Novel attempts to cultivate abundant microbes from marine surface water at Buzzards Bay, Woods Hole, MA

Miniproject during Microbial Diversity Summer Course 2003

by

a zoo of unknown microbes and

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Abstract

In many environmental habitats less than one percent of the microbial communities have been obtained in pure culture and a broad range of phylogenetic lineages belonging to the bacteria and archea have only been found by means of molecular methods. Obviously there is a lack of laboratory methods to cultivate those microbes. Recently there has been some success in culturing abundant microbial strains from the environment by the application of dilution techniques in combination with sensitive detection of microbial growth. The present study provides evidence for the presence of typical marine microbial phylotypes at Buzzards Bay surface water and additionally the enrichment of microbes in artificial medium with low concentrations and even without organic substrates is reported. The results suggest, that microbial growth in laboratory enrichments is possible even under very low nutrient conditions. Although growth may be too weak to be detected by conventional visual and microscopic methods.

1 Introduction

Traditionally microbiological investigations are based on the isolation of pure bacterial or archeal strains from different environments. A broard variety of bacteria have been investigated and described in this way. However, during the last decades the introduction of nucleic acid stains and a variety of molecular methods, have revealed a much higher total cell numbers and phylogenetic diversity in virtually all environments, than assumed by previous cultivation based investigations (Amann *et al.*, 1995). Depending on the study area the cultivation efficiency was determined to be as low as 0.001- 0.2 % of the total cell count in seawater, lake water, and soil. Only in wastewater treatment systems, with high nutrient levels up to 15 % of the total cell count could be detected also by cultivation-based methods (Amann *et al.*, 1995; Schut *et al.*, 1993). And in terms of diversity 13 out of 36 to 40 identified bacterial phyla remain to harbor no yet cultivated representatives (Hugenholtz *et al.*, 1998). A lot of research focuses on methods to investigate the phylogenetic and metabolic diversity by means of molecular methods (e.g. Lorenz & Schleper, 2002). However, the understanding of microbial metabolism and microbial genetics rely on laboratory investigations on living cells. Therefore novel approaches to the cultivation of microorganisms from the environment remain necessary to assess the yet unknown metabolic and phylogenetic diversity in the environment. Recently also in this respect new ideas have been developed using dilution procedures (Bruns *et al.*, 2003; Cho & Giovannoni, 2003; Connon & Giovannoni, 2002; Rappé *et al.*, 2002; Schut *et al.*, 1993) or diffusion chambers (Kaeberlein *et al.*, 2002). Most of these studies also took advantage of the availability of molecular methods, e. g. FISH (Amann *et al.*, 1990; DeLong *et al.*, 1989) or DGGE (Muyzer *et al.*, 1993) to monitor the enrichment procedures tracking the enrichment of novel strains.

The current mini project goes in the same line, trying to attempt the cultivation of abundant aerobic heterotrophic bacteria from a marine environment by applying two dilution techniques with different nutrient levels in combination with more sensitive systems for the detection of microbial growth. Based on the assumption that abundant microorganisms in the marine environment are well adapted to their environment, they might not grow up to cell densities beyond 10^7 cells ml^{-1} (e. g. Rappé *et al.*, 2002) detectable by conventional methods like light microscopy and may not sustain nutrient levels orders of magnitude higher than they find in their natural environment. Therefore, comparative microplate assays were set up, with basic artificial seawater or filtrated seawater and three different nutrient levels to allow growth of either low-

nutrient adapted as well as high nutrient depending microbes. These assays were monitored by measuring DNA-dependent fluorescence of the SybrGreenI dye in the beginning and after three weeks of incubation. If growth will be detectable in either of these assays the phylogenetic type of these enrichments as well as the phylogenetic composition of the environment are going to be tracked by means of molecular methods enabling an evaluation of the obtained enrichments as well as the used methods.

2 Material and Methods:

2.1 Sampling site and sampling procedure

For enrichments and molecular analyses a water sample was collected at Buzzards Bay on board "RV Hobbes" on July, 8th 2003. The sample was taken into acid washed 15 l cans, transported to the laboratory and processed for subsequent procedures soon as possible.

2.2 Total cell counts

A 50 ml sub sample was taken into a Falcon tube from the original water sample immediately after return to the laboratory, fixed with 3 ml formaldehyde (37 %, Fisher Scientific), and stored at 4 degree C until further treatment. Staining of cells was performed according to (Noble & Fuhrman, 1998). Briefly, 1 ml of the fixed sample was mixed with 100 µl staining solution (1:400 dilution of Sybr GreenI, Molecular Probes, Eugene) and incubated for 10 min in the dark. A 100 µl of this mixture was filtered onto a 0.2 µm pore size polycarbonate filter (Millipore, Eschborn), rinsed with 0.2 µm filtered PBS buffer, air dried, and fixed on a microscopic slide by the addition of 10 µl mounting solution (50 % glycerol, 50 % PBS, 0.1 % p-phenylenediamine [Sigma Aldrich]). Samples were counted using a Zeiss Axioscope microscope equipped with a 100 W mercury vapor lamp and filter set II (GP365, FT395 ,LP420). At least 20 fields and a total of 400 cells were counted.

2.3 Cultivation experiments

Enrichments of abundant microbes from Buzzards Bay surface water were set up in 1.5 ml micro titer plates following two different dilution strategies in combination with no nutrient addition, low level nutrient addition, and high level nutrient addition. A conventional Most Probable Number dilution technique (Bruns *et al.*, 2003) was used to dilute cells to extinction. Therefore one 96 well micro titer plate for each nutrient treatment was filled with 800µl artificial or filtrated

seawater supplemented with 1 ml of nutrient mix (see below) per l iter medium. Subsequently 200 µl of Buzzards Bay water sample were added to the first ($2x10^{-1}$) dilution and then diluted row by row to extinction.

A stock dilution technique was used to dilute cells to a specific cell density of 50 cells ml^{-1}. Therefore the medium was amended with nutrient and then spiked with a certain amount of water sample to dilute approx 50 cells into each ml of medium. Subsequently this inoculum was delivered into a 96 well micro titer plate for each nutrient treatment.

Artificial seawater was designed according to the concentrations of major and trace elements for seawater (Brown *et al.*, 1989). Filtrated seawater was prepared from Buzzards Bay water sample by filtration trough 0.2 µm pore size polycarbonate filter (Millipore) into a 2 l Erlenmeyer flask. All materials were autoclaved prior to usage. The filter papers were assumed to be sterile.

The two different nutrient mixes consisted of glucose, lactose, chitin, cellobiose, fructose, glucosamine, acetate, malate, succinate, tartrate, pyruvate, cysteine, methionine, leucine, proline, glycine, alanina, phenylalanine (each approx. 1 mM concentration) and the high nutrient mix received additionally 1.4 % (w/v) Marine Broth (Difco Laboratories).

Growth of microbial cells was monitored by quantification of cell density by SybrGreen I staining and measurement of batch fluorescence at the beginning and after 17 days of incubation. Therefore, from all of the 13 obtained microtiterplate-incubations (two media types x two dilution strategies x three nutrient regimes plus one control plate for the stock dilution exeperiment) immediately after setup and after 17 days subsamples of 200 µl volume were transferred to black 96 well micro titer plates (Corning Inc.), supplemented with 50 µl of a 1:1000 dilution of SybrGreen I nucleic acid gel stain (Molecular Probes, Eugene) in buffer (200 mM Tris, 50 mM EDTA, pH 8). Assay plates were incubated for at least one hour and subsequently the amount of fluorescence in the samples was determined in a micropate reader (Tecan SPECTRAFluor Plus, Tecan GmbH, Austria). Measurements were carried out at 480 nm exitation and 540 nm emission. Platereader settings were optimized empirically to 40 flashes at a gain of 80 and each plate was measured three times, in order to minimize deviations.

2.4 PCR, Cloning, and Sequencing

In order to get an idea of what the diversity of microorganisms at Buzzards Bay is, a E. coli clone library of bacterial 16S rRNA genes was established, sequenced and analyzed.

