Characterization of the microbiota within the water column and sediments of Salt Pond

Andrea Foster[1], Chris Francis[2], Andreas Kappler[3], Milva Pepi[4]

1) Stanford University, USA; 2) Scripps Institution of Oceanography, UCSD, USA; 3) University of Konstanz, Germany; 4) University of Siena, Italy

Abstract

Salt Pond is a seasonally stratified basin with an oxic-anoxic transition zone (OATZ). Samples were collected from 0.5 m depth intervals and at 3.5 m the water smelled of hydrogen sulfide gas and was visibly pink. Profiles of pH, conductivity, dissolved oxygen, and hydrogen sulfide were generated and allowed the identification of the approximate location of the OATZ zone, ranging from 3.5 to 4 m. Microscopic analysis of filtered Salt Pond water samples indicated a distinct increase in the proportion of autofluroescent bacteria in this zone. Sediments collected from the edge of the Salt Pond were used as the inoculum for various anaerobic enrichments. We found evidence for a wide spectrum of metabolic capabilities, including sulfate-reducers, methanogens, homoacetogens, and Fe(III)-reducers. Anoxygenic phototrophic bacteria were also enriched by using Winogradsky columns with selective light filters. Total DNA was extracted from the nearshore sediments, purified, and 16S rDNA was amplified, cloned, and sequenced. In addition, digoxygenin-labeled 16S rRNA amplicons were generated and used for checkerboard hybridization analysis. This analysis provided further evidence that these sediments are composed of a diverse array of microorganisms.

Characterization of the microbiota within the water column and sediments of Salt Pond

Andrea Foster[1], Chris Francis[2], Andreas Kappler[3], Milva Pepi[4]

1) Stanford University, USA; 2) Scripps Institution of Oceanography, UCSD, USA; 3) University of Konstanz, Germany; 4) University of Siena, Italy

Abstract

Salt Pond is a seasonally stratified basin with an oxic-anoxic transition zone (OATZ). Samples were collected from 0.5 m depth intervals and at 3.5 m the water smelled of hydrogen sulfide gas and was visibly pink. Profiles of pH, conductivity, dissolved oxygen, and hydrogen sulfide were generated and allowed the identification of the approximate location of the OATZ zone, ranging from 3.5 to 4 m. Microscopic analysis of filtered Salt Pond water samples indicated a distinct increase in the proportion of autofluroescent bacteria in this zone. Sediments collected from the edge of the Salt Pond were used as the inoculum for various anaerobic enrichments. We found evidence for a wide spectrum of metabolic capabilities, including sulfate-reducers, methanogens, homoacetogens, and Fe(III)-reducers. Anoxygenic phototrophic bacteria were also enriched by using Winogradsky columns with selective light filters. Total DNA was extracted from the nearshore sediments, purified, and 16S rDNA was amplified, cloned, and sequenced. In addition, digoxygenin-labeled 16S rRNA amplicons were generated and used for checkerboard hybridization analysis. This analysis provided further evidence that these sediments are composed of a diverse array of microorganisms.

Introduction

Salt Pond is a shallow (approximately 5.5 m deep), seasonally stratified marine basin near Woods Hole, MA (Scranton et al, 1984, Wakeham et al 1984, 1987). While aerobic processes dominate the epilimnion, the anaerobic hypolimnion generally has high concentrations of H_2S (up to 5 mM in summer), generated from sulfate reduction in the bottom waters and sediments. In summer, the oxic-anoxic transition zone (OATZ) often rises to about 3 m. Typical sulfide and oxygen profiles show that this zone represents the consumption zone for the oxygen diffusing from the surface and for the sulfide diffusing upwards from the bottom. The objectives of this project were as follows: (1) to enrich for and isolate a wide variety of aerobic and anaerobic bacteria from the Salt Pond sediments; (2) to use molecular techniques to explore the microbial diversity of these sediments; and (3) to locate the oxic/anoxic transition zone (OATZ) by generating a chemical profile of samples collected from the water column, and by microscopic examination of microorganisms with depth.

Materials and Methods

Water samples

Water samples were collected from the water column using a Niskin Bottle (a plexiglass tube that is closed from the pond surface by releasing a metal weight on a small rope after reaching the sampling depth). Water samples were poured directly into glass biological oxygen demand (BOD) bottles, minimizing exposure to oxygen. The BOD bottles are designed such that when completely full, the volume is equal to 300 ml, and that there is no gas headspace.

Sediment samples

Salt Pond grab sediment samples were collected from the upper 10 cm of a shallow nearshore region (~0.5m deep) of the pond. The sediments are characterized by two distinct layers: the top layer appears to be composed of an organic-rich flocculant (ooze) whose in situ thickness was not determined. The bottom later is composed of mainly inorganic material (pebbles). The samples were collected in plastic buckets, stored in the laboratory with a 2-3 inch covering layer of Salt Pond water (to maintain anoxic conditions in the sediments), and capped with aluminum foil. Sediments collected from approximately the same locality developed very different microbial communities in the overlying water, depending on the storage conditions. When incubated under ambient laboratory conditions, very little turbidity developed in the overlying water. However, after 2-3 days of storage in the 12°C refrigerator, the overlying waters developed a very dense suspension of microorganisms.

Chemical analysis

The sulfide and oxygen content of the water column samples were measured spectrophotometrically with Chemetrics® test kits. The pH and conductivity of each water sample were also determined.

Winogradsky column

Two Winogradsky columns were constructed in order to select for anoxygenic phototrophic sulfur bacteria that have been observed in the Salt Pond OATZ layer. Nearshore sediments were amended with powdered $CaSO_4$ (approx. 0.01g/g sediment) and added to glass columns closed with a rubber plug on the bottom. As previously mentioned, although sediments were added as a homogeneous slurry, density stratification resulted in the formation of the same two distinct layers of organic and inorganic material we observed in the Salt Pond itself. The column was then filled with Salt Pond water and wrapped in aluminum foil to shield it from

ambient light sources. Selective light filters were then placed on the top of each column: 715 nm cutoff to select for green sulfur bacteria, and 820 nm cutoff to select for purple sulfur bacteria. A 25 watt incandescent light bulb provided the input light for each column.

Enrichment s for anaerobic bacteria

Enrichments were carried out with sediments in anaerobic fresh- and saltwater mineral medium prepared according to the course handout. The media contained a bicarbonate-buffer and was reduced by sodium sulfide. Different substrates were added to the mineral media: ethanol [20 mM], ethanol [20 mM] plus $NaSO_4$ [20 mM], methanol [10 mM], methanol plus bromoethanesulfonate [10 mM], trimethylamine [10 mM], trimethylamine [10 mM] plus bromoethanesulfonate, Na-vanillate [10 mM], H_2/CO_2, H_2/CO_2 plus Na_2SO_4 [20 mM]. Approximately one gram of sediment was added to the media containing different substrates and bottles were incubated at 30°C. Enrichments showing growth were transferred into secondary enrichments and then used for isolation of pure cultures, by using the agar shake method, with the same mineral medium and the appropriate growth substrate.

Endospore-former enrichment

Salt Pond sediment was added to 5ml of peptone-freshwater (PFW) medium in a test tube, which was then incubated at 70°C for 10 minutes, followed by incubation on ice. This liquid was used to streak PFW plates which were incubated at room temperature either aerobically or anaerobically in gaspack chambers. Colonies were observed microscopically for the presence of spores and these colonies were streaked repeatedly to obtain pure cultures.

