0 + 14Fe^{3+} \longleftrightarrow 2FeS + 12Fe^{2+} + 3SO_4^{2-} + 24H^+ \qquad \textbf{(eq. 2)}$$

$\Delta G^{0'} \approx -70$ kJ mol sulfur $^{-1}$

- **Disproportionation of $S_2O_3^{2-}$ with either Fe^{2+} or Fe^{3+} as S^{2-} sink**

$$H_2O + Fe^{2+} + S_2O_3^{2-} \longleftrightarrow FeS + SO_4^{2-} + 2H^+ \qquad \textbf{(eq. 3)}$$

$$3H_2O + 2Fe^{3+} + 3S_2O_3^{2-} \longleftrightarrow 2FeS + 3SO_4^{2-} + 9H^+ + S^0 \qquad \textbf{(eq. 4)}$$

$\Delta G^{0'} \approx -72.8$ kJ mol sulfur $^{-1}$

The media was prepared under anoxic conditions inside a glove box (Plas Labs Inc., MI) with a gas mixture of H_2:CO_2:N_2 (10:5:85), right before the cruises. Media were kept anoxic throughout the whole growing process by incubating in sealed Hungate tubes.

The growth of microorganisms possessing oxidative or disproportionation metabolisms was monitored by visible production of turbidity in the cultures. Bacterial growth was later confirmed by acridine orange (AO) direct counts of subsamples (Hobbie

et al., 1977). Samples (10-15ml) were concentrated down to 5 ml volumes over a 0.2 μm pore-size black Poretics polycarbonate membrane filters and were stained 5min with acridine orange. Fluorescent microscopy was applied to enumerate the microorganism counting 10-15 fields within a slide.

IV.3. Enrichment cultures

Solutions for the media are described in Appendix I. Water samples were withdrawn from the N_2 pressurized glass bottle with a 1ml syringe. These 1ml samples were injected into the first set of screw cap tubes representing a 10^{-1} dilution. Subsequent dilutions were performed serially by withdrawing 1ml of inoculated media from the previous tube and injecting it into the next tube for a total of eight tubes in triplicate and a final dilution of 10^{-8}.

Enrichment cultivation of elemental sulfur oxidizing bacteria was performed during CAR-132, CAR-139, CAR-145 and CAR-153 cruises at varying depths within the redoxcline. Other depths within the oxic and anoxic zones were enriched as reference samples (control). Cultures were inoculated as described above into 18mL vented Hungate tubes and 30mL serum bottles containing selective media with elemental sulfur as the energy source. The Hungate tubes and serum bottles were inoculated with 1mL and 10mL of sea water from the depths of interest, respectively. Enrichments for thiosulfate disproportionating bacteria were prepared during CAR-132, CAR-139, CAR-145 and CAR-153 cruises within the redoxcline as described for sulfur oxidizers.

IV.4. Isolation of chemoautotrophic microorganisms involved in the sulfur speciation within the redoxcline

The main purpose of the isolation of environmental microorganisms is the identification and deeper study of their physiology under defined conditions. In order to accomplish the isolation of the sulfur-utilizing microorganisms within the Cariaco Basin redoxcline, I employed different techniques in pursuit of monocultures. The main objective of my thesis was to culture "isolates" with chemoautotrophic metabolisms involved in the sulfur speciation.

IV.4.1. Dilution to extinction

The first approach employed to obtain sulfur-oxidizing or thiosulfate disproportionating isolates from suboxic water samples involved repeated dilutions into fresh sterile media before culture purity was assessed. These repeated transfers consisted of between seventeen and thirty passages for all targeted cultures. This approach reduces the number of cells present in each subsequent tube as they are being diluted out. Eventually, aliquots will contain such small amounts of original sample that will translate into no microorganism in the most dilute inoculum. A 1mL aliquot from the last tube where growth was observed was withdrawn and added into a new set of 9 mL tubes and serially diluted down to 10^{-8}. Tubes were incubated for 3-5 weeks in darkness at 20°C. Positive tubes containing either elemental sulfur or thiosulfate disproportionating or oxidizing microorganisms were identified by changes in color of the media produced by iron sulfide. Dilutions were monitored for growth following initial enrichments. AO

microscopic counts were used to confirm growth, estimate concentrations and describe cell morphologies.

IV.4.2. Dilution in solid media

The second approach used to isolate elemental sulfur and thiosulfate-utilizing bacteria involved dilution series of selected cultures transferred into Hungate tubes containing 9 ml of molten agar. The isolation tubes contained 1 ml of inoculum in 9 ml of the selected media with 1.5% noble agar. The energy source was added according to the type of metabolism targeted (elemental sulfur or thiosulfate) as described by Finster *et al*. (1998). The agar was melted and allowed to sit in the tube at 40°C, while the dilutions on the media were done separately. Subsequently 1ml from each dilution was transferred into the molten agar and mixed well. Tubes were incubated for 6-8 weeks in darkness at 20°C. Tubes containing isolates were recognized by the presence of colonies identifiable as black spots in the agar as a result of iron sulfide precipitation.

Black spots in the agar representing putative "isolates" were withdrawn from the solid media with sterile Pasteur pipettes after breaking the tube open and transferred to liquid cultures containing thiosulfate or elemental sulfur media. After 6-8 weeks of incubation, cultures were examined by fluorescence microscopy after being stained with AO (Sieracki et al., 1985).

IV.4.3 Continuous culture

In the final stages of my research, an anaerobic chemostat was employed for the isolation of thiosulfate-utilizing bacteria. This bioreactor has been described as a

sequencing batch reactor or stirred tank reactor (Strous et al., 1998). The chemostat was kept under an anaerobic atmosphere by an attached gas line that split to the reservoir and the bioreactor. The gas was mixture of H_2:CO_2:N_2 (10:2:88) with 25 $\pm$ 5psi flow rate (Fig.4).

The continuous culture technique selects for populations that are supplied with appropriate substrate for their growth. The microorganisms grown in the bioreactor were expected to be chemolithotrophic thiosulfate disproportionaters that coupled the reaction to iron reduction. Cell numbers (AO direct counts) and pH were monitored throughout the experiment within the bioreactor in order to confirm that steady-state was maintained and that continuous CO_2 bubbling did not significantly alter the pH of the media. The microorganisms remaining at the end of a chemostat run were transferred to new sterile liquid media. Samples for suspended particulate organic carbon (POC) and particulate nitrogen (PN) measurements were filtered through precombusted 13mm GF/F filters four times during the experimental run in order to estimate the production of new biomass. Filters were stored frozen until analysis by the SoMAS, SUNY at Stony Brook facility using methods of chromatography. Typical uncertainty at this analyte level is +/-5% for N, +/-2% for C.

Two runs of continuous cultivation were performed with thiosulfate disproportionating media, the first run lasting 10 days and the second 12 days. The two runs differed in media dilution rates; 0.11 and 0.67 doublings per day in the first and second runs respectively. This approach should select for the fastest growing microorganisms as fresh media caused dilution and washout of slower growing organisms.

IV.5. Screening isolated ribotypes using standard 16S terminal restriction fragment length polymorphism (T-RFLP)

After obtaining putative isolates, T-RFLP was used to identify ribotypes amplified from the different isolation approaches. After continuous cultivation for 22 days (total for the two runs) of one previously "purified" culture under anaerobic conditions, the remaining microorganisms present in the bioreactor culture were subject to molecular processing involving DNA purification and amplification. This process provided information about the culture purity, i.e., true isolation. It included the description of the different ribotypes using standard 16S Terminal Restriction Fragment Length Polymorphism (T-RFLP), as described below.

IV.5.1. DNA extraction and purification

DNA samples were derived from 10-50 ml of cell suspension filtered onto a 13 mm 0.2 μm polycarbonate membrane (Pall Gelman Inc.) in a syringe filter holder using a 20 ml polypropylene syringe. Filters were placed in 600 μl microcentrifuge tubes, immersed in 200 μl of DNA lysis buffer (20 mM Tris HCl, pH 8.0, 2 mM EDTA, pH 8.0, 1.2% Triton X) and stored for 7-10 days at –20°C. Prior to extraction, DNA samples were thawed and incubated with 20 mg ml^{-1} lysozyme for 1 h at 37ºC. Subsequently 25 μl of Proteinase K (25 mg ml^{-1}) was added, and the samples were later treated according to the DNeasy tissue kit protocol for Gram-positive bacteria (Qiagen Inc. CA) in order to guarantee the lysis of gram-negative and gram-positive bacteria. High molecular weight DNA was confirmed electrophoretically on 1% agarose gels (Lin et al., 2006).

IV.5.2. Amplification of 16S rRNA genes

The amplification of the 16S rRNA genes was carried out by Polymerase Chain Reaction (PCR). The primers used for PCR amplification were 63F-FAM (5'-CAG GCC TAA CAC ATG CAA GTC -3') and 778R (5'-AGG GTA TCT AAT CCT GTT TGC -3'), which give a $\pm$ 1500 b product of the 16S rDNA (Lin et al., 2008). 63F-FAM was 5' labeled at the end with phosphoramidite fluorochrome 5-carboxyfluorescein (Integrated DNA Technologies Inc.). Each 50µl PCR reaction contained (final concentration) both primers at 0.2 µM, 1.5 mM $MgCl_2$, 1X PCR buffer (Qiagen), 250 µmol of each deoxynucleoside triphosphate (Roche Molecular Systems) and 1.25 units of Hotstart DNA polymerase (Qiagen). One nanogram of DNA extract was added to each PCR reaction. PCR was performed in a Stratagene Mx3000P real time thermocycler under the following conditions: an initial denaturation step of 95°C for 3 min, 30 cycles of at 95°C for 30s, annealing at 55°C for 30s, and extension at 72°C for 1 min. Cycling was completed by a final extension at 72°C for 7 min. PCR product was confirmed in 1.0% agarose gel in 1x TAE (40 mM Tris-acetate, 1 mM EDTA) containing ethidium bromide (0.50 mg ml^{-1}) (Lin et al., 2006). Three replicate PCR products per sample were pooled and purified with a Qiaquick PCR Purification Kit (Qiagen). DNA was precipitated by adding 2.5 volumens of 3M sodium acetate (pH 5.2) and two volumes of 95% chilled ethanol. The mixture was incubated at -80°C for 15 min and centrifuged at 4 °C and max of 20,000g for 20 min. The DNA pellet was washed with 70% ethanol and left to dry in a PCR-prep laminar flow hood for 1 h. It was resuspended in 20 µL of PCR grade water and stored at -20 °C.

IV.5.3. Terminal restriction fragment length polymorphism (T-RFLP)

This method is based on presence or absence of specific terminal restriction fragments (T-RFs) after cleavage with specific endonucleases (see Figure 5 for an overview of the technique). PCR of the 16S rRNA gene from 42 isolates was carried out with fluorescently labeled primers as described above.

Despite the high resolution of T-RFLP, there is the possibility that two different species could have the same restriction site in the 16S rDNA, resulting in an identical overlapping peak. Therefore, I performed three independent restriction digests using different enzymes *Alu*I (AG/CT), *Msp*I (C/CGG), *Rsa*I (GT/AC), (Lin et al., 2007), to increase the information for a single sample. Restriction digestions were conducted using 100 ng of purified PCR product for each sample and contained 10 µl of a cleaned PCR product, 10 Units of the restriction enzyme and the appropiate restriction buffer. The reaction was brought to a final volume of 30 µl with PCR grade water. Digestions were conducted at 37°C for 6 h. Restriction endonucleases were deactivated by heating the reaction mixture to 65°C for 10 min after reaction was completed.

Fragments were size-separated on an automated capillary sequencer (ABI 3100). Before the capillary electrophoresis, a mix of Hi-Di formamide and MapMarker Rox1000 size standard (BioVenture Inc.) was added to 1uL of each sample (10 µL final volume). Only the labeled terminal fragments (T-RFs) are detected and quantified by this technique (Kitts, 2001; Egert and Friedrich, 2003) (Fig. 5). GeneMapper V3.7 software was used to analyze terminal fragments in the 30-500 b range and a minimum peak

threshold was set at 50 arbitrary units. Size alignment and grouping of peaks was manually accomplished within $\pm$ 1b fragment size (Clement et al., 1998).

V. Results

V.1 Abundance of elemental sulfur and thiosulfate oxidizing and disproportionating microorganisms by MPN

Eight serial 10-fold dilutions of triplicate samples were performed to estimate the abundance of elemental sulfur oxidizers and thiosulfate disproportionating bacteria using selective media under anaerobic conditions. Abundance estimates for sulfur oxidizing and thiosulfate disproportionating microorganisms were performed for a total of four cruises (CAR-132, CAR-139, CAR-145 and CAR-153). A change in the color of the media occurred after a 2 month incubation period for both thiosulfate and sulfur media. During these incubations, a black precipitate formed (presumably FeS) which was interpreted as the indicator of growth for targeted microorganisms. These cruises also included water column profile measurements of elemental sulfur, thiosulfate and sulfite concentrations (Xiaona Li, 2009), total bacterial counts using fluorescence microscopy and chemoautotrophic production with and without sulfur amendments for depths in the redoxcline (Fig.7, 8).

MPN estimations for chemoautotrophs oxidizing elemental sulfur during CAR-132 cruise presented positive growth in every tube inoculated. This suggests a possible contamination problem preparing serial dilutions or a higher number of microorganisms than the method can estimate for this particular cruise (Table 1). These results should be viewed skeptically. Thiosulfate disproportionating MPN estimates were highest at 250 m, with samples reaching a MPN of 9.2×10^5 cells L^{-1}. MPN estimates decreased deeper in the redoxcline by an order of magnitude to 3.7×10^4 cells L^{-1}. MPN estimates for

thiosulfate disproportionaters accounted for almost 1% of the total prokaryotes at 250 m (Fig.7.a).

CAR-139 MPN estimates observed at 300 m were the highest in the redoxcline for both media ($2.7x10^5$ cells L^{-1} for S^0 oxidizing and $3.4x10^5$ cells L^{-1} for $S_2O_3^{2-}$ disproportionaters). Highest MPN estimates for both groups of microorganisms coincided with low concentrations of sulfur species at that same depth (Fig. 7, 8). Thiosulfate disproportionating microorganisms appeared to be more abundant than elemental sulfur oxidizers for every depth sampled within the redoxcline (Fig. 7, 8).

In May 2008, CAR-145 MPN estimates appeared to vary inversely with sulfur species concentrations (S^0 and $S_2O_3^{2-}$). This may indicate S^0 and $S_2O_3^{2-}$uptake by the microorganisms which would therefore diminish the respective concentrations. Elemental sulfur oxidizing media yielded an increase in abundance almost by an order of magnitude from 250 m to 350m, $4.2x10^4$ to $2.7x10^5$ cells L^{-1} respectively (Table 3). Contrary to what was found in the previous cruise, elemental sulfur was at a minimum concentration at this depth (0.01μM). At 350 m, sulfur oxidizing microorganisms accounted for as much as 0.5% of the total bacterial counts (Fig.8).

Abundances of elemental sulfur oxidizing prokaryotes increased from $3.6x10^3$cells L^{-1} at 220m to $2.7x10^6$ cells L^{-1} at 275m during CAR-153 cruise (Table 4). This MPN represents 1.6% of the total prokaryotic direct counts at 275 m (Fig. 8). A sample taken from deep waters (900m) during the same cruise yielded similar abundances to those taken from the redoxcline. A sample from 1300 m (CAR-145) was also inoculated to check for growth of the targeted microorganisms in deep waters. This sample did, in fact,

exhibit microbial growth which confirmed the presence of viable cells at this depth (Fig. 11). A control culture was also prepared with no inoculum to confirm that no oxidation or sulfur disproportionation occurred abiotically (Fig. 11.a). During CAR-139 and CAR-145 cruises, surface water samples were also taken to represent control enrichments as I wanted to test for the presence of $S_2O_3^{2-}$ and S^0 disproportionaters or oxidizers in the oxygenated photic zone waters. Growth was not evident for any of these samples implying that physiologies that the used media selected for were only able to develop under dark anaerobic conditions.

Data from CAR-153 showed a peak in thiosulfate disproportionaters at 250 m amounting to $1.5x10^5$cells L^{-1}. Thiosulfate concentrations also varied inversely with abundances of thiosulfate disproportionating microorganisms, yielding low values when MPN abundances presented the highest in the redoxcline (Fig. 7, 8). Dissolved inorganic carbon uptake (chemoautotrophy) and bacterial net production (heterotrophy) were also measured for all cruises to examine total community activity throughout the water column. In general high MPN abundances for both sulfur species corresponded to peaks in chemoautotrophy within the redoxcline (Fig. 9, 10).

In parallel with routine chemoautotrophic assays, duplicate samples were amended with 50 μM (final concentration) of thiosulfate or elemental sulfur and incubated at 17˚C in darkness. These experiments assessed the influence of the two sulfur species of interest on autotrophic production (Fig. 9, 10). A common feature among all four cruises was an increase in dissolved inorganic carbon assimilation in the presence of additional thiosulfate compared to control incubations.

Thiosulfate stimulation of dissolved inorganic carbon assimilation was highest during CAR-145, with thiosulfate enrichments doubling fixation over controls at 280m, reaching 1.7 μMC d^{-1} (Fig.10.a). Elemental sulfur amendments also showed a strong influence on chemoautotrophic fixation. Inorganic carbon assimilation rates increased by 20 to 500 μMC d^{-1} whenever amendments were performed. At 355m, CAR-139 carbon fixation was stimulated almost 10 times (fig.9.b).