Briefly, 300 ml of water sample was filtrated onto a 0.2µm pore size polycarbonate filter. The filter was cut into small pieces with a sterile razor blade, and introduced into a DNA extraction kit

(MoBio soil DNA extraction kit, MoBio, Solana Beach, CA) and treated according to the instructions of the manufacturer. Additionally a 3-fold freeze/thaw cycle was introduced after the bead beating step. The purified DNA was finally stored in 10 mM Tris-Buffer at –30 °C.

Polymerase chain reaction amplifications were set up with 49 µl PCR mastermix (45 µl Platinum PCR Supermix [Invitrogen, Carlsbad, CA], 1 µl of each primer [10µM] and 2 µl dH_2O) and 1 µl of 1:100 diluted DNA-extract. The primers used were bacteria specific 8Forward and 1492R. PCR reactions were carried out in a gradient thermocycler (Eppendorff Tgradient) using annealing temperatures of 56, 58, and 60 °C. The PCR program consisted of 5 min initial denaturation at 95 °C, followed by 30 cycles of 60 sek denaturation at 95 °C, 60 sek annealing at 56, 58, 60 °C, and 90 sek of primer extension at 72 °C, and a final extension step for 8.5 min at 72 °C. PCR products were finally analyzed on a 1.5 % agarose gel electrophorese.

Subsequently the PCR product obtained with 58 C annealing temperature was chosen for the cloning experiment using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Briefly, 2 µl of PCR product was mixed with 1 µl salt solution, 2 µl dH_2O, and 1 µl of vector pCR2.1-TOPO and the ligation reaction was carried out at room temperature for exact 30 min. Afterwards the vector construct was transformed into cells of the chemically competent *E. coli* strain TOP10, provided along the kit according to the instructions of the manufacturer. Incubation on ice were carried out for 20 min, and cells were heated to 42 °C for exact 30 sek and placed back on ice. After initial 1 hour incubation at 37 °C, 20 µl of cell suspension was plated onto two prewarmed agar plates, containing ampicillin and 40 µl X-Gal (40 mg/ml) and incubated over night. Plates were screened for colorless clones, indicating insertion of PCR product into the vector.

Colonies of 96 clones were picked and lysed in dH_2O for 10 min at 95 °C in a PCR thermocycler. For sequencing the vector insert was reamplified using the M13 primers specific for DNA-fragments up and downstream of the insert. Sequencing reactions were then sent out for sequencing reaction. Out of 96 reactions 53 sequences were obtained and 41 were included into following analyses.

Phylogenetic analyses. The obtained 16S rRNA gene sequences were analysed by using the ARB software package (Ludwig *et al.*, submitted). The program Fast Aligner V1.03 was used for automatic sequence alignment, which was subsequently checked and corrected manually at the 5' and 3' end of the sequences. Sequences were then added to the database "philrdp.16Sab.arb" obtained from the RDP II homepage (http://rdp.cme.msu.edu/html/alignments.html) using the parsimonious tool. After this closely related sequences found in the database were used to proof the obtained alignments. Finally, sequences were selected from the database and phylogenetic trees were computed using a Neighbor Joining algorithm combined with a Jukes Cantor

correction and a filter for including only positions of bases, which occur in more than 40 % of the chosen sequences. Based on the different length of sequences obtained from the clone library a minimum of 300 positions were included in tree calculation. The most important reason for choosing this library is that it covers representatives of all known phyla and subgroups, but is much smaller than up-to-date complete databases. It appears to be not possible to work with those databases at workstations with 256 MB memory because the quality of alignment results was observed to go down dramatically without obtaining error messages.

3 Results and Discussion

3.1 Phylogenetic distribution of 16S rRNA gene sequences

Even though the clone library established from Buzzards Bay surface water consisted only of about 41 analyzed clones, the phylogenetic distribution appears to be very interesting and to some extent even representative. Clones belonged to four different phyla of the bacterial domain and different typical sequences were obtained which usually are found in marine waters.

In spite of the fact, that the clone library was built using primers specific for the domain Bacteria no information is available concerning the abundance of archea. Typically they appear to do not contribute in significant numbers to the microbial community of coastal surface waters.

It appeared to be surprising that out of 14 clones representing sequences belonging to the α-subclass of the Proteobacteria, seven clones belonged to the SAR11 subcluster (Fig. 1). Two other clones represented sequences belonging to an arctic marine clone group and additional five clones belonged to the Roseobacter-Ruegeria group. Interestingly these groups show the sequences with the closest cultivated relatives present in the database. Additionally one clone groups closely together with the marine clone PLY43-Rochelle, which appeared to be the closest relative to the first yet cultured phylotype of this cluster (Rappé *et al.*, 2002).

Only one clone was obtained containing a sequence of the β-subgroup of the Proteobacteria. This clone belonged to a clone group related to the Methylophilus-Methylobacillus group (Fig. 2). This clone group appears to harbor marine as well as freshwater clones and could therefore represent bacteria, which origin from marine or freshwaters. One could state, that more such clones could have been obtained, if the library itself was much larger.

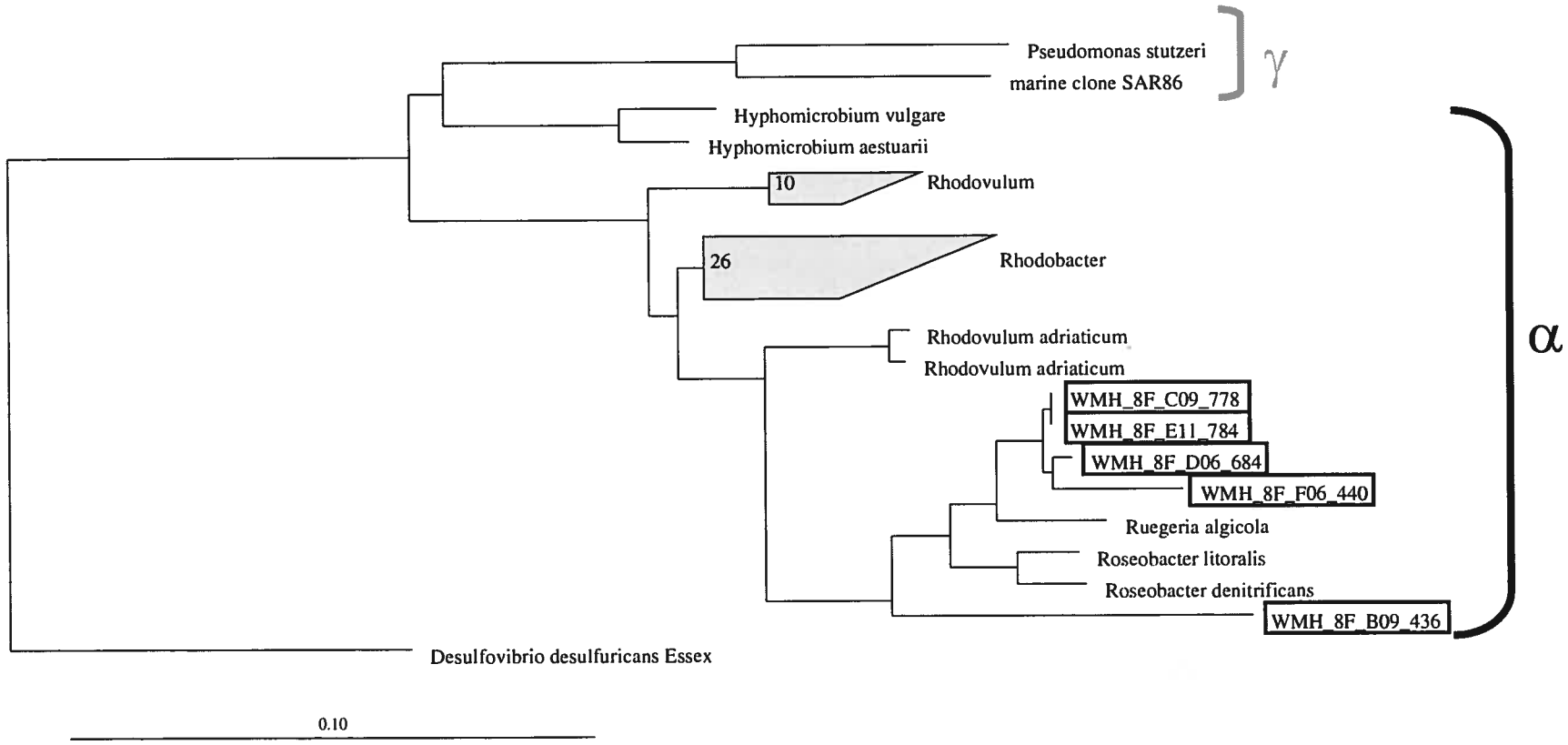

Fig.1 Phylogenetic distribution of clones belonging to the α-subclass of the Proteobacteria.