Methylotroph enrichment

For the enrichment of organisms capable of growing aerobically using methanol as the carbon and energy source, Salt Pond sediment was used to inoculate nitrate mineral salts (NMS) media containing 0.2% (vol/vol) methanol, and cyclohexamide, with shaking at room temperature. After there was visible turbidity, 200 µl of this culture was spread onto NMS plates with 0.05% methanol. Individual colonies from these plates were streaked numerous times to obtain pure cultures. Colonies from pure cultures were used directly in PCR reactions and RFLP was used to determine whether these were different organisms.

Enrichments and agar shakes for isolating Fe-reducing bacteria

Primary enrichments were carried out with acetate [10 mM] as electron source and amorphous $Fe(OH)_3$ [40 mM] as electron acceptor with non-reduced freshwater medium. About 3 ml inoculum were used for the first enrichments. Growth was followed by measuring the

Fe(II) with the ferrozine assay (course handout). In subsequent cultures, AQDS (Anthraquinone disulfonate) was used as terminal electron acceptor respectively as electron carrier to amorphous iron hydroxide. Growth was followed by the absorption of the formed AHQDS (Anthrahydroquinone disulfonate) at 450 nm. After the primary enrichments and transfer to further liquid cultures with $Fe(OH)_3$ or AQDS as electron acceptor, agar shakes with $Fe(OH)_3$ were prepared. Because of the high concentration of the $Fe(OH)_3$ [40 mM], it was rather difficult to pick single colonies for further purposes. Therefore, agar shakes with soluble iron-NTA were prepared. From these shakes, single colonies were picked for microscopy and for phylogenetic characterization.

Phylogenetic characterization of the iron reducing bacteria

For DNA extraction, single colonies from the iron-NTA agar shakes were picked and transferred to 100 µl of Tris buffer. The DNA extraction procedure was carried out according to the course handout. PCR was carried out without further purification with 1 µl of the undiluted sample and of dilutions 1:10, 1:100 and 1:10,000. The 16S rDNA sequences were amplified with the Universal primers 8f and 1492r. A 55°C temperature program as described in the course handout was used for amplification. Detection of the PCR products was carried out on a 0.8% agarose gel with 1.75 µl of GelStar (FMC BioProducts) nucleic acid stain. Sequencing was carried out by the Forsythe Dental Center. The phylogenetic placement of the 16S rDNA sequences was determined with the BLAST program.

Molecular analysis of sediments

Total DNA was extracted from approximately 1 ml of sediment using the bead beater protocol (Microbial Diversity Course 1998) and the extracted DNA was fu ther purified using the Wizard Purification system, modified by eluting with 70°C water. The PCR reaction was carried out by using the universal primers 8f and 1492r, and by running the standard PCR reaction with a 55°C annealing temperature. PCR products were visualized by running 0.8% agarose gels as described above. PCR products were cloned using the TOPO-TA cloning kit (Invitrogen). Sequencing was carried out by the Forsythe Dental Center. The phylogenetic placement of the 16S rRNA sequences was determined with the BLAST program. The sequences were aligned and a phylogenetic tree was generated using TreeCon.

Checkerboard hybridization

For checkerboard hybridization, digoxygenin-labeled 16S rRNA amplicons were generated from Salt Pond sediment DNA by using a DIG labeled forward primer, the universal 1492r primer, and by running the reaction with a 50°C annealing temperature. PCR products

were visualized by running 0.8% agarose gels and these PCR products were then used for the checkerboard hybridization procedure.

Results and Discussion

Anaerobic enrichments

The Salt Pond sediments showed growth in enrichments conducted in the presence of trimethylamine with and without bromoethanesulfonate; ethanol plus bromoethanesulfonate; H_2/CO_2 mixture plus Na_2SO_4; and a H_2/CO_2 mixture. All these enrichments showed growth in the secondary enrichments, with the same mineral media and the appropriate growth substrate. Agar shakes from the secondary enrichments showed the presence of single colonies in all the different cultures. Phase contrast microscopic analyses showed rod shaped cells in colonies grown in the presence of H_2/CO_2 mixture, and observation with fluorescence light at 420 nm indicated the presence of methanogenic bacteria in Salt Pond sediments (Fig. 1). Pure colonies of sulfate-reducing bacteria were also isolated from these sediments, which is in accordance with previous reports of bacterially-produced H_2S in Salt Pond sediments (Wakeham, 1984).

Winogradky columns

After 2-3 weeks, the phototrophs in the Winogradsky columns were visible to the naked eye. A green layer of bacteria developed in the 715 nm cutoff column, and some evidence for a nascent purple layer was seen. With more time (1 week), the green layer thickened, but the purple layer did not. Both layers were not suspended in the water column, but instead were located at the sediment/water interface. Putative sulfide-oxidizing organisms were observed to form white, pillow-type structured colonies in the water column. When examined under the microscope, these organisms appear to swim and rotate very fast, and are filled with refractile globules which are probably elemental sulfur.

Water column analysis

Chemical analysis of the water column samples allowed the approximate location of the oxic/anoxic transition zone (OATZ) to be determined (see Fig. 2). Oxygen was highest at the surface and dropped off at approximately 3.5 m depth, while sulfide became detectable at around 4 m and increased with depth down to the bottom (5 m). The trends in oxygen depletion and sulfide enrichment match our observation that the water from the 3.5-4 m interval was pink colored (Fig. 3). The increase in coloration of the water samples at this depth interval is clearly evident in bottles 12 to 15 in Fig. 3, which shows water samples collected from increasing depths

in the water column. In order to observe changes in the microbial community of the Salt Pond water column with depth, aliquots of samples collected at 0.5 m intervals were filtered onto black 0.22 μm polycarbonate membrane filters and subsequently stained with DAPI. The filters were viewed under standard DAPI and rhodamine wavelengths for comparison of all cells (stained with DAPI) and autofluorescent cells, respectively. Two images were made for each depth interval (left and right panels of Fig. 4).

Despite potential sampling bias due to the filtration procedure, we were able to obtain clear evidence for a dramatic increase in the proportion of autofluorescent cells at the 3.5-4.0 m depth interval (Fig. 4). This position coincides well with our chemical profiles of dissolved oxygen and HS^- and suggests that the OATZ zone can be located with a variety of techniques. Unfortunately, the high proportion of autofluorescent cells precluded FISH analysis, because the labeled-oligonucleotide probes fluoresce at the same wavelength as the autofluorescent cells. However, in the future, if the probes are labeled with an alternative fluorochrome, this problem could be circumvented.

Iron reducing bacteria

It was possible to enrich for iron reducing bacteria from the Salt Pond sediment through first and secondary enrichments on $Fe(OH)_3$ and acetate. After transfer to liquid cultures with AQDS (identification of AHQDS), agar shakes with $Fe(OH)_3$/acetate (dark, big colonies) and iron-NTA/acetate were prepared (single colonies). Morphological characterization with phase contrast microscopy showed oval shaped rods. The partial seque ces of the 16S rRNA gene, amplified from colonies from agar shakes, showed strong identity to two sequences related to Cytophaga/Flexibacter species. Related organisms, from the Salt Pond, were also found in agar shakes with other substrates (Fig. 5). For more detailed analysis of the Fe(III)-reducers, further purification of the isolated bacteria is necessary.