The influence of different electron acceptors was tested along with thiosulfate amendments. During CAR-153 $Fe^{3+}$and Mn $^{4+}$ were added separately to thiosulfate amendments at a 50μM final concentration. The purpose of this test was to differentiate which element would influence the reactions the most. Mn $^{4+}$ additions were associated with greater carbon fixation than Fe^{3+} (almost 200 times higher) (Fig.10.b).

V.2 Enrichment cultures

MPN cultures exhibiting positive growth by change in color due to formation of FeS were used for further isolation attempts. These cultures were transferred in a series of dilution passages in selective media in order to isolate axenic cultures. T-RFLP was conducted after many dilutions to extinction passages. After 20-28 passages had been performed, cultures appeared morphologically uniform under the microscope. Selected cultures of "putative isolates" were allowed to grow for several weeks in larger volume serum bottles (50-70 mL). Larger volumes were necessary in order to obtain enough biomass for subsequent DNA isolation and processing (Fig. 11). A total of 42 cultures for both media yielded putative isolates, after being subjected to 20-28 transfers. However, these probably didn't represent pure cultures as a putative isolate from 300 m (CAR-139)

yielded more than one clone, as demostrated by the presence of more than one terminal fragment peak in the corresponding electropherogram.

Samples originating from 250 and 350 m during the CAR-145 cruise yielded the highest number of putative isolates with metabolisms involving elemental sulfur. All surface samples inoculated into SOD and TDB media failed to exhibit growth, indicating that surface waters didn't support growth or survival of these ecophysiotypes. The average time for elemental sulfur cultures to show visible signs of growth was five weeks after inoculating the media. Once growth was confirmed, direct counts indicated a range between 0.56 to 8.95 x 10^7 cells ml^{-1} for elemental sulfur oxidizing bacteria which includes all cultures derived from the four cruises studied.

Preparation of elemental sulfur oxidizing media presented a problem as S^0 is insoluble in water. After 2 to 3 week of incubation of this media, a white film was observed on the yellow surface of elemental sulfur which was assumed to be bacterial growth. After the film was formed and 3 to 4 weeks had passed, the white film transformed into a black precipitate which was assumed to be due to FeS formation. Among all observed morphologies, bacilli and filamentous bacteria were the most abundant. The bacilli presented bright granules that are likely to be sulfur granules. It is well known that sulfur in bacteria is mostly stored as elemental sulfur granules that can either be deposited intra or extracellularly (Fig. 12).

Thiosulfate disproportionating bacteria were also enriched following the same protocol as for elemental sulfur oxidizers. MPN cultures that showed FeS formation were used as inocula for isolation series (Fig.13). The most diluted positive cultures were

transferred and diluted into new selective media and the growth was confirmed by direct counts and observations under the microscope after two to three weeks of incubation. Dilution series in thiosulfate media were conducted more frequently than those in elemental sulfur media as growth seemed to be faster than in cultures provided with elemental sulfur as the energy source (Fig. 14). Dilution series in thiosulfate media yielded a total of 42 "putative isolates" compared to 25 for sulfur oxidizers. The molecular T-RFLP technique was performed on all "putative isolates" to check the isolation status of the cultures. In all cases, terminal fragment analysis showed the presence of more than one peak per digest. These results indicate that clonal isolates were not obtained for either S^0 oxidizing or $S_2O_3^{2-}$ disproportionating bacteria. Results indicate that more extended isolation approaches are necessary to produce pure cultures. Even though single species isolation was not accomplished, many peaks were shared among different cultures after grouping all the fragment peaks obtained from S^0 and $S_2O_3^{2-}$ media (Table 5). Several peaks appeared to be common in most cultures after the restriction *Alu*I digest including 10, 111, 156 and 470 bp fragments, 10, 455 and 769 bp for *Msp*I and 45 and 401 bp for *Rsa*I digest. The most repeated fragments include 85, 298, 470 and 580 bp for the *Alu*I digestions; 137, 214, 256 and 435 pb for *Msp*I restriction enzyme; and 45, 626 and 750 bp fragments (Table 5).

T-RFLPs shown in Figures 16, 17 and 18 are examples of bacterial fingerprints resulting from S^0 or $S_2O_3^{2-}$ cultures that had passed through a high number of dilutions series. Elemental sulfur disproportionating "isolates" originating from 300 m during CAR-139 yielded many more fragments after four dilution series passages compared to that found in a sample from 280 m during CAR-145 for the same media after a total of

eight dilution series passages. For the *Msp*I restriction digest, a prominent fragment of 725 bp was detected in both samples with a strong fluorescence value above 500 fluorescent units (40% of total peak area). Electropherograms from thiosulfate disproportionating isolates also share similarities that suggest the presence of what could be the same microorganism (Fig. 17). For example, *Alu*I digests of TDB isolates derived from CAR-139 and CAR-145 cruises have four prominent common peaks that are 170, 185, 280 and 470 bases long (Fig. 18). However, the *Msp*I digests did not share any significant fragments (Fig.17.b). Four peaks are shared between these two samples suggesting that the same microorganism from different samples has proliferated in this medium. Common peaks include 195, 435, 626 and 730 base fragments that prevail in the *Alu*I digestion after several successive dilutions (Fig.16 and 17). A third set of electropherograms shows T-RFs from an isolate derived from a 230 m sample collected during CAR-153. Successive dilution series passages (more than 5 series of dilutions) seemed to have decreased the number of peaks present in a sample, suggesting that fewer operational taxonomic units remained as the samples were transferred to fresh sterile media. Persistence in the dilution to extinction technique might have led to a pure culture after several more passages if time had allowed.

V.3 Isolation of chemoautotrophic microorganisms involved in the sulfur speciation within the redoxcline

Once putative isolates were obtained, different protocols were performed in order to obtain axenic cultures including regular enrichment cultures, series of dilutions to extinction in selective media and continuous anoxic cultivation for thiosulfate

disproportionaters. An alternative approach for isolation was employed only for samples taken during CAR-132 cruise at depths of 305 and 340 m. For theses samples a similar protocol of dilution to extinction in liquid media was performed, but for this experiment cells were isolated and immobilized through the use of solid media (agar) (Fig. 15). Growth was not evident until 3 months after inoculation when a change in color of the agar was present in every tube of the dilution series. The growth in every tube indicated that higher dilutions should have been performed. Only a few tubes produced single isolated colonies of microorganisms as black spots within the agar (Fig.15.b). Eight colonies were selected to isolate elemental sulfur oxidizing bacteria, and three were available for thiosulfate disproportionating bacteria. All eleven colonies were retrieved from the solid media and inoculated into a liquid medium; dilution to extinction series were performed 3 or 4 more times allowing the growth of the isolated species in a selective liquid media.

The final approach I employed for isolation of thiosulfate disproportionaters was incubation of a sample under anoxic continuous cultivation. This sample originated from 350 m collected during CAR-145 and had been subjected to 27 dilution passages before entering continuous cultivation in the anaerobic chemostat. A total of two chemostat runs were performed. The first run of the chemostat started with a flow rate of 50 ml d^{-1} into a total bioreactor volume of 450ml (0.11 d^{-1} turnover rate). Once steady-state had been achieved, the dilution rate was increased until it reached 0.9 day^{-1} by the end of the second run. As dilution rates increased, the continuous culture theoretically selected for the fastest growing microorganisms able to use thiosulfate (either by oxidation or disproportionation) under anaerobic conditions (Fig. 19). When the end of the continuous

cultivation was reached (22 days total), molecular fingerprints were analyzed in order to assess the purity of the culture. Figures 21, 22 and 23, represent digestions of samples collected at three time points during the chemostat run. Electropherograms shown in figure 21 represent terminal restriction fragments obtained after digestions with *Alu*I restriction endonuclease. *Alu*I electropherograms reveal the presence of at least two or more fragment sizes which confirms the presence of more than one organism, meaning that pure clonal culture had not been obtained after the bioreactor had run for 22 days. Even though this information confirms that clonal isolation was not achieved, it also shows a decrease in number of peaks as cultivation proceeded of the mixed culture. Figure 21 shows a decrease in the number of peaks as the bioreactor ran for three, eleven and twenty days. The total number of peaks after three days in the run was 53, decreasing to 27 by the eleventh day and 6 at the end of the experiment. Peaks of 140, 170, 256, 270, 455 and 470 bp were common among all electropherograms. *Msp*I endonuclease digests also contained higher numbers of peaks at the beginning of the run (Fig. 22), and only three major peaks were present at the last day of cultivation. T-RFs of 195, 710 and 760 bp prevailed throughout the run. T-RFLp patterns of *Rsa*I digests after three days showed the fewest peaks compared with the other restriction enzymes (Fig. 23), yielding only fifteen detectable peaks. Digestions at different stages suggest a change in the culture populations as the number of peaks diminish from day 3 to 20. By the end of the continuous cultivation run, only the 760 b T-RF retrieved from the *Rsa*I digest was common among all digests performed for this endonuclease.

V.I. Discussion

Thiosulfate disproportionating bacteria have been detected and isolated from a variety of habitats, including marine and freshwater sediments (Jackson and McInerney, 2000). No pure cultures were isolated in this research, and therefore no physiological explanation for dark carbon assimilation coupled to sulfur uptake is available from this research. Enrichment cultivation has been performed previously on thiosulfate-oxidizing manganese-reducing bacteria from Cariaco water samples in selective media. However no isolates were acquired by these authors (Madrid et al., 2000). The literature reports that different genera disproportionate thiosulfate, including *Desulfotomaculum* which is able to form endospores indicating its resistance to extreme conditions. *D. thermobenzoicum* also disproportionates thiosulfate in freshwaters and/or hydrothermal vents (Jackson and McInerney, 2000).

Dilutions in solid media (agar) set up a greater possibility of separating colonies, as growth of individual cells happens away from other colonies as the sample is diluted and mixed into a solid matrix. Two important considerations are significant in this process for selecting chemolitothrophs. The first concern is that the solid physical barrier represented by the agar may also provide an organic carbon source that can be metabolized by agarolytic microorganisms present in the sample, if any. Therefore, the growth of exclusively chemoautotrophs cannot be assumed under these conditions. The second concern is the temperature required for the agar to stay in liquid state for inoculation. The elevated temperature of the media could change the microbial habitat so much, that it could have inactivated or killed the microorganisms targeted for this study. *In situ* growth

temperature for the targeted chemoautotrophs is likely ± 20˚C, as they grow at mid and deeper waters in the Basin. On the other hand, optimal temperature for agar to be kept in liquid state is ± 40˚C. Unless thiosulfate disproportionates or sulfur oxidizers developing in the Cariaco Basin's redoxcline are able to form spores or can survive these temperatures, it seems likely that this technique is not the most appropriate for this system.

Dilution to extinction is an appropriate method for simplifying the complexity of microbial communities as the growth continuously comes from a smaller fraction of the initial community. However, this is a long process as more passages are required to validate purity and the growth rate of microorganisms is so slow that delays the process takes at least 1 or 2 months. This technique represents a good initial approach for cultivation; however, it needs to be complemented by a molecular tool that gives rapid results about the status of the isolation as more than twenty successive serial dilution passages need to be done before the isolation point, representing at least two years into the growing and serial dilution transferring process.

Continuous cultivation was the most successful approach used in pursuit of isolates among the three methods performed. Bioreactors provide one of the few environments that can be a fully controlled experiment for testing microbial growth. The chemostat represents a basic ecosystem were thiosulfate chemoautotrophs are the main objective and growth rates, nutrient supply and uptake can be controlled and measured (Smith and Waltman, 1995). In the 22 day period that the chemostat was run for the cultivation of thiosulfate disproportionaters, the mixed culture became significantly less complex in the

amount of microorganisms that appeared as a decreased in the amount of terminal fragments from the original sample and yielded a simpler community, as shown in electropherograms. Anaerobic bireactors are artificial growing environments that must be set up with extreme care and concern about concentration of nutrients, turnover rates, gas composition, pH and cell numbers among other characteristic. This project specifically, implemented an anaerobic bioreactor that was initially purged with an oxygen-free gas mixture. The use of oxygen-free gas was thought to be enough to maintain the anoxic environment in the reservoir and bioreactor. However; anoxic conditions were not tested over time to make sure that thiosulfate was not reacting with oxygen traces or that oxygen could have leaked inside the vessels (reservoir and bioreactor). The possibilities of thiosulfate being abiotically oxidized may explain why its concentrations in both the reservoir and the bioreactor were smaller than the amount provided at the beginning.

T-RFLP electropherogram interpretation must be done with caution as PCR reactions can introduce bias in the amplification process (Crosby and Criddle, 2003). Another common misinterpretation happens if the T-RFLP method accounts only for those dominant species disregarding the less common but important microorganisms present in the sample that could be significant in the anoxic disproportionation or oxidation processes. By the employment of T-RFLP as a molecular technique, it is possible to recover community structures even to genus level. However, more than 3 restriction endonucleases must be used for the digest process in order to obtain higher accuracy in the retrieved information. Another factor that usually causes misleading results is the production of pseudo-terminal fragments as a consequence of improper or incomplete

digest with endonucleases (Clement et al., 2003; Marsh et al., 2000). Constructing clone libraries along with T-RFLP analyses would yield much more information (expected TRFs), than can be retrieved by terminal fragments alone. However, constructing 16S rDNA clone libraries is more laborious, time-consuming and expensive (DeSantis et al., 2003). A 16S rDNA library was developed from CAR-132 (305, 320 and 340 m) samples that were considered potential isolates at the initial stages of my research. This analysis yielded a small database and a phylogenetic tree that showed the presence multiple ribotypes (Appendix I, Fig. 25) samples. In this case, the construction of a 16S rDNA library was not the most suitable technique. The 16S rDNA tree was constructed using the Neighbor-Joining method and Jukes-Cantor algorithms and the percentage of replicate trees represented the associated taxa clustered together using the bootstrap test. The phylogenetic tree showed that the Cariaco isolations were still mixed communities of different microbial species. These sequences shared high level of identity and some were closely affiliated with a *Marine bacterium* BS12. The 16S rDNA library also showed the presence of *Pseudoalteromonas sp.* indicating an evident enrichment of this heterotrophic group where only autotrophs were intentionally enriched for as the target microorganisms. The presence of heterotrophic microorganisms in the cultures implied that some organic content may have been introduced or released into the selective "minimal organic" media. Possible sources of organic carbon could have been the lysis of cells from the community growing in culture, the ability to digest agar in the solid media used for these samples. After those observations, I prepared a new selective media with filtered and UV oxidized Cariaco seawater. The organic content of the new media was found to be 340 μMC of dissolved organic carbon, which was higher than expected.

Previous studies in the Cariaco Basin have evaluated inorganic carbon assimilation response to additions of sulfur species, including $S_2O_3^{2-}$, S^0 and SO_3^{2-}. Amendment experiments have been done in order to assess their role of sulfur species in the chemoautotrophic metabolism. In general, thiosulfate seems to stimulate chemoautotrophy more in the deeper portions of the redoxcline (Li et al., 2009). Thiosulfate appears to be the most stimulating substrate of the two sulfur species leading to the hypothesis that its uptake is favored for chemolithotrophs residing in the basin's redoxcline (Fig. 9 and 10). The role of thiosulfate metabolizers is also consistent with persistent elevations in MPN abundance estimates, as numbers reached highest concentrations for sulfur utilizing bacteria in the redox transition zone (Morris et al. 1985) (Fig. 7, 8). Increases in the assimilation of dissolved inorganic carbon were more pronounced when thiosulfate was spiked into chemoautotrophic incubations than in the case of S^0. One explanation for this preference is a midwater enrichment of thiosulfate concentrations which suggests its availability as energy source. The CAR-139 cruise also exhibited higher MPN estimations whenever thiosulfate concentrations were lower in the redoxcline suggesting its exhaustion possibly by the microbial uptake. Thiosulfate exhaustion and availability could also be explained by the existence of defined microbial communities residing at different depths in the redoxcline, with microbial communities in the upper anoxic waters which are not the same as the ones developing at shallow depths in the redoxcline. Microbial communities residing in the redoxcline can be active but also subject to variation from one depth to the next (Lin et al., 2007). Similarly, in the redoxcline higher MPN estimations coincide with lower S^0 concentrations. Cells capable of both types of metabolisms, anoxic thiosulfate disproportionation and sulfur oxidation,

accounted for a small percentage of the total prokaryotic numbers at specific depths within the chemocline (≈ 1%). However, their importance for this ecosystem is yet to be determined as isolation in the laboratory becomes a reality.

VII. Conclusion and recommendations

The study was able to enrich and describe terminal fragments from microorganisms that reside in the Cariaco Basin redoxcline. However, isolation was not accomplished in this process. Little is known about the possible causes that could have interfered with the isolation process that have led to a pure culture. Community linked metabolism and interdependent processes (syntrophy) are the most likely reasons for failed isolation.

Continuous cultivation was the most efficient method used for enriching thiosulfate disproportionaters as it gave the lower terminal fragments in a sample after 22 days in the run. This process should be considered as the main approach for future research if the experiment can be run for at least one month continuously. Prolongation of continuous cultures must be the main focus of future experiments as it showed the most efficient way to reduce microbial diversity for anaerobic disproportionating thiosulfate cultures. However, a sterile control run under truly anoxic conditions must be performed before a new set of isolation with microorganisms is attempted. A control set up would be beneficial in monitoring continuous anoxic conditions as well as other possible abiotic reactions of the nutrients within the reservoir and/or the bioreactor. Careful set up and maintenance of an oxygen-free environment is key element in the isolation process of specific sulfur metabolizers from the Cariaco Basin's redoxcline.

Further research needs to be done in order to understand the role and importance of these still unknown prokaryotes prevailing and metabolizing sulfur in the Cariaco's redoxcline.