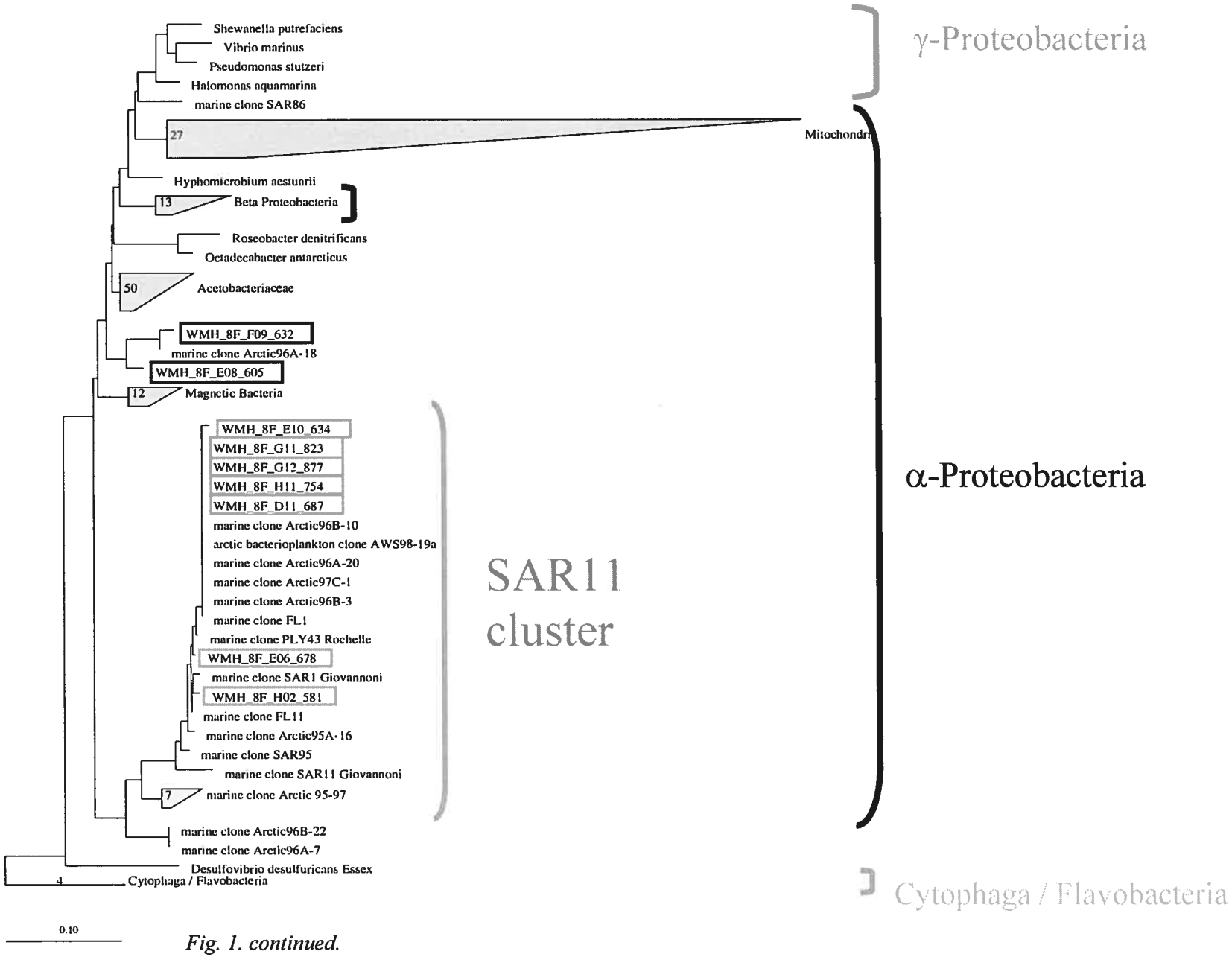

Fig. 1. continued.

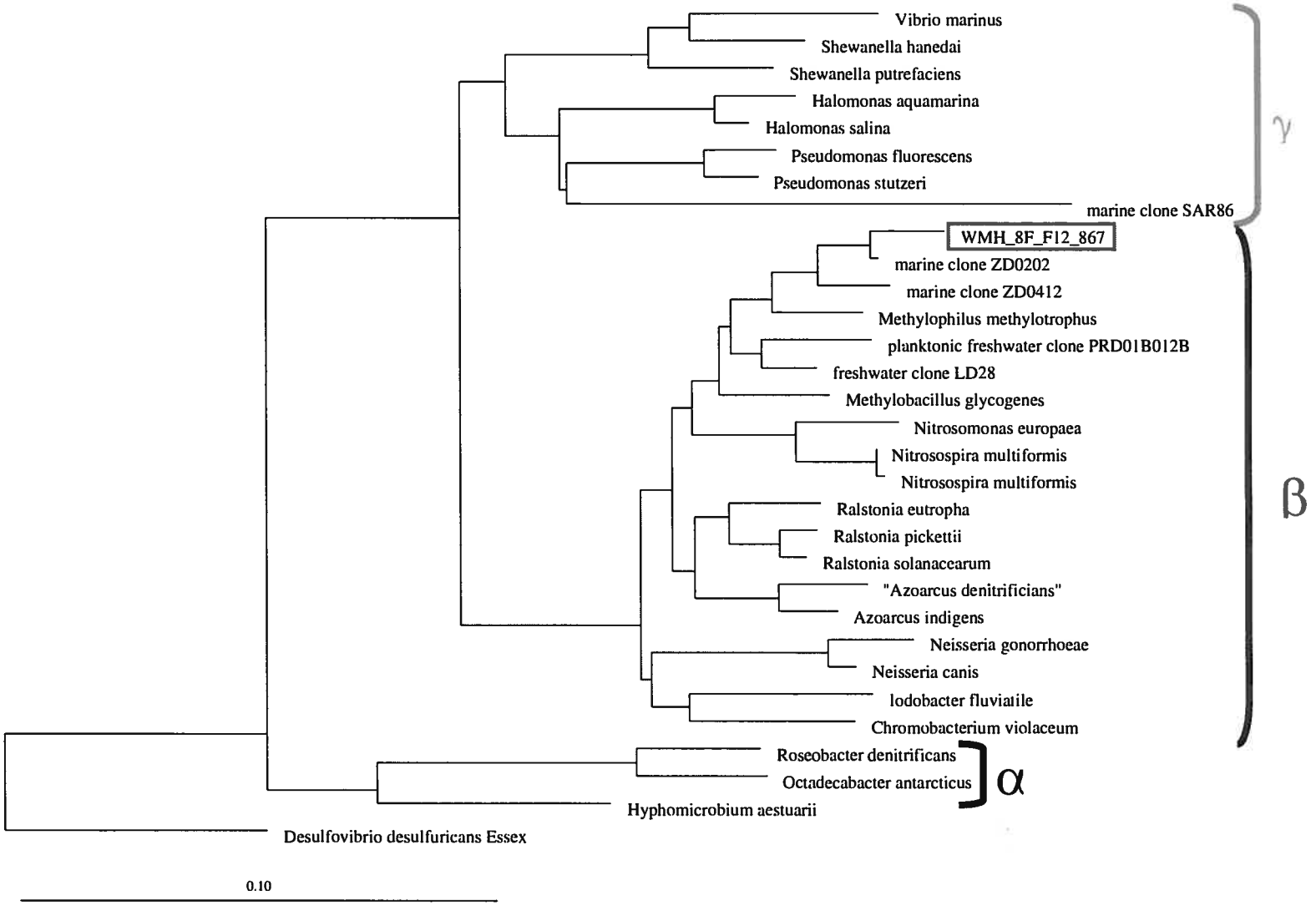

Fig. 2. Phylogenetic position of a clone belonging to the Methylophilus group of the β-subclass of Proteobacteria.