Endospore-former enrichment

Three different aerobic spore-formers were isolated from the Salt Pond sediments on PFW plates, while no anaerobic or marine spore-formers were obtained. The three freshwater organisms could be easily distinguished by their colony color and morphology. One of the organisms formed whitish/opaque colonies and the others formed light orange and dark orange colonies, respectively.

Methylotrophic bacteria

Two different organisms capable of growing on 0.2% methanol were successfully isolated from the Salt Pond sediments. On NMS-methanol plates, one of the organisms produced

large opaque colonies, while the other organisms produced yellowish/green colonies. PCR products were obtained from each of these organisms and RFLP analysis revealed different banding patterns, indicating that these are different organisms. The methylotroph oligonucleotide probe used in the checkerboard hybridization, designed to be specific for methylotrophs using the serine pathway, hybridized with our sediment DIG-PCR product. Thus, it is possible that this probe hybridized with the 16S genes from these organisms. Sequencing of these 16S rRNA genes will allow determination of the phylogenetic relationship of these organisms to each other as well as to other methylotrophic organisms.

Salt Pond sediment sequences

Of the 5 clones sent off for sequencing, 4 partial 16S rRNA gene sequences were obtained and these sequences were analyzed by BLAST and RDP. They are listed below:

Clone	Closest Relative	% Identity
WH18	Pirellula (Planctomyces)	< 87%
WH19	Desulfobacula/Desulfobacter (δ-proteo)	< 90%
WH20	Magnetospirillum/Azospirillum (α-proteo)	< 88%
WH22	Putative Gram-positive organism (?)	< 76%

All of these sequences appear to be fairly distinct and different from other organisms in the databases, suggesting that they may represent new species. As expected, different sequence analysis programs (e.g., BLAST, RDP, TreeCon) yielded different phylogenetic placement of these sequences. Phylogenetic trees were generated for both WH19 and WH20 (Fig. 6 and 7). WH19 clusters with various other sulfate-reducing organisms, while WH20 is closest to *Magnetospirillum magnetotacticum*. Although the WH20 sequence is not close enough to infer that it is also a magnetotactic organism, it is worth noting that magnetotactic organisms have been previously obtained from the Salt Pond.

Checkerboard hybridization

Checkerboard hybridization with the DIG-labeled PCR product amplified from the Salt Pond sediment DNA confirmed that this community was composed of a wide variety of organisms (Fig. 8, lane 16). The probes that gave positive signals were as follows: Berry "Purple Sulfur", Betas-all, Enterics/some Gammas, Methylotrophs, Alphas-all, Berry "Plastid", SRB2/some deltas, SRB/some deltas, LoGC Gram positive, *Planctomyces*-all, *Spirochetes*, Flavos-all, Cyanobacteria, and Universal. Overall, these probing results appear to be consistent with our enrichment data as well as the other 16S rRNA gene sequences obtained from these

sediments. In the future, it would be interesting to perform checkerboard hybridization analysis on PCR products amplified from DNA extracted from different depth intervals within the water column. This would allow comparison of the microorganisms present in the oxic, anoxic, and oxic/anoxic transition zones of the Salt Pond.

References

Bazylinski D., et al. (1997) Microbial biomineralization of magnetic iron minerals: Microbiology, magnetism, and environmental significance. *In* Geomicrobiology: Interactions between Microbes and Minerals. J.F. Banfield et al., Eds. Mineralogical Society of America, Washington, D.C., 448 p.

Scranton, M.I. et al. (1984). The distribution and cycling of hydrogen gas in the waters of two anoxic marine environments. Limnol. Oceanog. **29**: 993-1003.

Wakeham S.G. et al. (1984) Dimethyl sulfide in a stratified coastal pond. Nature **310:** 770-772.

Wakeham S.G. et al (1987) Biogeochemistry of dimethylsulfide in a seasonally stratified coastal salt pond. Geochim. Cosmochim. Acta 51: 1675-1684