VIII. Figures

Figure 1. Location of the Cariaco Basin site in the Caribbean Sea, position of the CARIACO time series station (circle) and location of the sub-basins (ovals)

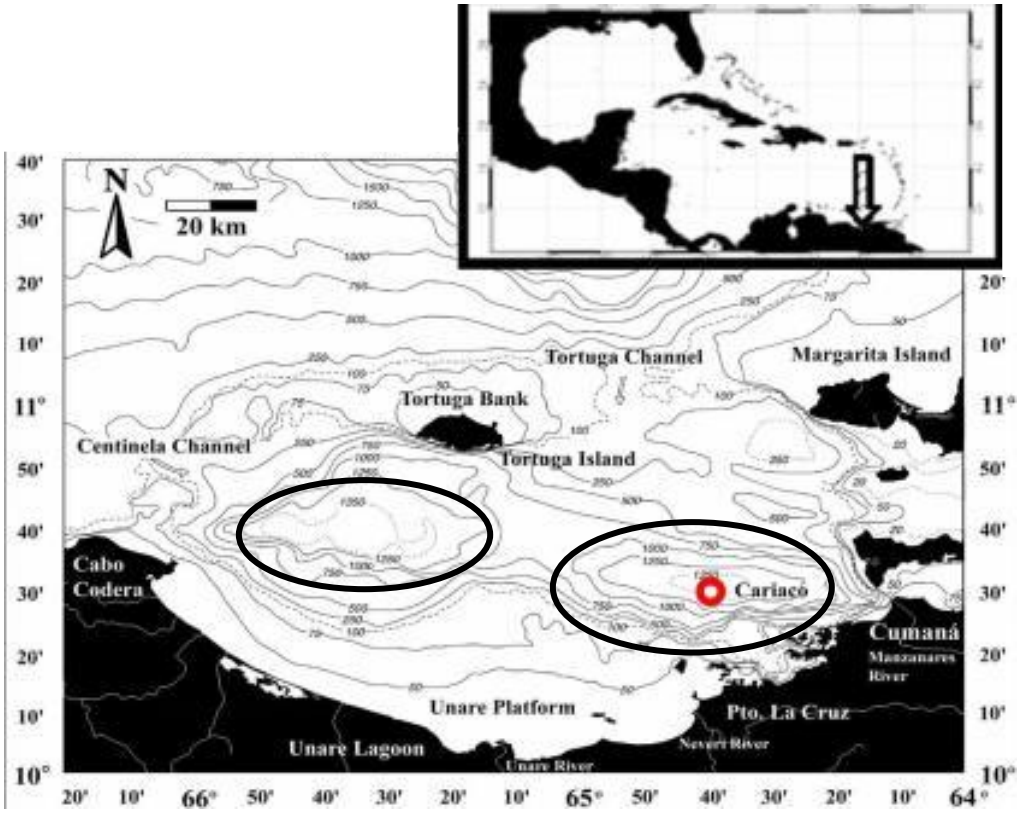

N
20 km
Tortuga Channel
Margarita Island
Tortuga Bank
Centinela Channel
Tortuga Island
Cabo
Codera
Cariaco
Cumaná
Manzanares
River
Unare Platform
Pto. La Cruz
Unare Lagoon
Unare River
Neverí River
40'
30'
20'
10'
11°
50'
10°
66°
65°
64°

Figure 2. Vertical distributions of physical and chemical properties of the water column during CAR-145 cruise (21-22 May 2008) at the Time Series Station. a) Potential temperature (θ), Salinity (S), water density (σ_θ). b) Concentration of dissolved oxygen and H_2S (μM)

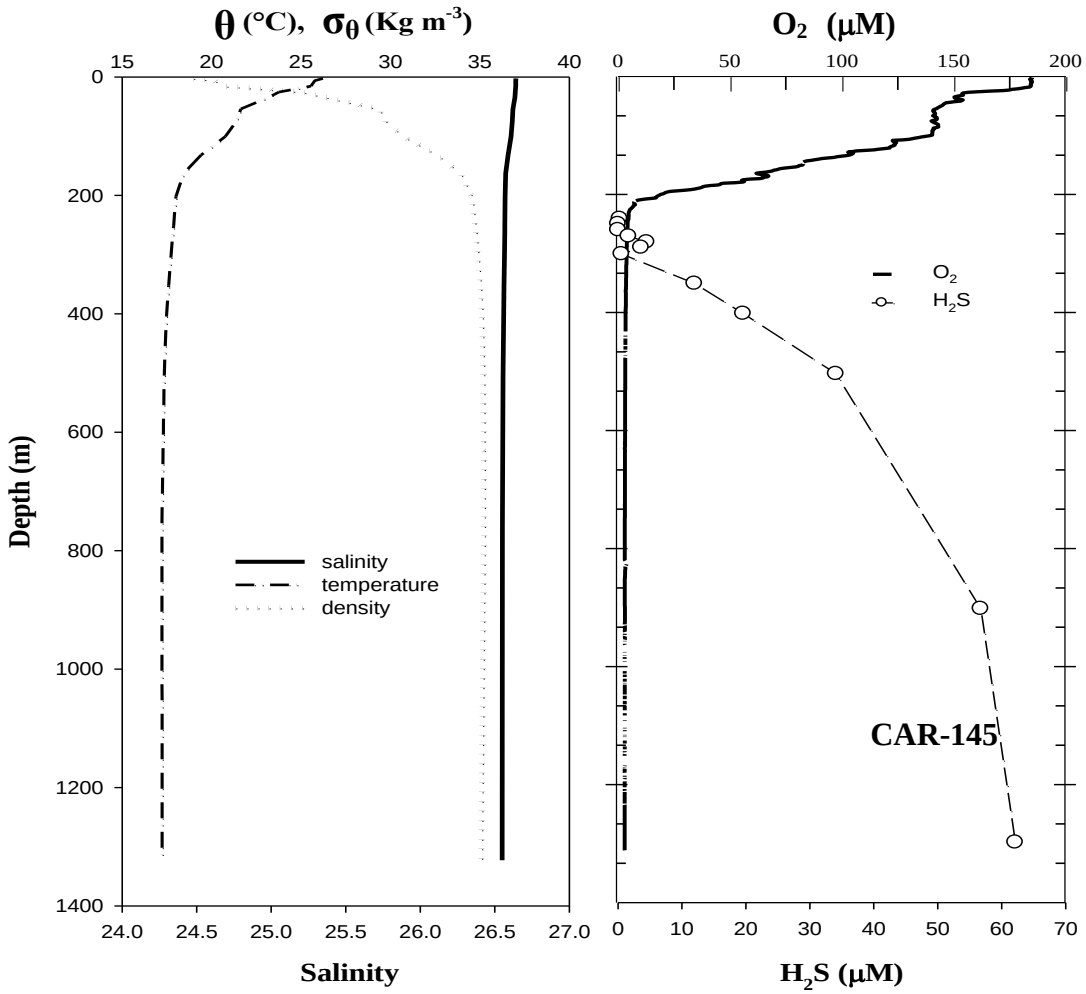

θ (°C), σθ (Kg m-3)
O2 (μM)
Depth (m)
salinity
temperature
density
O2
H2S
CAR-145
Salinity
H2S (μM)

Figure 3. Vertical distributions of a) elemental sulfur and thiosulfate concentrations and b) dark inorganic carbon assimilation with and without sulfur species amendments within the Cariaco Basin water redoxcline during CAR-145 (21-22 May 2008) (courtesy of G.T. Taylor). Note that EGME (Ethylene Glycol Monomethyl Ether) was used to sulubilized S^0 and thus a separate control was needed.

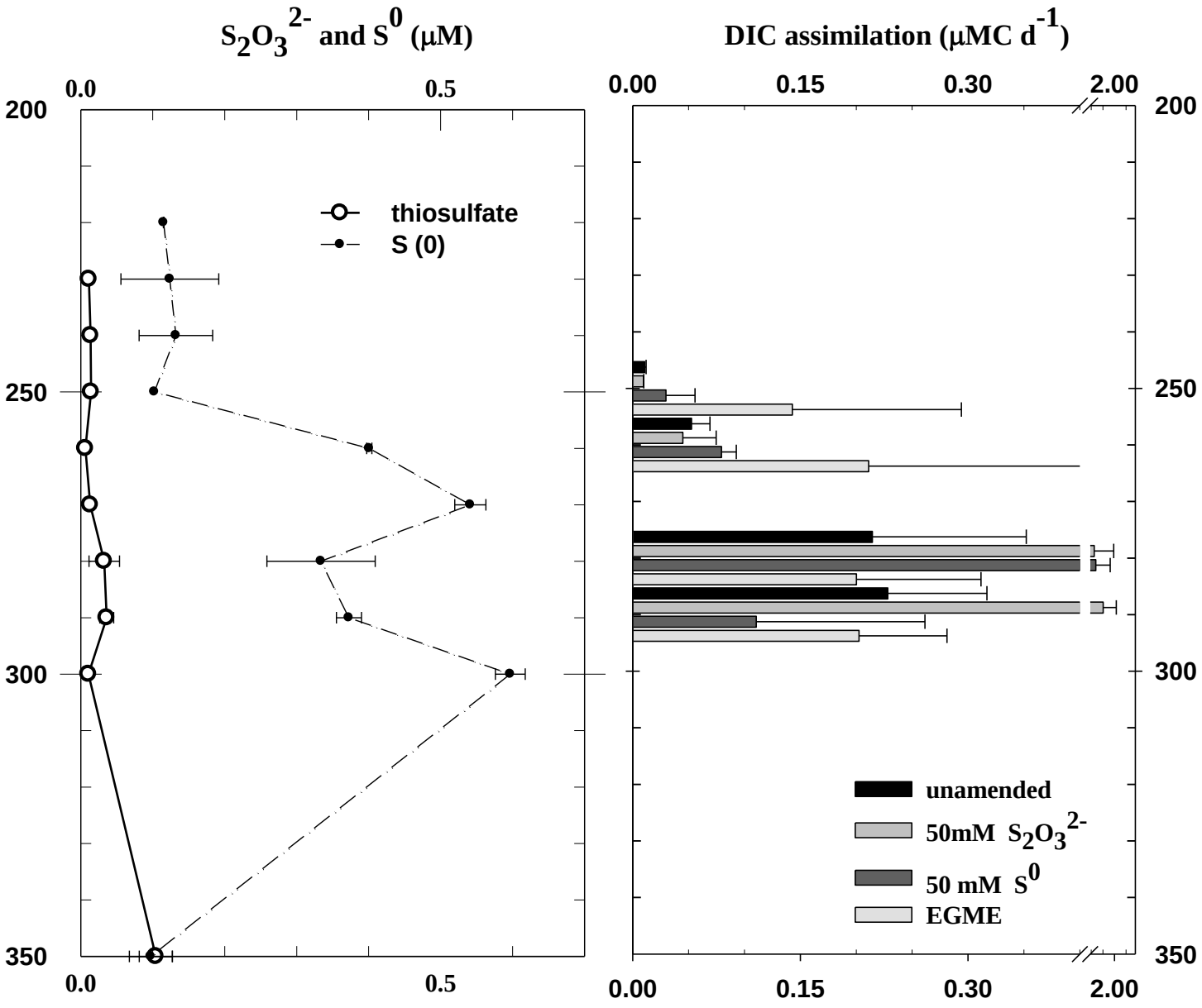

$S_2O_3^{2-}$ and S^0 (μM)
DIC assimilation (μMC d^{-1})
0.0
0.5
0.00
0.15
0.30
2.00
200
250
300
350
thiosulfate
S (0)
unamended
50mM $S_2O_3^{2-}$
50 mM S^0
EGME

Figure 4. Anaerobic chemostat set up in the laboratory for continuous cultivation of thiosulfate disproportionating chemoautotrophs as described by Strous et al. (2002)

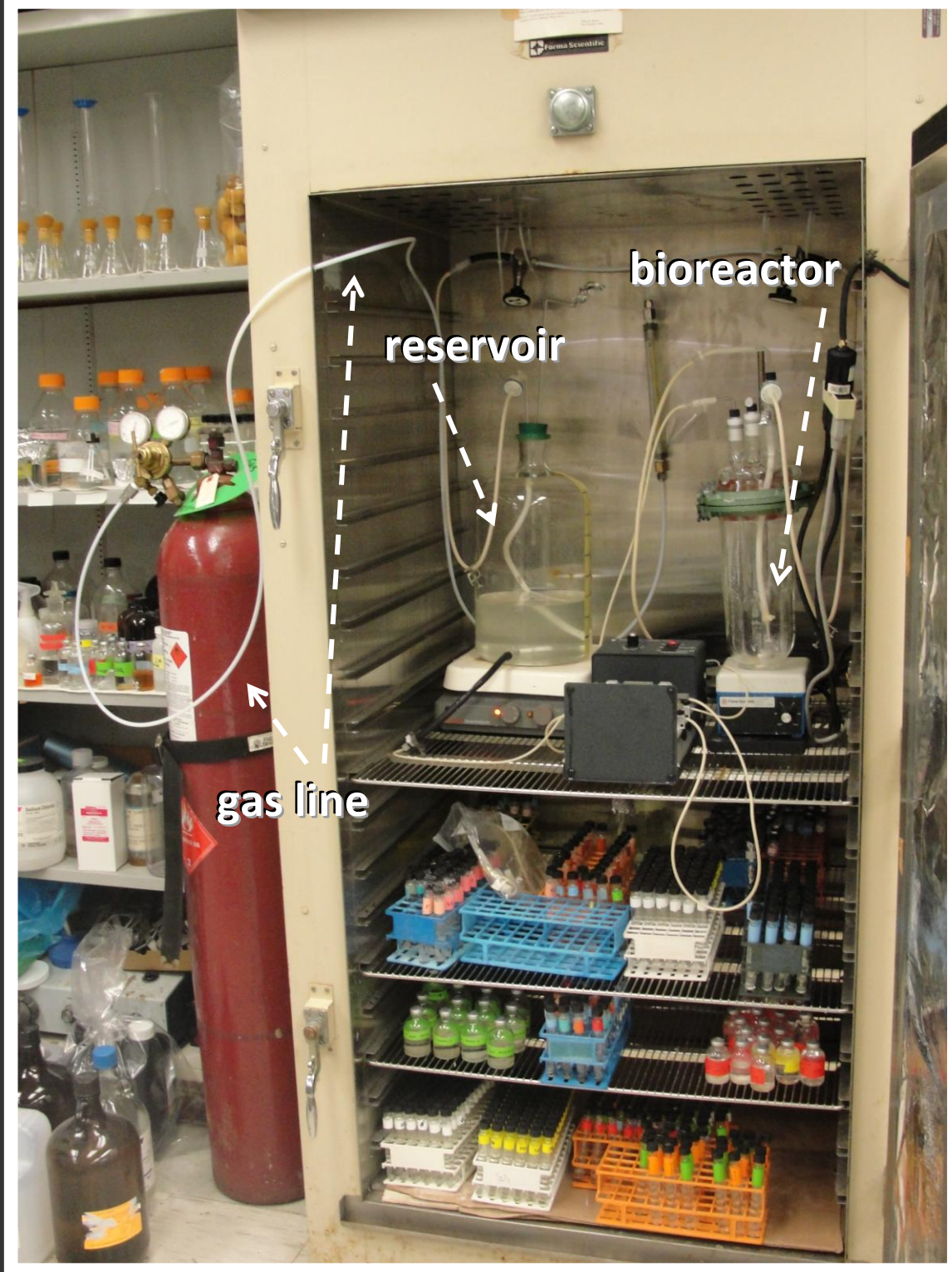

bioreactor
reservoir
gas line

Figure 5. Schematic diagram of Terminal Restriction Fragment Length Polymorphism (TRFLP) protocol. (1) DNA isolation from bacterial culture; (2) PCR 16S rRNA gene amplification using fluorescent primer; (3) DNA digestion reaction by restriction endonucleases; (4) Capillary electrophoresis; (5) Data analysis. (reprinted from Liu,W-T. et al., 1997)