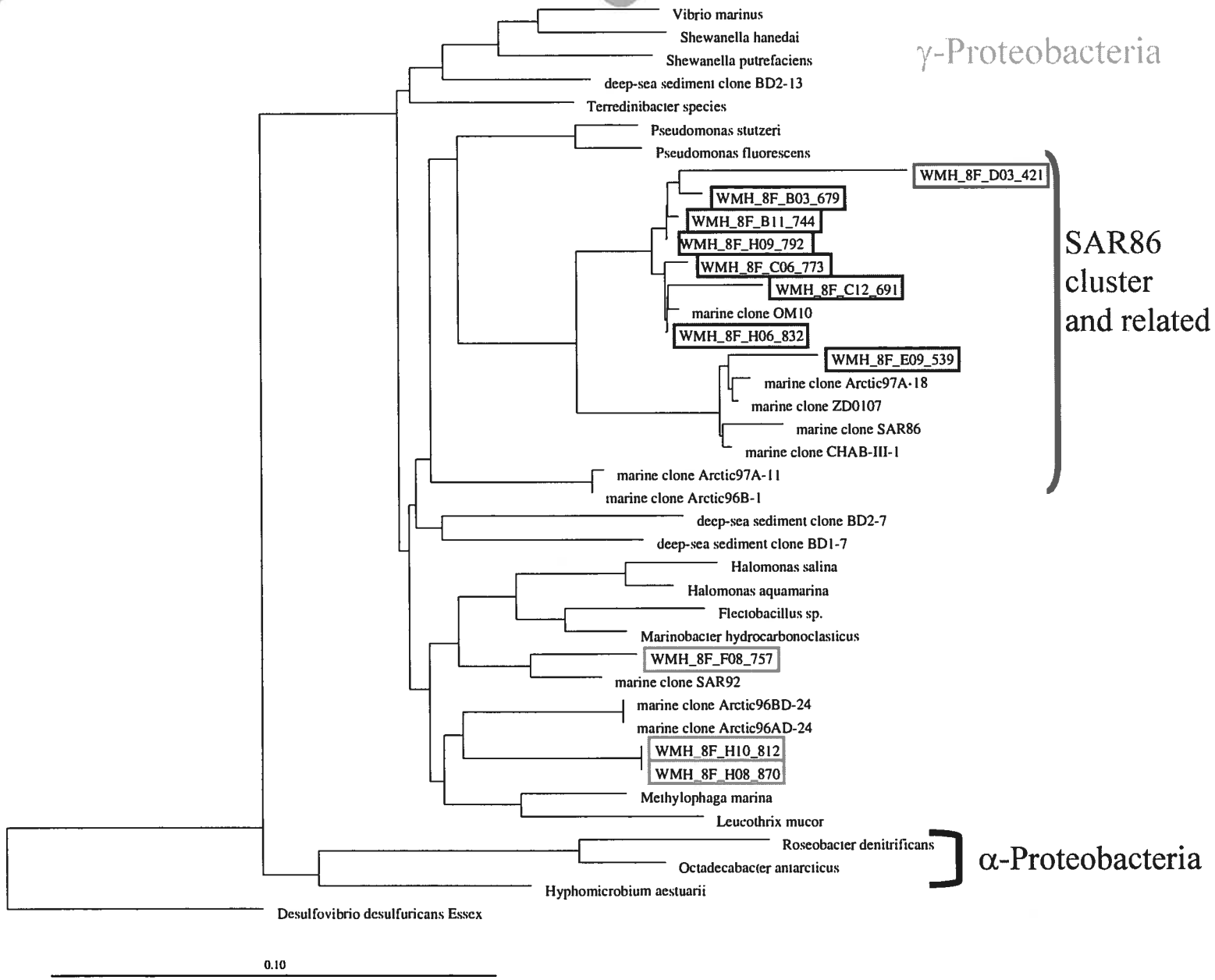

Fig. 3. Phylogenetic position of eight clones belonging to the SAR86 cluster and related clones of the γ-subclass of Proteobacteria.

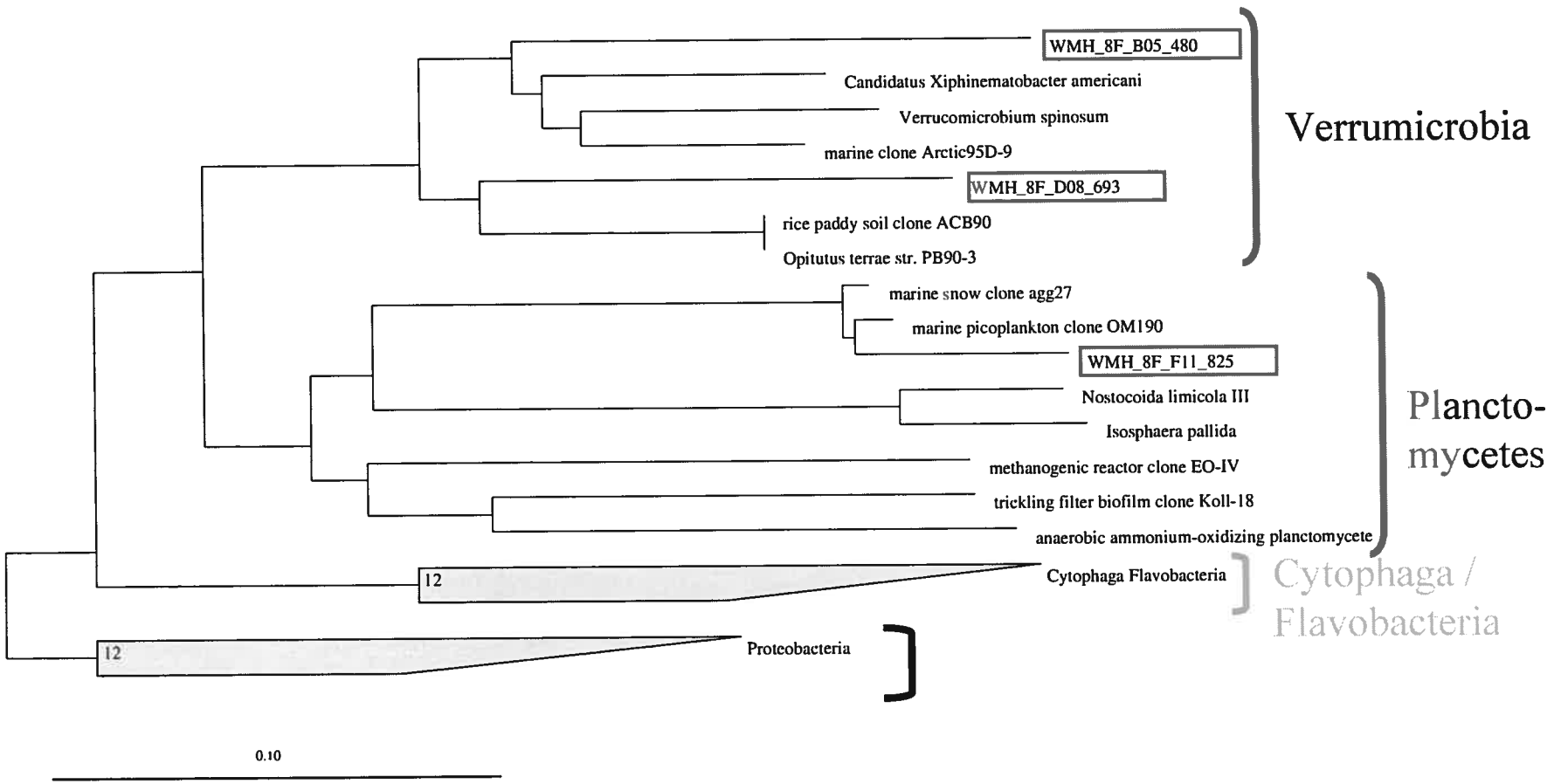

Fig. 4. Phylogenetic position of clones related to the phyla Verrumicrobia and Planctomycetes.