Figure 1

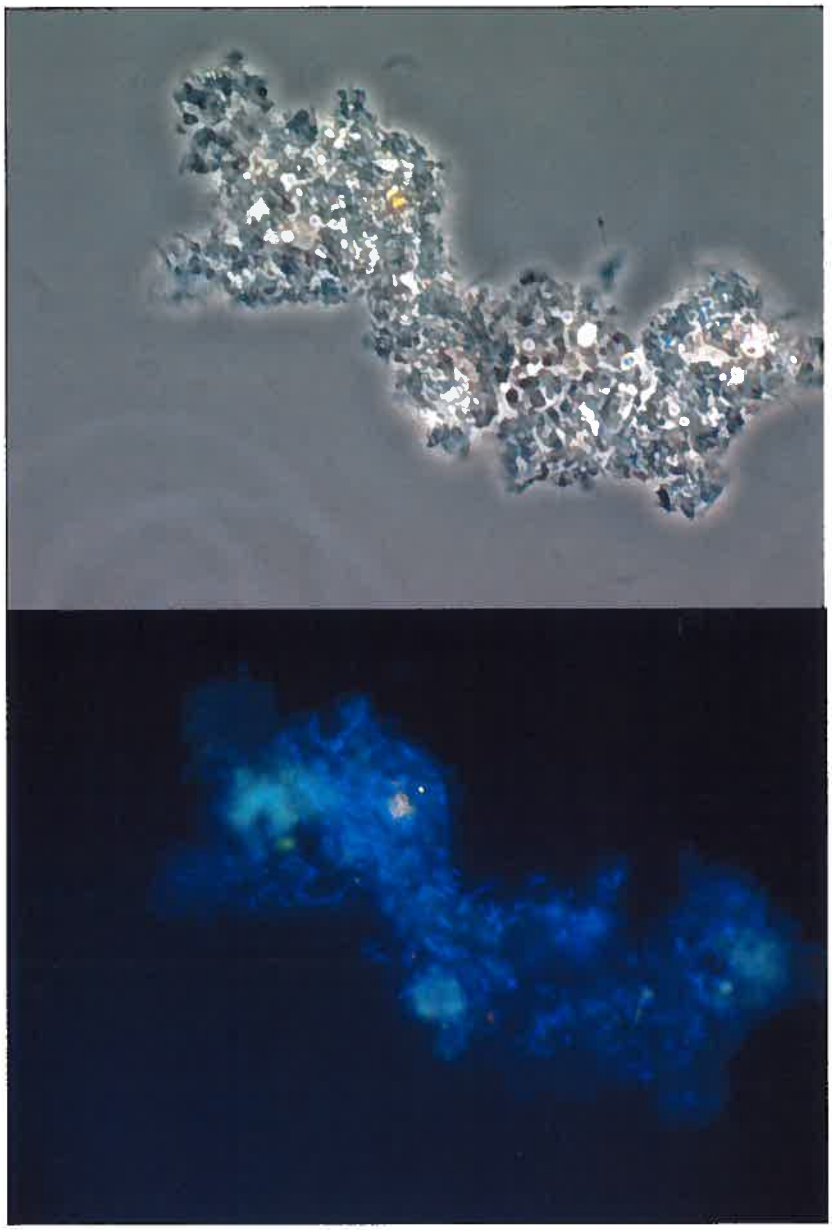


Figure 2

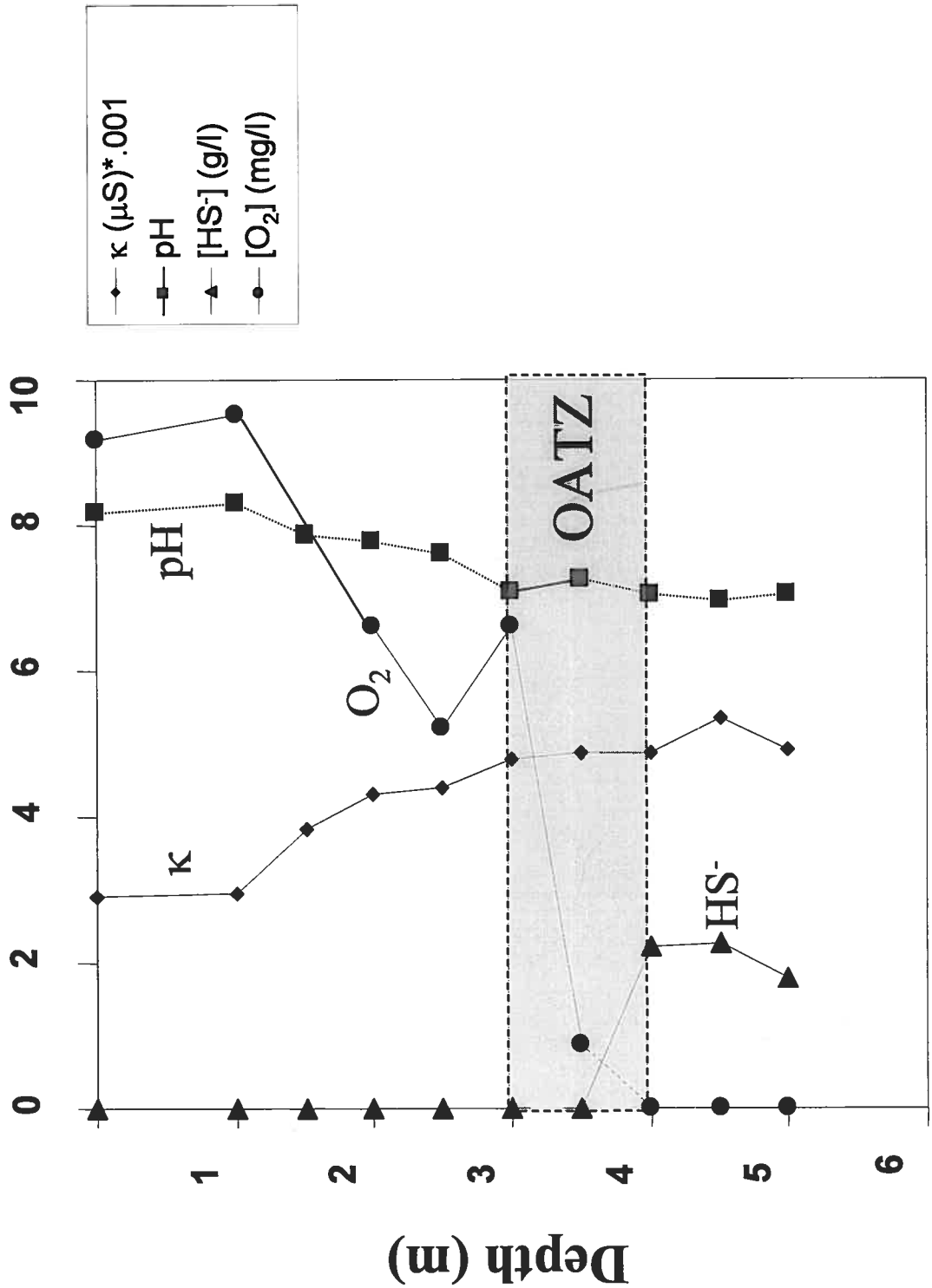


Figure 3

Figure 4a

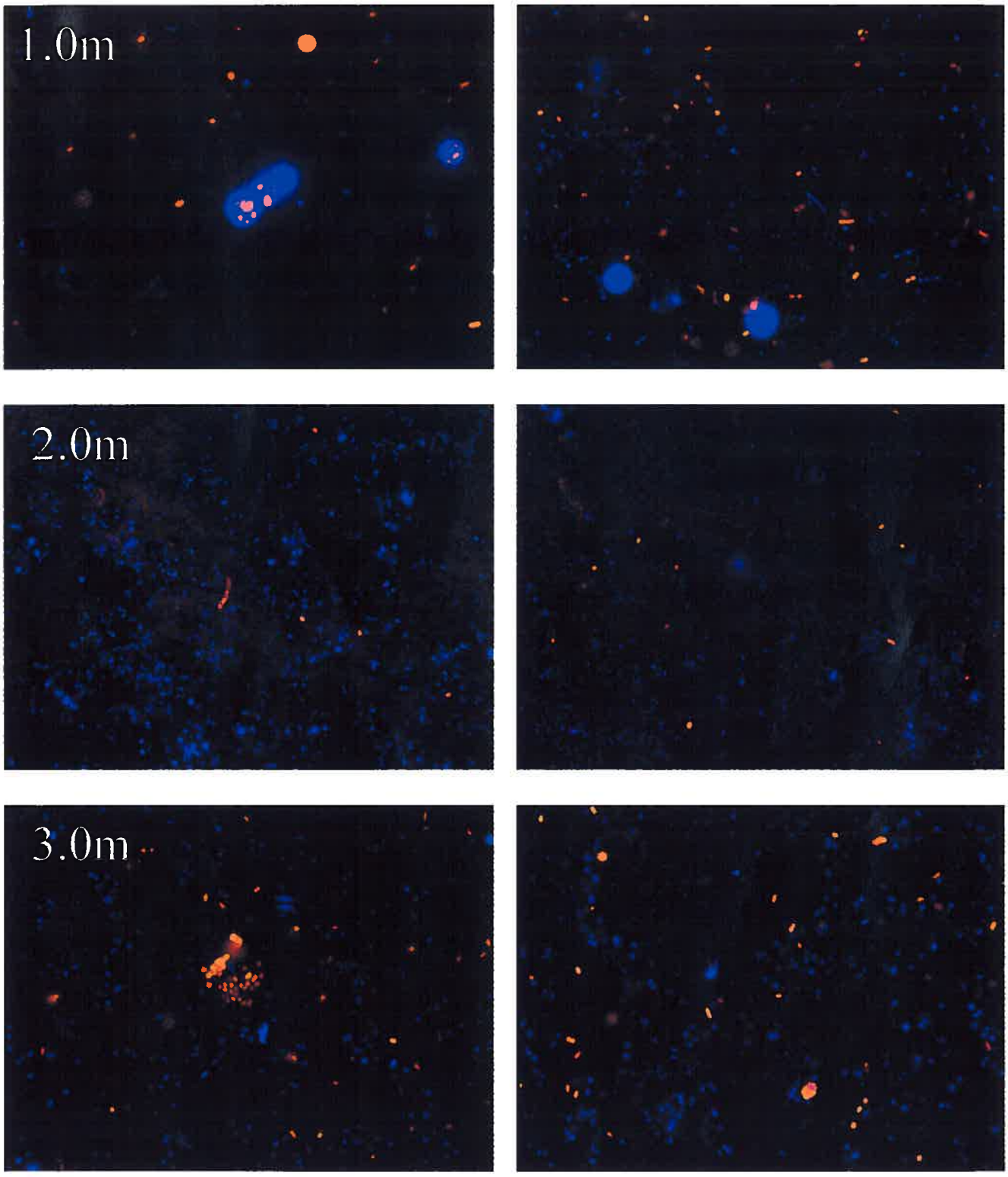


Figure 4 b

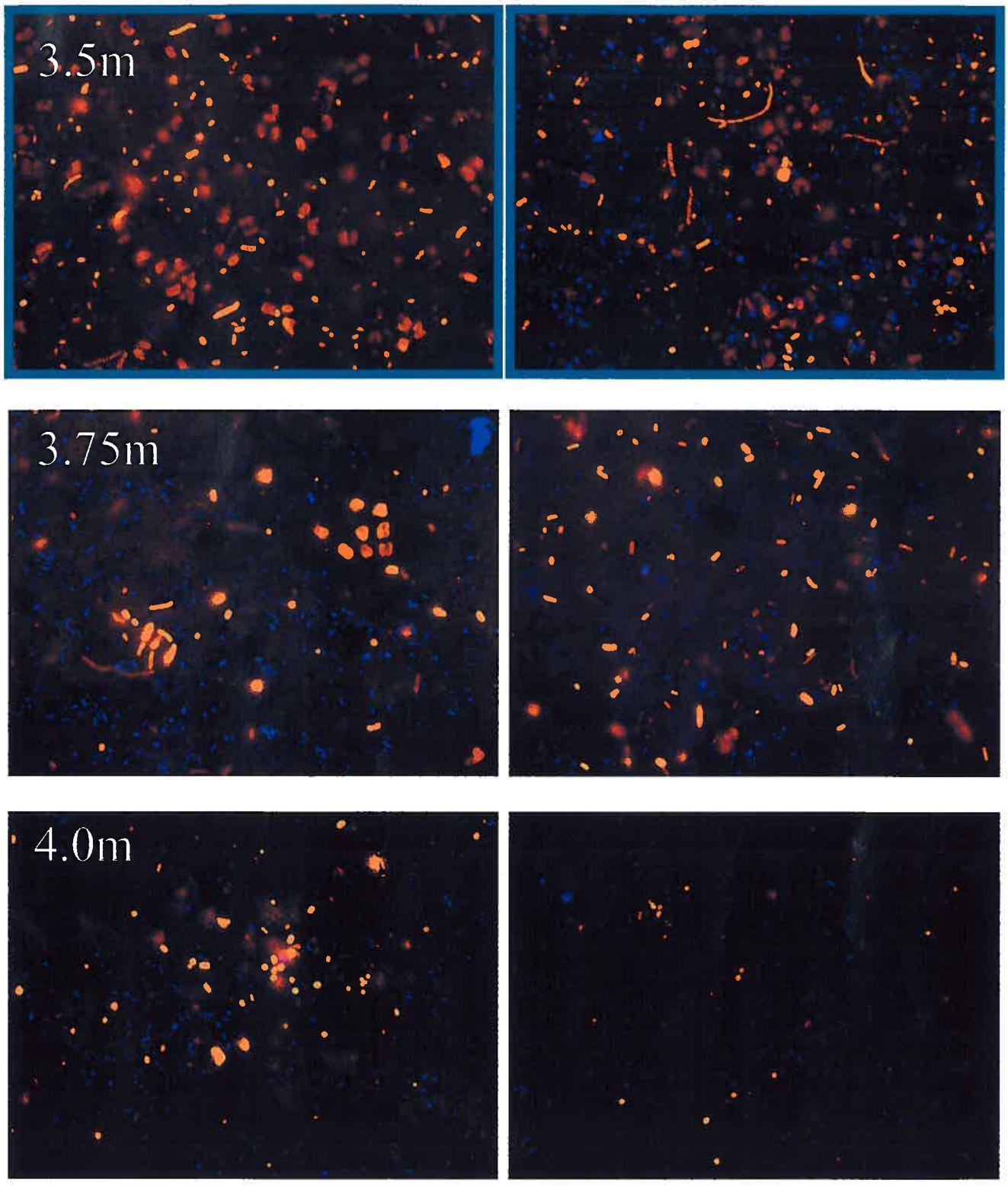


Figure 4 c

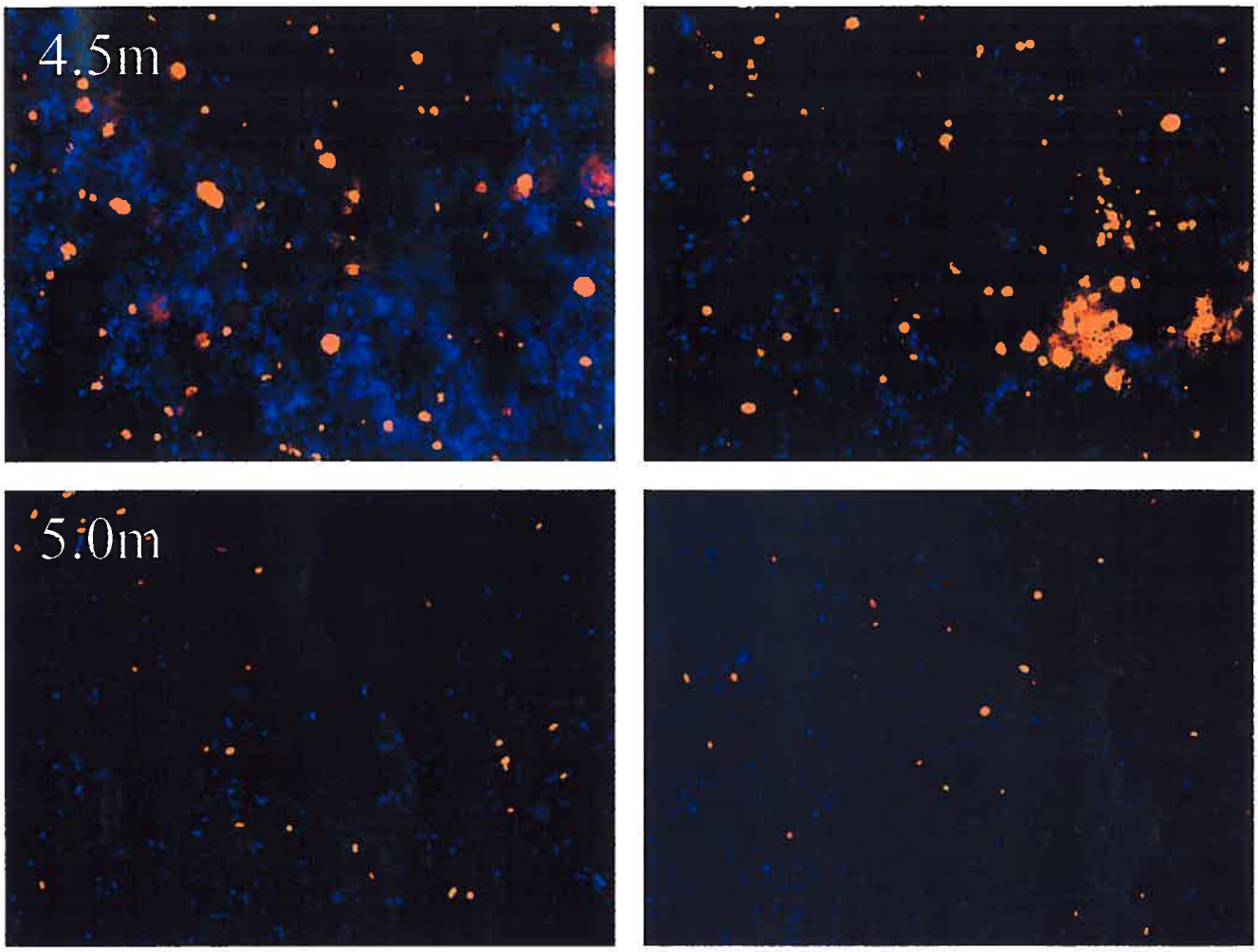


Figure 4 d

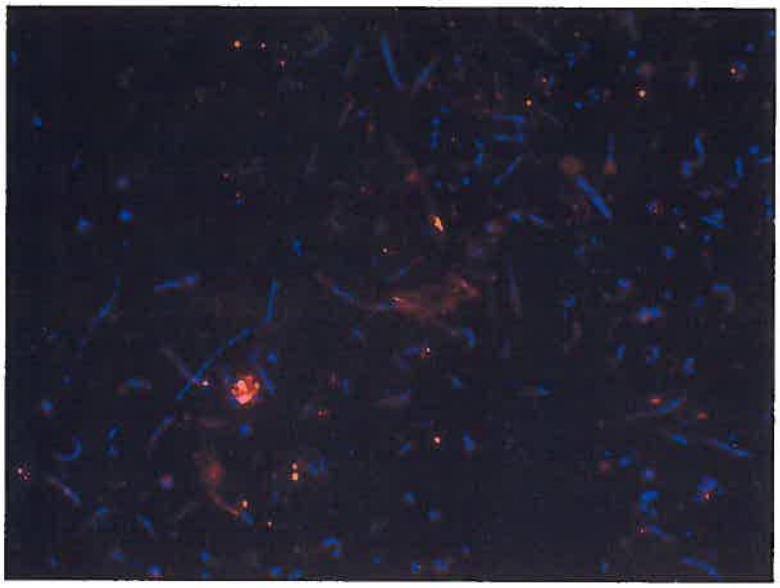
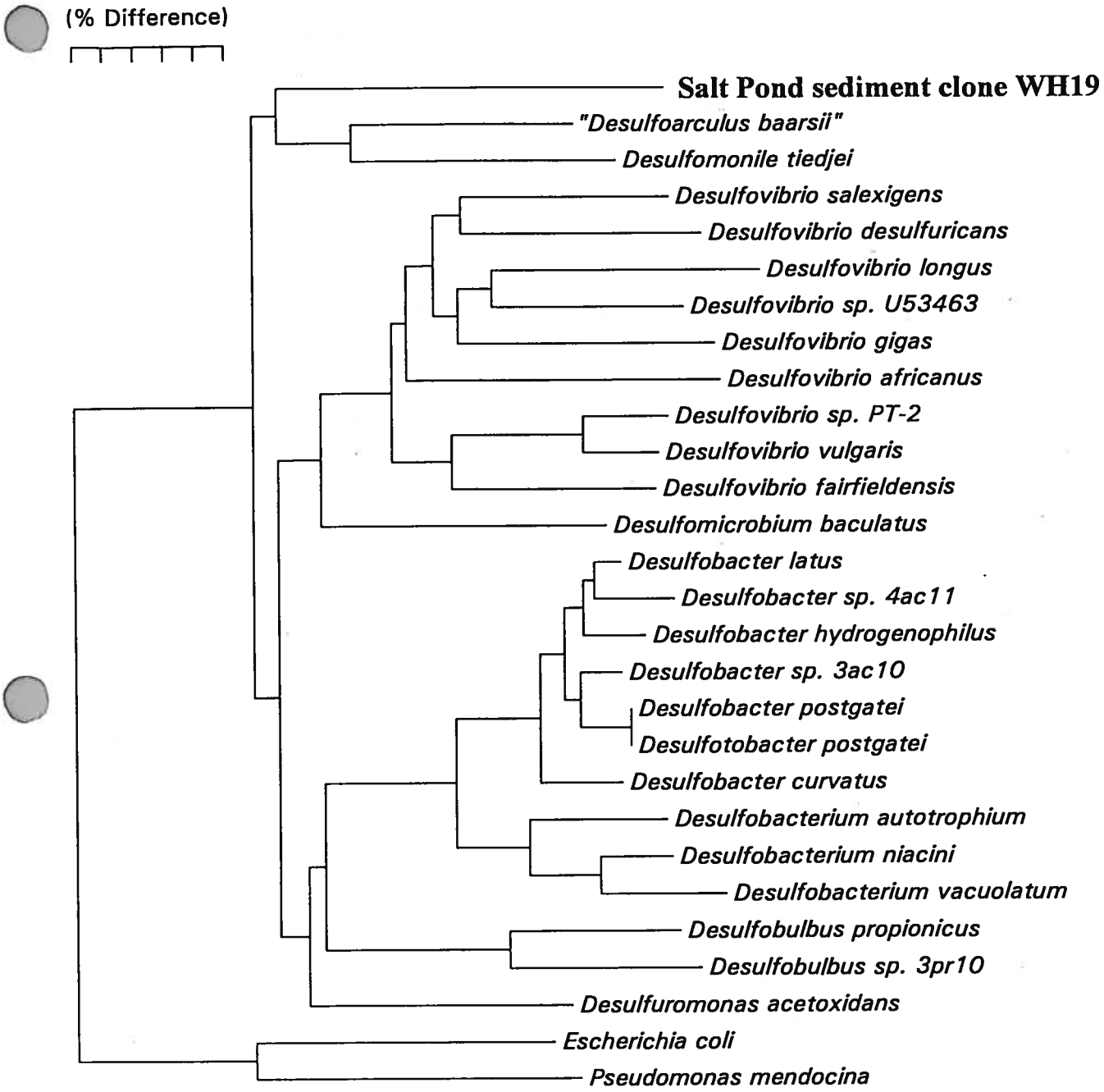


Figure 6


(% Difference)
Salt Pond sediment clone WH19
"Desulfoarculus baarsii"
Desulfomonile tiedjei
Desulfovibrio salexigens
Desulfovibrio desulfuricans
Desulfovibrio longus
Desulfovibrio sp. U53463
Desulfovibrio gigas
Desulfovibrio africanus
Desulfovibrio sp. PT-2
Desulfovibrio vulgaris
Desulfovibrio fairfieldensis
Desulfomicrobium baculatus
Desulfobacter latus
Desulfobacter sp. 4ac11
Desulfobacter hydrogenophilus
Desulfobacter sp. 3ac10
Desulfobacter postgatei
Desulfotobacter postgatei
Desulfobacter curvatus
Desulfobacterium autotrophium
Desulfobacterium niacini
Desulfobacterium vacuolatum
Desulfobulbus propionicus
Desulfobulbus sp. 3pr10
Desulfuromonas acetoxidans
Escherichia coli
Pseudomonas mendocina