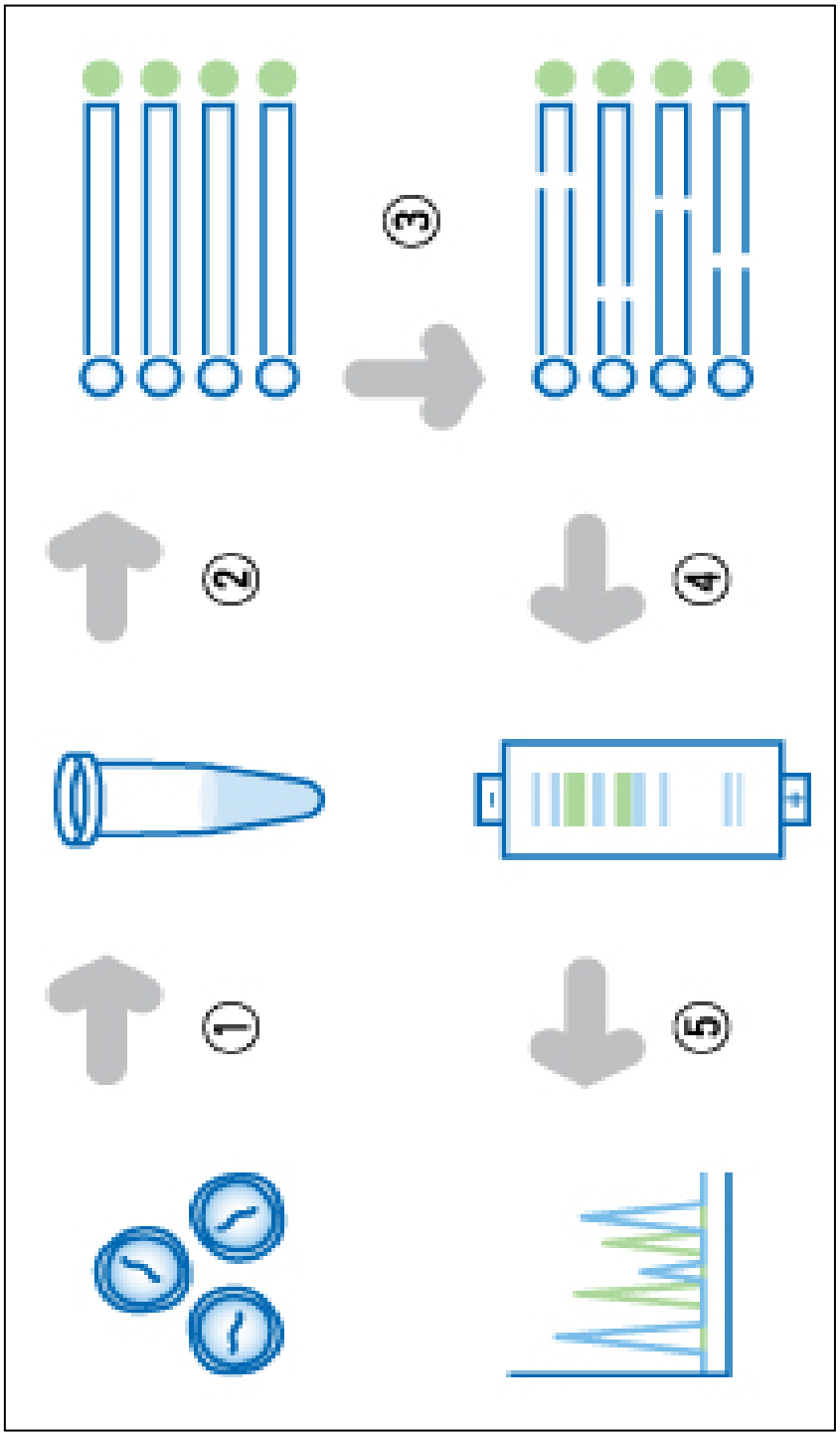

3
2
4
1
5

Figure 6. Example of serial dilution in elemental sulfur oxidizing media showing positive growth in five of ten dilutions of sample from 305 m during CAR-132 (11-12 April 2007)

Figure 7. Elemental sulfur, sulfite and thiosulfate concentrations (courtesy of X. Li) (a, d); Most probable number (MPN) estimations for elemental sulfur and thiosulfate utilizing bacteria (b, e); Total prokaryotes counts in the Cariaco Basin's water column (c, f) during CAR-132 (11-12 April 2007) and CAR-139 (30 Nov-01 Dec 2007)

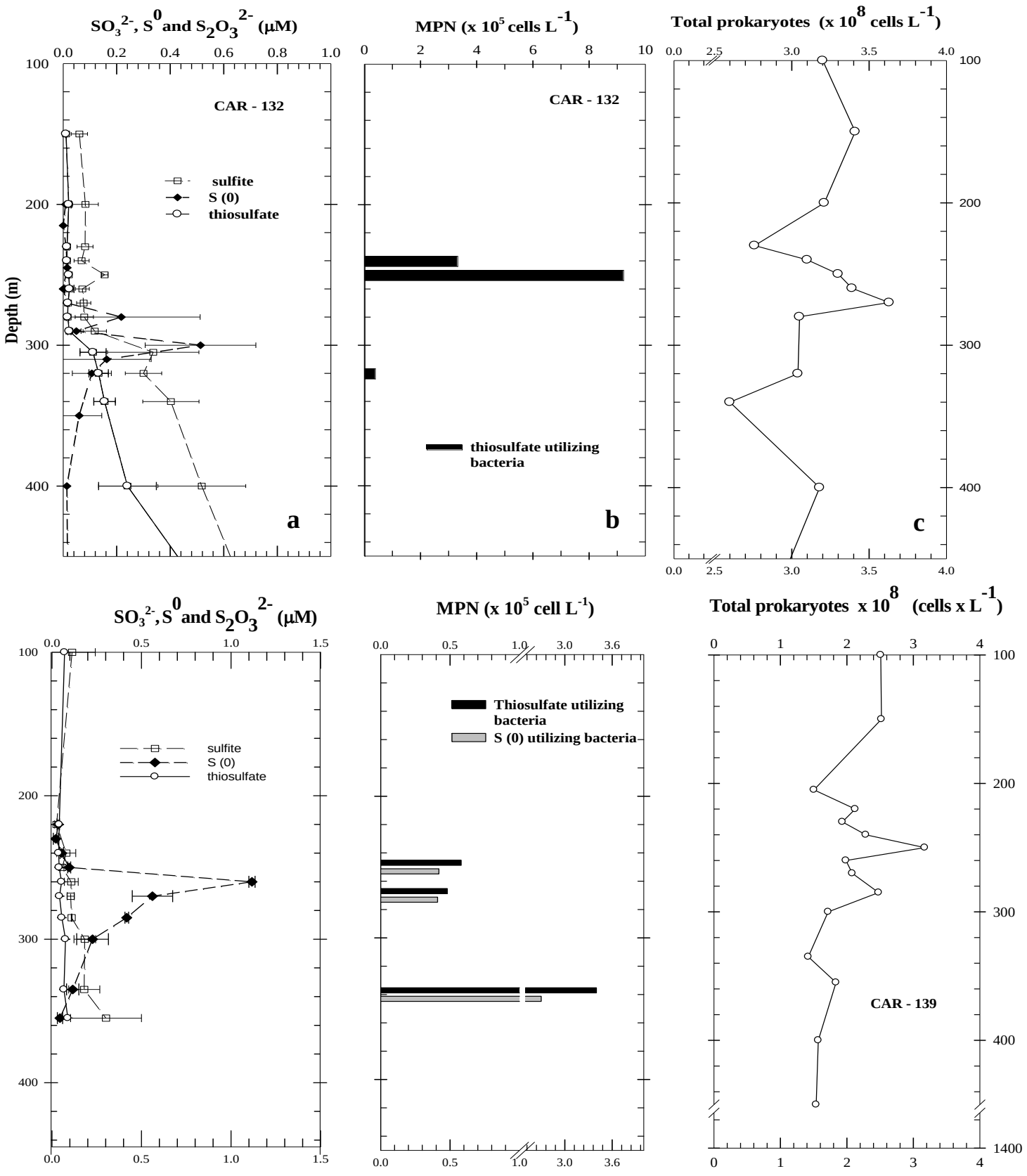
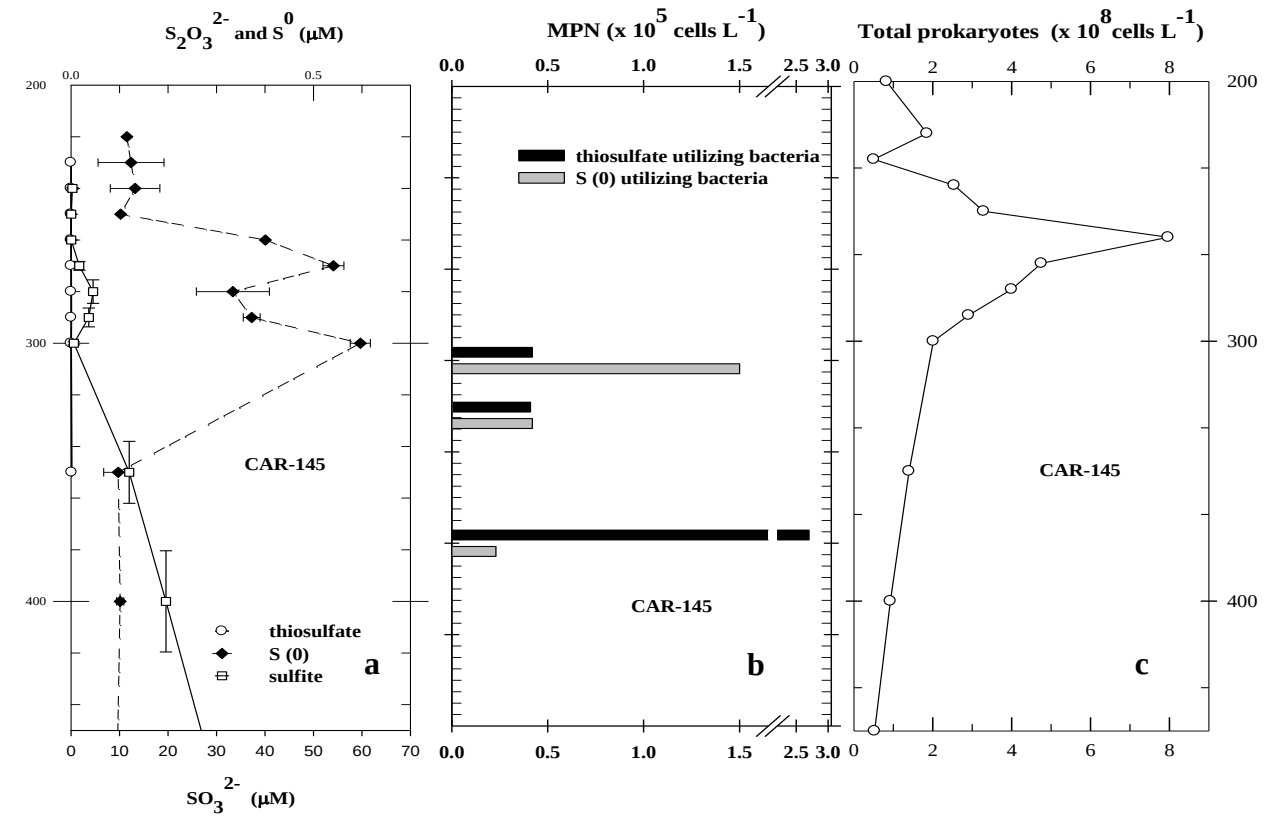

SO3 2-, S0 and S2O3 2- (µM)
MPN (x 10^5 cells L^-1)
Total prokaryotes (x 10^8 cells L^-1)
Depth (m)
CAR - 132
sulfite
S (0)
thiosulfate
thiosulfate utilizing bacteria
a
b
c
SO3 2-, S0 and S2O3 2- (µM)
MPN (x 10^5 cell L^-1)
Total prokaryotes x 10^8 (cells x L^-1)
Thiosulfate utilizing bacteria
S (0) utilizing bacteria
CAR - 139

Figure 8. Elemental sulfur, sulfite and thiosulfate concentrations (courtesy of X. Li) (a, d); Most probable number (MPN) estimations for elemental sulfur and thiosulfate utilizing bacteria (b, e); Total prokaryotes counts in the Cariaco Basin's water column (c, f) during CAR-145 (22-23 May 2008) and CAR-153 (19-20 January 2009)


$S_2O_3^{2-}$ and S^0 (μM)
MPN (x 10^5 cells L^{-1})
Total prokaryotes (x 10^8 cells L^{-1})
thiosulfate utilizing bacteria
S (0) utilizing bacteria
CAR-145
thiosulfate
S (0)
sulfite
a
b
c
SO_3^{2-} (μM)

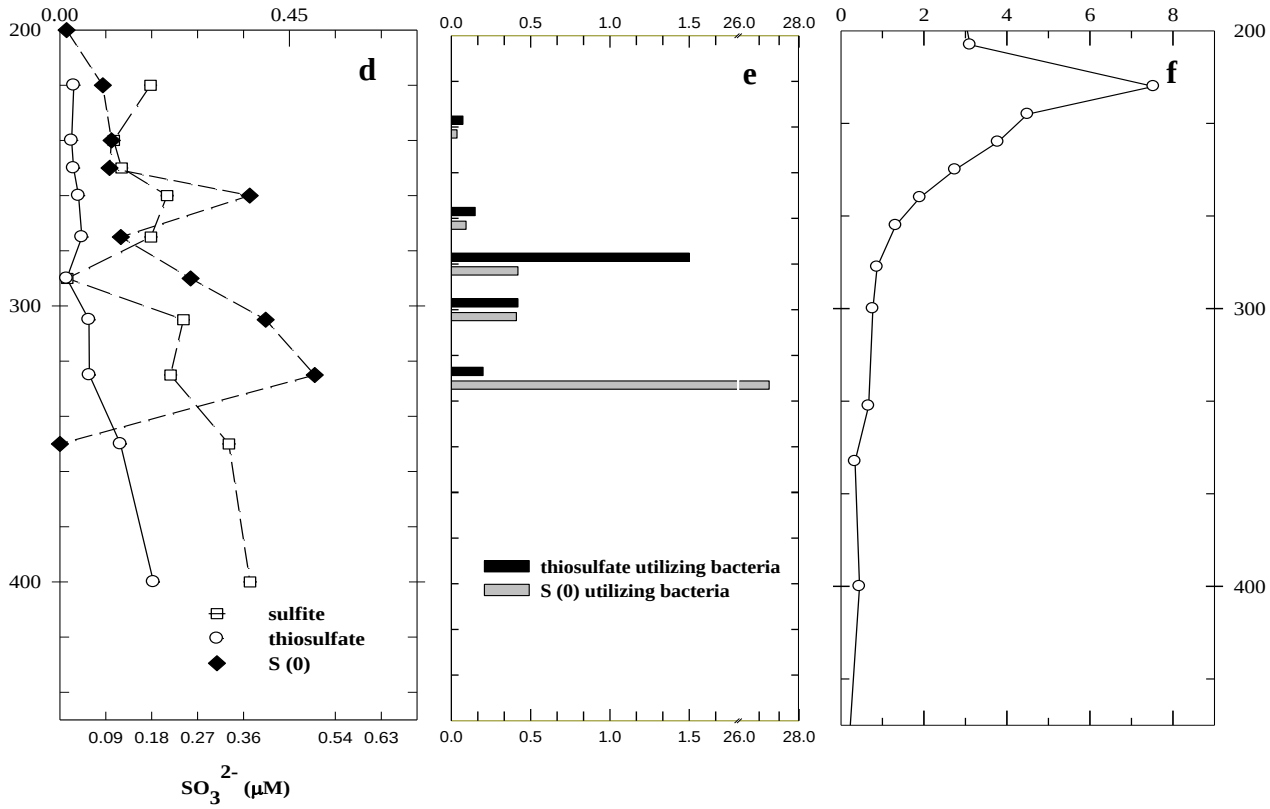

$S_2O_3^{2-}$ and S^0 (μM)
MPN (x 10^5 cell L^{-1})
Total prokaryotes (x10^8 cells L^{-1})
d
e
f
thiosulfate utilizing bacteria
S (0) utilizing bacteria
sulfite
thiosulfate
S (0)
SO_3^{2-} (μM)

Figure 9. a) Verical profiles of heterotrophic and chemoautotrophic bacterial production during CAR-132 (11-12 April 2007) and CAR-139 (30 Nov. to 01 Dec 2007) (a,c). Effects of 50 μM $S_2O_3^{2-}$ and S^0 amendments on chemoautotrophic production during same cruises (b,d)