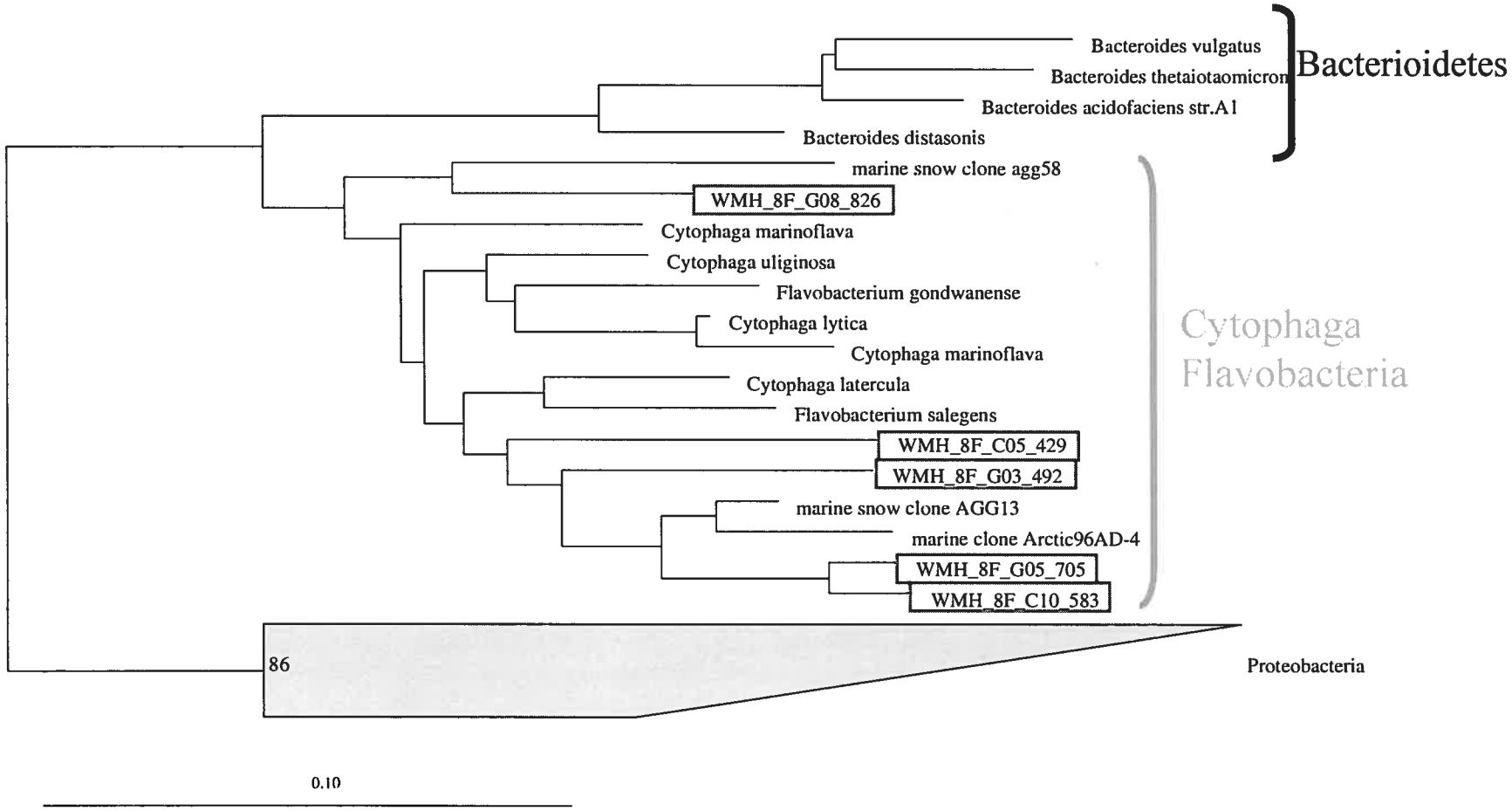

Fig. 5. Phylogenetic position of clones related to the phylum Cytophaga- Flavobacteria.

A group of eleven clones falls into the γ-subgroup of the Proteobacteria. Eight of them appear to be most related to the marine clone cluster SAR86 (Fig. 3). One sequences groups together with the marine clone group SAR92 and two sequences form a distinct branch near clone sequences of arctic marine water samples.

Two of the obtained sequences group into the phylum Verrumicrobia and one sequence was obtained related to a clone group of the phylum Planctomycetes.

In general some sequences form quite deep branches in the presented trees (e.g. D_03_421, C_12_691, and E09_539, Fig.3). This seems to be due to the length of the sequences. Generally, this was one of the most important problems, concerning this phylogenetic approach that a lot of sequences were to short to allow a reasonable classification. Most of those sequences were omitted from the treeing procedure, but still some of the included sequences form such branches according to a lack of informative positions. Because short sequences provide only poor informative positions, the tree itself generated including some short sequences always becomes less reasonable. So that one should tend to include only full sequences into the analysis. Therefore it appears to be nice that a most of the sequences matched pretty well to known sequences in the database, although short sequences were present in some of the presented phylogenetic trees. Other sequences, which group only distantly with known sequences need to be revisited. The most important thing is to obtain all sequence information available from the clones. Additionally a closer look should be taken onto more recently submitted sequences available at common databases. Nevertheless this small library represents typical marine bacterial lineages found in most parts of the oceans and therefore appears at least to some extent representative. An experiment concerning the representation of the bacterial diversity at Buzzards Bay in this clone library by tRFLP failed and no information on this is available.

3.2 Cultivation approaches

One of the goals of this study was to examine different dilution treatments for the enrichment of abundant marine bacteria. Since single strains obtained in the past, which represent abundant members of the bacterial community in seawater show only weak growth under laboratory conditions (Kaeberlein *et al.*, 2002; Rappé *et al.*, 2002). It was necessary to use a very sensitive system to detect microbial growth. Here a simple but sensitive microplate assay was applied, which allows the detection of cells down to a cell density of 5×10^4 cells/ml (Fig. 6). This method showed a linear correspondence of fluorescence to the cell density over about four orders of

magnitude. Thus, this method seemed to appear suitable for a sensitive detection of microbial growth.

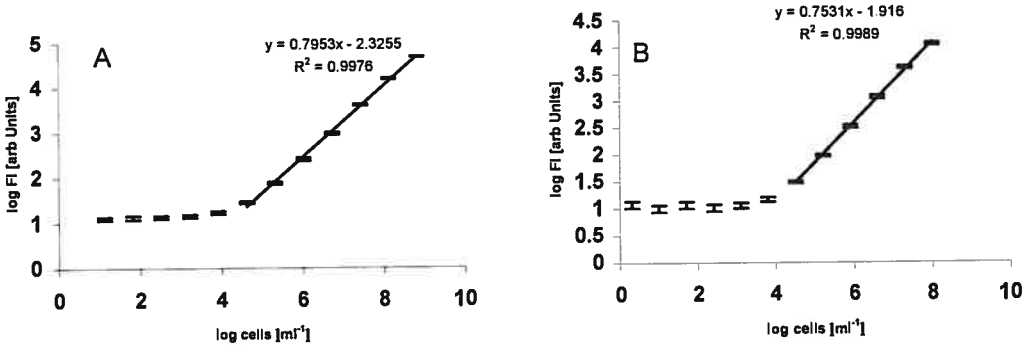

Fig. 6. Comparison of fluorescence emission after SybrGreenI staining of dilutions from a fresh grown Rhizobium sp. strain (A) and an one-year rested culture of Shewanella oneidensis (B).