Cytophaga Flexibacter Bacteriodes (CFB) Division

Microbial Diversity 1998

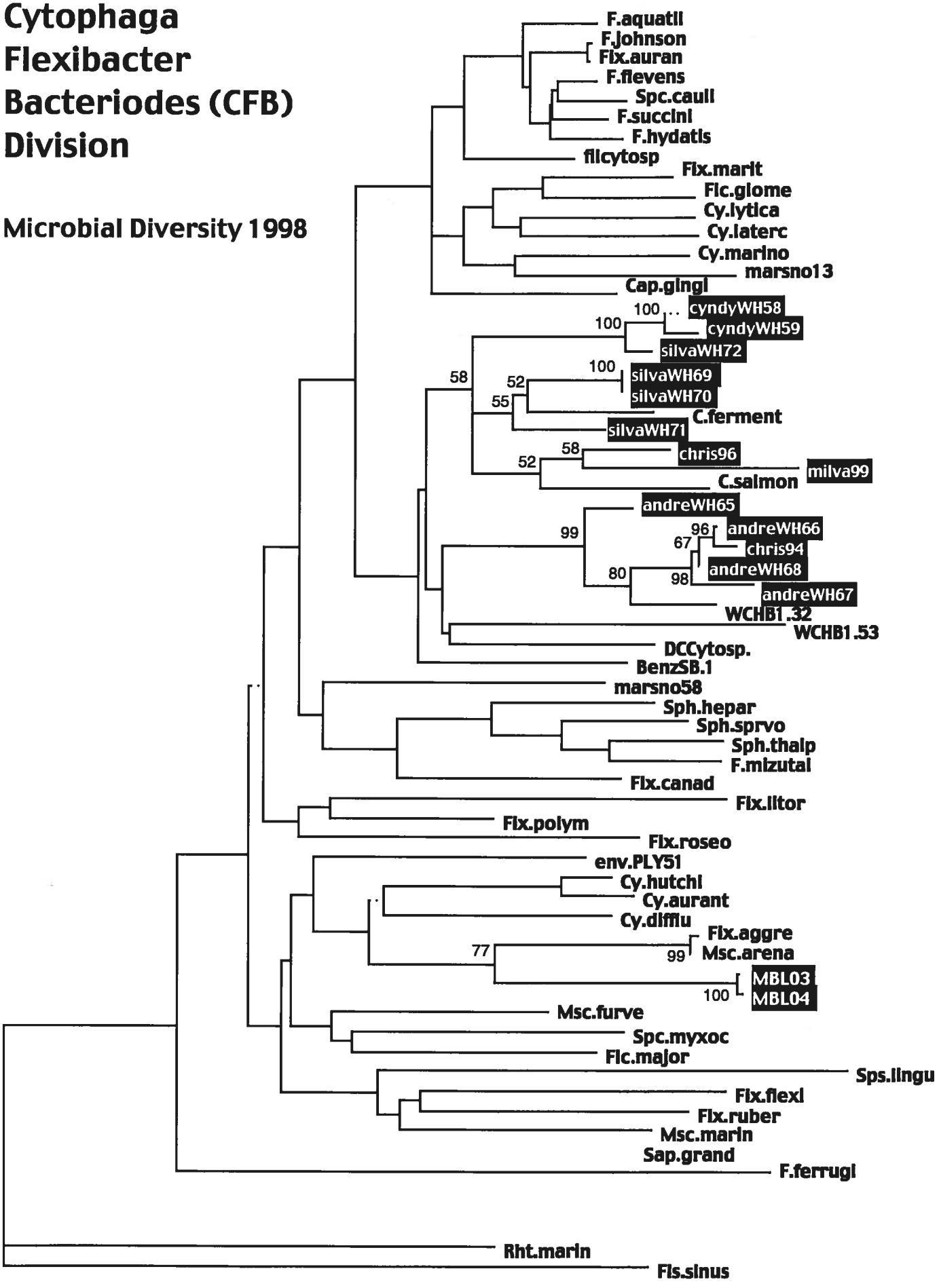
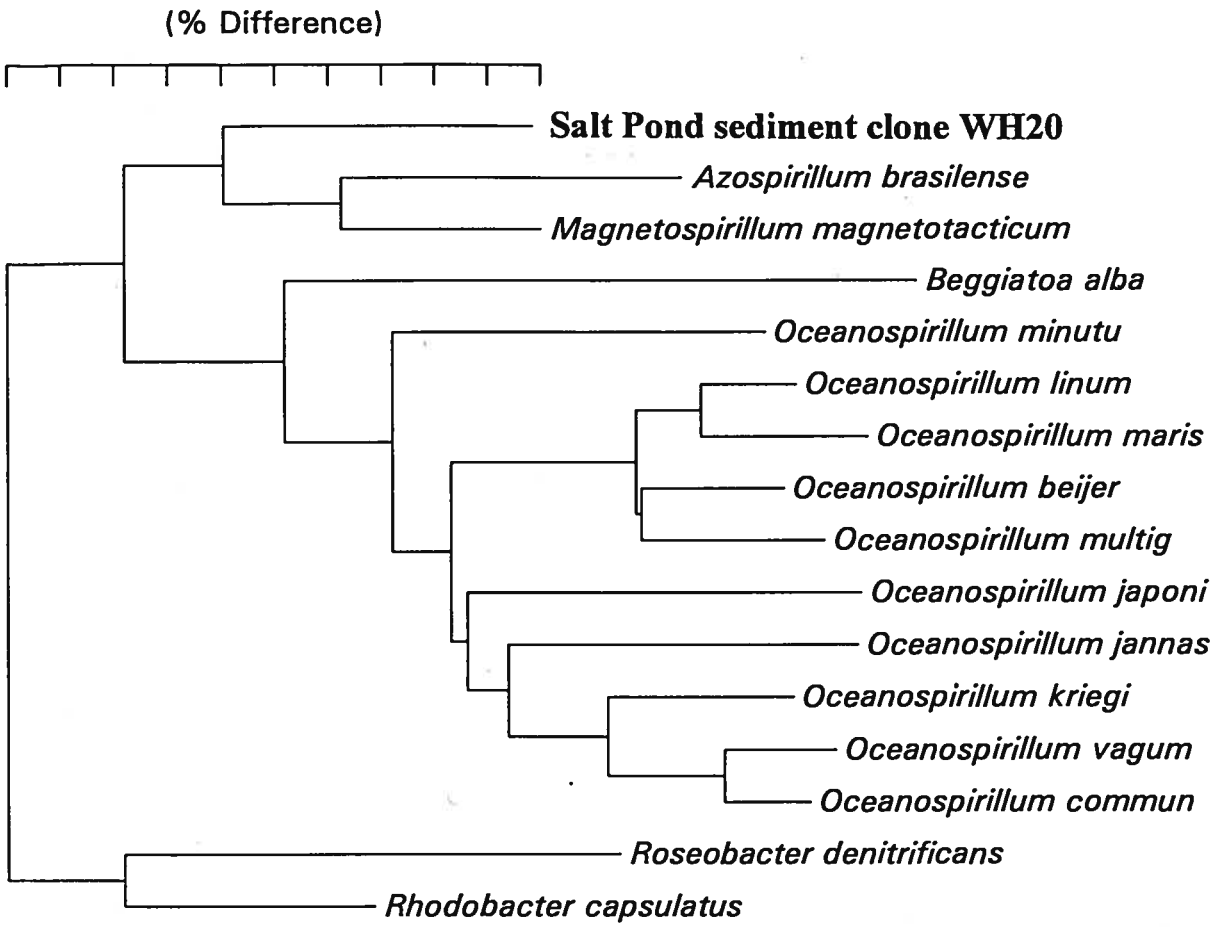