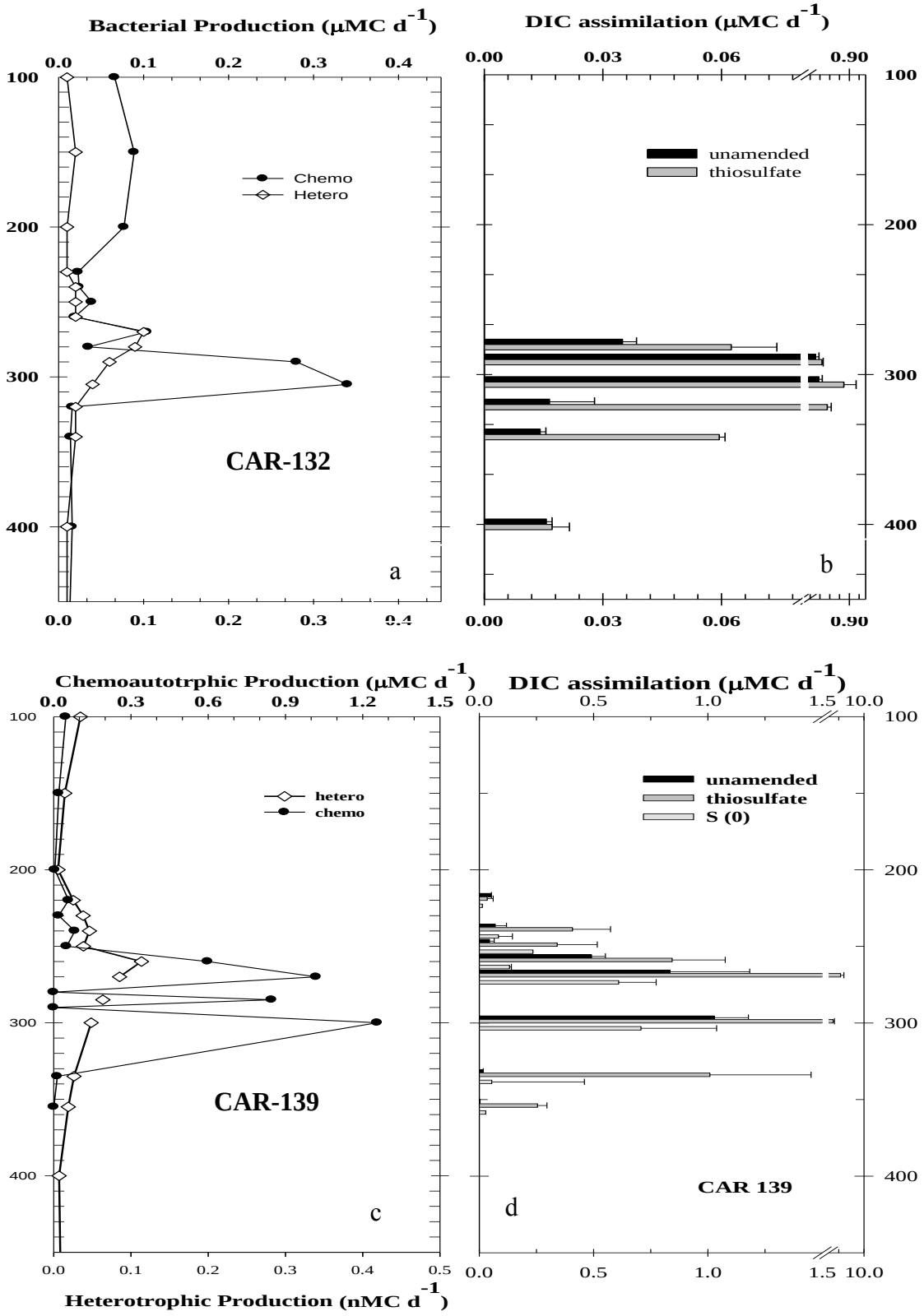
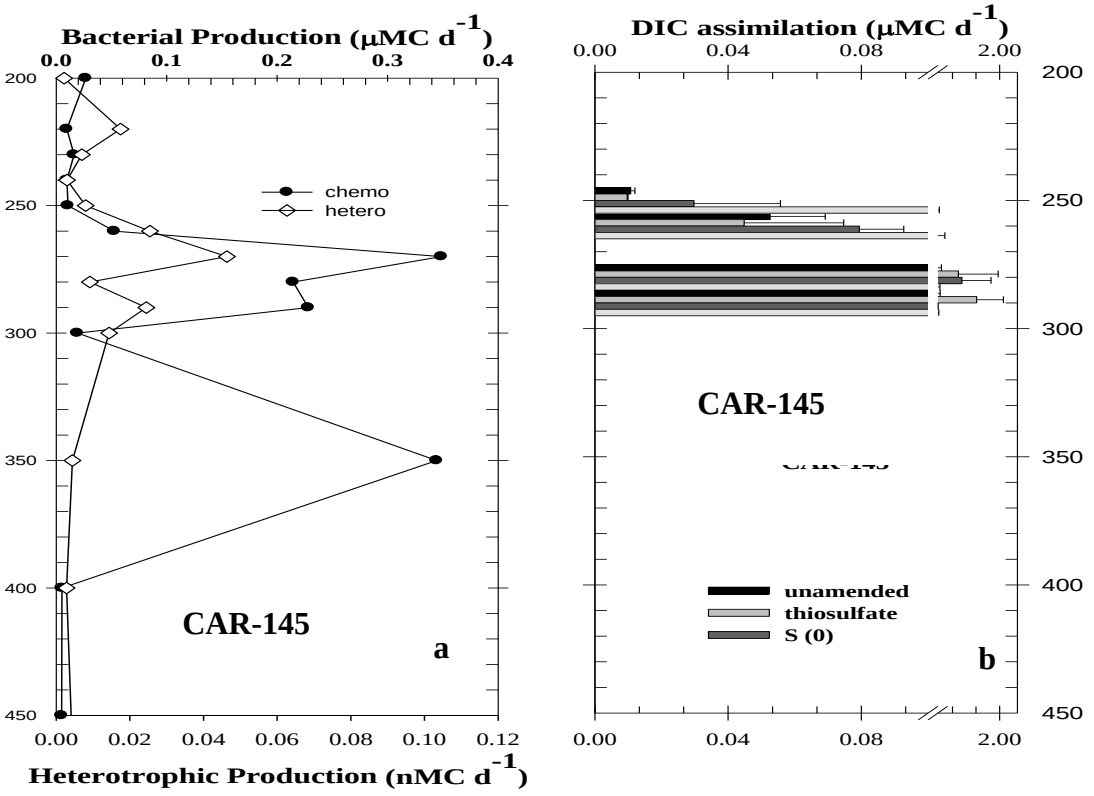

Bacterial Production (μMC d-1)
DIC assimilation (μMC d-1)
Chemo
Hetero
CAR-132
a
unamended
thiosulfate
b
Chemoautotrphic Production (μMC d-1)
hetero
chemo
CAR-139
c
DIC assimilation (μMC d-1)
unamended
thiosulfate
S (0)
CAR 139
d
Heterotrophic Production (nMC d-1)

Figure 10. Verical profiles of heterotrophic and chemoautotrophic bacterial production during CAR-145 (22-23 May 2008) and CAR-153 (19-20 January 2009) (a,c). Effects of 50 μM $S_2O_3^{2-}$ and S^0 amendments on chemoautotrophic production during same cruises (b, d)


Bacterial Production (µMC d-1)
0.0
0.1
0.2
0.3
0.4
200
250
300
350
400
450
chemo
hetero
CAR-145
a
0.00
0.02
0.04
0.06
0.08
0.10
0.12
Heterotrophic Production (nMC d-1)
DIC assimilation (µMC d-1)
0.00
0.04
0.08
2.00
CAR-145
unamended
thiosulfate
S (0)
b

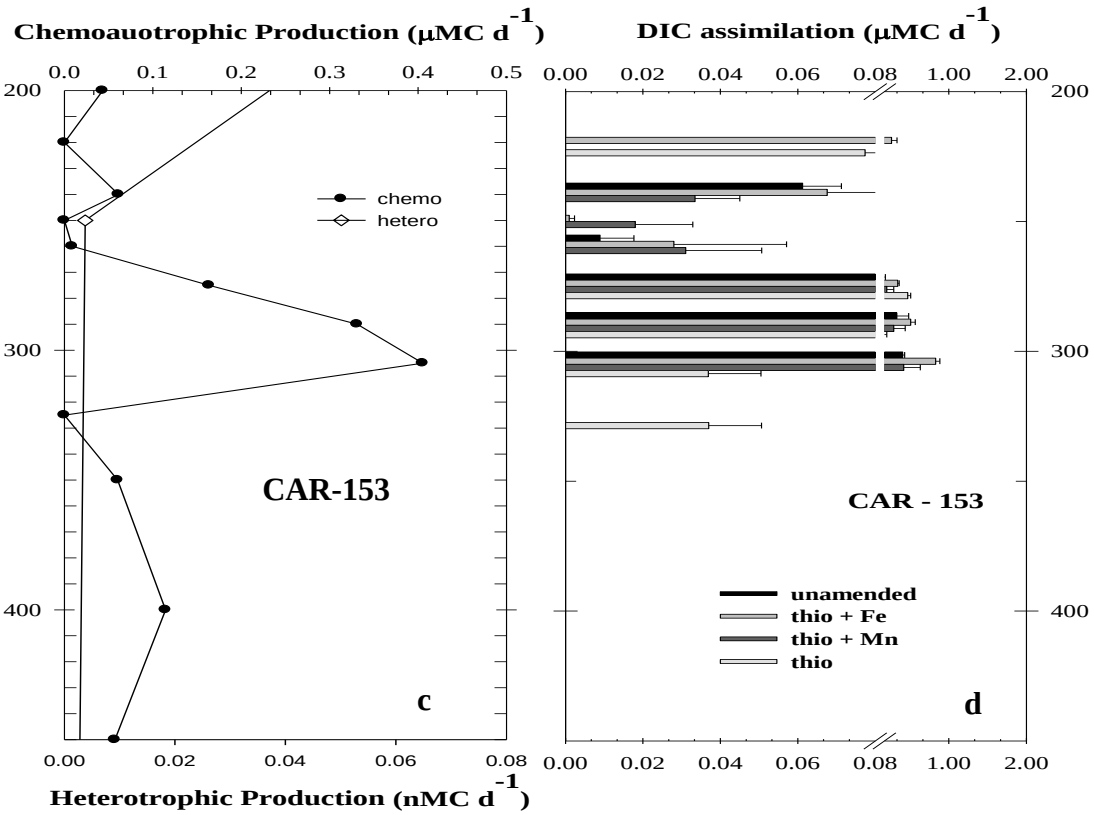
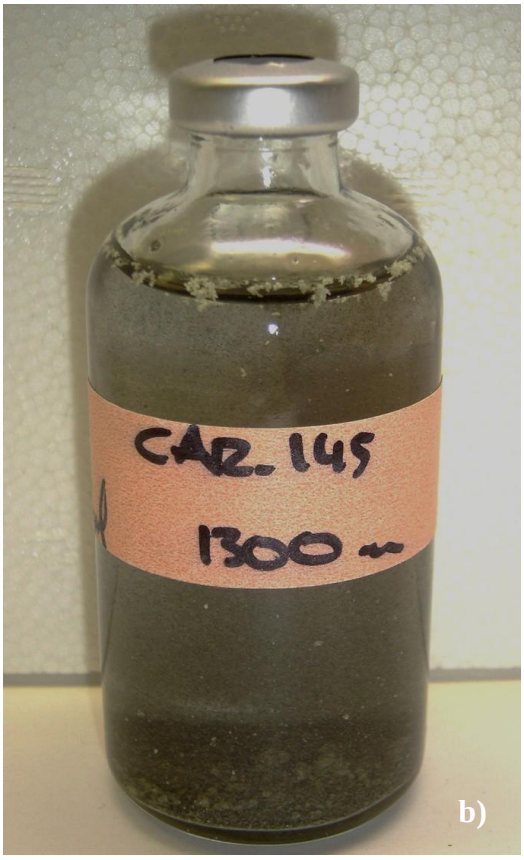

Chemoauotrophic Production (µMC d-1)
0.0
0.1
0.2
0.3
0.4
0.5
200
300
400
chemo
hetero
CAR-153
c
0.00
0.02
0.04
0.06
0.08
Heterotrophic Production (nMC d-1)
DIC assimilation (µMC d-1)
0.00
0.02
0.04
0.06
0.08
1.00
2.00
CAR - 153
unamended
thio + Fe
thio + Mn
thio
d

Figure. 11. Examples of enrichment cultures for elemental sulfur oxidizing bacteria from 1300 m during CAR-145 in high volume bottles. Cultures were inoculated under anoxic atmosphere with a $H:CO_2:N_2$ (10:2:88) mixture headspace for 6 weeks

a)



b)

Figure 12. Bacterial morphologies in an elemental sulfur enrichment after 16 isolation passages (300m, CAR-139).

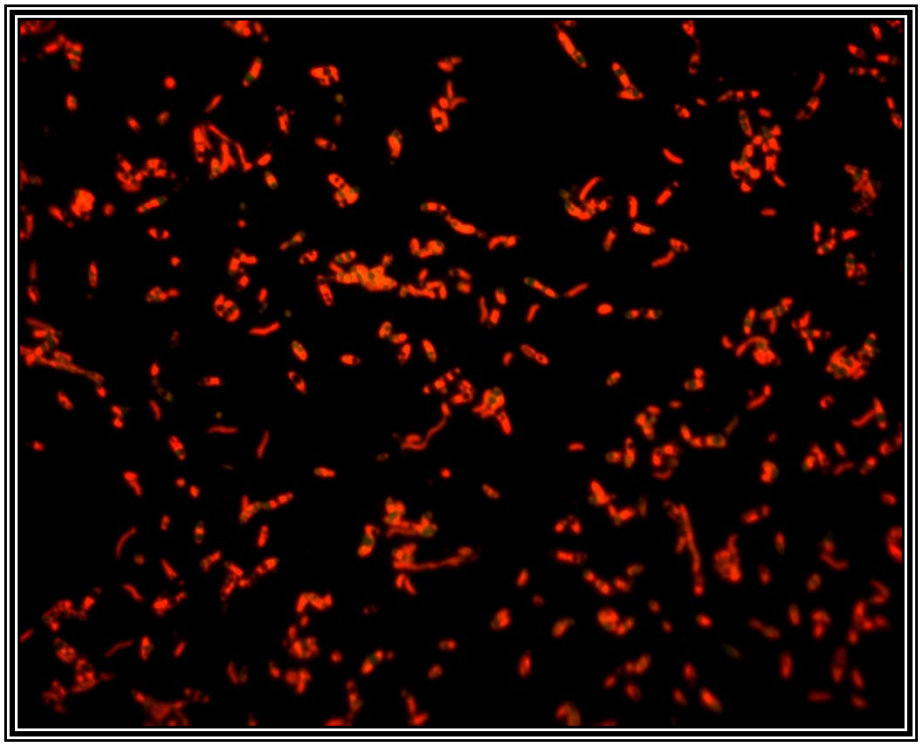


Figure 13. Example of serial dilution for thiosulfate disproportionating microorganisms from 340 m during CAR-132 (11-12 April 2007). Black residue indicates the formation of FeS by the microorganisms growing in the culture after 6 week of incubation.

Figure 14. Bacterial morphologies in thiosulfate enrichment culture from 230m, CAR-139 (30 Nov to 01 Dec, 2007) after 5 isolation passages. Circles represent different morphologies within the same sample, coccoid and sulfur bacilli.

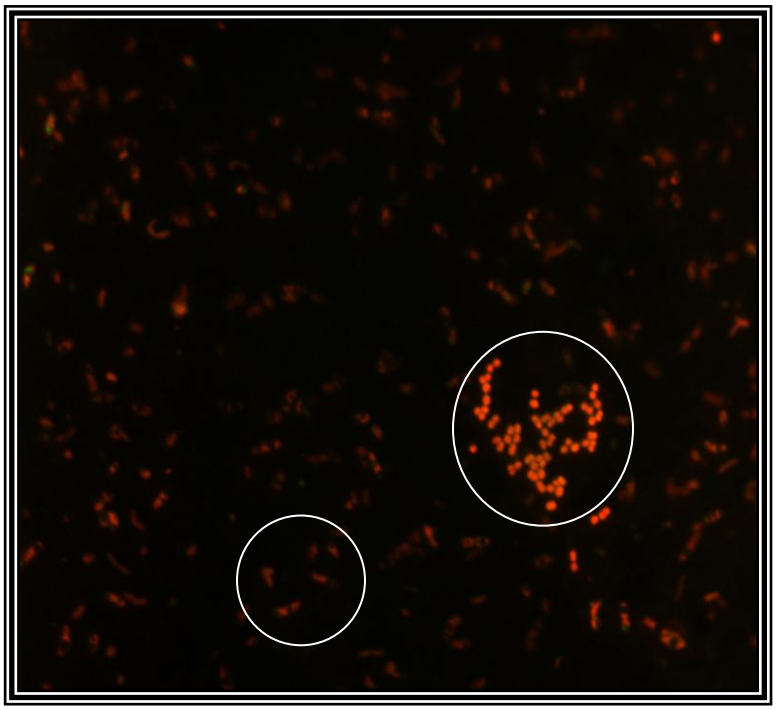


Figure 15. Example of deep agar dilutions from 305 m during CAR-132 (11-12 April 2007) for enriched cultures of S^0 oxidizing microorganisms. a) Deep agar dilution series (10 dilutions). b) Detail of single bacterial colony in the deep agar. c) Confluent bacterial growth in agar.