This method was then applied for the quantitative monitoring of all enrichments.

One part of the incubations was carried out in 0.2 µm sterile filtrated seawater. This filtration procedure was not successful. An even distribution of growth was obtained in all these microtiter plates including the controls, depending on the amount of substrate amended to the medium (data not shown). Therefore these experiments were omitted from further analyses.

Another part of the enrichments was arranged with a stock dilution of approx. 50 cells per ml medium. This experiment also showed an even distribution of growth over all wells of the respective plates. This leads to the conclusion, that either more than 50 cells were diluted into each well or that the limit of 50 cells was to high in order to obtain significant differences between the different wells.

In the third part of the experiments enrichments were set up using artificial seawater in a dilution series on each plate with ether no, low or high nutrient level. These experiments yielded interesting different growth patterns (Fig.7-9).

Apart from growth, which appeared in the assay plates at T 0, as a result of two days storage of the (non-sterile) assay plates prior to measurements, a significant increase in cell numbers was recognized in all parallels of the high level nutrient experiment down to a dilution of approx. 10 to 100 cells/ml (Fig. 7C). Interestingly most of the wells last in the end nearly the same amount of cells, as estimated by fluorescence intensity after 17 days leading to the conclusion, that may be the amount of nutrients added limits further growth at this point. Further analyses will point on this in more detail. May be it is possible to gain insights into carbon usage of these enrichments. Microscopic observations indicate that there are a lot of cells in this treatment after 17 days, indicating intense growth, as expected by fluorescence measurements (Fig. 10).

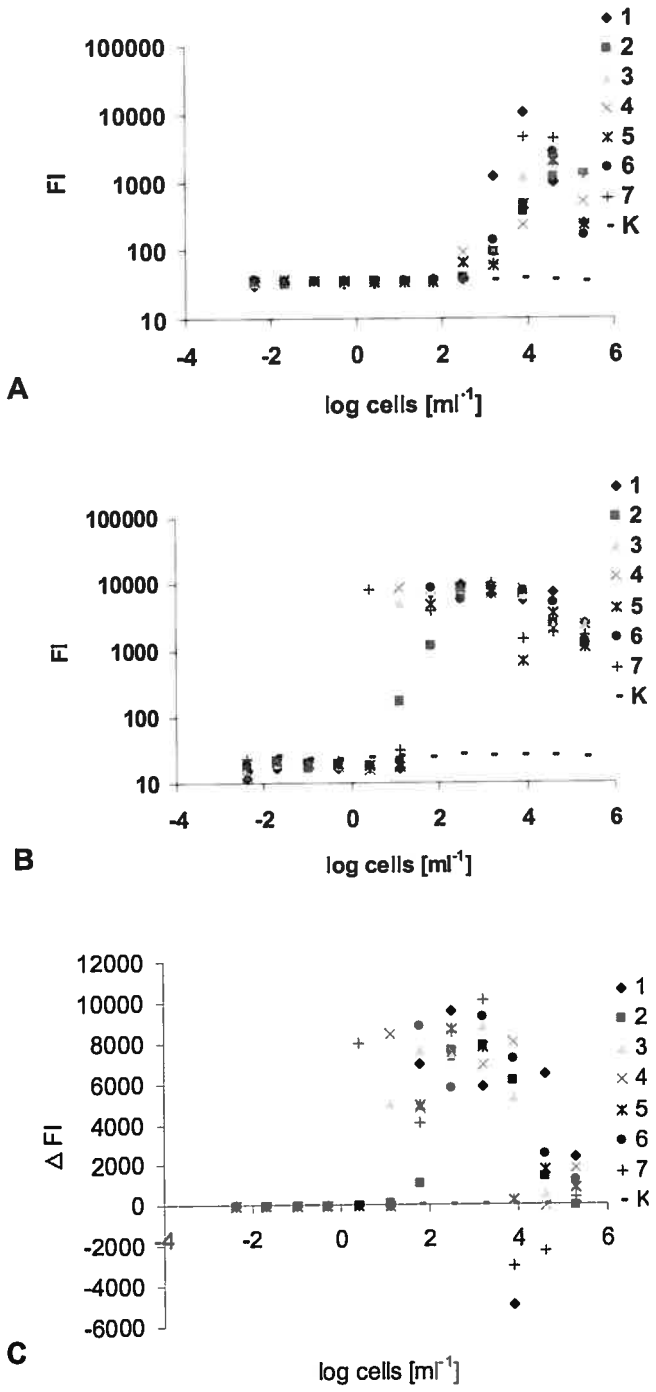

Fig. 7. Growth of environmental enrichments in MPN dilution series with artificial seawater and high nutrient level. Graphs A and B represent cell density measured by SybrGreenI fluorescence at T 0 and after 17 days, respectively. Graph C gives the difference in fluorescence between both time points, indicating growth of cells in the assay. Numbers 1 through 7 represent seven independent parallels and K denotes an un-inoculated control. Cell numbers on the x-axis represent the expected average cell number in each dilution.

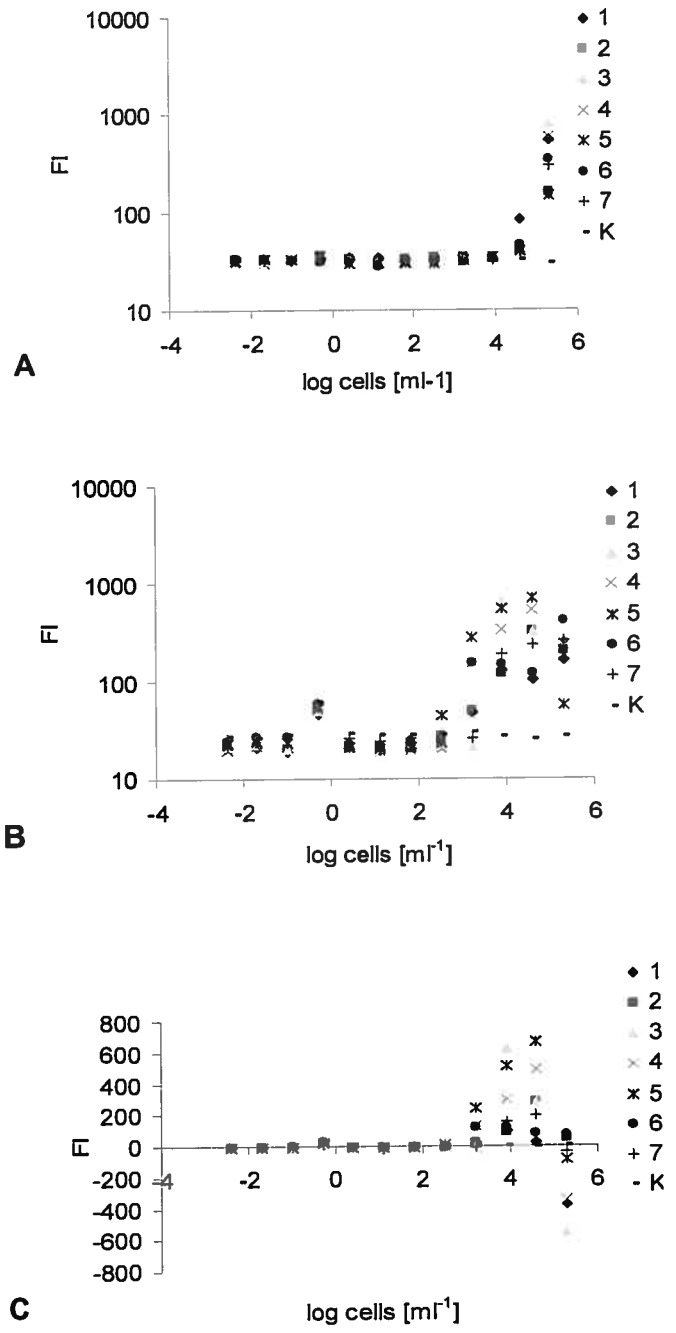

Fig. 8. Growth of environmental enrichments in MPN dilution series with artificial seawater and low nutrient level. Graphs A and B represent cell density measured by SybrGreenI fluorescence at T 0 and after 17 days, respectively. Graph C gives the difference in fluorescence between both time points, indicating growth of cells in the assay. Numbers 1 through 7 represent seven independent parallels and K denotes an uninoculated control. Cell numbers on the x-axis represent the expected average cell number in each dilution.