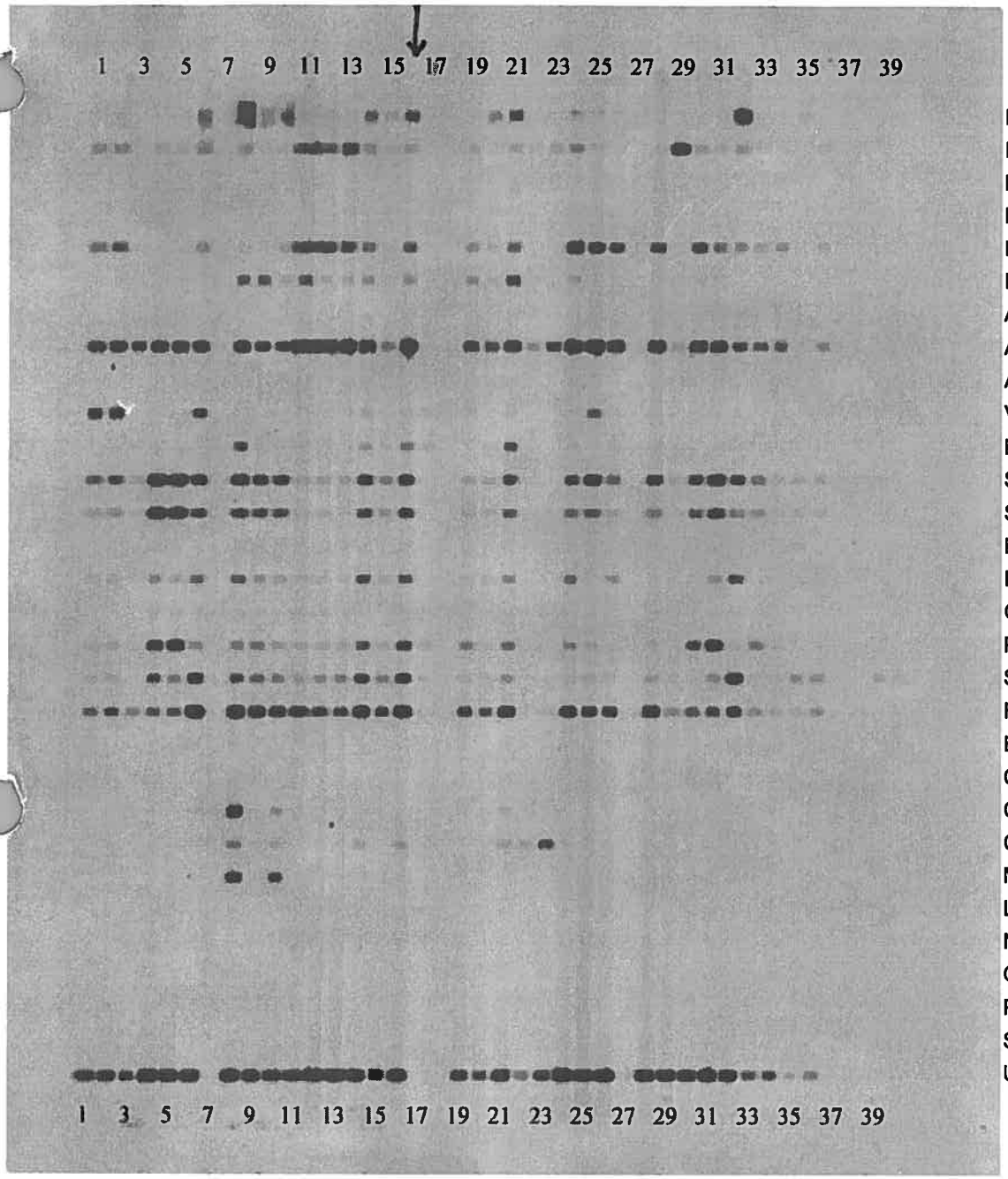
Figure 7


(% Difference)
Salt Pond sediment clone WH20
Azospirillum brasilense
Magnetospirillum magnetotacticum
Beggiatoa alba
Oceanospirillum minutu
Oceanospirillum linum
Oceanospirillum maris
Oceanospirillum beijer
Oceanospirillum multig
Oceanospirillum japoni
Oceanospirillum jannas
Oceanospirillum kriegi
Oceanospirillum vagum
Oceanospirillum commun
Roseobacter denitrificans
Rhodobacter capsulatus

Checkerboard hybridization

Figure 8

7/24/98



Berry "Purple Sulfur "
Betas-all
Nitrosolobus/Nitrosovibrio
Nitrosomonas spp
Enterics / some gammas
Methylotrophic bacteria
ANG Alteromonas sp.
Alphas-all
ANG Shewanella sp.
Vibrio gazogenes
Berry "Plastid"
SRB2, some deltas
SRB, some deltas
Berry SRB clones C16/C18
LoGC Gram Positive
Green Sulfur-all
Planctomyces-all
Spirochetes-
Flavos-all
Bacteroides-
Cytophaga berry C10
Cytophaga berry C9
Cyanobacteria
Microcoleus sp.
Lyngbia sp.
Nostoc sp.
Oscillatoria sp.
Phormidium ectocarpi .
Synechococcus sp.
Universal

Key:

1. Silvana-salt pond 1
2. Silvana-salt pond 2
3. John oyster
4. Group I- ED
5. Group I--LD
6. Group I-MD
7. Group I-G1 [No good]]
8. Group I-P2
9. Group I-G3
10. Group I-B4
11. Milva enrich $t_0 + O_2$
12. Milva enrich $t_2 + O_2$
13. Milva enrich $t_4 + O_2$
14. Milva enrich $t_8 + O_2$
15. Milva enrichment t_6 w/o O_2
16. Grp III sediment
17. Grp III sediment [No good]
18. [No good]
19. Patti Sip. Hi Nitrogen plot
20. Patti Sip. Low Nitrogen plot
21. Patti Sip. Control plot
22. Karin Cyan. Assoc.
23. Karin Cyan. Pure
24. Scott- Begg. Environ
25. Andreas Deep Sea
26. Andreas Deep Sea
27. Andreas [no good]
28. Andreas Deep Sea
29. Yoshiko ThioS
30. Yoshiko-H_2/Fe
31. Grp II--PSB
32. Grp II Cyto/PS
33. Grp II SRB
34. Yoshiko-H_2/Fe
35. Pond Berry
36. Pk sand berry 1/10
37. Pk sand berry 1/100
38. Brown berry
39. Pink sand 1/10
40. Mussel 1/10