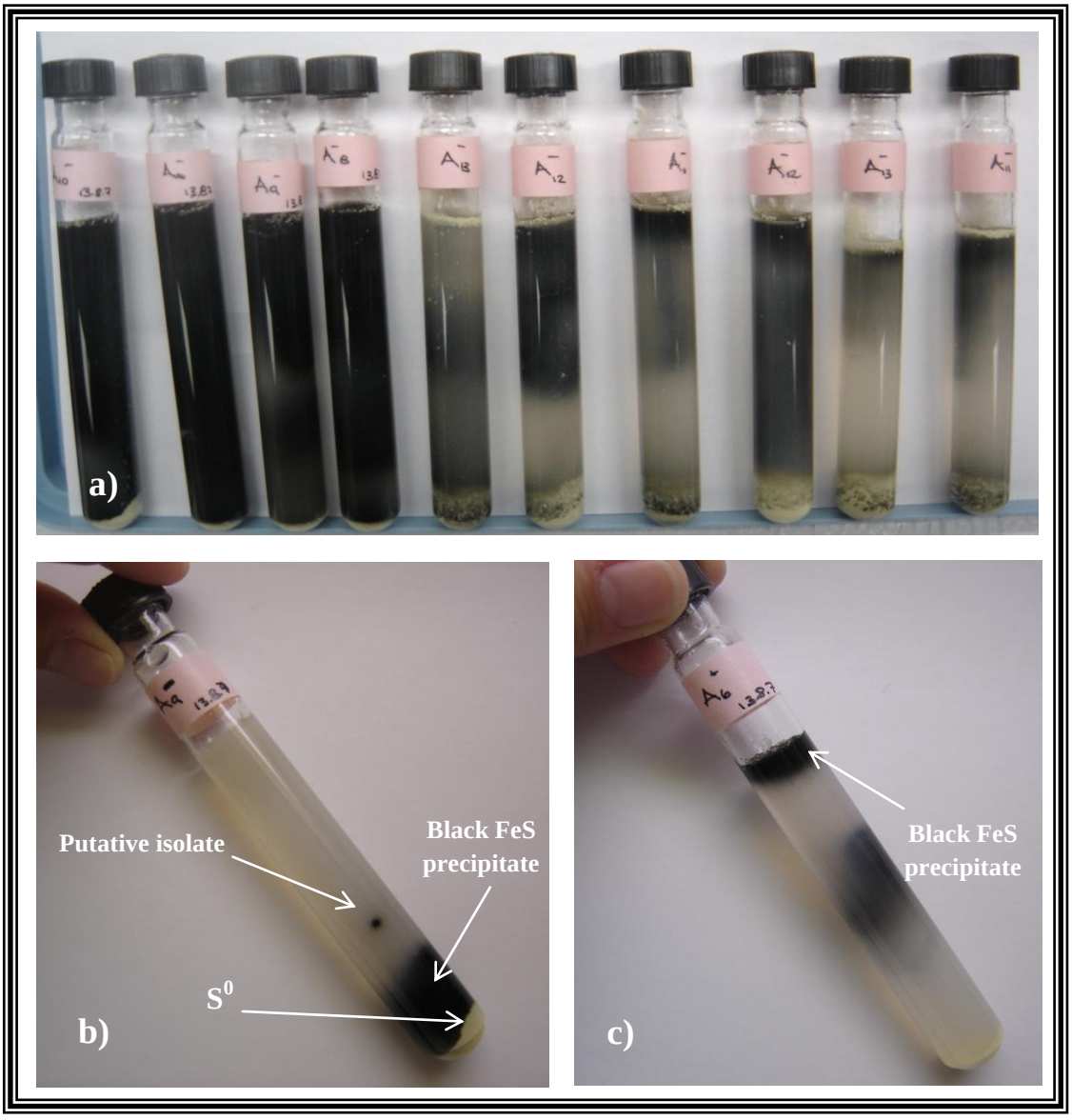

a)
Putative isolate
Black FeS precipitate
S^0
b)
Black FeS precipitate
c)

Figure 16. Terminal restriction fragments length polymorphisms (T-RFLP) electropherograms showing 16S rDNA fingerprint of S^0 oxidizing cultures after successive dilutions to extinction. a) Terminal restriction fragments (T-RF) remaining after eleven series of dilutions to extinction of sample originating from CAR-139 at 300m after four series of dilutions to extinction, b) Terminal restriction fragments (T-RF) remaining after eleven series of dilutions to extinction of sample originating from 280 m during CAR-145 after eight series of dilutions to extinction. DNA fragments are shown in the horizontal axes in base pairs after *Alu*I endonuclease and *Msp*I endonuclease digests. Fuschia shading represents peaks that showed a higher fluorescence signal than the rest of the peaks in the electropherograms.

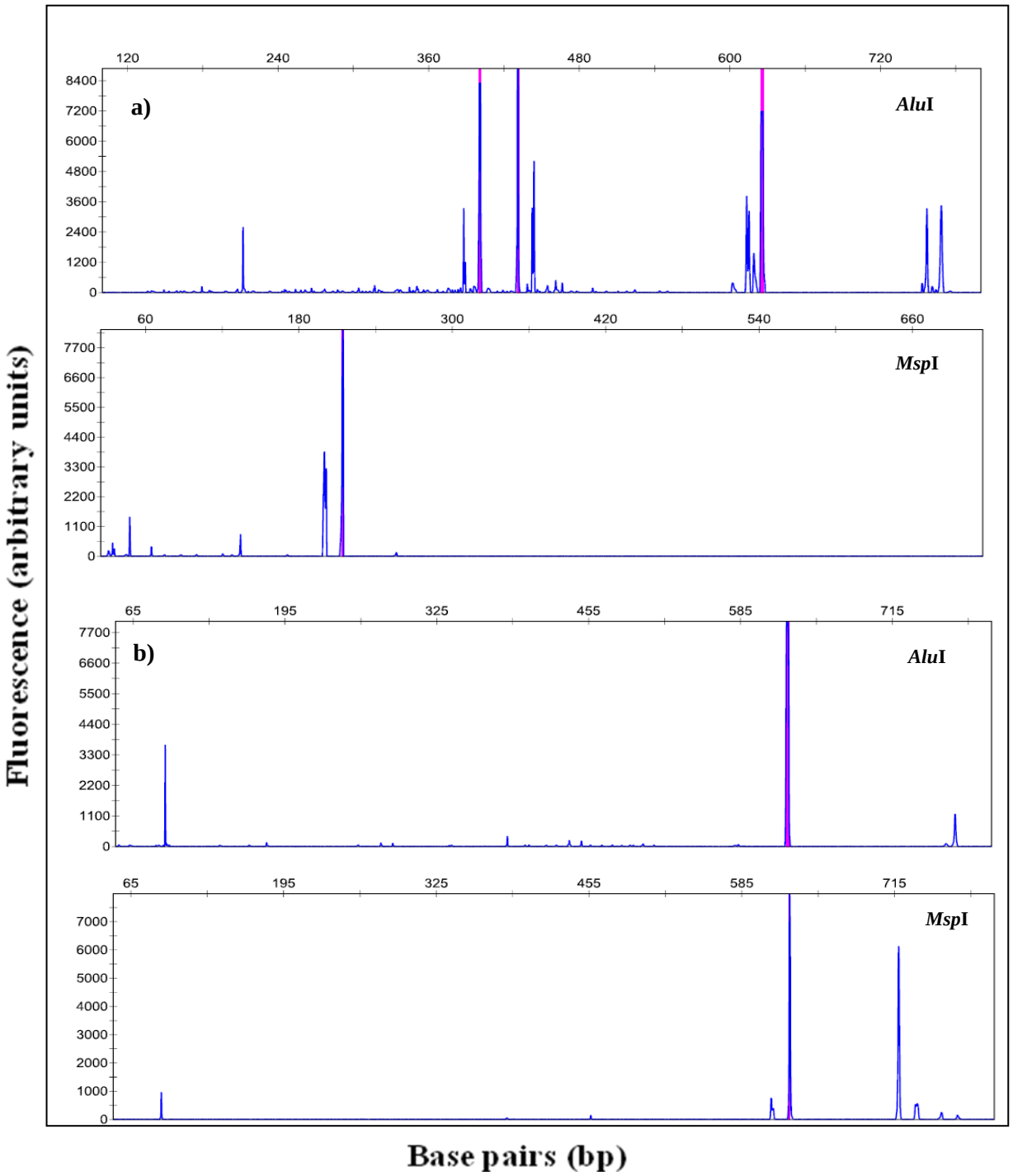

a)
AluI
MspI
b)
AluI
MspI
Fluorescence (arbitrary units)
Base pairs (bp)

Figure 17. Terminal restriction fragments length polymorphisms (T-RFLP) electropherograms showing 16S rDNA fingerprint of $S_2O_3^{2-}$ disproportionaters after successive series of dilutions to extinction. a) Terminal restriction fragments (T-RF) remaining after eleven series of dilutions to extinction of sample originating from 270 m during CAR-139 cruise b) Terminal restriction fragments (T-RF) remaining after eleven series of dilutions to extinction of sample originating from 280 m during CAR-145 after twenty two dilution to extinction passages. DNA fragments are shown in the horizontal axes in base pairs after digestions with *Alu*I and *Msp*I endonuclease digests. Fuschia shading represents peaks that showed a higher fluorescence signal than the rest of the peaks in the electropherograms.

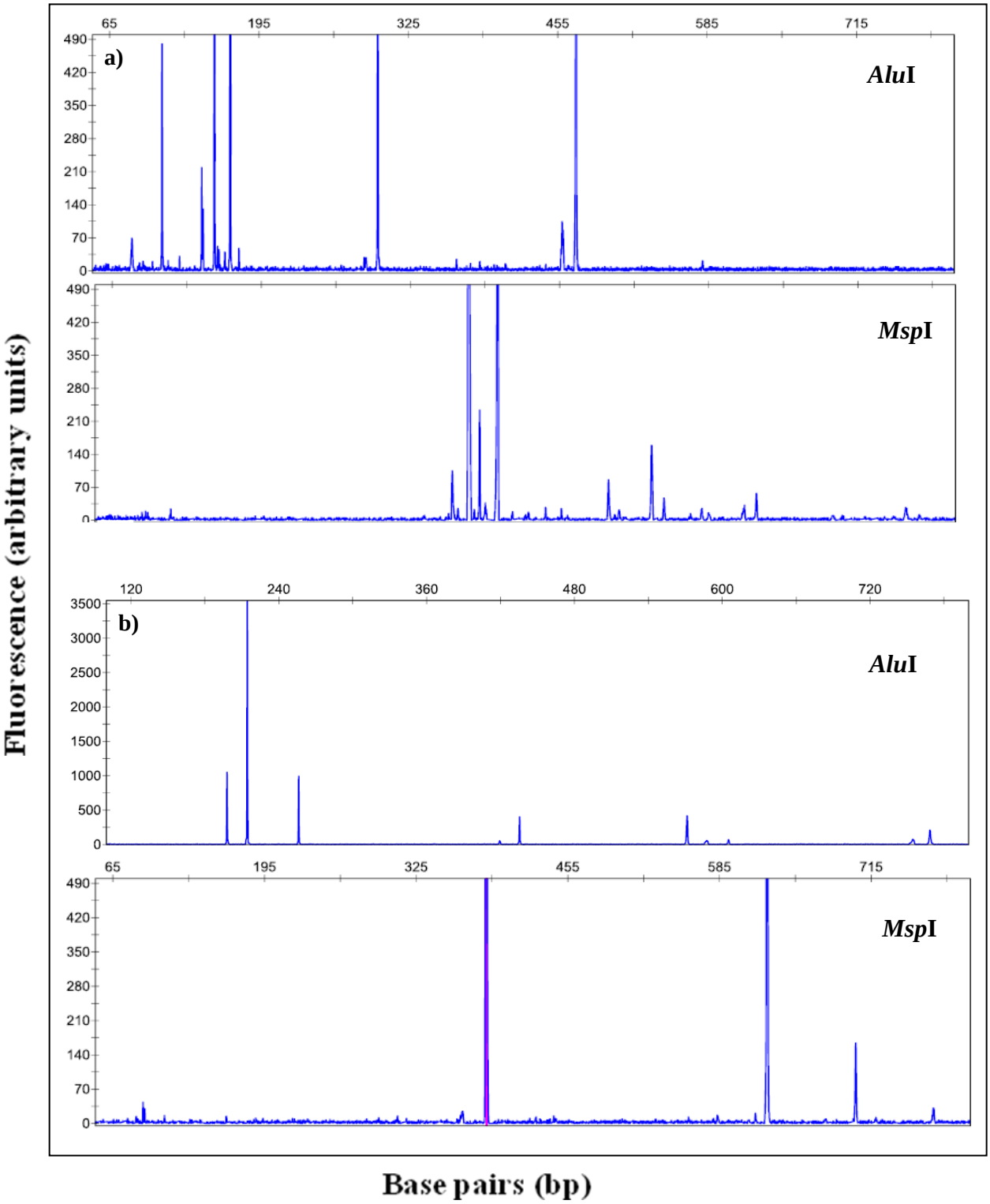

a)
AluI
MspI
b)
AluI
MspI
Fluorescence (arbitrary units)
Base pairs (bp)

Figure 18. Terminal restriction fragments length polymorphisms (T-RFLP) electropherograms showing 16S rDNA fingerprint from S^0 oxidizing cultures after successive dilutions to extinction. a) Terminal restriction fragments (T-RF) remaining after eleven series of dilutions to extinction of sample originating from 230 m during CAR-153 after five dilutions to extinction passages. DNA fragments are shown in the horizontal axes in base pairs after digestions with *Alu*I, *Msp*I and *Rsa*I endonucleases. Fuschia shading represents peaks that showed a higher fluorescence signal than the rest of the peaks in the electropherograms.

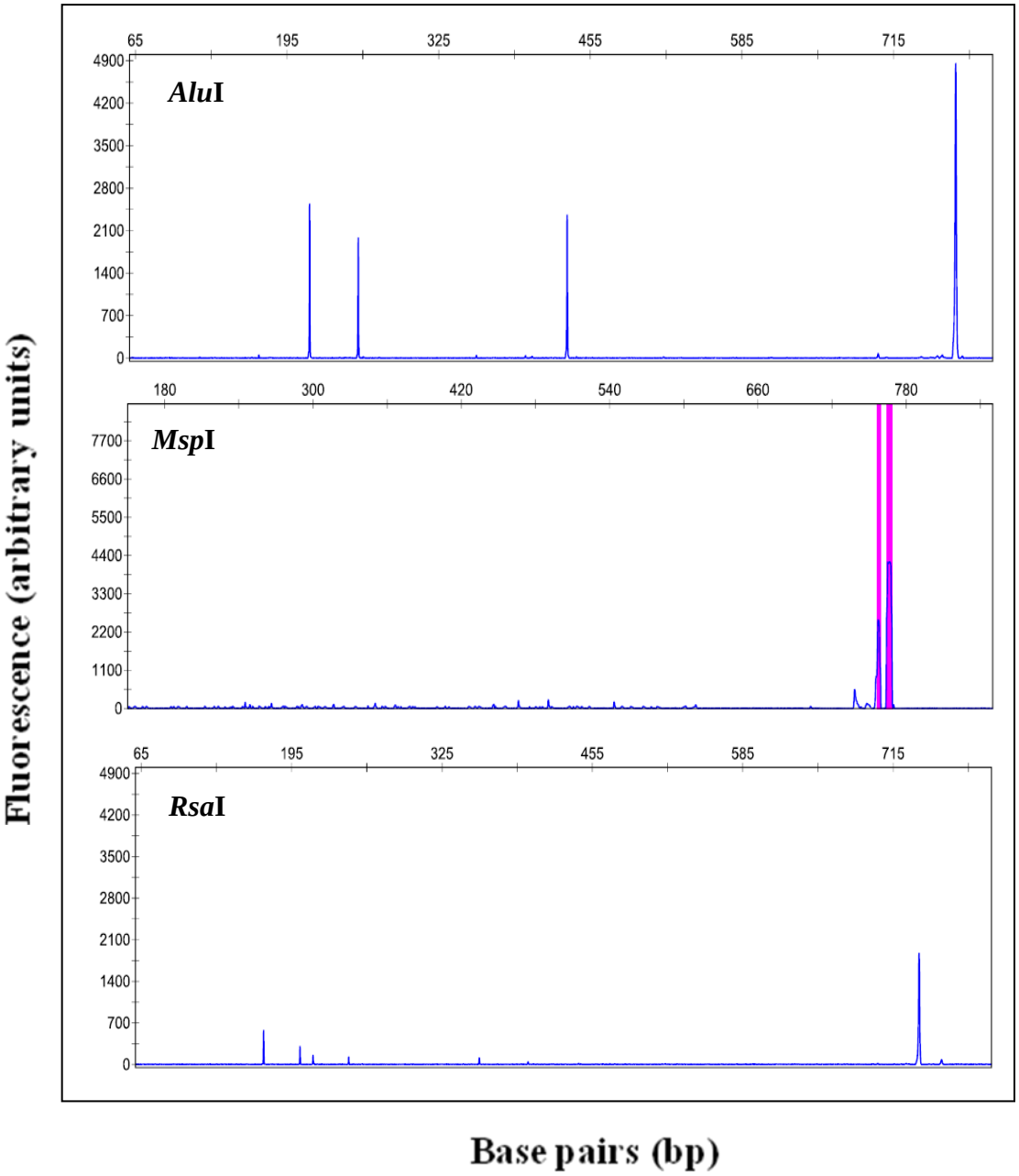

Fluorescence (arbitrary units)
AluI
MspI
RsaI
Base pairs (bp)

Figure 19. Epifluorescent microscopic enumeration of suspended cells in bioreactor culture with thiosulfate as energy source over the 22 days of incubation. Dashed lines indicate change in media dilution rates in the bioreactor

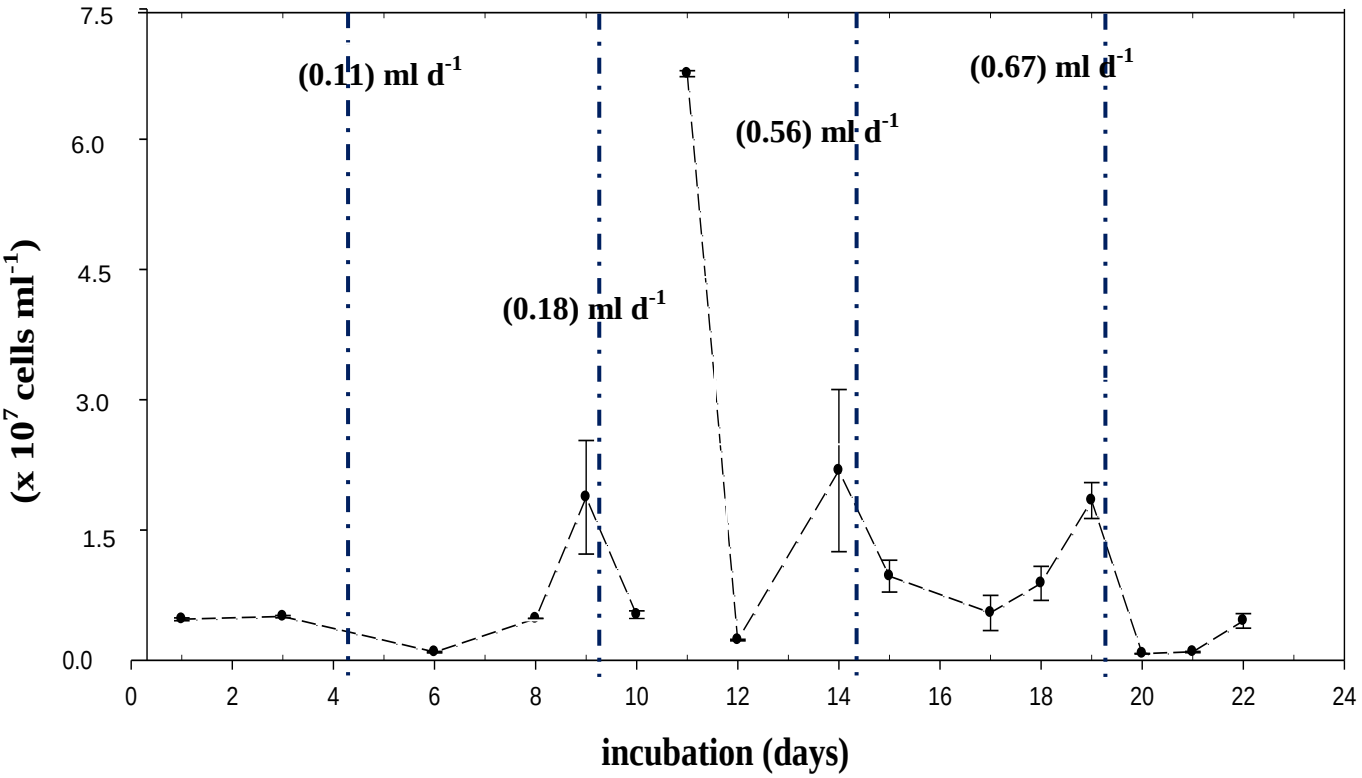

(0.11) ml d-1
(0.18) ml d-1
(0.56) ml d-1
(0.67) ml d-1
(x 10^7 cells ml^-1)
incubation (days)
0.0
1.5
3.0
4.5
6.0
7.5
0
2
4
6
8
10
12
14
16
18
20
22
24