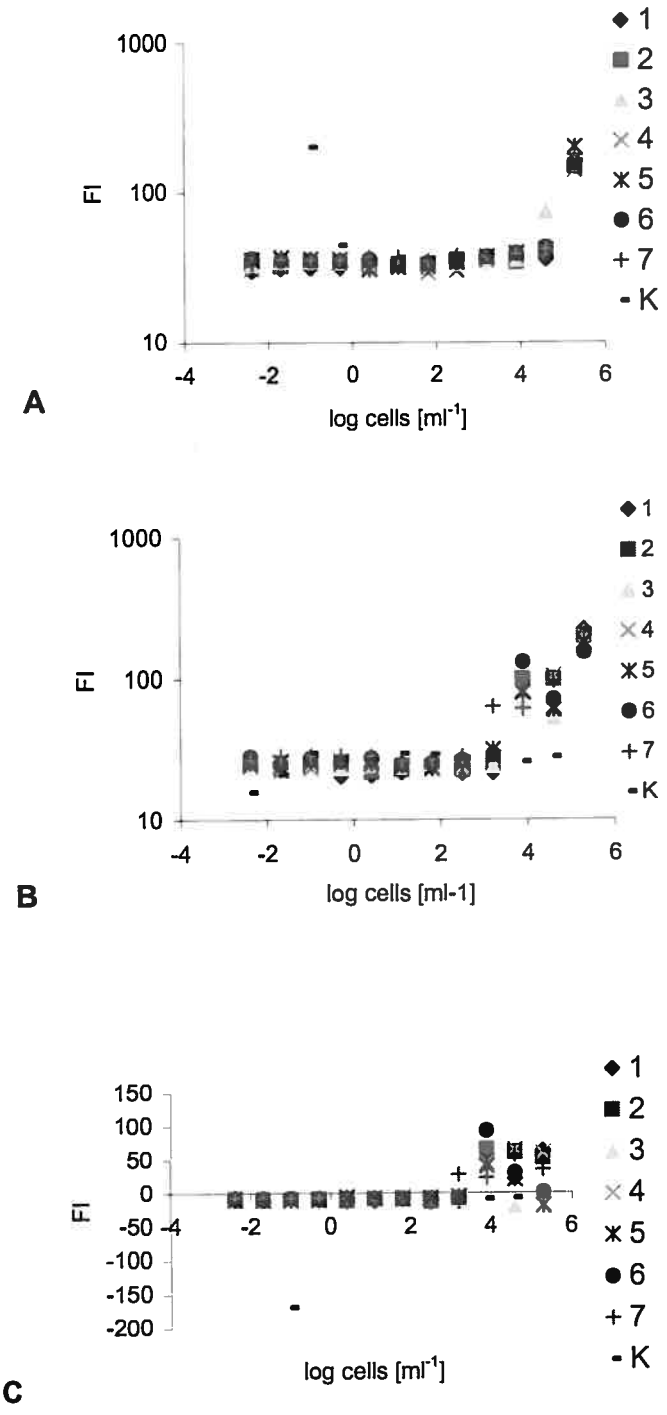

Fig. 9. Growth of environmental enrichments in MPN dilution series with artificial seawater without nutrient addition. Graphs A and B represent cell density measured by SybrGreenI fluorescence at T 0 and after 17 days, respectively. Graph C gives the difference in fluorescence between both time points, indicating growth of cells in the assay. Numbers 1 through 7 represent seven independent parallels and K denotes an uninoculated control. Cell numbers on the x-axis represent the expected average cell number in each dilution.

But according to the propidiumiodine stain (Schumann *et al.*, 2003) also some cells are present, which seem to be not in a good physiological state anymore.

As expected the low nutrient treatment and the treatment without nutrient yielded significantly lower cell densities within 17 days. This makes sense concerning the different amounts of carbon and energy source added to these treatments.

The maximum cell density obtained in the low nutrient treatment was 10-fold smaller compared to the high nutrient treatment (Fig. 8C). Furthermore the number of positive dilutions appeared to be smaller than in the high nutrient treatment. This would indicate a lower cultivation success. But according to the dilutions and lower nutrient concentrations this could be a transient effect, caused by a too short incubation time. This could be revisited, if it was possible to perform additional measurements after four, eight or more weeks of incubation. After this periods possibly more wells would show positive growth. If this would be the case, it would be interesting to gain insights, which type of microbes grows under such conditions.

Another interesting observation concerns the growth behavior in different dilution steps. In all three treatments weak or nearly now growth was observed in the first (1:5) dilution of the water sample although the same amount of substrate added lasts significantly more growth in higher dilutions. It needs to be analyzed, if this effect is due to artifacts caused by storage of the samples at T 0 or if this results from the growth behavior of the cells in these low dilutions. In this respect it would be interesting to find out, if the majority of cells is may be inhibited by the addition of organic nutrients and therefore only a subpopulation growth up during the incubation period.

Surprisingly a weak but significant growth was also observed in the treatment without nutrients (Fig. 9C). In this case the number of positive dilutions was even smaller than in the low nutrient treatment and the final cell density did not exceed 200 Fluorescence Units in these measurements. This appears to be a very exciting result. These low growth patterns could never be detected microscopically without significantly concentrating the cells by filtration or centrifugation. According to the standard curve the fluorescence signal would equal approx. 600,000 to 700,000 cells/ml enrichment culture, which is well in the range of natural seawater environments with the same low nutrient concentration. Therefore this should not be surprising in principle. Moreover this matches well with observations by Rappé et al. (2002) on the only yet described culture belonging to the marine clone cluster SAR11.

Due to the low cell density it is not possible by now to state, if this is really a growth effect of members of the natural bacterioplankton community. Attempts to follow up the enrichments by molecular analyses could not get finished successfully in time. But it was possible to find single cells in the highest positive dilution after concentrating cells 100-fold by centrifugation (Fig. 11).