Figure 20. Comparison of cell, particulate organic carbon (POC) and thiosulfate concentrations in the continuous culture apparatus over the course of the experiment. Thiosulfate was the sole energy source added to the reservoir media at 3 mM (final concentration)

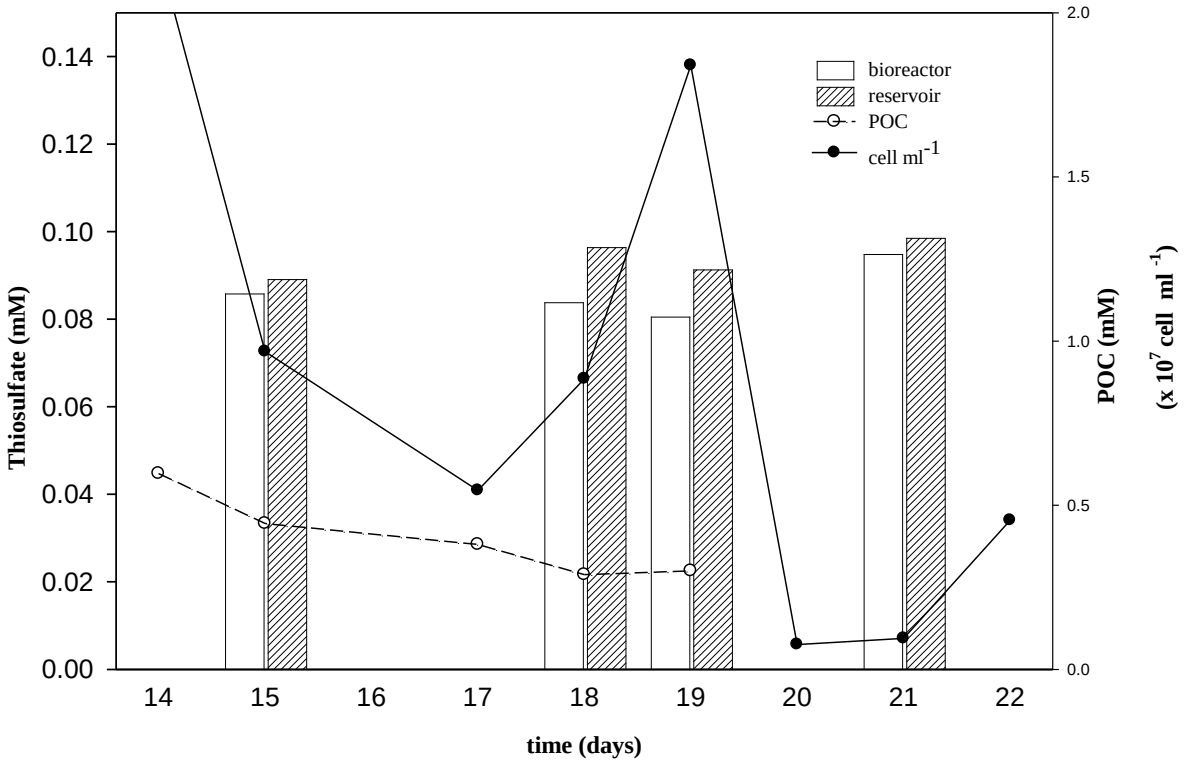

bioreactor
reservoir
POC
cell ml-1
Thiosulfate (mM)
POC (mM)
(x 10^7 cell ml^-1)
time (days)
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.0
0.5
1.0
1.5
2.0
14
15
16
17
18
19
20
21
22

Figure 21. Terminal restriction fragments length polymorphisms (T-RFLP) electropherograms showing 16S rDNA fingerprint of cultures in the bioreactor with thiosulfate as energy source sampled on three different days. a) TRFLP fingerprint after three days of the run (14 march 2009), b) after eleven days of the run (27 april 2009) and c) last day of the run, 20 days total (12 may 2009). DNA fragment length are shown on the horizontal axes as base pairs after *Alu*I digest. Fuschia shading represents peaks that showed a higher fluorescence signal than the rest of the peaks in the electropherograms.

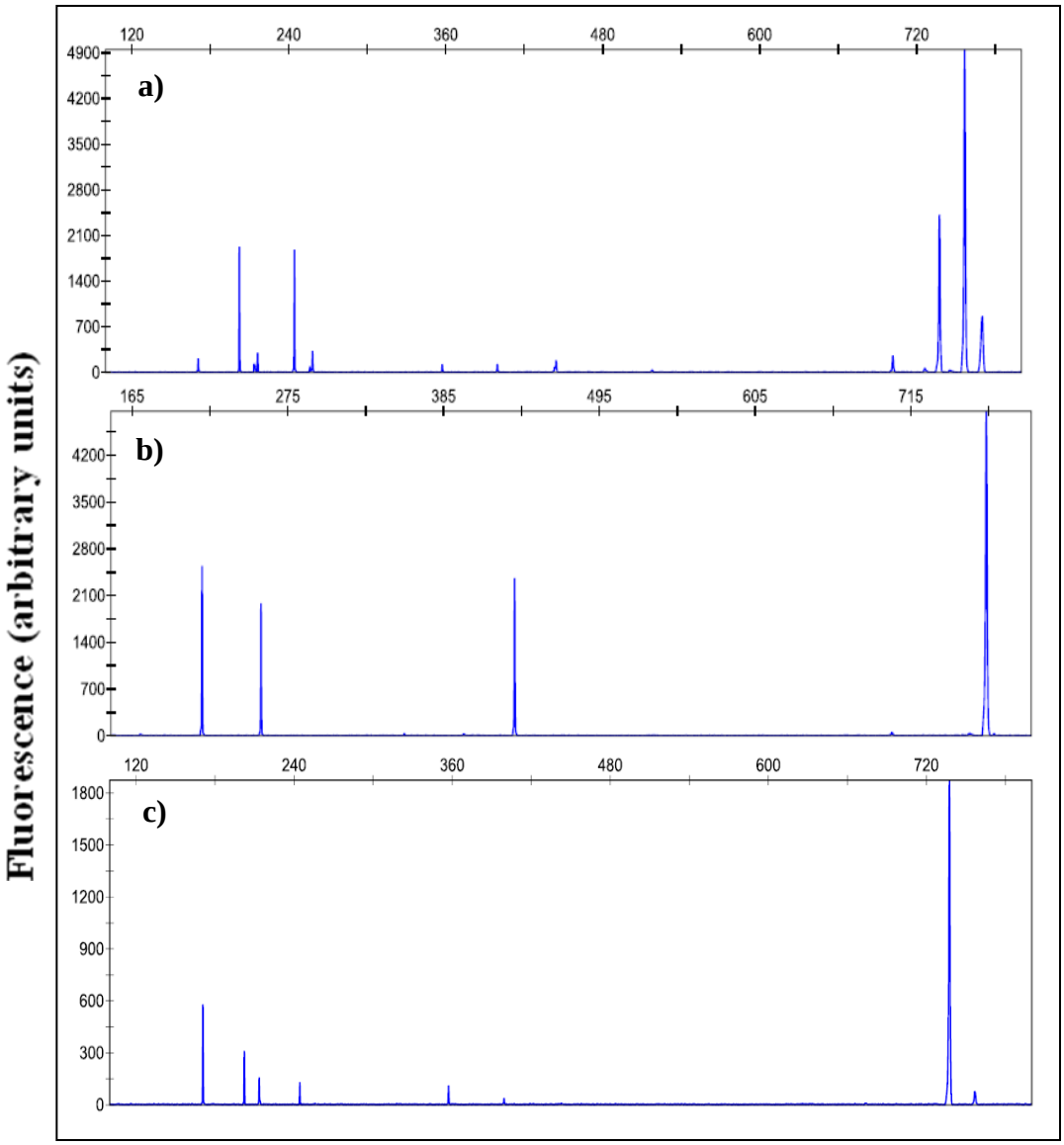

a)
b)
c)
Fluorescence (arbitrary units)
Base pairs (bp)

Figure 22. T-RFLP electropherograms showing 16S rDNA fingerprint of cultures in the bioreactor with thiosulfate as energy source sampled on three different days a) TRFLP fingerprint after three days of the run (14march 2009), b) after eleven days of the run (27 april 2009) and c) last day of the run, 20 days total (may 12 2009). DNA fragments are shown in the horizontal axes in base pairs after *Msp*I digest, Fuschia shading represents peaks that showed a higher fluorescence signal than the rest of the peaks in the electropherograms.

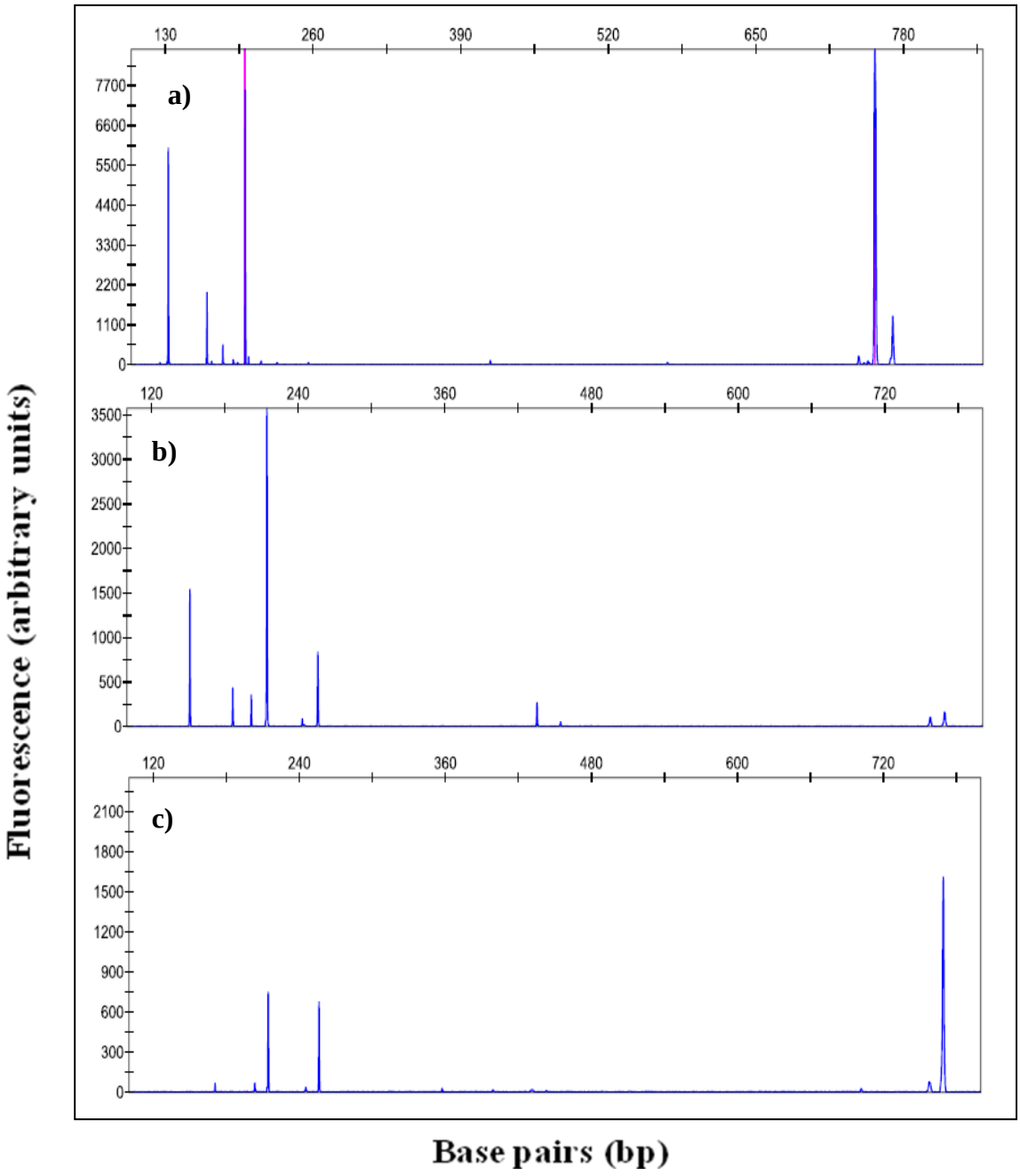
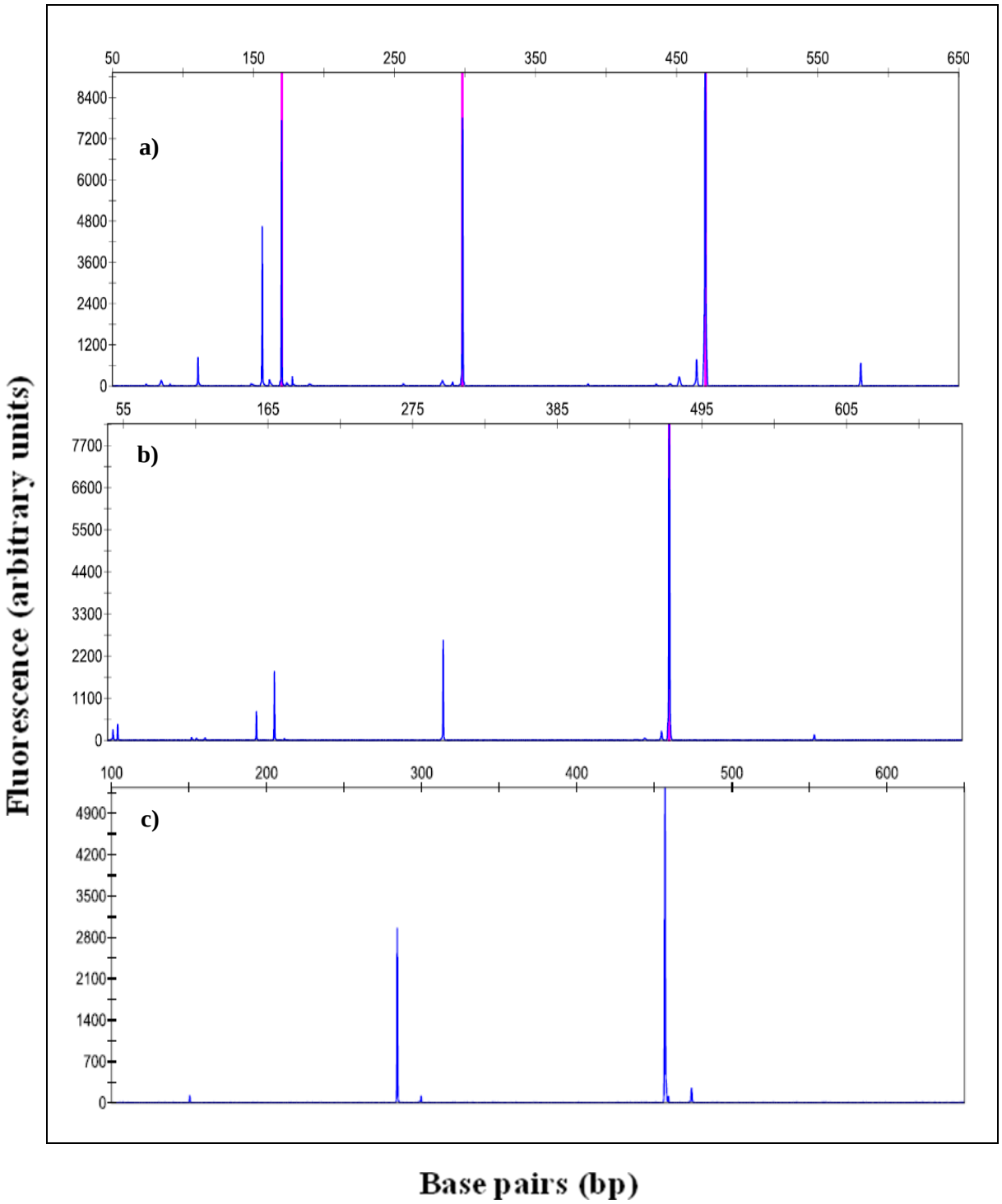

a)
b)
c)
Fluorescence (arbitrary units)
Base pairs (bp)

Figure 23. T-RFLP electropherograms showing 16S rDNA fingerprint of cultures in the bioreactor with thiosulfate as energy source sampled on three different days a) TRFLP fingerprint after three days of the run (14 march 2009), b) after eleven days of the run (27 april 2009) and c) last day of the run, 20 days total (12 may 2009). DNA fragments are shown in the horizontal axes in base pairs after *Rsa*I digest. Fuschia shading represents peaks that showed a higher fluorescence signal than the rest of the peaks in the electropherograms.


Fluorescence (arbitrary units)
a)
b)
c)
Base pairs (bp)

IX. Tables

Table 1. Dilution series of microbial communities into SOB (Elemental Sulfur Oxidizing Bacteria) and TDB (Thiosulfate Disproportionating Bacteria) media. Counts of positive tubes and Most Probable Numbers for CAR-132 cruise (11-12 April 2007)

MPN of Sulfur Oxidizing/Disproportionating media										
Depth	**Positive tubes per dilution**							**MPN**	**Lower**	**Upper**
	10^{-1}	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**10^{-7}**	**(cells L^{-1})**	**95% C.I.**	**95% C.I.**
290 m	3	3	3	3	3	3	3	> $1.1x10^{7}$	-	-
305 m	3	3	3	3	3	3	3	> $1.1x10^{7}$	-	-
340 m	3	3	3	3	3	3	3	> $1.1x10^{7}$	-	-
MPN of Thiosulfate Disproportionating media										
Depth	**Positive tubes per dilution**							**MPN**	**Lower**	**Upper**
	10^{-1}	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**10^{-7}**	**(cells L^{-1})**	**95% C.I.**	**95% C.I.**
240 m	3	2	1	-	-	2	1	3.3×10^{5}	$8.8x10^{4}$	$1.2x10^{6}$
250 m	3	3	2	-	-	-	-	9.2×10^{5}	$2.3x10^{5}$	$3.7x10^{6}$
320 m	1	3	3	1	1	-	-	3.7×10^{4}	$9.6x10^{3}$	$1.5x10^{5}$

Table 2. Dilution series of microbial communities into SOB (Elemental Sulfur Oxidizing Bacteria) and TDB (Thiosulfate Disproportionating Bacteria) media. Counts of positive tubes and Most Probable Numbers for CAR-139 cruise (30 Nov. to 01 Dec 2007)

MPN of Sulfur Oxidizing/Disproportionating media										
Depth	**Positive tubes per dilution**							**MPN**	**Lower**	**Upper**
	10^{-1}	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**10^{-7}**	**(cells L^{-1})**	**95% C.I.**	**95% C.I.**
100 m	1	-	-	-	-	-	-	3.6×10^{3}	5.1×10^{3}	2.5×10^{4}
250 m	2	2	1	2	2	-	-	4.2×10^{4}	1.0×10^{4}	1.7×10^{5}
270 m	2	1	2	1	1	-	-	4.1×10^{4}	1.0×10^{4}	1.6×10^{5}
300 m	3	2	1	-	1	1	-	2.7×10^{5}	7.5×10^{4}	9.5×10^{5}
MPN of Thiosulfate Disproportionating media										
Depth	**Positive tubes per dilution**							**MPN**	**Lower**	**Upper**
	10^{-1}	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**10^{-7}**	**(cells L^{-1})**	**95% C.I.**	**95% C.I.**
250 m	2	2	2	1	1	1	-	5.8×10^{4}	1.3×10^{4}	2.5×10^{5}
270 m	2	1	2	1	1	1	-	4.8×10^{4}	1.1×10^{4}	2.0×10^{5}
300 m	3	2	2	-	1	1	-	3.4×10^{5}	9.1×10^{4}	1.3×10^{6}

Table 3. Dilution series of microbial communities into SOB (Elemental Sulfur Oxidizing Bacteria) and TDB (Thiosulfate Disproportionating Bacteria) media. Counts of positive tubes and Most Probable Numbers for CAR-145 cruise (May 21-22 2008)

MPN of Sulfur Oxidizing/Disproportionating media										
Depth	**Positive tubes per dilution**							**MPN**	**Lower**	**Upper**
	10^{-1}	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**10^{-7}**	**(cells L^{-1})**	**95% C.I.**	**95% C.I.**
250 m	3	2	1	-	-	-	-	1.5×10^5	4.1×10^4	5.2×10^5
280 m	3	1	-	-	-	-	-	4.2×10^4	1.0×10^4	1.7×10^5
350 m	3	-	-	-	-	-	-	2.3×10^4	6.6×10^3	8.0×10^4
MPN of Thiosulfate Disproportionating media										
Depth	**Positive tubes per dilution**							**MPN**	**Lower**	**Upper**
	10^{-1}	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**10^{-7}**	**(cells L^{-1})**	**95% C.I.**	**95% C.I.**
100 m	-	-	-	-	-	-	-	-	-	-
250 m	2	2	1	1	1	-	-	4.2×10^4	1.0×10^4	1.7×10^5
280 m	2	1	2	1	1	-	-	4.1×10^4	1.0×10^4	1.6×10^5
350 m	3	2	1	-	1	1	-	2.7×10^5	7.5×10^4	9.5×10^5

Table 4. Dilution series of microbial communities into SOB (Elemental Sulfur Oxidizing Bacteria) and TDB (Thiosulfate Disproportionating Bacteria) media. Counts of positive tubes and Most Probable Numbers for CAR-152 cruise (19-20 Jan 2009)

MPN of Sulfur Oxidizing/Disproportionating media										
Depth	Positive tubes per dilution							MPN	Lower	Upper
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	(cells L^{-1})	95% C.I.	95% C.I.
100 m	-	-	-	-	-	-	-	-	-	-
220 m	1	-	-	-	-	-	-	3.6×10^{3}	5.1×10^{3}	2.5×10^{4}
240 m	2	-	-	-	-	-	-	9.2×10^{3}	2.3×10^{3}	3.7×10^{4}
250 m	2	2	1	1	1	1	-	4.2×10^{4}	1.0×10^{4}	1.7×10^{5}
260 m	2	1	2	1	1	1	-	4.1×10^{4}	1.0×10^{4}	1.6×10^{5}
275 m	3	2	1	-	1	1	-	2.7×10^{6}	7.5×10^{4}	9.5×10^{5}
900 m	2	1	-	-	-	-	-	1.5×10^{4}	4.1×10^{3}	5.2×10^{4}
MPN of Thiosulfate Disproportionating media										
Depth	**Positive tubes per dilution**							**MPN**	**Lower**	**Upper**
	10^{-1}	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**10^{-7}**	**(cells L^{-1})**	**95% C.I.**	**95% C.I.**
100 m	-	-	-	-	-	-	-	-	-	-
220 m	1	1	-	-	-	-	-	7.3×10^{3}	1.7×10^{3}	3.3×10^{4}
240 m	2	1	-	-	-	-	-	1.5×10^{4}	4.1×10^{3}	5.2×10^{4}
250 m	3	2	1	-	-	-	-	1.5×10^{5}	4.1×10^{4}	5.2×10^{5}
260 m	3	1	-	-	-	-	-	4.2×10^{4}	1.0×10^{4}	1.7×10^{5}
275 m	2	1	1	-	-	-	-	2.0×10^{4}	5.9×10^{3}	7.1×10^{4}
900 m	2	2	-	-	-	-	-	2.1×10^{4}	6.1×10^{3}	0.3×10^{4}

Tables 5. Frequency of peak occurrences among the 67 samples for the two media subjected to three endonuclease digests and T-RFLP analyses

Elemental sulfur disproportionating - oxidizing media					
***Alu*I**		***Msp*I**		***Rsa*I**	
Size	**freq**	**Size**	**freq**	**Size**	**freq**
10	28	275	11	45	8
111	8	470	16	385	5
156	9	435	13	401	7
165	3	769	9	626	7
298	20	137	14	769	11
470	17	10	13		
580	14				
Thiosulfate disproportionating media					
***Alu*I**		***Msp*I**		***Rsa*I**	
Size	**freq**	**Size**	**freq**	**Size**	**freq**
10	14	10	6	11	3
47	12	11	14	47	6
85	11	47	18	347	4
298	10	171	7	401	3
470	10	214	19	443	3
156	9	256	18		
170	9	435	4		
111	8	769	9		
580	7				
165	6				

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Appendix

A. Enrichment media (Thamdrup et al, 1993).

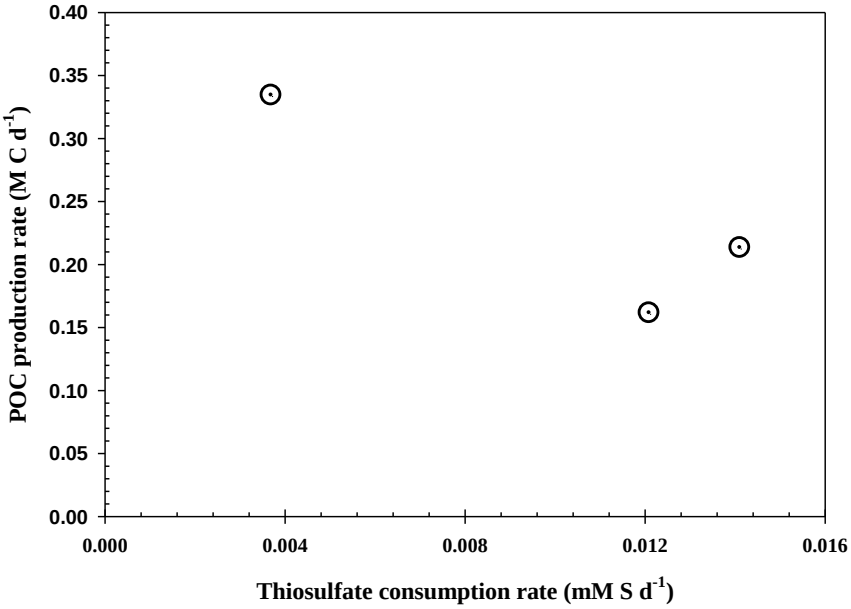
- **Sulfur oxidizing bacteria** 1:1 saline solution and aged Cariaco's seawater filtered through 0.2 μM membrane. Add the following solution after sterilizing by autoclave (final concentrations): 4.6 μM resazurin, 0.19 μM $FeCl_3.6H_2O$, 1.06 mM $CaCl_2.2H_2O$, 5.95 mM $NaHCO_3$, 1 ml of SL-8 trace element solution, 0.062 M sulfur, pH=7.5.
- **Sulfur disproportionating bacteria** 1:1 saline solution and aged Cariaco's seawater filtered through 0.2 μM membrane. Add the following solution after sterilizing by autoclave (final concentrations): 0.6 μM resazurin, 0.19 μM $FeCl_3.6H_2O$, 1.06 mM $CaCl_2.2H_2O$, 5.95 mM $NaHCO_3$, 1 ml of SL-8 trace element solution, 0.062 M sulfur, pH=7.5.
- **Thiosulfate disproportionating bacteria:** 1:1 saline solution and aged Cariaco's seawater filtered through 0.2 μM membrane. Add the following solution after sterilizing by autoclave (final concentrations): 4.6 μM resazurin, 0.36 mM $FeSO_4.7H_2O$, 0.19 μM $FeCl_3.6H_2O$, 1.06 mM $CaCl_2.2H_2O$, 5.95 mM $NaHCO_3$, 1 ml of SL-8 trace element solution , 3mM $Na_2S_2O_3.5H_2O$, pH=7.5.

Saline Solution: 0.51 M NaCl, 0.037 mM NH_4Cl, 9.84 mM $MgCl_2.6H_2O$, 3.8 mM KH_2PO_4 , 4 mM K_2HPO_4 , 1.0 L distilled H_2O.

Trace element solution SL-8: 5.20 g Na_2-EDTA, 1.50 g $FeCl_2.4\ H_2O$, 70mg $ZnCl_2$, 100 mg $MnCl_2.4\ H_2O$, 62 mg H_3BO_3, 190 mg $CoCl_2.6\ H_2O$, 17 mg $CuCl_2.2H_2O$, 24 mg $NiCl_2.6H_2O$, 36 mg $Na_2MoSO_4.2\ H_2O$, 1000 ml distilled water.

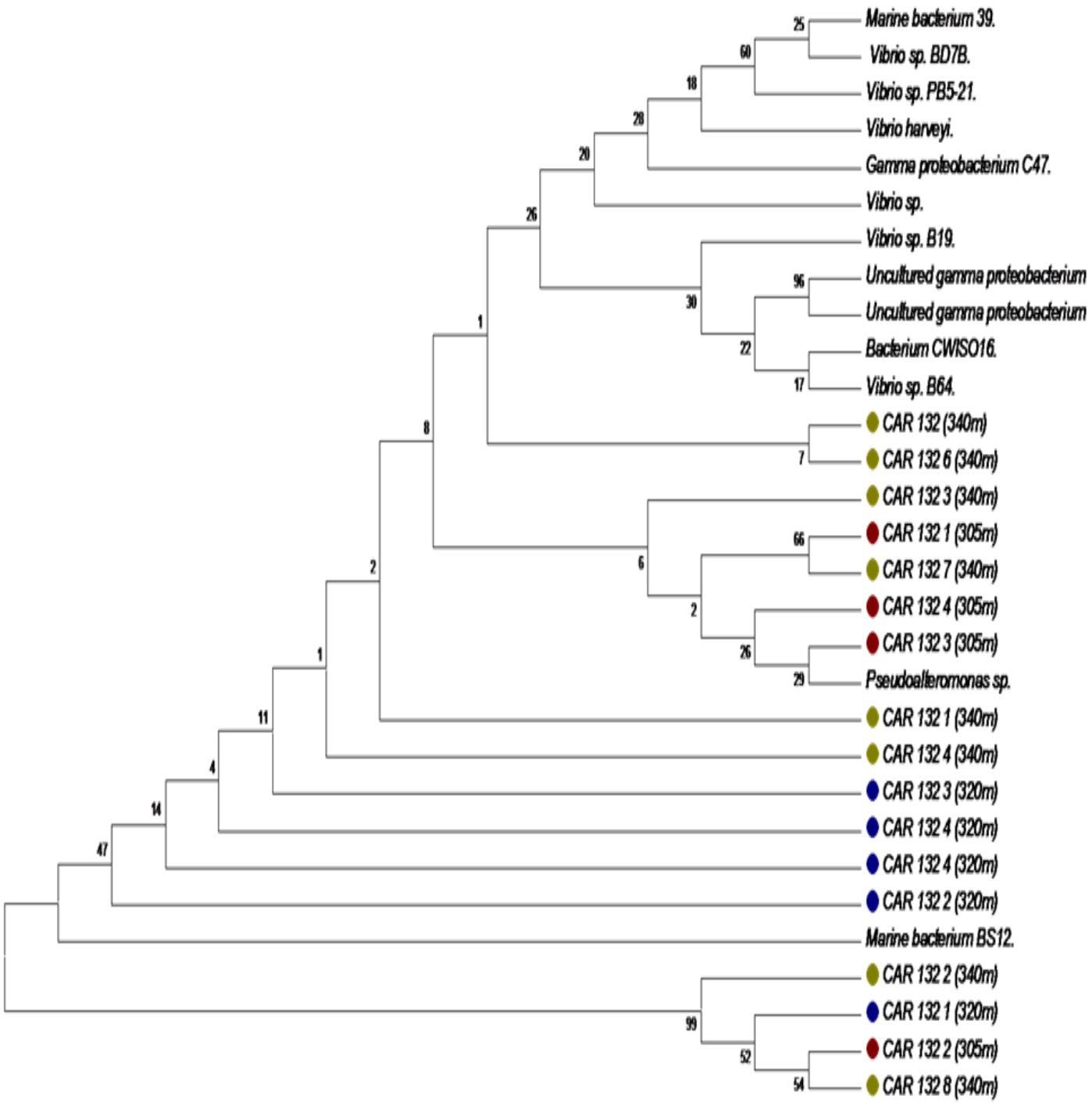
B.

Figure 24. Thiosulfate consumption rate ($Thio_{res}$ - $Thio_{bio}$) (mM S d^{-1}) vs. particulate organic carbon (POC) production rate (M C d^{-1}); used as biomass proxy.


0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
POC production rate (M C d-1)
0.000
0.004
0.008
0.012
0.016
Thiosulfate consumption rate (mM S d-1)

C.

Figure 25. Phylogenetic relationships of partial SSU rDNA sequences from anoxic S^0 oxidizing microorganisms residing in the Cariaco Basin's redoxcline after 12 series of dilutions to extinction


Marine bacterium 39.
Vibrio sp. BD7B.
Vibrio sp. PB5-21.
Vibrio harveyi.
Gamma proteobacterium C47.
Vibrio sp.
Vibrio sp. B19.
Uncultured gamma proteobacterium
Uncultured gamma proteobacterium
Bacterium CWISO16.
Vibrio sp. B64.
CAR 132 (340m)
CAR 132 6 (340m)
CAR 132 3 (340m)
CAR 132 1 (305m)
CAR 132 7 (340m)
CAR 132 4 (305m)
CAR 132 3 (305m)
Pseudoalteromonas sp.
CAR 132 1 (340m)
CAR 132 4 (340m)
CAR 132 3 (320m)
CAR 132 4 (320m)
CAR 132 4 (320m)
CAR 132 2 (320m)
Marine bacterium BS12.
CAR 132 2 (340m)
CAR 132 1 (320m)
CAR 132 2 (305m)
CAR 132 8 (340m)