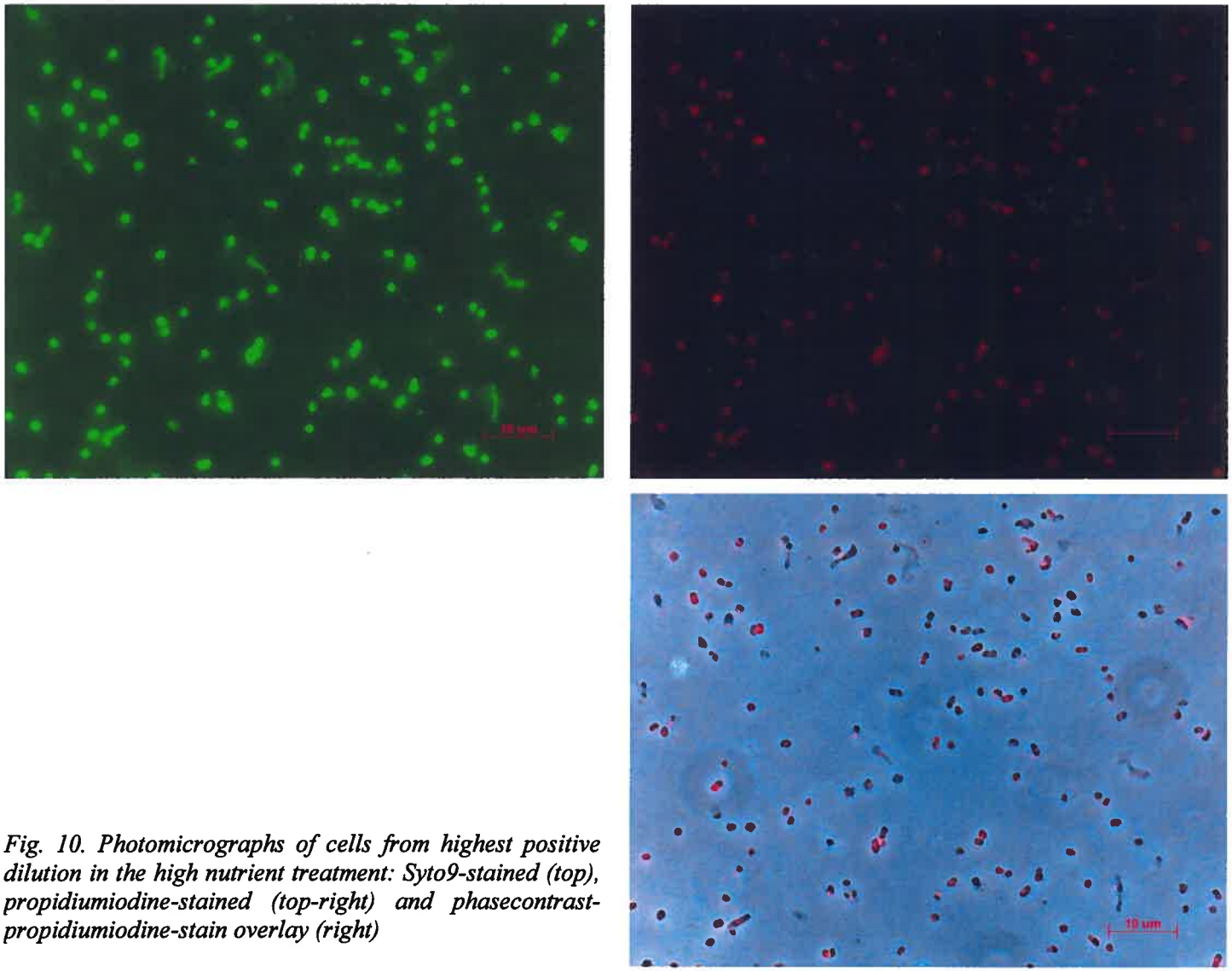

Fig. 10. Photomicrographs of cells from highest positive dilution in the high nutrient treatment: Syto9-stained (top), propidiumiodine-stained (top-right) and phasecontrast-propidiumiodine-stain overlay (right)

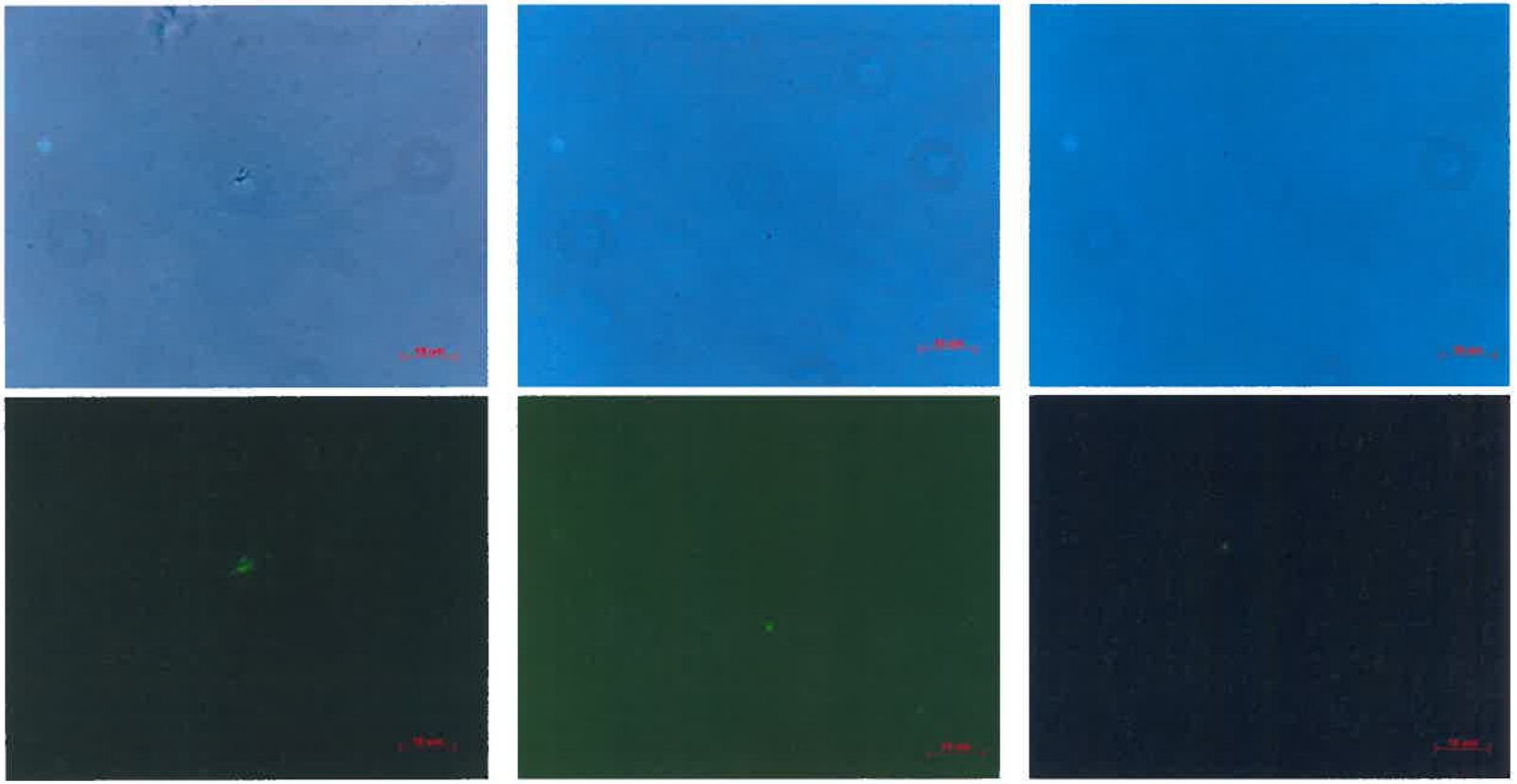

Fig. 11.Phasecontrast- (top) and Syto9-fluorescence-photomicrographs of cells observed in the highest positive dilution of the enrichment treatment without nutrient addition. Cells appeared to be very small and showed up very weak fluorescence.

3.3 General conclusions

During this study Buzzards Bay surface water was found to harbor typical marine micro-organisms, that are found to be ubiquitous in marine environments (e.g. Fuhrman & Davis, 1997; Morris *et al.*, 2002; Rappé *et al.*, 2002).

The major goal of this project, however, was to enrich for abundant microbes of Buzzards Bay surface water. Interesting observations have been made by applying a simple and sensitive fluorescence-based technique for the quantification of microbial cells in enrichment procedures. This could perhaps serve as one more useful tool for future studies in this field. Enrichments could be obtained under high and low nutrient conditions, and even under conditions without nutrients a weak increase of fluorescence was observed, possibly indicating enrichment without nutrient addition. It remains to be resolved, whether or not these enrichments represent known or unknown strains of microorganisms. This could be addressed by the application of PCR-based techniques.

In general this miniproject appeared to deal with one of the most important and difficult problems in microbial ecology. And by far the goal of this study was not achieved. Up to now the role of a major part of microbial communities in diverse habitats remain elusive. But may be, this study possibly contributed a few small ideas towards new discoveries, which remain to be made in future (and may be here in Woods Hole) for understanding microbial life in nature.

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